THE EFFECTS OF ACCLIMATION TEMPERATURE ON HYPOXIA TOLERANCE OF MUMMICHOG KILLIFISH (*FUNDULUS HETEROCLITUS*)

THE EFFECTS OF ACCLIMATION TEMPERATURE ON HYPOXIA TOLERANCE OF MUMMICHOG KILLIFISH (*FUNDULUS HETEROCLITUS*)

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TITLE: The effects of acclimation temperature on hypoxia tolerance of mummichog killifish (*Fundulus heteroclitus*)

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LAY ABSTRACT

Human disturbance is leading to increased variability of environmental temperature as well as increased incidence and severity of aquatic hypoxia (low oxygen conditions). The effect of elevated temperatures on hypoxia tolerance is well studied, however little is known about how environmentally relevant temperature variability impacts hypoxia tolerance in fish. To address this research gap, I examined the effects of constant temperature and fluctuating temperature regimes on the hypoxia tolerance of mummichog killifish. I provide evidence that acclimation temperature impacts hypoxia tolerance, gill structure, haematology and energy stores in the brain. My thesis demonstrates that thermal history can alter temperature-sensitive physiological traits that fish use to cope with stressors such as low oxygen. These findings contribute to the existing body of work that documents the interactive effects of combined stressors in changing ecosystems on the physiology of fish.

ABSTRACT

Climate change is leading to rapid change in aquatic environments, increasing the mean and variability of temperatures, and increasing the incidence of hypoxia. We investigated how acclimation to constant temperatures or to diel temperature fluctuations affect hypoxia tolerance in mummichog killifish (*Fundulus heteroclitus*). Killifish were acclimated to constant cool (15˚C), constant warm (25˚C), or a diel temperature cycle (15˚C at night, 25˚C during day) for 6 weeks. I then measured hypoxia tolerance (time to loss of equilibrium in severe hypoxia, t_{LOE}; critical O_2 tension, P_{crit}), whole-animal metabolism, gill morphology, haematology, and tissue metabolites at 15˚C and 25˚C in a full factorial design. Among constant temperature groups, t_{LOE} was highest and P_{crit} was lowest in fish tested at their acclimation temperature. Warm acclimated fish had lower metabolic rate at 25˚C and greater gill surface area (less coverage of lamellae by interlamellar cell mass, ILCM), but cool acclimated fish had greater brain glycogen stores. Therefore, effects of constant temperature acclimation on hypoxia tolerance were temperature specific and not exhibited broadly across test temperatures, and they were associated with different underlying mechanisms. Hypoxia tolerance was less sensitive to test temperature in fish acclimated to fluctuating temperatures compared to fish acclimated to constant temperature. Acclimation to fluctuating temperatures also increased haemoglobin-O_2 affinity of the blood (decreased P_{50}) compared to constant temperature groups. Therefore, acclimation to fluctuating temperatures helps maintain hypoxia tolerance across a broader range of temperatures, and leads to some distinct physiological adjustments that are not exhibited by fish acclimated to constant temperatures.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CT_{max}	Critical thermal maximum
CT_{min}	Critical thermal minimum
GTP	Guanosine triphosphate
Hb	Haemoglobin
Hct	Haematocrit
ILCM	Interlamellar cell mass
LLT	Lower lethal temperature
LOE	Loss of equilibrium
MCHC	Mean corpuscular haemoglobin concentration
MO_2	Metabolic rate; oxygen consumption rate
O_2	Oxygen
P_{50}	Oxygen tension when haemoglobin is 50% saturated with oxygen
P_{crit}	Critical oxygen tension
PO_2	Partial pressure of oxygen
RMR	Resting metabolic rate
SEM	Standard error of the mean
t_{LOE}	Time to loss of equilibrium
T_{opt}	Thermal optimum
TPC	Thermal performance curve
ULT	Upper lethal temperature

THESIS ORGANIZATION AND FORMAT

This thesis is organized in "sandwich" format, as recommended by my supervisory committee. It consists of three main chapters. Chapter one is a general introduction and outlines relevant background information and discusses the justification for the subsequent objectives and hypothesis of my thesis research. Chapter two is a manuscript in review at a peer-reviewed scientific journal. Chapter three is an overview of the major findings of the thesis, their implications in fish physiology and suggestions of future directions of research.

CHAPTER ONE: **GENERAL INTRODUCTION**

CHAPTER TWO: **CONSTANT TEMPERATURE AND FLUCTUATING TEMPERATURE HAVE DISTINCT EFFECTS ON HYPOXIA TOLERANCE IN KILLIFISH (*Fundulus heteroclitus*)**

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CHAPTER THREE: **GENERAL DISCUSSION**

CHAPTER ONE: GENERAL INTRODUCTION

1.1 Anthropogenic stress in aquatic environments

Global climate change has, and will continue to alter aquatic ecosystems through changes in multiple interacting abiotic factors (e.g. temperature, O_2 availability, salinity and pH). Rising global temperatures have increased the temperature of the ocean's upper layers by 0.6˚C over the past 100 years (Hoegh-Guldberg and Bruno, 2010), leading to rapid changes in species distribution, phenologies, physiology and fitness (Deutsch et al., 2015). Species may relocate to avoid warming temperatures where possible, however species isolated in habitats near thermal tolerance limits may disappear (Covich et al., 1997). In addition to its independent effects, increased temperature commonly co-occurs with another important environmental stressor; reduced O_2 availability (i.e. hypoxia). Environmental hypoxia (i.e. low or depleted oxygen) occurs naturally in many aquatic ecosystems, however, it can also occur as a result of anthropogenic disturbance such as eutrophication due to expansion of agricultural activity, increasing population size and urbanization (Diaz, 2001; Diaz and Rosenberg, 2008). Human impacts have exacerbated the occurrence of aquatic hypoxia, including habitats not historically effected by hypoxic events (Diaz, 2001; Diaz and Rosenberg, 2008; Rabalais et al., 2010). Hypoxia presents a significant challenge to aquatic organisms, as it threatens the balance of O_2 supply and demand. Fueling aerobic metabolism, oxygen is essential for the production of nearly all of the cellular energy (ATP) required to sustain biological processes thus, reduced availability of oxygen has the potential to result in detrimental effects on physiological function.

The mean and variability of environmental temperature and the incidence of hypoxic events are increasing in many aquatic ecosystems, resulting in profound effects on aquatic organisms (Diaz and Rosenberg, 2008; Hoegh-Guldberg and Bruno, 2010; Vasseur et al., 2014). However, we have a relatively poor understanding of how environmental variability, including fluctuations in environmental temperature, impact an organism's capacity to withstand additional environmental stressors such as hypoxia. My thesis aims to examine the effect of acclimation temperature and temperature fluctuation on hypoxia tolerance as well as various underlying respiratory and metabolic traits in an estuarine fish model.

1.2 Environmental temperature in aquatic ecosystems

Temperature has pervasive effects on the rates of biochemical processes such as aerobic metabolism, thus dictating the distribution and abundance of fish species. Therefore, understanding the effects of increased environmental temperature is critical for predicting how species will respond to future global change. Natural variation in water temperature is characteristic of many aquatic ecosystems such as coastal aquatic environments, and many fish have evolved to cope and sometimes thrive under such conditions (Schulte, 2007). However, climate change continues to increase mean aquatic temperatures as well as temperature variability, leading to unpredictable and potentially inhospitable conditions for aquatic organisms (Schulte, 2007). Changes in environmental temperature can cause complex patterns of disturbance, with changes to overall productivity of an ecosystem, migration patterns and seasonal shifts, as well as changes in the physico-chemical variables such as dissolved oxygen content, pH and salinity (Harley et al., 2006). Projected increases in water temperature may displace whole populations or result in extirpation from the historical range of some species living near their thermal tolerance limits (Dillon et al., 2010; Eliason et al., 2011; Pinsky et al., 2019). Predicting how increased temperature variability will impact aquatic organisms presents a unique challenge, as few studies have incorporated environmental variability akin to what exists in natural ecosystems.

1.2.1 Thermal tolerance

The thermal biology of ectotherms shapes species distributions and abundance through thermal tolerance and temperature sensitive effects on performance. Acute increases in water temperature results in an increase in the metabolic rate of ectotherms, however excessive increases surpassing the thermal tolerance of a species may cause more serious disturbances to various performance traits or cellular damage (Schulte et al., 2011). The effect of temperature on performance can be examined through a thermal performance curve (TPC; see Fig. 1.1), which typically shows an increase in performance with an increase in temperature up to a maximum intermediate temperature (T_{opt}), followed by a rapid decrease in performance at temperatures surpassing T_{opt} (Schulte et al., 2011). T_{opt} can therefore be defined as the temperature at which performance is highest. The points at which performance is equal to zero are termed the critical thermal minimum (CT_{min}) and maximum (CT_{max}; (Becker and Genoway, 1979). Both are a

measure of thermal tolerance among ectotherms and highlight the temperature at which an organism's locomotory activity becomes uncoordinated and would be unable to escape danger (Becker and Genoway, 1979). These metrics are different than the upper and lower lethal temperatures (ULT and LLT), which are the temperatures at which all function ceases and the individual cannot survive (Golovanov, 2012). A TPC can be modified through adaptive evolution or phenotypic plasticity to change the height of the curve (e.g. increased performance), the position of the curve (e.g. a shift in the T_{opt}) or the width (e.g. increased thermal breadth). Thermal breadth, or the range of temperatures a species can maintain performance, can range from narrow (i.e. stenothermal) for groups such as Antarctic marine ectotherms, whose CT_{min} and CT_{max} may only differ by a few degrees (Wilson et al., 2001), to wide (i.e. eurythermal) in species such as goldfish (*Carassius auratus*) whose CT_{min} and CT_{max} are thought to be 0˚C and 42˚C respectively (Ford and Beitinger, 2005). Factors such as age, life stage or phenotypic plasticity also influence thermal breadth, however selection has resulted in a correlation between a species' zone of tolerance and their thermal environment in the wild (Schulte et al., 2011). Within their respective zones of tolerance, temperature also impacts various performance traits at the whole-animal level (e.g. growth, fecundity and metabolic rate) and the sub-organismal level (e.g. heart rate, respiration rate and enzyme activity; Schulte et al., 2011). Understanding how thermal tolerance can change is becoming increasingly important to accurately predict the response of populations or species to future climate change.

1.2.2 Thermal acclimation

Thermal acclimation has the capacity to alter thermal tolerance, in which prolonged exposure to elevated or reduced temperatures can lead to plastic adjustments of respiratory and metabolic traits to improve function during changes in temperature (Lagerspetz, 2006). Effects of temperature may be subject to a generalist-specialist trade-off, in which maximum performance at an optimum temperature decreases with increased thermal breadth (Seebacher et al., 2015). Acclimation to a constant elevated temperature has been observed to improve performance at acclimation temperature, demonstrating plastic changes in the thermal optimum temperature, or a change in the position of a TPC (Schulte et al., 2011). However, the effect of constant temperature acclimation on thermal breadth is unclear. Acclimation to fluctuating temperatures may be expected to modify the width of a TPC, increasing the thermal breadth of a given

performance trait. However, the possibility of a generalist-specialist trade-off has received little previous attention.

1.2.3 Physiological response to elevated temperature

Fish may use various behavioural and physiological strategies to avoid or cope with stressors such as warm temperatures. Fish are known to seek out their preferred temperature to maximize performance (Larsson, 2005; Nivelle et al., 2019). For example, pelagic fish such as yellowfin tuna can utilize vertical thermoclines to optimize ambient water temperatures to sustain higher levels of activity, while species living in coastal aquatic environments such as estuaries live in shallow systems and cannot utilize the same thermal buffer capacity that exists in the open ocean (Brill et al., 1999; O'Reilly et al., 2015). Given the physical restrictions of their environment, species that dwell in shallow systems have limited dispersal capacity, and cannot escape high temperature conditions (Woodward et al., 2010). If elevated temperatures cannot be avoided, fish must make physiological adjustments to maintain balance between tissue O_2 supply and demand. Fish may undergo rapid physiological adjustments to cope with acute increases in temperature and subsequent increase in aerobic metabolism, such as increased ventilation rate to increase water flow across the gill allowing for increased gas-exchange (Crawshaw, 1977; Maricondi-Massari et al., 1998). In response to elevations in temperature, many species of fish increase cardiac output through elevation of heart rate to increase internal oxygen convection and improve oxygen delivery to the tissues (Farrell, 2009; Farrell et al., 1996; Gollock et al., 2006; Steinhausen et al., 2008), or re-direct blood to fuel more oxygen-sensitive tissues such as the brain (Yoshikawa et al., 1997). These strategies may be useful during acute exposure to elevated temperature, but may be insufficient to maintain performance, such that additional physiological plasticity may be important.

With chronic exposure to high temperatures (i.e. warm acclimation), fish may make plastic adjustments along the oxygen cascade to further improve the extraction of oxygen from the environment, oxygen transport to the tissues, and improve oxygen utilization. For example, many species have demonstrated the ability to increase exposed gill surface area through the regression of a mass of cells referred to as the interlamellar cell mass (ILCM; McBryan et al., 2016; Nilsson, 2007; Sollid and Nilsson, 2006). Originally observed in the crucian carp (*Carassius carassius*) and goldfish (*Carassius auratus*) and later in other species such as killifish

(*Fundulus heteroclitus* and *Kryptolebias marmoratus*), some fish species possess the ability to reversibly remodel their gills in response to environmental stimuli such as hypoxia and changes in temperature (McBryan et al., 2016; Ong et al., 2007; Sollid and Nilsson, 2006; Sollid et al., 2003; Sollid et al., 2005a; Turko et al., 2011; Turko et al., 2012). In response to hypoxia, the gills of *C. carassius* transition from having lamellae which are embedded in a mass of cells, to the reduction of the ILCM to reveal protruding lamellae (Sollid et al., 2003). This increase in the proportion of protruding lamellae is useful when fish are exposed to low oxygen conditions, as it allows for increased extraction of O_2 from the surrounding water. This form of gill remodelling is also highly responsive to changes in temperature, whereby an increase in temperature and O_2 demand results in the retraction of the ILCM to reveal a higher surface area for O_2 diffusion and improves O_2 uptake (Sollid et al., 2005a). Fish can increase O_2 carrying capacity of the blood by increasing hemoglobin (Hb) content and hematocrit via erythropoiesis (DeWilde and Houston, 1967; Houston and Cyr, 1974). Finally, through warm acclimation, many species can reduce the thermally-driven increase in metabolic rate, resulting in lower routine O_2 requirements (Sandblom et al., 2014; Seebacher et al., 2014). Plastic changes in response to chronic exposure to high temperatures vary among species, and the magnitude of the response contributes to a fish's thermal tolerance and their capacity to cope with elevated water temperature.

1.3 The interaction between environmental temperature and hypoxia

Elevated temperatures and low oxygen concentrations are two stressors that often reinforce each other, as an increase in environmental temperature leads to a decrease in oxygen solubility (Garcia and Gordon, 1992) and an increase in cellular respiration rate of aquatic ectotherms such as fish (Schulte, 2015). Many aquatic ecosystems such as estuarine habitats, stratified or ice-covered lakes experience natural fluctuations in oxygen availability (Diaz and Rosenberg, 2008). However, more severe or frequent oxygen depletion can also occur as a result of climate change induced increases in water temperature alone (Jankowski et al., 2006; Stefan and Fang, 1994; Stefan et al., 1996) or through the addition of nutrients (i.e. eutrophication) from increased agricultural activity, fisheries and wastewater treatment (Diaz and Rosenberg, 2008). Eutrophication has also resulted in the depletion of oxygen from environments where hypoxia was not historically present, and its consequences can be detrimental to aquatic ecosystems (Diaz, 2001). In addition to the stress of low oxygen, increased algae production, decreased

water clarity and accumulation of toxic substances as a result of eutrophication can be lethal to aquatic organisms, leading to considerable fish kill events (Breitburg et al., 2009; Diaz and Rosenberg, 2008; Friedrich et al., 2014). Even for hypoxia-tolerant organisms, a decrease in dissolved O_2 imposes potential restrictions on organismal aerobic metabolism (Deutsch et al., 2015). Fish must therefore undergo various physiological changes in order to mitigate the disparity in the oxygen availability and demand when exposed to a combination of high temperatures and hypoxia (Claësson et al., 2016; Nilsson et al., 2010; Schulte, 2007; Slesinger et al., 2019). The incidence and severity of warming temperatures and aquatic hypoxia are predicted to increase with climate change and the rise of human disturbance (Diaz, 2001), which will require physiological adjustments if fish are to overcome this challenge.

1.3.1 Hypoxia tolerance

The hypoxia tolerance of aquatic ectotherms such as fish can be measured using several whole-animal indices that reflect an organism's capacity to withstand low oxygen conditions. Hypoxia tolerance can be variable across species, such that hypoxia tolerant organisms can maintain function over a broader range of partial pressure of oxygen (PO_2) than organisms which are less hypoxia tolerant (Farrell and Richards, 2009; Richards, 2009). Common indices used to determine hypoxia tolerance include critical oxygen tension (P_{crit}) and the ability to resist loss of equilibrium in severe hypoxia (LOE). P_{crit} describes the PO_2 at which an organism shifts from oxyregulation (i.e. relatively constant oxygen consumption rate independent of external oxygen levels) to oxyconformation (i.e. oxygen consumption rate is dependent and declining with external oxygen levels; Rogers et al., 2016). Species with a lower P_{crit} can maintain resting oxygen consumption rates to fuel aerobic metabolism at a lower PO_2 and as such, are considered to be more hypoxia tolerant. Other common indices such as PO_2 at LOE or time to LOE (t_{LOE}) assess an organism's ability to resist LOE (i.e. remain dorsoventrally upright in the water column) during severe hypoxia (Becker and Genoway, 1979), rather than the maintenance of MO_2 in acute hypoxia associated with P_{crit}. Hypoxia tolerant species will have lower PO_2 at LOE or will maintain equilibrium longer than less hypoxia tolerant species (Mandic et al., 2009; Mathers et al., 2014; Speers-Roesch et al., 2012). LOE represents a proxy for ecological death, as fish lose the ability to move and function normally, and although the mechanisms of LOE are not completely understood, LOE is believed to be associated with an imbalance of ATP

homeostasis in hypoxia-sensitive tissues such as the brain (Mandic et al., 2013; Speers-Roesch et al., 2013). The PO_2 used in both LOE indices are substantially lower than the P_{crit} of an individual and can approach near anoxic levels in some hypoxia tolerant species.

1.3.2 Thermal plasticity of hypoxia tolerance

Environmental temperature and thermal acclimation have been known to affect hypoxia tolerance. The capacity of an organism to cope with hypoxia is dependent on the ability to maintain sufficient O_2 supply to tissues to meet metabolic demands (Hughes, 1973; Mandic and Regan, 2018). An increase in water temperature may be expected to reduce hypoxia tolerance of ectotherms through thermal acceleration of metabolic rate and a decrease in oxygen solubility. Acute warming (i.e. short-term exposure to an elevated test temperature) is known to reduce hypoxia tolerance, characterized by a decrease in the capacity to maintain body posture and alertness (decreased t_{LOE}) during exposure to severe hypoxia in several species (Borowiec et al., 2016; Jung et al., 2020; McBryan et al., 2016). This decline in hypoxia tolerance may be due, in part, to the rapid increase in metabolic rate following acute temperature change, causing an imbalance between the O_2 demand and supply to the tissues. However, evidence suggests that prolonged exposure to warm temperatures has the capacity to modify hypoxia tolerance through shared plastic traits which underpin responses to warming and hypoxia.

Acclimation to elevated temperatures has been observed to improve hypoxia tolerance when tested at warm temperatures. In many species such as the triplefin fish (*Bellapiscis lesleyae*), crucian carp (*Carassius carassius*) and goldfish (*Carassius auratus*), warm acclimation has been observed to improve hypoxia tolerance, demonstrated by a lower P_{crit}, relative to their cool acclimated counterparts (Hilton et al., 2008; Sollid et al., 2005a). Additionally, killifish (*Fundulus heteroclitus*) and salmon (*Salmo salar*) exhibited the same improvements following warm acclimation, demonstrated by an increase in t_{LOE} relative to cool acclimated fish when tested at a common, elevated temperature (Anttila et al., 2015; McBryan et al., 2016). These findings suggest that acclimation to warm temperatures leads to general improvements in hypoxia tolerance, however relatively few studies have examined the impacts of test temperature and acclimation temperature in a full factorial design, preventing the possibility of determining how acute temperature effects interact with acclimation effects in

these species, and it remains unclear whether the benefits of warm acclimation are restricted to warm temperatures or apply broadly across a range of test temperatures (Collins et al., 2021).

Warm acclimation is known to alter a variety of physiological processes along the oxygen cascade (i.e. O_2 uptake, transport and utilization) which may contribute to improved hypoxia tolerance at warm temperature. In combination with improved hypoxia tolerance, several species have demonstrated an increase in total lamellae surface area of the gill, through plastic changes in the ILCM, suggesting that thermal plasticity of gill morphology may contribute to improved hypoxia tolerance. An increase in exposed gill surface area allows for increased O_2 uptake across the gills and help fuel the increased demand for O_2 by the tissues. Improvements in hypoxia tolerance of warm acclimated salmon was observed in combination with changes in the oxygen supply and delivery system (increased compact myocardium thickness and capillary density), likely improving oxygen transport to the tissues (Anttila et al., 2015; Klaiman et al., 2011). These finding suggest that prolonged exposure to warm temperatures has the capacity to modify hypoxia tolerance through shared physiological mechanisms which underpin responses to warming and hypoxia.

Finally, in many aquatic ecosystems such as estuaries and salt marshes, water temperature and dissolved oxygen are rarely constant, and aquatic organisms often experience wide fluctuations in these and other abiotic factors. However, very little is known about how acclimation to realistic diel cycles of temperature impact hypoxia tolerance, even though these conditions are more relevant to what is experienced in the wild. Acclimation to fluctuating temperatures, a more ecologically relevant temperature regime, may favour a more generalist strategy as it relates to thermal tolerance, while acclimation to constant temperature may favour thermal specialization at their acclimation temperature, however this is relatively poorly understood. When considering the expected increase in complexity of interacting abiotic factors with future climate change, studies incorporating fluctuating conditions are becoming increasingly relevant areas of study.

1.4 Model organism

An ideal model for this study is the mummichog killifish (*Fundulus heteroclitus*). As an estuarine species, *F. heteroclitus* experiences complex patterns of abiotic stressors which can vary in both temporal and spacial scales. Marshes and estuaries are expected to be particularly

affected by future climate change, exposing the organisms that inhabit these already challenging habitats to increasingly variable environmental conditions (Scavia et al., 2002). This species is highly abundant along the Atlantic coast of North America and can be found as far north as Nova Scotia and as far south as Florida (Schulte, 2007). A steep thermal gradient exists across their latitudinal range, estimated to equal 1˚C change in mean annual temperature per degree change in latitude where, at the southern end of the species range, summer temperatures reach 35˚C and the northern range temperatures rarely surpass 25˚C (Schulte, 2007). Northern populations are likely to encounter extensive ice cover in the winter months while southern populations will not. However, at both ends of their distribution range, *F. heteroclitus* will experience significant seasonal variation in temperature, where winter temperatures are over 10˚C lower than average summer temperatures (Schulte, 2007). Finally, due to the tidal effects present in coastal environments, temperatures can change drastically over short time scale; sometimes 10˚C over the course of a few hours (Schulte, 2007). As a result of their thermally challenging habitats, *F. heteroclitus* is highly thermally tolerant and their thermal tolerance is highly plastic. For example, when acclimated to 2.5˚C, their CT_{max} is 28.5˚C, but can reach 41.5˚C when acclimated to 30˚C (Fangue et al., 2006). In response to warm acclimation, *F. heteroclitus* has demonstrated the capacity to increase maximum heart rate and maintain regular heart function 10˚C higher compared to cool acclimated fish (Safi et al., 2019). Additionally, warm acclimation results in significant remodelling of the gill lamellae, demonstrated by a significant regression of the ILCM (McBryan et al., 2016). Accordingly, this eurythermal species is an excellent model for elucidating the physiological mechanisms involved in thermal acclimation.

In addition to experiencing variations in temperature, *F. heteroclitus* routinely experiences dynamic fluctuations many other abiotic factors while living in coastal aquatic environments such as oxygen availability, carbon dioxide, pH and salinity, making them highly tolerant of many environmental stressors. Estuaries and salt marshes in which *F. heteroclitus* occur can experience oxygen concentrations ranging from oxygen supersaturation to anoxia and have been known to survive in waters where dissolved oxygen is as low as 1.5-2.5 ppm (Greaney et al., 1980) making them extremely hypoxia tolerant. In response to prolonged or intermittent hypoxia, *F. heteroclitus* demonstrates several physiological adjustments used to cope with low oxygen. Following constant hypoxia acclimation, *F. heteroclitus* increases blood haemoglobin content (Borowiec et al., 2015). During intermittent hypoxia, both haemoglobin content and

hematocrit increases, but this is reversed this during reoxygenation demonstrating their capacity to modulate haematology rapidly to balance O_2 supply and demand (Borowiec and Scott, 2021). Metabolic adjustments are also used to reduce O_2 demand following prolonged hypoxia exposure, whereby MO_2 decreases by 50% with little recruitment from anaerobic metabolism, suggesting metabolic depression (Borowiec et al., 2018). Generally appreciated to have a broad range of environmental tolerance, *F. heteroclitus* is known to exhibit high levels of plasticity, which makes them an excellent model for studying multi-stressor responses (Borowiec et al., 2015; Burnett et al., 2007).

1.5 Objectives & hypotheses

For my thesis, the aim of my research was to investigate how interacting abiotic stressors impact fishes in a warming world. More specifically, I sought to determine how the thermal history and thermal environment of fish impacts their capacity to cope with low oxygen availability. Using a full factorial design, my work had several objectives:

(i) Compare the effects of acclimation to constant cool, constant warm, and fluctuating temperatures on hypoxia tolerance, using a full-factorial design at both cool and warm test temperatures.

(ii) Investigate the mechanisms that underlie variation in hypoxia tolerance.

I hypothesized that thermal history alters hypoxia tolerance through thermal plasticity of various respiratory and metabolic traits. I predicted that acclimation to a constant temperature would lead to greatest hypoxia tolerance at that acclimation temperature, but poor hypoxia tolerance at a different test temperature. I also predicted that acclimation to fluctuating temperatures would induce a more generalist strategy, in which hypoxia tolerance is better maintained over a broader range of cool and warm temperatures when compared to fish acclimated to constant temperature.

Considering the ability of *F. heteroclitus* to adapt and thrive in challenging estuarine habitats, they are a valuable model to investigate the effect of thermal history on sensitivity to low oxygen, two ecologically relevant, co-occurring stressors. Previous studies have investigated the effects of temperature acclimation on hypoxia tolerance; however, it is vital that we understand the interactive effects of dynamic fluctuations of abiotic factors in order to predict how fish will respond to future global change. This work will provide valuable insight into fish

population management in the future, as global change continues to impact aquatic environments.

1.6 Figures

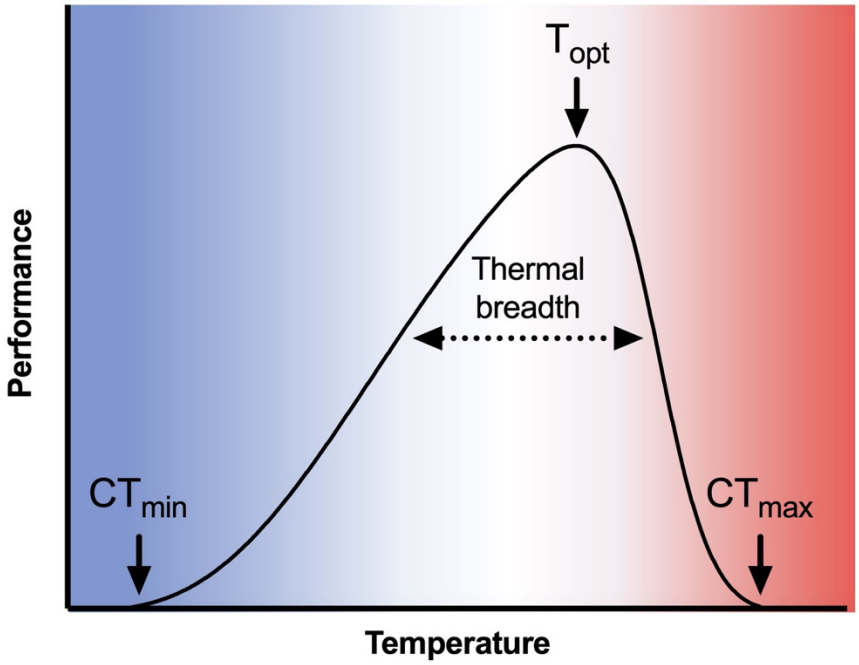
T opt
Thermal breadth
CT min
CT max
Performance
Temperature

Fig. 1.1. Hypothetical thermal performance curve (TPC). The temperature at which performance is optimized is T_{opt}, the range of temperatures at which performance is maintained above an arbitrary threshold (usually 80% of performance at T_{opt}) represents the thermal breadth. The points at which performance is equal to zero are the critical thermal minimum (CT_{min}) and maximum (CT_{max}).

CHAPTER TWO: CONSTANT TEMPERATURE AND FLUCTUATING TEMPERATURE HAVE DISTINCT EFFECTS ON HYPOXIA TOLERANCE IN KILLIFISH (*Fundulus heteroclitus*)

2.1 Abstract

Climate change is leading to rapid change in aquatic environments, increasing the mean and variability of temperatures, and increasing the incidence of hypoxia. We investigated how acclimation to constant temperatures or to diel temperature fluctuations affect hypoxia tolerance in mummichog killifish (*Fundulus heteroclitus*). Killifish were acclimated to constant cool (15˚C), constant warm (25˚C), or a diel temperature cycle (15˚C at night, 25˚C during day) for 6 weeks. We then measured hypoxia tolerance (time to loss of equilibrium in severe hypoxia, t_{LOE}; critical O_2 tension, P_{crit}), whole-animal metabolism, gill morphology, haematology, and tissue metabolites at 15˚C and 25˚C in a full factorial design. Among constant temperature groups, t_{LOE} was highest and P_{crit} was lowest in fish tested at their acclimation temperature. Warm acclimated fish had lower metabolic rate at 25˚C and greater gill surface area (less coverage of lamellae by interlamellar cell mass, ILCM), but cool acclimated fish had greater brain glycogen stores. Therefore, effects of constant temperature acclimation on hypoxia tolerance were temperature specific and not exhibited broadly across test temperatures, and they were associated with different underlying mechanisms. Hypoxia tolerance was less sensitive to test temperature in fish acclimated to fluctuating temperatures compared to fish acclimated to constant temperature. Acclimation to fluctuating temperatures also increased haemoglobin-O_2 affinity of the blood (decreased P_{50}) compared to constant temperature groups. Therefore, acclimation to fluctuating temperatures helps maintain hypoxia tolerance across a broader range of temperatures, and leads to some distinct physiological adjustments that are not exhibited by fish acclimated to constant temperatures.

2.2 Introduction

As global climate change continues, it is becoming increasingly critical to understand how the interaction between rising temperatures and other anthropogenic stressors impact the natural world. The mean and variability of environmental temperatures are increasing in many aquatic ecosystems, which is having profound negative effects on species abundance and

distribution (Deutsch et al., 2015; Parmesan, 2006; Parmesan and Yohe, 2003; Sunday et al., 2012). Environmental hypoxia occurs naturally in some aquatic ecosystems, but it is also becoming increasingly prevalent across the globe as a result of rising temperatures and anthropogenic disturbance (Ficke et al., 2007). Thus, understanding the interactive impacts of temperature and hypoxia will be critical for predicting how aquatic organisms, such as fish, will respond to future global change. However, relatively few past studies on this issue have incorporated environmental variability akin to what exists in natural ecosystems. We have a relatively poor understanding of how ecologically relevant fluctuations in temperature contribute to variation in an organism's capacity to withstand other environmental challenges such as hypoxia.

Environmental temperature has pervasive impacts on fish physiology (Schulte, 2015). Acute increases in water temperature tend to increase metabolic rate and influence performance traits due to thermal effects on biochemical reaction rates, but excessive increases in temperature can lead to declines in performance and induce cellular damage (Schulte et al., 2011). These effects can be altered by thermal acclimation, in which prolonged exposure to elevated temperatures can lead to plastic adjustments of respiratory and metabolic traits and can improve function and performance at warmer temperatures (Lagerspetz, 2006). In general, effects of temperature may be subject to a generalist-specialist trade-off, in which maximum performance at an optimal temperature decreases with an increase in thermal breadth (Seebacher et al., 2015). Acclimation to fluctuating temperatures might be expected to induce plastic adjustments that increase thermal breadth and lead to a more generalist strategy, but this possibility has received relatively little previous attention.

Environmental temperature and thermal acclimation are known to affect hypoxia tolerance. The ability of fish to survive hypoxia is strongly influenced by the ability to maintain sufficient rates of tissue O_2 supply to meet metabolic O_2 demands (Hughes, 1973; Mandic and Regan, 2018). Increases in temperature might therefore be expected to reduce hypoxia tolerance by increasing metabolic rate and reducing aquatic oxygen solubility. Indeed, acute warming (i.e. short-term exposure to a warm test temperature) has been shown to reduce hypoxia tolerance, as reflected by decreased time to loss of equilibrium in severe hypoxia, in several species (Borowiec et al., 2016; Jung et al., 2020; McBryan et al., 2016). However, evidence in several species suggests that warm acclimation can offset such effects of warm temperature on hypoxia

tolerance. In mummichog killifish (*Fundulus heteroclitus*), for example, warm acclimation improved hypoxia tolerance compared to fish acclimated to cooler temperature when each was tested and compared at a common warm temperature, and this improvement was associated with an increase in gill surface area (McBryan et al., 2016). Similarly, in the triplefin fish (*Bellapiscis lesleyae*), warm acclimation reduced critical oxygen tension (P_{crit}) compared to cool-acclimated fish when tested at a common warm temperature (Hilton et al., 2008). These and other findings have led to suggestions that warm acclimation leads to general improvements in hypoxia tolerance (Anttila et al., 2015; Hilton et al., 2008; McBryan et al., 2016; Sollid et al., 2005a). However, relatively few studies have comprehensively examined the impacts of test temperature and acclimation temperature in a full factorial design, and it remains unclear whether the benefits of warm acclimation are restricted to warm temperatures or apply broadly across cool and warm temperatures (Collins et al., 2021). Furthermore, very little is known about how acclimation to realistic diel cycles of temperature impact hypoxia tolerance, even though diel temperature fluctuations are often more relevant to what is experienced in the wild.

The objectives of this study were (i) to compare the effects of acclimation to constant cool, constant warm, and fluctuating temperatures on hypoxia tolerance, using a full-factorial design at both cool and warm test temperatures, and (ii) to investigate the mechanisms that underlie variation in hypoxia tolerance. We used the mummichog killifish (*F. heteroclitus*), an estuarine species that routinely experiences fluctuations of many abiotic factors in their native environment, including temperature and oxygen levels, and is generally appreciated to have a broad range of environmental tolerance (Borowiec et al., 2015; Burnett et al., 2007). On a temporal scale, killifish experience significant seasonal and daily fluctuations in temperature, which, at its extreme, can result in a 10˚C change over a matter of hours (Schulte, 2007). Killifish can also experience periods of significant hypoxia in their estuarine habitat (Tyler et al., 2009). We tested the general hypothesis that thermal history alters hypoxia tolerance through thermal plasticity of various respiratory and metabolic traits. We predicted that acclimation to a constant temperature would lead to greatest hypoxia tolerance at that acclimation temperature, but poor hypoxia tolerance at a different test temperature. We also predicted that acclimation to fluctuating temperatures would induce a more generalist strategy, in which hypoxia tolerance is better maintained over a broader range of cool and warm temperatures when compared to fish acclimated to constant temperature.

2.3 Materials and methods

2.3.1 Study animals

Adult mummichog killifish (*Fundulus heteroclitus;* Linnaeus 1766) were wild caught by a commercial supplier (Aquatic Research Organisms, Hampton, NH, USA) and shipped to McMaster University in Hamilton, Ontario, Canada. Fish were kept in 300 l fibreglass tanks containing well-aerated and charcoal-filtered brackish water (4 ppt) at room temperature (~20˚C), and maintained on a 12 h: 12 h light:dark photoperiod until they were used in the temperature acclimations described below. Fish were fed to satiation 5 days per week with commercial pellets (AgloNorse Complete Fish Feed, 0.6-0.9 mm, Tromsø, Norway). All animal protocols were developed in accordance with guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board.

2.3.2 Temperature acclimations

Fish were acclimated for 6 weeks to each of three acclimation treatment groups (Fig. 2.1): constant water temperature of 15˚C ('cool acclimation group'), constant water temperature of 25˚C ('warm acclimation group'), or a diel temperature cycle of 15˚C during the night-time dark phase and 25˚C during the daytime light phase ('fluctuating acclimation group'). Other husbandry conditions (salinity, photoperiod, and feeding) were the same as described above. Acclimations were carried out in a multi-stressor exposure system (Aquabiotech, Coaticook, QC, Canada) that constantly monitored and adjusted temperature using water chillers and heaters based on feedback from salinity-resistant temperature probes. Transitions between daytime and night-time temperatures occurred over 4 h beginning at 0600 (10˚C increase) and 1800 (10˚C decrease) local time. We chose this pattern of temperature fluctuation because it represents a reasonable estimation of the diel patterns of temperature experienced by this species in estuaries along the east coast of North America (Schulte, 2007). Each acclimation group had 8-9 tank replicates with 9-12 fish per exposure tank. Following 6 weeks of acclimation, 3-4 fish from each tank replicate were used to measure time to loss of equilibrium in severe hypoxia, 3-4 separate fish were used for respirometry and critical O_2 tension measurements (8-9 tank replicates), and many of the remaining fish from each tank replicate were sampled for tissues (8-9 tank replicates) (see details below). These *in vivo* measurements as well as tissue sampling

were carried out at 25˚C or 15˚C in a full factorial design (see Fig. 2.1 for a graphical representation of the experimental design and treatment groups).

2.3.3 Hypoxia tolerance and respirometry

Time to loss of equilibrium in severe hypoxia (t_{LOE}) was measured as a metric of hypoxia tolerance. Fish were first fasted for 48 h, then transferred before 1800 local time to a 40 L glass aquarium containing well-aerated water at 4 ppt. Fish were placed in a single aquarium in batches of 3-4 fish but each individual was held in a separate plastic chamber, each of which had mesh sides to allow for water flow from the surrounding aquarium and to also prevent aquatic surface respiration. Bubble wrap was placed on the water surface to prevent O_2 diffusion from the air. Fish were first maintained at the overnight temperature appropriate for their acclimation group. The following morning at 0600, water temperature was set to the desired test temperature (either 15˚C or 25˚C). At 1100, after ~1 h of exposure to the test temperature, the O_2 pressure (PO_2) in the aquarium was rapidly reduced to 0.35 kPa over the course of ~30 min. Fish were continuously monitored using a camera positioned underneath the glass tank while minimizing visual disturbance by covering sides with dark plastic. Time to loss of equilibrium was determined as the duration of time at 0.35 kPa until fish were unable to maintain an upright position and were unresponsive to stimuli.

Resting metabolic rate (RMR) and critical O_2 tension (P_{crit}) were measured using stop-flow respirometry. Fish were first fasted for 48 h and then transferred before 1800 local time to individual 90 mL cylindrical acrylic chambers held within a darkened buffer tank containing well-aerated water (4 ppt) at the appropriate overnight temperature for their acclimation group. Each of these respirometry chambers were connected to a flush pump, which pumped water from the buffer tank through the chamber, making a ‘flushing circuit’. A second ‘measurement circuit’ used a circulating pump to continuously pump water from the chamber across a fibre-optic O_2 sensor (FireSting FSO2-4, PyroScience GmbH) in a closed loop. Both the flushing and recirculating circuits were active during the overnight adjustment period in the respirometry chambers. The following morning at 0600, water temperature was set to the desired test temperature (either 15˚C or 25˚C). At 1100, after ~1 h of exposure to the test temperature, sequential activation and deactivation of the flush pumps allowed for repeated measurements of O_2 consumption rate (MO_2). During 5 min measurement periods, the flush pumps were

deactivated, and the circulating pump continued to pass water across the O_2 sensor to measure the decline in PO_2 over time. Measurement periods were interspersed with 5 min flush periods, during which both pumps were activated to allow oxygenated water from the buffer tank to enter the chamber. RMR was determined as the lowest MO_2 measured during measurement periods between 1100-1200 in normoxia. We then measured MO_2 throughout a progressive stepwise hypoxia protocol, where the PO_2 of the buffer tank was reduced from ~20 kPa to 2 kPa in 2 kPa steps (10 min per step) as previously described (Borowiec et al., 2020). P_{crit} was determined by plotting MO_2 versus PO_2, fitting a linear regression at low PO_2 for MO_2 datapoints that fell below the RMR value for that individual, and then calculating P_{crit} as the PO_2 at which this regression equalled RMR. This method conforms to recent calls for a standardized curve-fitting approach for determining P_{crit} (Reemeyer and Rees, 2019).

2.3.4 Sampling of fish tissues

In a subset of fish from each acclimation group, water temperature was set to the desired test temperature (either 15˚C or 25˚C) at 0600. At 1100, after ~1 h of exposure to the test temperature, fish were sampled under resting conditions. Fish were euthanized with a sharp blow to the head followed by spinal transection, and the tail was severed for blood collection. Blood was collected in a heparinized syringe. A portion of blood (10 µl) was added to Drabkin's reagent for determining hemoglobin concentration according product instructions (Sigma-Aldrich, St. Louis, MO, USA). Oxygen dissociation curves were generated at either 15˚C or 25˚C using a Hemox Analyzer (TCS Scientific, New Hope, PA, USA) using 10 µl of whole blood in 5 ml of buffer (100 mmol l^{-1} Hepes, 50 mmol l^{-1} EDTA, 100 mmol l^{-1} NaCl, 0.1% bovine serum albumin and 0.2% antifoaming agent). Buffer pH was set to 8.0 when running samples at 15˚C and 7.8 when running samples at 25˚C to account for the expected variation in blood pH with temperature (Cameron, 1978). The remaining blood was collected in a heparinized capillary tube and centrifuged at 12,700 g for 5 min to determine hematocrit. Brain, liver, and axial white muscles were dissected and immediately freeze-clamped in liquid nitrogen (within 1 min of euthanasia), then stored at -80˚C for later analysis of metabolites. Gills were dissected, fixed in 10% formalin for 24 h, then stored in 0.2 mol l^{-1} PBS (274 mmol l^{-1} NaCl, 30.4 mmol l^{-1} Na_2HPO_4, 5.4 mmol l^{-1} KH_2PO_4; pH 7.8) at 4˚C for later analysis of gill morphology.

2.3.5 Gill histology

After fixation, the first gill arch was immersed in embedding medium (Shandon Cryomatrix, Fisher Scientific, Runcorn, Cheshire, UK), rapidly frozen in liquid N_2-cooled isopentane, and stored at -80˚C for at least 24 h before sectioning. Frozen blocks were sectioned at 10 μm thickness using a cryostat maintained at -20˚C (Leica CM 1860). Sections were mounted on glass microscope slides, dried overnight at room temperature, then stored at -80˚C until staining. Sections were stained by incubating in Gills II haematoxylin for 10 min and then in eosin for 2 min, rinsing in distilled water between each step. After staining, sections were dehydrated through progressively increasing concentrations of ethanol (50%, 70%, 95%) for 30 s each before being placed in 100% ethanol for 1 min. Sections were then placed in xylene for at least 1 min before being mounted in Permount Mounting Medium (Fisher Scientific). For each individual, brightfield images were taken of sections throughout the whole gill arch tissue using a Nikon Eclipse E800 microscope (Nikon Instruments, Melville, NY, USA). ImageJ (v.1.53q) software (Rasband, 2008) was used to measure heights of lamellae and the interlamellar cell mass (ILCM).

2.3.6 Tissue metabolite assays

Frozen samples of brain, liver and muscle were ground into a fine powder using an insulated mortar and pestle that was pre-cooled with liquid nitrogen, and the powder was stored at -80˚C until homogenization. Powdered tissue (20-30 mg) was homogenized for 20 s in 10 μl per mg tissue of ice-cold 6% $HClO_4$, using the highest setting of a PowerGen 125 electric homogenizer (Fisher Scientific, Whitby, ON, Canada). The homogenate was vortexed, and a 100 μl aliquot was frozen in liquid nitrogen and stored at -80˚C for future analysis of glucose and glycogen. The remaining, unfrozen aliquot was centrifuged at 4˚C for 10 min at 10,000 g, and 200 μl of the supernatant was transferred to a new microcentrifuge tube, neutralized using 3 M K_2CO_3 to $6.8 \leq pH \leq 7.2$ and centrifuged again under the same conditions. The resulting supernatant was used for quantification of lactate under the following initial assay condition: 2.5 mmol l^{-1} NAD^+ in glycine buffer (0.6 mol l^{-1} glycine, 0.5 mol l^{-1} hydrazine sulphate, pH 9.4). An initial absorbance measurement was made at 340 nm before adding lactate dehydrogenase (LDH) in excess (5 U ml^{-1}). After a 30-min incubation period, a second absorbance measurement was

made. The increase in NADH concentration in the well was calculated using the molar extinction coefficient for NADH (6.22 $mmol^{-1}$ cm^{-1}). Given that the assay couples lactate to NADH via the LDH reaction, the increase in NADH concentration equals the original molar concentration of lactate in the sample. The assay was run in triplicate at 37˚C on a Synergy H1 hybrid multimode microplate reader (Biotek Instruments, VT, USA).

To measure glucose and glycogen, the frozen aliquot of acidified homogenate was thawed on ice, and then 100 µl of 400 mmol l^{-1} Na acetate buffer (pH 4.8) and 50 µl of 1 mol l^{-1} K_2CO_3 were added. For liver, samples were also centrifuged (1000 g, 4˚C, 5 min) and any debris present on top of the supernatant was removed before the following step. For digestion, 100 µl of each sample was digested with 7 µl of amyloglycosidase (suspended at 4 U l^{-1} in 300 mmol l^{-1} Tris-HCl, 4.05 mmol l^{-1} $MgSO_4$; pH 7.5) for 2 h in a 40˚C water bath (vortexing every 15 min), while another 100 µl aliquot from each sample was kept undigested on ice for 2 h. Both digested and undigested fractions were neutralized to $6.8 \leq pH \leq 7.2$ with 1 mol l^{-1} K_2CO_3, centrifuged at 4˚C for 10 min at 10,000 g, and then assayed. Initial assay conditions were as follows: 1 mmol l^{-1} ATP, 0.5 mmol l^{-1} $NADP^+$, 5 mmol l^{-1} $MgCl_2$, 20 mmol l^{-1} imidazole (pH 7.4). All samples were first depleted of glucose-6-phosphate by adding excess glucose-6-phosphate dehydrogenase (5 U ml^{-1}) and incubating for 30 min, after which an initial NADPH absorbance reading was taken at 340 nm. Glucose content was then determined in both digested samples (containing endogenous free glucose and glucose produced via the breakdown of glycogen) and undigested samples (containing only endogenous free glucose) by measuring the increase in NADPH absorbance elicited 50 min after the addition of the coupling enzyme hexokinase in excess (5 U ml^{-1}). The difference between the glucose contents measured in the digested and undigested samples was used to calculate total glycogen content of the tissue.

2.3.7 Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 9.4.1, San Diego, California, USA). We tested for main and interactive effects of acclimation group and test temperature using two-way AVOVA. Data were log or square root transformed, if necessary, to meet the assumptions of the statistical tests. Using the Bonferroni correction to adjust for multiple comparisons, we looked for significant differences between acclimation groups within each test temperature as well as between test temperatures within each acclimation group. A

significance level of $P<0.05$ was used for all statistical analyses. All data are reported as means ± SEM.

2.4 Results

2.4.1 Hypoxia tolerance

There were strong effects of thermal acclimation and test temperature on time to loss of equilibrium (t_{LOE}), a key metric of hypoxia tolerance (Fig. 2.2). This was reflected by significant main effects of acclimation group ($P=0.0207$) and test temperature ($P<0.0001$) as well as a significant interaction ($P<0.0001$) (Table 1). Hypoxia tolerance in the cool acclimation group collapsed when tested at warm temperature, demonstrated by an 11.6-fold decrease in t_{LOE} at 25˚C relative to 15˚C. The warm acclimation group had greater hypoxia tolerance at 25˚C but much worse hypoxia tolerance at 15˚C than the cool acclimation group. The fluctuating acclimation group displayed hypoxia tolerance that was similar to the cool acclimation group when tested at 15˚C, and intermediate between constant acclimation groups when tested at 25˚C. Therefore, acclimation to a constant temperature improved hypoxia tolerance at that temperature, whereas acclimation to diel cycles of temperature may have helped maintain hypoxia tolerance across a broader range of test temperatures.

2.4.2 Metabolic rate and critical oxygen tension

Thermal acclimation also had significant effects on resting metabolic rate (RMR), measured as the resting rate of O_2 consumption (Fig. 2.3A; Table 1). RMR was 2.1- to 2.9-fold higher when fish were tested at 25˚C relative to when they were tested at 15˚C, which drove a significant effect of test temperature ($P<0.0001$). However, there was also a significant interaction between acclimation group and test temperature ($P=0.007$) and a significant main effect of acclimation group ($P<0.0001$), due to significantly greater RMR in the cool group than in both warm and fluctuating groups at 25˚C but not at 15˚C. This suggested that the increase in RMR following acute exposure to elevated temperature, as illustrated in the cool acclimation group, was attenuated over time with acclimation to constant warm or fluctuating temperature regimes.

We also exposed fish to progressive stepwise hypoxia to examine how O_2 consumption rate (MO_2) varied across a range of PO_2 (Fig. S2.1). Consistent with the effects of test

temperature on RMR (Fig. 2.1), MO_2 was higher on average across a range of high PO_2 in fish tested at 25˚C relative to 15˚C (Fig. S2.1). MO_2 declined in more severe levels of hypoxia (Fig. S2.1), as expected, and the data for MO_2 as a function of PO_2 for each individual were used to calculate the critical O_2 tension (P_{crit}). There was a significant interaction (P<0.0001) between acclimation group and test temperature for P_{crit} (Fig. 2.3B; Table 1). When tested at 15˚C, cool acclimated fish demonstrated significantly lower P_{crit} compared to the warm acclimation group. At 25˚C, warm acclimated fish had the lowest P_{crit}, which was significantly lower than the cool acclimation group, and the fluctuating group were intermediate between the constant acclimation groups. Therefore, the pattern of variation in P_{crit} in the constant acclimation groups was akin to the pattern of variation for t_{LOE}, with both warm and cool groups demonstrating significantly lower P_{crit} when tested at their acclimation temperature, whereas the fluctuating group was intermediate and exhibited less variation in P_{crit} across test temperatures.

2.4.3 Gill morphology

Thermal acclimation had a significant impact on gill morphology (Fig. 2.4; Table 2.1), reflected by a main effect of acclimation group (P=0.002) but not test temperature (P=0.508) on the proportion of the lamellae that was covered by interlamellar cell mass (ILCM). This effect appeared to be driven by a reduction in ILCM coverage in the warm group compared to the cool group when fish were sampled at either 15˚C or 25˚C, but the pairwise differences between groups did not reach significance. The fluctuating group displayed ILCM coverage that was not significantly different from either the cool or warm acclimation groups, and was intermediate on average between constant acclimation groups when sampled at 25˚C.

2.4.4 Hematology

Thermal acclimation affected haemoglobin-O_2 affinity, measured in intact erythrocytes as the PO_2 at 50% saturation (P_{50}) (Fig. 2.5A; Table 2.1). This was reflected by a significant effect of acclimation group on P_{50} (P=0.027), driven by lower overall values of P_{50} in the fluctuating group compared to the constant groups. There was also a significant main effect of test temperature (P<0.0001) on P_{50}, such that P_{50} was generally lower at 15˚C than at 25˚C, and the pairwise difference between temperatures was significant in the warm acclimation group. This

expected decrease in Hb-O_2 affinity with test temperature is a direct effect of the overall exothermic nature of Hb oxygenation (Weber and Jensen, 1988).

Thermal acclimation had some other haematological effects (Fig. 2.5; Table 2.1). There were significant main effects of acclimation group (P=0.0002 and P=0.049) and test temperature (P=0.0009 and P<0.0001) on both haematocrit (Fig. 2.5B) and blood hemoglobin content (Fig. 2.5C), respectively. Haematocrit and blood hemoglobin content were higher at 25˚C compared to 15˚C in the fluctuating group, reflecting some capacity to rapidly modulate these haematological traits in response to acute changes in temperature. Although the overall pattern of variation appeared to differ slightly between these traits, there was no significant variation in mean corpuscular hemoglobin concentration (MCHC; calculated as the quotient of blood hemoglobin content and hematocrit) across acclimation groups and test temperatures (Fig. 2.5D).

2.4.5 Tissue metabolites

Thermal acclimation affected glycogen stores in the brain (acclimation effect, P<0.0001), with highest values in the cool acclimation group (Fig. 2.6A). However, thermal acclimation had no effect on glycogen stores in the white muscle (Fig. 2.6B) or liver (Fig. 2.6C), and glycogen stores were largely unaffected by test temperature (Table 2.1). There was also significant variation in some other tissue metabolite measurements (Table 2.2). Brain lactate was elevated in the cool acclimation group (main effect of acclimation group, P<0.0001) but also tended to be higher overall at test temperatures of 25˚C compared to 15˚C (main effect of test temperature, P<0.0001). There was also a significant acclimation×test temperature interaction on free glucose in the brain (P=0.024), largely driven by elevated values in the cool acclimation group acutely transferred to 25˚C. In the muscle, lactate was highest in fish acclimated to fluctuating temperatures (main effect of acclimation group, P=0.002), and there was variation in free glucose across acclimation groups that differed between test temperatures (acclimation×test temperature, P=0.017). In the liver, acclimation group had a significant effect on lactate levels (P=0.046), which appeared to be driven by higher values in the warm acclimation group, but the pairwise differences between groups were not significant.

2.5 Discussion

Climate change is leading to rapid change in aquatic environments, increasing the mean and variability of aquatic temperatures, and increasing the incidence of hypoxia. This study examines how thermal history affects the ability of fish to cope with hypoxia through plastic changes in key physiological traits associated with balancing O_2 supply and demand. Thermal history had a strong influence on how hypoxia tolerance changed with acute changes in temperature. Fish acclimated to constant temperatures had greatest t_{LOE} and lowest P_{crit} at their acclimation temperature, but lowest t_{LOE} and highest P_{crit} following acute temperature change (Figs. 2.2, 2.3), suggesting thermal specialization to the acclimation temperature. Effects of acute temperature change on t_{LOE} and P_{crit} were somewhat reduced in fish acclimated to fluctuating conditions, suggesting that they may acquire a more generalist thermal strategy. Variation in these metrics of hypoxia tolerance were associated with variation in physiological and morphological traits associated with O_2 uptake, circulatory O_2 transport, and anaerobic metabolism (Figs. 2.4-2.6). Taken together, these data contribute to the growing appreciation that thermal history and thermal environment can have strong effects on hypoxia tolerance in fish.

2.5.1 Acclimation to constant temperatures leads to thermal specialization and changes in O_2 uptake

Killifish acclimated to constant temperatures appeared to be thermal specialists with respect to hypoxia tolerance, based on our finding that highest values of t_{LOE} and lowest values of P_{crit} were observed in fish tested at their acclimation temperature (Figs. 2.2, 2.3). Therefore, in contrast to some previous suggestions (Anttila et al., 2015; Hilton et al., 2008; McBryan et al., 2016; Sollid et al., 2005a), warm acclimation does not lead to general improvements in hypoxia tolerance across temperatures, at least in killifish. If this were the case, warm acclimated fish would have exhibited highest t_{LOE} and lowest P_{crit} at both test temperatures. However, many previous studies did not use a full factorial design to test cool and warm acclimated fish at cool temperatures (Anttila et al., 2015; Hilton et al., 2008; McBryan et al., 2016), precluding the ability to assess whether effects of warm acclimation on hypoxia tolerance are temperature specific.

Improvements in hypoxia tolerance at 25˚C were associated with physiological adjustments in warm acclimated fish that may reduce discrepancies between O_2 supply and O_2

demand in hypoxia. Firstly, warm acclimation led to an apparent regression of the interlamellar cell mass (ILCM) compared to cool acclimated killifish (Fig. 2.4), as previously observed (McBryan et al., 2016). Regression of the ILCM has often been observed with acclimation to warm temperatures (Mitrovic and Perry, 2009; Sollid and Nilsson, 2006; Sollid et al., 2005b), which should increase the functional surface area of the gills and thus increase the capacity for branchial O_2 uptake (Evans et al., 2005; Nilsson and Östlund-Nilsson, 2008). Warm acclimation also reduced resting metabolic rate at 25˚C (Fig. 2.3) (McBryan et al., 2016), which may help reduce O_2 demands in hypoxia. Some other important determinants of O_2 transport were similar between constant acclimation groups, such as the O_2 affinity and content of haemoglobin in the blood (Fig. 2.5). The lack of any effect of thermal acclimation on Hb-O_2 affinity contrasts previous findings, in which killifish acclimated to 33˚C had lower P_{50} than killifish acclimated to 15˚C across each test temperature (Chung et al., 2017). This distinction could suggest that Hb-O_2 affinity exhibits relatively little plasticity with acclimation between 15-25˚C but increases above this range. Nevertheless, improvements in O_2 supply from an increased gill surface area coupled with reduced O_2 demands from a lower resting metabolic rate are likely important for reducing P_{crit} and prolonging t_{LOE} at 25˚C in warm acclimated killifish.

Improvements in hypoxia tolerance in cool acclimated fish at 15˚C cannot be explained by similar mechanisms to those improving hypoxia tolerance in warm acclimated fish at 25˚C. Indeed, cool acclimated fish had greater ILCM coverage, similar O_2 affinity and content of haemoglobin in the blood, and similar metabolic rate at 15˚C as compared to warm acclimated fish. It is possible that other determinants of O_2 transport are enhanced in cool acclimated fish at 15˚C. For example, acute exposure to reduced temperatures can reduce maximal heart rates, but this effect can be partially overcome by acclimation to reduced temperatures (Driedzic and Gesser, 1994; Gilbert and Farrell, 2021; Graham and Farrell, 1989). If acute exposure to cool temperature limits heart rate and cardiac output in hypoxia, prolonged acclimation to cool temperature could offset this limitation and lead to more effective perfusion of the gills. It is also possible that thermal history alters the strategy killifish use to cope with hypoxia, akin to the shifts in such strategies that occur with acclimation to different patterns of hypoxia exposure (Borowiec et al., 2018). For example, cool acclimation could increase reliance on anaerobic metabolism for coping with periods of O_2 deprivation. In this regard, our observation that cool acclimated fish had higher levels of glycogen in the brain could suggest that they possess greater

fuel reserves to sustain anaerobic glycolysis in this tissue (Fig. 2.6A). The brain is an energetically demanding organ and is highly susceptible to decreases in O_2 (Soengas and Aldegunde, 2002), and many studies have linked glycogen stores, enzyme activities, and the maintenance of ATP levels in the brain to hypoxia sensitivity (Dunn and Hochachka, 1986; Mandic et al., 2013; Saez et al., 2014; Speers-Roesch et al., 2013). In contrast, we found that acclimation temperature had no effects on glycogen levels in the liver or muscle (Fig. 2.6B, C), consistent with previous studies showing no correlation between these traits and hypoxia tolerance across species (Mandic et al., 2013).

2.5.2 Acclimation to diel cycles of temperature reduces thermal sensitivity of hypoxia tolerance

Acclimation to diel cycles of temperature appeared to shift killifish towards becoming thermal generalists with respect to hypoxia tolerance. This was supported by our findings that t_{LOE} in the fluctuating group was intermediate or nearly as high as constant groups tested at their acclimation temperature (Fig. 2.2), and that P_{crit} was not affected by test temperature in this group (Fig. 2.3). In many respects, fish in the fluctuating group had similar physiological characteristics as warm acclimated fish, with similar resting metabolic rates, ILCM coverage, and glycogen contents. However, in contrast to the warm acclimation group, fish acclimated to fluctuating temperatures demonstrated rapid and pronounced changes in blood hemoglobin content and haematocrit in response to diel temperature change (Fig 2.5B, C). Rapid hematological responses to warm temperature have been observed previously (Kapila et al., 2002; Lowe and Davison, 2005; Muñoz et al., 2018), and may help improve O_2 carrying capacity and blood O_2 transport in hypoxia as long as cardiac output is not overly constrained by increases in blood viscosity (Weber and Jensen, 1988). These increases in blood hemoglobin content in the fluctuating group at warm temperature are too rapid to be caused by erythropoiesis, and are likely attributable to splenic contraction, which is known to enable rapid modulation of blood hemoglobin content in killifish and many other species (Borowiec and Scott, 2021; Fänge and Nilsson, 1985; Lai et al., 2006; Yamamoto, 1987; Yamamoto et al., 1985). Increases in Hb-O_2 affinity may also help improve hypoxia tolerance across temperatures, based on the significant effect of acclimation group on P_{50} that was driven by lower P_{50} values in the fluctuating group (Fig. 2.5A). The latter finding suggests that acclimation to diel cycles of temperature fluctuation leads to some mechanisms of thermal plasticity that are distinct from fish acclimated to constant

temperatures, and may contribute to improving hypoxia tolerance across a broad range of temperatures.

2.5.3 Conclusions

Our findings suggest that the interaction between thermal history and thermal environment has significant effects on the ability of fish to cope with hypoxia. Fish experiencing different thermal histories may utilize different physiological mechanisms to cope with hypoxia and exhibit different temperature sensitivities of hypoxia tolerance. Acclimation to constant temperatures appears to result in thermal specialization, as demonstrated by enhanced hypoxia tolerance at the acclimation temperature but lower tolerance following acute temperature change. Contrastingly, fish acclimated to fluctuating temperatures demonstrate a strategy more consistent with a thermal generalist. Our results thus demonstrate that thermal fluctuations can elicit physiological adjustments that are challenging to predict from those exhibited by fish in constant thermal environments. Appreciating the impacts of thermal fluctuations and how they interact with other stressors associated with climate change will be vital for predicting how aquatic organisms will cope with environmental change in the future.

2.6 Tables and Figures

Table 2.1. Statistical results of two-way ANOVA

	Main effect of acclimation		Main effect of test temperature		Interaction	
Trait	*F*	*P*	*F*	*P*	*F*	*P*
t_{LOE}	$F_{2, 76} = 4.083$	0.0207	$F_{1, 76} = 146.0$	<0.0001	$F_{2, 76} = 12.85$	<0.0001
RMR	$F_{2, 60} = 14.88$	<0.0001	$F_{1, 60} = 167.3$	<0.0001	$F_{2, 60} = 5.356$	0.0072
P_{crit}	$F_{2, 57} = 0.8244$	0.4436	$F_{1, 57} = 0.0591$	0.8088	$F_{2, 57} = 15.15$	<0.0001
ILCM	$F_{2, 48} = 7.418$	0.0016	$F_{1, 48} = 0.4439$	0.5084	$F_{2, 48} = 0.5441$	0.5839
P_{50}	$F_{2, 71} = 3.803$	0.0270	$F_{1, 71} = 21.75$	<0.0001	$F_{2, 71} = 0.1416$	0.8682
Hct	$F_{2, 67} = 9.848$	0.0002	$F_{1, 67} = 12.01$	0.0009	$F_{2, 67} = 1.955$	0.1496
[Hb]	$F_{2, 71} = 3.148$	0.0490	$F_{1, 71} = 21.36$	<0.0001	$F_{2, 71} = 0.9527$	0.3906
MCHC	$F_{2, 66} = 1.455$	0.2407	$F_{1, 66} = 5.770$	0.0191	$F_{2, 66} = 0.2663$	0.7670
Glycogen						
Brain	$F_{2, 63} = 11.44$	<0.0001	$F_{1, 63} = 0.0731$	0.7877	$F_{2, 63} = 0.1172$	0.8896
Liver	$F_{2, 44} = 0.1504$	0.8608	$F_{1, 44} = 0.6075$	0.4399	$F_{2, 44} = 0.09126$	0.9130
Muscle	$F_{2, 62} = 1.746$	0.1829	$F_{1, 62} = 1.416$	0.2386	$F_{2, 62} = 0.4724$	0.6257
Glucose						
Brain	$F_{2, 62} = 1.198$	0.3087	$F_{1, 62} = 3.211$	0.0780	$F_{2, 62} = 3.949$	0.0243
Liver	$F_{2, 44} = 2.339$	0.1083	$F_{1, 44} = 2.217$	0.1436	$F_{2, 44} = 1.066$	0.3530
Muscle	$F_{2, 62} = 5.118$	0.0088	$F_{1, 62} = 2.317$	0.1330	$F_{2, 62} = 4.345$	0.0171
Lactate						
Brain	$F_{2, 64} = 15.34$	<0.0001	$F_{1, 64} = 19.39$	<0.0001	$F_{2, 64} = 0.6402$	0.5305
Liver	$F_{2, 64} = 0.6296$	0.5361	$F_{1, 64} = 0.4290$	0.5148	$F_{2, 64} = 0.2917$	0.7480
Muscle	$F_{2, 60} = 7.258$	0.0015	$F_{1, 60} = 2.103$	0.1522	$F_{2, 60} = 1.913$	0.1565

Hct, hematocrit. [Hb], hemoglobin concentration.

Table 2.2. Effect of acclimation temperature on metabolites in killifish

Metabolite	Test temperature	Acclimation group		
		Constant cool	Constant warm	Fluctuating
Brain				
Lactate	15˚C	16.04±0.92 (12)^{a}	10.53±0.98 (11)^{b}	11.47±0.72 (11)^{b}
	25˚C	18.09±1.23 (12)^{a}	14.37±0.55 (12)^{b*}	15.18±0.78 (12)^{ab*}
Glucose	15˚C	1.82±0.14 (11)	2.14±0.27 (11)	2.85±0.54 (11)
	25˚C	3.19±0.34 (12)*	2.09±0.43 (12)	2.45±0.26 (12)
Liver				
Lactate	15˚C	3.26±0.37 (10)	4.39±0.33 (8)	3.93±0.48 (9)
	25˚C	3.30±0.57 (7)	4.01±0.40 (10)	2.43±0.19 (6)
Glucose	15˚C	3.70±0.37 (10)	3.09±0.62 (8)	4.57±1.05 (9)
	25˚C	6.56±1.25 (7)	3.90±0.66 (10)	3.61±0.43 (6)
Muscle				
Lactate	15˚C	17.27±1.31 (11)^{ab}	12.23±1.07 (11)^{a}	22.88±2.85 (12)^{b}
	25˚C	19.03±3.82 (9)	18.07±1.50 (11)	23.98±2.85 (12)
Glucose	15˚C	3.26±0.34 (11)^{a}	3.62±0.28 (12)^{ab}	5.45±0.85 (12)^{b}
	25˚C	5.55±0.68 (10)*	3.44±0.29 (11)	5.00±0.58 (12)

Metabolite contents are reported as means ± SEM, with the sample size in brackets, and are expressed in units of μmol per g tissue. Dissimilar letters indicate a significant difference in pairwise comparisons between acclimation groups when compared within each test temperature, and asterisks indicate significant pairwise differences between test temperatures within acclimation groups.

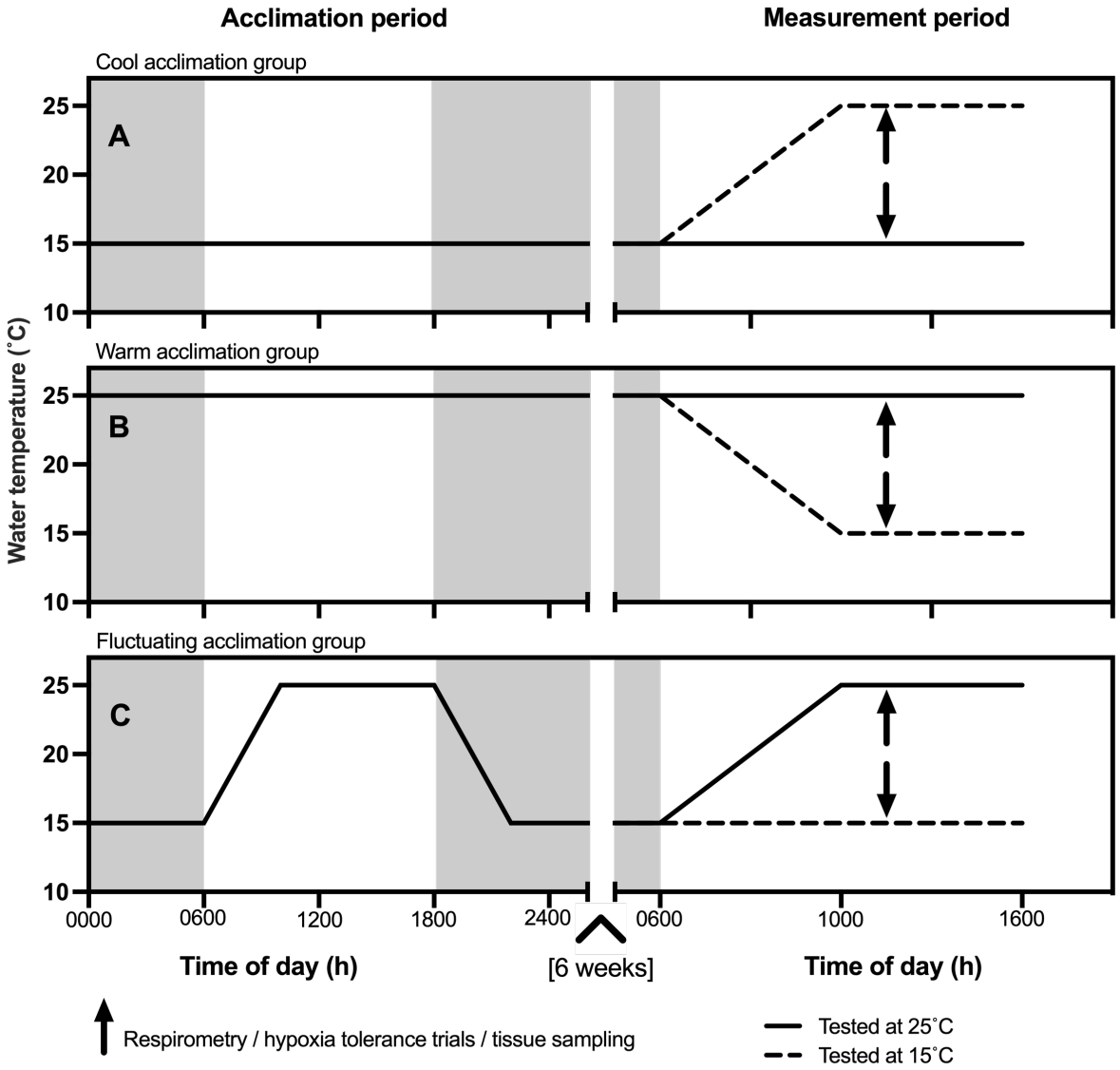
Acclimation period
Measurement period
Cool acclimation group
A
Warm acclimation group
B
Fluctuating acclimation group
C
Water temperature (˚C)
25
20
15
10
0000
0600
1200
1800
2400
0600
1000
1600
Time of day (h)
[6 weeks]
Time of day (h)
Respirometry / hypoxia tolerance trials / tissue sampling
Tested at 25˚C
Tested at 15˚C

Fig. 2.1. Experimental design. Killifish were first acclimated for 6 weeks to constant cool (15˚C), constant warm (25˚C), or diel cycles of temperature (reaching 25˚C during the daytime light phase and 15˚C during the night-time dark phase; the latter shown by grey shading). These acclimation conditions are shown to the left of the break in the x-axis. Following 6 weeks of acclimation, *in vivo* whole-animal measurements and *in vitro* tissue measurements were made in a full-factorial design at both 15˚C and 25˚C, starting at the times marked by arrows. This was achieved by holding fish overnight at the temperature appropriate for their acclimation group, and then transitioning them over the course of 4 hours to the desired test temperature (either the acclimation temperature, solid lines, or the alternative temperature, dashed lines) before experimental measurements or tissue sampling. See Material Methods for additional details.

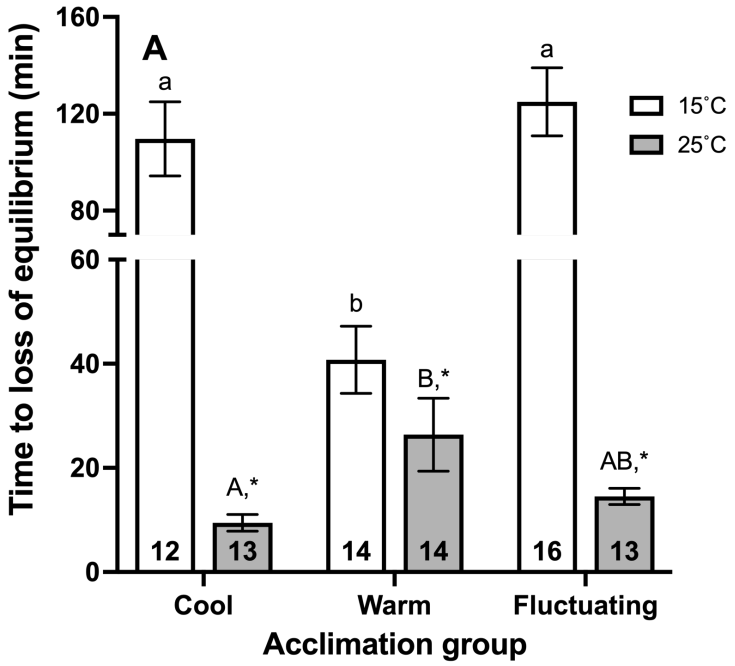
A
a
a
b
B,*
A,*
AB,*
15˚C
25˚C
Time to loss of equilibrium (min)
160
120
80
60
40
20
0
12
13
14
14
16
13
Cool
Warm
Fluctuating
Acclimation group

Fig. 2.2. Acclimation group and test temperature affected time to loss of equilibrium (t_{LOE}) in severe hypoxia. Different test temperatures are distinguished by white (15˚C) and grey (25˚C) bars. Dissimilar letters indicate a significant difference in pairwise comparisons between acclimation groups when compared within each test temperature, and asterisks indicate significant pairwise differences between test temperatures within acclimation groups. Data are presented as mean ± SEM, with samples sizes indicated within each bar.

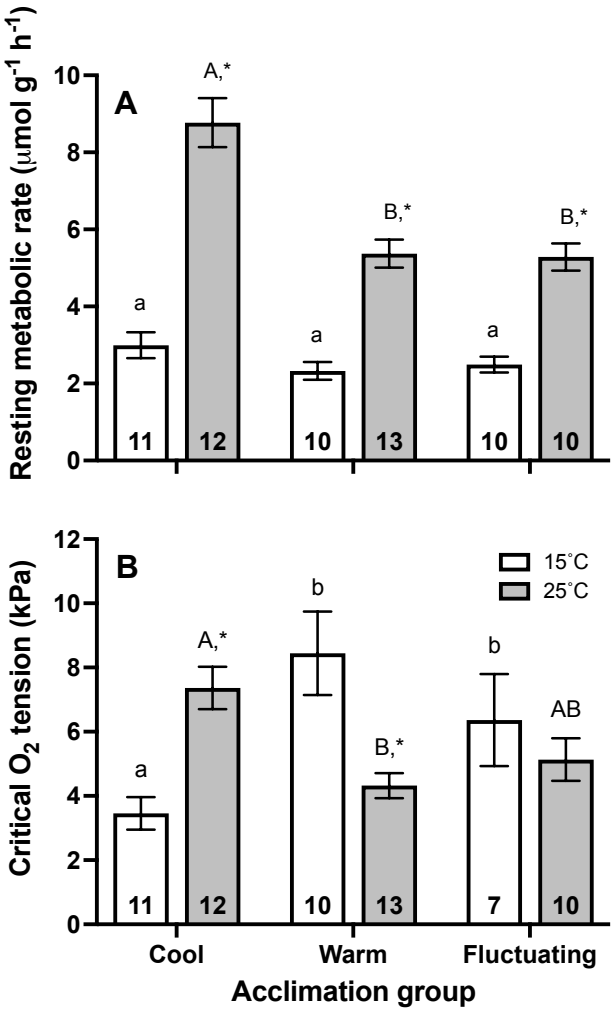
A
Resting metabolic rate (μmol g-1 h-1)
10
8
6
4
2
0
A,*
B,*
B,*
a
a
a
11
12
10
13
10
10
B
Critical O2 tension (kPa)
12
10
8
6
4
2
0
15˚C
25˚C
b
A,*
b
B,*
AB
a
11
12
10
13
7
10
Cool
Warm
Fluctuating
Acclimation group

Fig. 2.3. Resting metabolic rate (RMR) and critical O_2 tension (P_{crit}) varied across acclimation groups and test temperatures. (A) RMR was measured as O_2 consumption rate (μmol O_2 per g body mass per h) in normoxia. (B) P_{crit} was calculated from measurements of O_2 consumption rate during exposure to stepwise progressive hypoxia (full set of measurements are shown in Fig. S1). Different test temperatures are distinguished by white (15˚C) and grey (25˚C) bars. Dissimilar letters indicate a significant difference in pairwise comparisons between acclimation groups when compared within each test temperature, and asterisks indicate significant pairwise differences between test temperatures within acclimation groups. Data are presented as mean ± SEM, with samples sizes indicated within each bar.

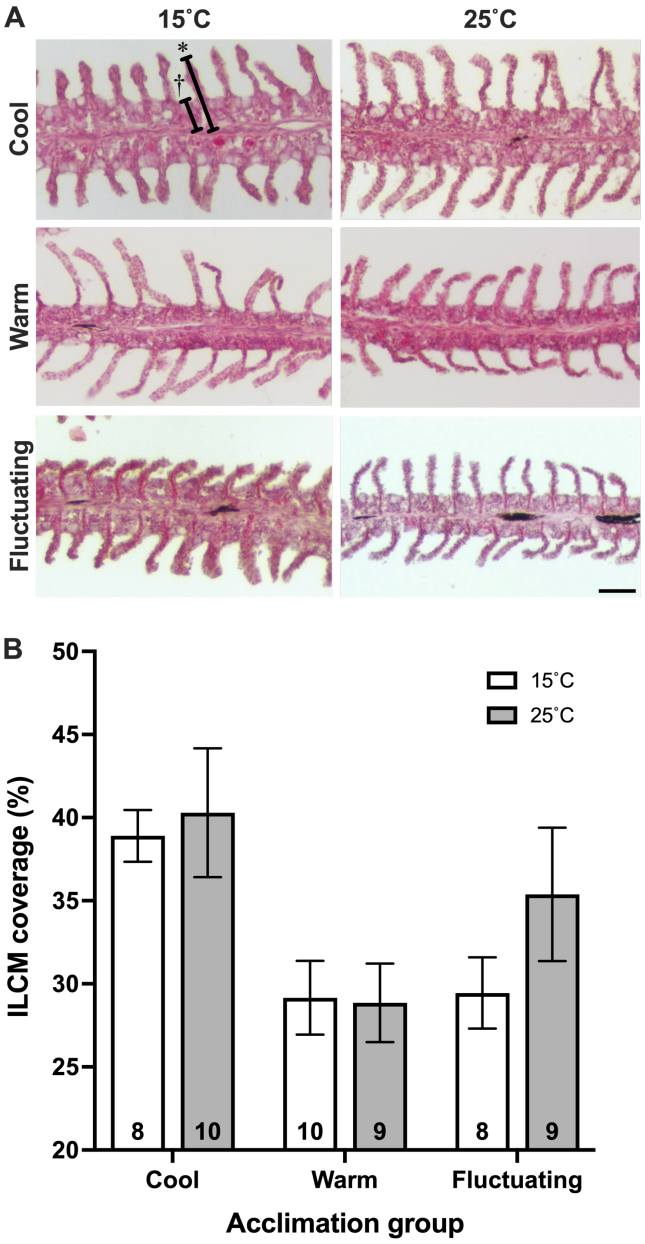
A
15˚C
25˚C
Cool
Warm
Fluctuating
*
†
B
50
45
40
35
30
25
20
ILCM coverage (%)
15˚C
25˚C
8
10
10
9
8
9
Cool
Warm
Fluctuating
Acclimation group

Fig. 2.4. Gill morphology varied between acclimation groups. (A) Representative images of gills from killifish from each acclimation group (cool, warm, or fluctuating) sampled at 15˚C or 25˚C. Slides are stained with haematoxylin and eosin. †ILCM height, *total lamellar height. Scale bar represents 50 μM and all images are shown at the same scale. (B) Interlamellar cell mass (ILCM) coverage (%) was the quotient of ILCM height and total lamellar height. Different test temperatures are distinguished by white (15˚C) and grey (25˚C) bars. There was a significant main effect of acclimation temperature on ILCM coverage (P=0.0016), but the pairwise comparisons between acclimation groups were not significant. Data are presented as mean ± SEM, with samples sizes indicated within each bar.

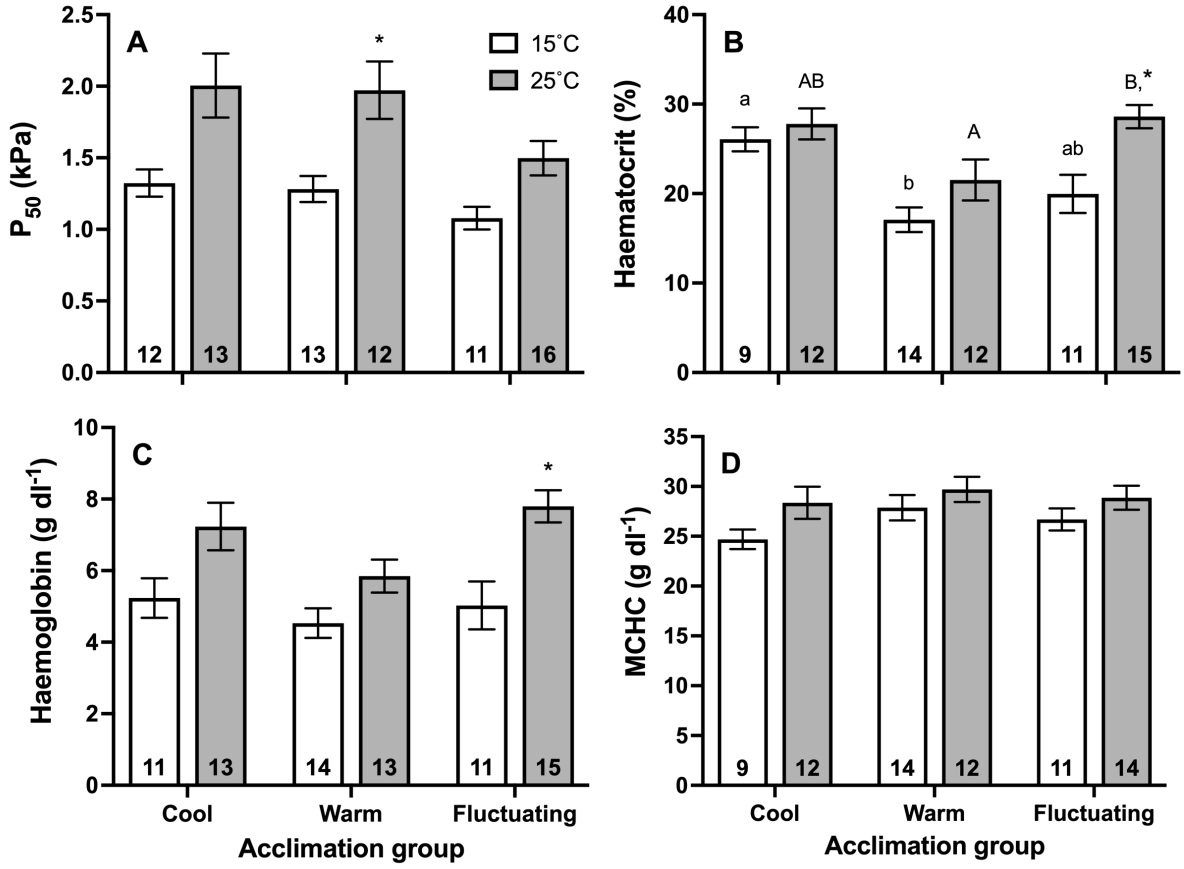
A
15˚C
25˚C
P_{50} (kPa)
2.5
2.0
1.5
1.0
0.5
0.0
*
12
13
13
12
11
16
B
Haematocrit (%)
40
30
20
10
0
a
AB
b
A
ab
B,*
9
12
14
12
11
15
C
Haemoglobin (g dl^{-1})
10
8
6
4
2
0
*
11
13
14
13
11
15
Cool
Warm
Fluctuating
Acclimation group
D
MCHC (g dl^{-1})
35
30
25
20
15
10
5
0
9
12
14
12
11
14
Cool
Warm
Fluctuating
Acclimation group

Fig. 2.5. Haemoglobin-O_2 affinity, haematocrit, and blood haemoglobin content varied across acclimation groups and test temperatures. (A) Whole cell O_2 tension at 50% haemoglobin saturation (P_{50}), (B) Haematocrit, (C) whole-blood haemoglobin content (g per dl of blood), and (D) mean corpuscular haemoglobin concentration (MCHC; g per dl of erythrocytes). Different test temperatures are distinguished by white (15˚C) and grey (25˚C) bars. Dissimilar letters indicate a significant difference in pairwise comparisons between acclimation groups when compared within each test temperature, and asterisks indicate significant pairwise differences between test temperatures within acclimation groups. Data are presented as mean ± SEM, with samples sizes indicated within each bar.

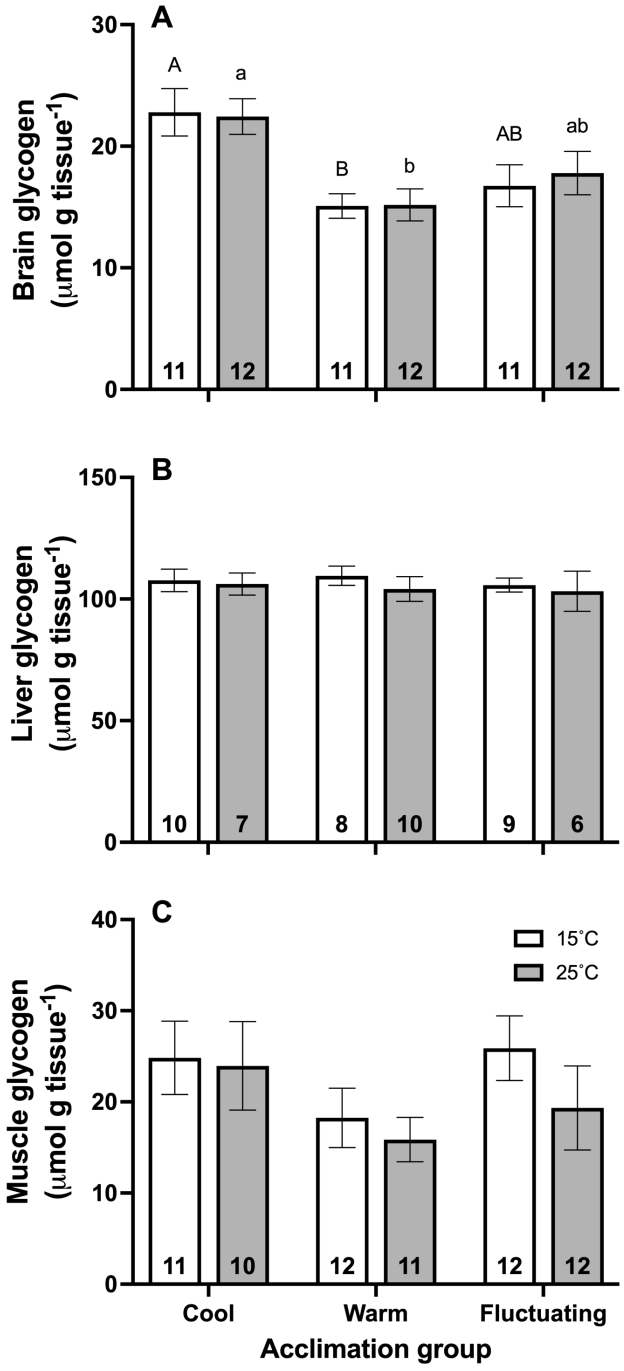
A
Brain glycogen (μmol g tissue-1)
30
20
10
0
A
a
B
b
AB
ab
11
12
11
12
11
12
B
Liver glycogen (μmol g tissue-1)
150
100
50
0
10
7
8
10
9
6
C
Muscle glycogen (μmol g tissue-1)
40
30
20
10
0
15˚C
25˚C
11
10
12
11
12
12
Cool
Warm
Fluctuating
Acclimation group

Fig. 2.6. Brain glycogen content was elevated in the cool acclimation group. Glycogen content (μmol per g tissue) in the (A) brain, (B) liver, and (C) white axial muscle. Different test temperatures are distinguished by white (15˚C) and grey (25˚C) bars. Dissimilar letters indicate a significant difference in pairwise comparisons between acclimation groups when compared within each test temperature. Data are presented as mean ± SEM, with samples sizes indicated within each bar.

2.7 Supplementary Materials

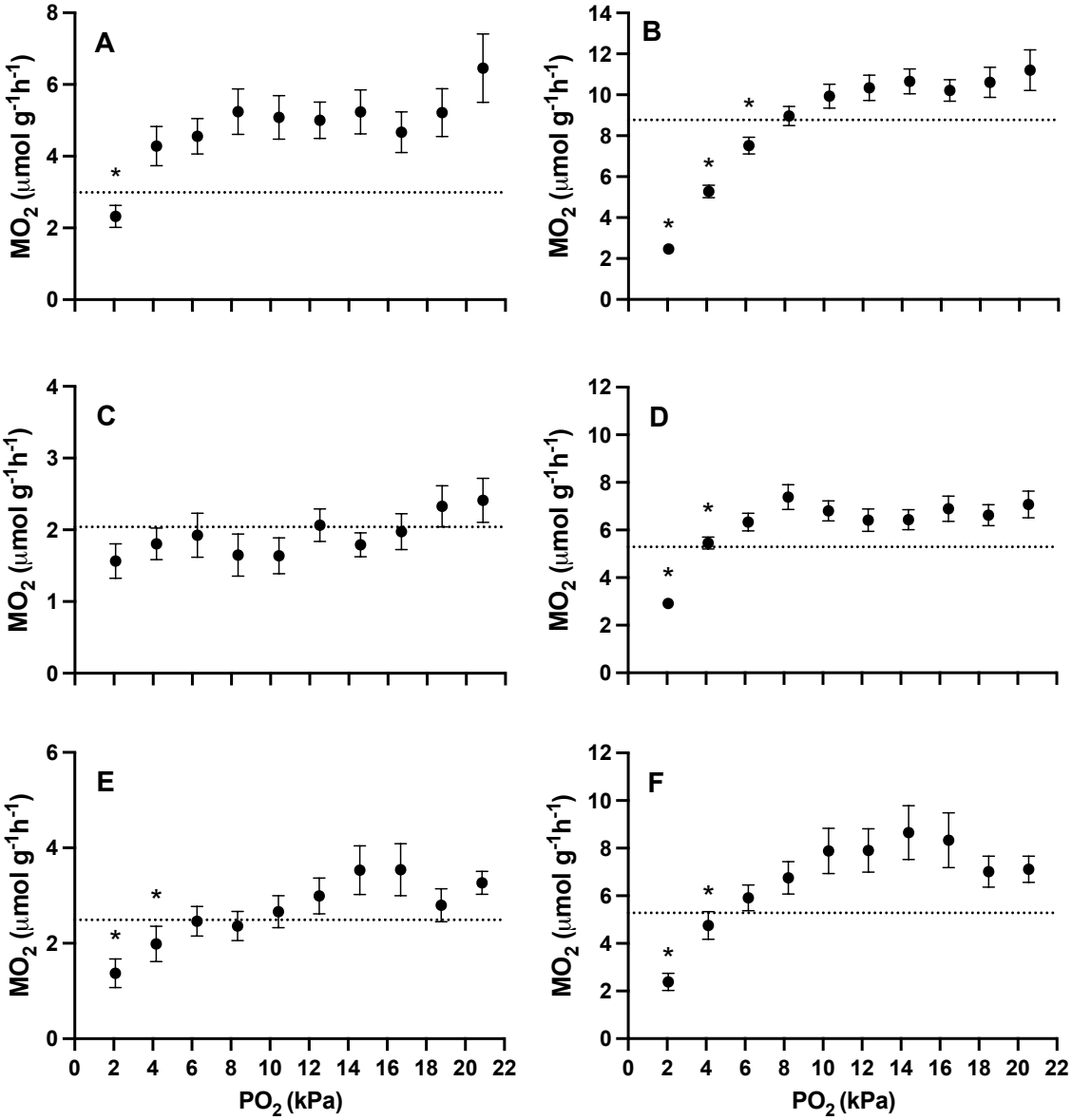

Fig. S2.1. Relationships between oxygen consumption rate (MO_2) and oxygen tension (PO_2) during exposure to progressive stepwise hypoxia. Within each treatment, the effects of PO_2 on MO_2 were compared using one-way ANOVA. (A,B) Cool acclimation group tested at (A) 15˚C (main effect of PO_2, $P<0.0001$) or (B) 25˚C (main effect of PO_2, $P<0.0001$). (C,D) Warm acclimation group tested at (C) 15˚C (main effect of PO_2, $P=0.16$) or (D) 25˚C (main effect of PO_2, $P<0.0001$). (E,F) Fluctuating acclimation group tested at (E) 15˚C (main effect of PO_2, $P<0.005$) or (F) 25˚C (main effect of PO_2, $P<0.0001$). Data are represented as means ± SEM, with the average resting metabolic rate of each group (from Fig. 3A in main paper) shown as dashed lines. *Significant pairwise difference from MO_2 at 20 kPa O_2 using Dunnett post-hoc tests to make pairwise comparisons within each group ($p<0.05$).

CHAPTER THREE: GENERAL DISCUSSION

3.1 Overview

The aim of my thesis was to investigate how the thermal history and thermal environment of fish impacts their capacity to cope with low oxygen availability. I used a full factorial design to achieve my objectives, which were two-fold: 1) To compare the effects of acclimation to constant cool, constant warm, and fluctuating temperatures on hypoxia tolerance at both cool and warm test temperatures, and 2) to investigate the mechanisms that underlie variation in hypoxia tolerance between groups. The overarching hypothesis stated that thermal history alters hypoxia tolerance through thermal plasticity of various respiratory and metabolic traits. I predicted that acclimation to a constant temperature would lead to greatest hypoxia tolerance at that acclimation temperature, but poor hypoxia tolerance at a different test temperature. I also predicted that acclimation to fluctuating temperatures would induce a more generalist strategy, in which hypoxia tolerance is better maintained over a broader range of cool and warm temperatures when compared to fish acclimated to constant temperature. My results show that 1) effects of constant temperature acclimation on hypoxia tolerance were temperature specific and not exhibited broadly across test temperatures and hypoxia tolerance was less sensitive to test temperature in fish acclimated to fluctuating temperatures, and 2) acclimation to constant versus fluctuating temperatures were associated with different underlying mechanisms. Overall, this work demonstrates that thermal history has the capacity to modify hypoxia tolerance, and the range of temperatures experienced by an individual can impact the thermal sensitivity of these changes in hypoxia tolerance. This thesis contributes to the body of work that examines the interactive effects of abiotic factors and how complex patterns of stressors impact the physiology and fitness of fish.

3.2 Acclimation to constant temperatures leads to thermal specialization

3.2.1 Warm acclimation does not lead to general improvements in hypoxia tolerance across temperatures

The results in Chapter 2 showed that acclimation to constant temperatures appeared to induce thermal specialization in mummichog killifish, based on the finding that the highest values of t_{LOE} and lowest values of P_{crit} were observed in fish tested at their acclimation temperature. This result contrasts previous suggestions that warm acclimation improves hypoxia

tolerance broadly across temperatures (Anttila et al., 2015; Hilton et al., 2008; McBryan et al., 2016; Sollid et al., 2005a), at least in this species. Warm acclimation has previously been observed to improve hypoxia tolerance in *F. heteroclitus* when tested at warm temperature (McBryan et al., 2016), however hypoxia tolerance at cool temperature was not tested, precluding the ability to assess whether the effects of warm acclimation on hypoxia tolerance are temperature specific. Similar improvements of hypoxia tolerance were observed in arctic char (*Salvelinus alpinus*), salmon (*Salmo salar*), crucian carp (*Carassius carassius*) and goldfish (*Carassius auratus*), but without measures of the response to hypoxia at cool temperatures, it remains unclear whether this improvement occurs over a broad range of temperatures in fish (Anttila et al., 2015; Sollid et al., 2005a). However, broad improvement of hypoxia tolerance across temperatures may occur in other aquatic organisms. For example, evidence in zebra mussel (*Dreissena polymorpha*) provides convincing support that warm acclimation improves their ability to withstand severe hypoxia across temperatures (Matthews and McMahon, 1999). Specifically, this study utilized a full factorial design to test survival time under severe hypoxia following acclimation to one of three environmentally relevant temperatures (5˚C, 15˚C and 25˚C) and found that the greatest hypoxia tolerance when tested at 15˚C or 25˚C was observed in warm acclimated mussels (25˚C), who performed better at 15˚C than mussels that were acclimated to 15˚C. This study also highlights the importance of testing the interactive effects of acclimation temperature and test temperature, as survival time in hypoxia decreased with increased temperature when individuals were tested at their acclimation temperature. This result could have misinterpreted the effect of warm acclimation as detrimental if fish were not tested over a range of temperatures to demonstrate that warm acclimation has the capacity to buffer against the negative effects of acute warming.

3.2.2 Acclimation to constant warm temperature leads to adjustments in metabolism and O_2 uptake

Improvements in hypoxia tolerance at 25˚C were associated with physiological adjustments to affecting metabolism and O_2 uptake. Warm acclimation reduced metabolic rate at 25˚C which may reduce O_2 demand under hypoxic stress. Given the thermodynamic effects of temperature on metabolic processes, acute increases in temperature are expected to result in an acute increase in metabolic rate. This is consistent with what I observed, as cool acclimated fish

acutely exposed to warm temperatures demonstrated a significant 2.9-fold increase in resting metabolic rate, however this was attenuated following six weeks of acclimation to 25˚C. The capacity for fish to adjust metabolism has been observed in many species (Seebacher et al., 2014; Sumner and Doudoroff, 1938) and the purpose is often to counter the direct thermal effects to allow consistent function over a larger thermal range (Jutfelt, 2020).

Warm acclimation led to an apparent regression of the interlamellar cell mass (ILCM) relative to cool acclimated fish. This result is consistent with what has been observed previously in this species (McBryan et al., 2016). Remodelling of the ILCM is highly responsive to changes in temperature or oxygen availability (Mitrovic and Perry, 2009; Sollid and Nilsson, 2006; Sollid et al., 2005a) and can be reversable. It is accomplished through increased apoptotic and decreased miotic activity in the cells of the ILCM and results in a higher proportion of exposed gill lamellae and a large surface area for branchial O_2 uptake (Sollid and Nilsson, 2006; Sollid et al., 2003). Plastic gill remodelling is believed to contribute to improved hypoxia tolerance following warm acclimation and hypoxia acclimation, as it improves O_2 uptake when O_2 demand is high or O_2 availability is low. However, increased gill surface area introduces an additional energetic cost of osmoregulation through what is referred to as the osmorespiratory compromise (Giacomin et al., 2019). A significant portion of the total energy budget of fish can be attributed to maintaining osmotic balance, depending on species and salinity conditions (Bushnell and Brill, 1992; Furspan et al., 1984; Nordlie, 1978; Nordlie and Leffler, 1975; Nordlie et al., 1991; Rao, 1968; Toepfer and Barton, 1992), therefore fish with larger surface area for O_2 diffusion may also need to contend with increased cost of osmoregulation. In the present study, fish were acclimated to hypoosmotic brackish water (4 ppt), which may limit gill remodelling to account for such a trade-off. However, under hypoxic stress, this cost is likely outweighed by the improved extraction efficiency of increased gill surface area due to the more immediate need of O_2 to sustain metabolic processes. At warm temperatures, this may be more challenging long-term, emphasizing the benefit of reduced metabolic rate following warm acclimation discussed previously.

In both constant acclimation groups, some determinants of O_2 transport were similar, such as Hb-O_2 affinity and haemoglobin content in the blood. The absence of any effect of thermal acclimation on Hb-O_2 affinity contrasts what had been found in this species previously, in which warm acclimation to 33˚C resulted in lower P_{50} than those acclimated to 15˚C across

each temperature (Chung et al., 2017). This suggests that Hb-O_2 affinity exhibits little plasticity with acclimation temperatures between 15-25˚C but may increase above this range. Overall, improvements in O_2 supply through increased gill surface area and reduced O_2 demand through reduced resting metabolic rate likely contribute to the observed reduction of P_{crit} and prolonging t_{LOE} in warm acclimated fish.

3.2.3 Underlying mechanisms of improved hypoxia tolerance are temperature-specific

Greater hypoxia tolerance in cool acclimated fish at 15˚C does not appear to be regulated by the same mechanisms as warm acclimated fish at 25˚C. Cool acclimated fish had greater ILCM coverage, while maintaining a similar metabolic rate at 15˚C relative to warm acclimated fish. Greater ILCM coverage is expected based on previous results which show that following acclimation to warm temperatures, the ILCM regresses to allow for increased O_2 uptake and the maintenance of metabolic demand for O_2 (Sollid and Nilsson, 2006; Sollid et al., 2005a). With the same level of metabolic demand for O_2 as warm acclimated fish at 15˚C, it is possible that cool acclimated fish have increased O_2 supply to the tissues at cool temperatures through other adjustments along the oxygen cascade. For example, although acute exposure to reduced temperatures can reduce maximal heart rates, acclimation to reduced temperatures can overcome these effects (Driedzic and Gesser, 1994; Gilbert and Farrell, 2021; Graham and Farrell, 1989). If acute exposure to cool temperature limits heart rate and cardiac output in hypoxia, prolonged exposure (i.e. acclimation) to cool temperature could offset this limitation and lead to greater perfusion of the gills and systemic tissues. It is also possible that thermal history may alter the strategy killifish use to cope with hypoxia, akin to the shifts in such strategies that occur with acclimation to different patterns of hypoxia exposure (Borowiec et al., 2018). For example, cool acclimated fish may rely more on anaerobic metabolism to cope with periods of O_2 deprivation. This would support the results of the present study which showed that cool acclimated had higher levels of glycogen in the brain. This suggests that they possess greater fuel reserved to sustain anaerobic glycolysis in this tissue, which may account for prolonged t_{LOE}. LOE is suspected to occur as a result of limited fuel availability in hypoxia-sensitive tissues such as the brain, therefore brain glycogen stores may limit the time a fish can cope with severe hypoxia (Dunn and Hochachka, 1986; Mandic et al., 2013; Saez et al., 2014; Speers-Roesch et al., 2013).

3.3 Effects of fluctuating temperature on hypoxia tolerance

3.3.1 Acclimation to diel cycles of temperatures reduces thermal sensitivity of hypoxia tolerance

Acclimation to diel cycles of temperature appear to shift killifish towards being thermal generalists with respect to hypoxia tolerance. Fish exposed to diel cycles of temperature performed as well as cool acclimated fish when tested at 15˚C, demonstrating that the negative effect of acute temperature change seen in the warm acclimated group was attenuated by exposure to diel cycles of temperature. At 25˚C, t_{LOE} appeared to be intermediate relative to both constant groups. Previous work seems to indicate that fish acclimate to either the maximum temperature or the mean temperature experienced during a diel cycle (Brett, 1944; Nilsson-Örtman et al., 2012). Time to loss of equilibrium appears to be more consistent with an acclimation to the mean temperature experienced daily, as t_{LOE} demonstrates intermediate values or values that are not significantly different from either constant group.

In contrast to both constant acclimation groups, P_{crit} was not affected by test temperature in the fluctuating group. This result suggests that exposure to a wider range of temperatures reduces temperature sensitivity of hypoxia tolerance, at least in this species. Reduced sensitivity to environmental change is characteristic of a generalist phenotype, which is beneficial to individuals that experience variability of environmental conditions (Nilsson-Örtman et al., 2012). Though the effect of fluctuating temperatures on hypoxia tolerance has received little previous attention, exposure to fluctuations in temperature has been shown to reduce the thermal sensitivity of other phenotypes in many species (Brett, 1944; Hubbs, 1964; Lowe and Heath, 1969; Robert Feldmeth et al., 1974). For example, in pupfish (*Cyprinodon nevadensis amargosae*), acclimation to a fluctuating temperature regime increase the thermal tolerance range of this fish significantly relative to those acclimated to constant temperatures (Robert Feldmeth et al., 1974). An increased scope of thermal tolerance is likely indicative of physiological adjustments which allow fish exposed to fluctuations in temperatures to remain metabolically active over a broader range of temperatures (Robert Feldmeth et al., 1974). These adjustments might therefore explain why fish exposed to fluctuating temperatures maintained aerobic metabolic activity during progressive hypoxia across a broader range of temperatures and demonstrated reduced temperature sensitivity of hypoxia tolerance.

3.3.2 Changes in O_2 transport that may be favourable under fluctuating temperatures

Fish acclimated to fluctuating temperatures demonstrated some unique mechanisms to maintain hypoxia tolerance. In contrast to the warm acclimated group, they displayed rapid and pronounced changes in haematocrit and haemoglobin content in the blood in response to temperature change. This is consistent with what has been observed previously, where blood haemoglobin content and haematocrit are modulated to match O_2 demand (Kapila et al., 2002; Lowe and Davison, 2005; Muñoz et al., 2018). An increase in blood haemoglobin content and haematocrit helps to improve O_2 carrying capacity and blood O_2 transport in hypoxia, however parallel increases in viscosity may set upper limits to this response in order to prevent constraints on cardiac output (Weber and Jensen, 1988). Mean corpuscular haemoglobin concentration was unchanged demonstrating that swelling or shrinking of blood cells are not responsible for this increase in haemoglobin content, rather this increase can likely be attributed to an increase in circulating erythrocytes. The increases in blood haemoglobin content and haematocrit in the fluctuating group at 25˚C are too rapid to be caused by erythropoiesis, and are likely attributable to splenic contraction, which is known to enable rapid modulation of blood hemoglobin content in killifish and many other species (Borowiec and Scott, 2021; Fänge and Nilsson, 1985; Lai et al., 2006; Yamamoto, 1987; Yamamoto et al., 1985), and may be a less energetically inexpensive way of adjusting blood O_2 transport (Weber and Jensen, 1988). Increases in Hb-O_2 affinity may also help improve hypoxia tolerance across temperatures, based on the significant effect of acclimation group on P_{50} that was driven by lower P_{50} values in the fluctuating group. In contrast to both constant temperature groups, Hb-O_2 affinity was not affected by test temperature, indicating a decrease in thermal sensitivity. Hb-O_2 binding affinity is sensitive to several conditions that can be exploited by fish during changes in temperature, notably the concentration of allosteric modifiers such as ATP and GTP and intracellular pH in erythrocytes. Greaney and Powers (1977, 1978) demonstrated that *F. heteroclitus* decrease ATP concentrations within erythrocytes resulting in increased Hb-O_2 affinity following warm acclimation. Additionally, reduced thermal sensitivity of Hb-O_2 affinity has been observed in regional heterotherms such as billfish through changes in concentration of ATP in erythrocytes (Weber et al., 2010), a mechanism which may contribute to the reduced temperature sensitivity of Hb-O_2 affinity in the fluctuating group. Although an increase in Hb-O_2 affinity as observed in the fluctuating group

may be expected to hamper O_2 unloading at the tissues, these consequences may be compensated for by the parallel increases observed in haematocrit and haemoglobin content of the blood.

RMR, ILCM coverage and glycogen stores of fish acclimated to fluctuating temperatures demonstrated similarities to warm acclimated fish. RMR for both the warm and fluctuating groups demonstrated the same pattern of variation, however the fluctuating group demonstrated an increased capacity to maintain this MO_2 at a lower range of PO_2 than the warm acclimated group when tested at 15˚C, which may be due to the increased affinity of hemoglobin for oxygen observed in this group. Additionally, the timeline of gill ILCM remodelling is largely unknown, but previous work in carp and goldfish indicates that acclimation to elevated temperatures alone results in significant changes in ILCM coverage after only 5 days and may occur more rapidly, but earlier timepoints would need to be explored (Sollid et al., 2005a). In response to hypoxia, retraction of the ILCM was visible after only 1 day and following one week in normoxia, this change was completely reversed (Sollid et al., 2003). When combined with an increase in temperature, this retraction of the ILCM occurred in the first 6 hours of hypoxia exposure (Sollid et al., 2005a), indicating that the rate of ILCM remodelling is highly dependent on temperature. It is unlikely that this rate of response could be accomplished through apoptosis alone, and it is therefore suspected that the ILCM may become detached from the lamellae as a rapid emergency response, at least in these species. Given the proposed timeline, modulation of gill coverage to match diel fluctuations in temperature would require nearly constant remodelling, but this did not appear to be the case here. Indeed, the fluctuating group exhibited ILCM coverage that appeared lower in fish sampled at 15˚C relative to 25˚C (Fig. 2.4). However, follow-up measurements in the fluctuating group of gill structure across the diel cycle showed no diel variation in ILCM coverage (Fig. 3.1). It may be that repeated daily remodelling of the ILCM is energetically inefficient, especially when more rapid strategies can be extorted such as haematological adjustments. Haematological changes are energetically inexpensive and can be accomplished rapidly to increase oxygen carrying capacity of the blood. This may be a more favourable strategy for fish experiencing daily fluctuations in temperature, since it allows them to maintain sufficient O_2 transport to the tissues when necessary, but is highly reversable during times of low O_2 demand (Borowiec and Scott, 2021). Finally, brain glycogen content was not different than either constant group, but on average appears to be intermediate relative to both warm and cool acclimated fish, suggesting that fuel availability to sustain anaerobic metabolism is not as

limiting in this group than it appears to be for cool acclimated fish. Overall, significant changes in O_2 transport in this group suggests that acclimation to diel cycles of temperature fluctuation leads to some mechanisms of thermal plasticity that are distinct from fish acclimated to constant temperatures and may contribute to improving hypoxia tolerance across a broad range of temperatures.

3.4 Thermal specialist versus generalist strategies

Taken together, the results of Chapter 2 demonstrate that thermal history has the capacity to modify hypoxia tolerance and give rise to thermal specialist and generalist strategies following acclimation to constant and fluctuating temperatures respectively. The shape of a TPC and their plasticity determine how individuals respond to environmental variability (da Silva et al., 2019). Theoretically, an increase in thermal breadth decreases maximum performance, leading to a specialist-generalist trade-off (Seebacher et al., 2015). In fish who experience historically stable environmental conditions, the generalist phenotype is not useful. Instead, maximizing performance at environmental conditions consistent with their thermal history makes an individual specialized to perform well at a narrower range of temperatures (Seebacher et al., 2015). However, in habitats where conditions can be variable and unpredictable, increased thermal breadth in key. This concept is consistent with what I have found in mummichog killifish, which suggest that acclimation to fluctuating temperatures may contribute to improved hypoxia tolerance across a broader range of temperatures. The effect of thermal history has been previously observed to result in divergent strategies to cope with low oxygen. For example, upon examination of two closely related intertidal species, *Bellapiscis medius* and *Bellapiscis lesleyae*, which occupy distinct thermal niches (the former occupying areas exposed to higher extremes of temperature and dissolved oxygen relative to the latter), distinct levels of tolerance were observed in response to hypoxia (Hilton et al., 2008). *B. medius* demonstrated consistently higher hypoxia tolerance relative to *B. lesleyae* in response to acute warming and following warm acclimation, demonstrating their ability to maintain hypoxia tolerance over a broader range of temperatures (Hilton et al., 2008). Although *B. lesleyae* did display improved hypoxia tolerance in response to warm acclimation, this species was still less hypoxia tolerant as its sister species. These results show a clear link between thermal history and hypoxia tolerance. The species which inhabits rock pools with greater fluctuations in temperature displays general

improvements in hypoxia tolerance across test temperatures relative to its more thermally stable counterpart (Hilton et al., 2008). This demonstrates that even in closely related species, ecological divergence is an important factor when examining physiological tolerance of fish species.

3.4 Implications and future directions

Overall, my findings suggest that the interaction between thermal history and other abiotic stressors such as hypoxia may have significant effects on the physiology and fitness of fish. This thesis showed that acclimation to constant versus fluctuating temperatures resulted in: 1) Strategies consistent with thermal specialists and thermal generalists respectively and 2) the utilization of different physiological mechanisms to cope with hypoxia. Thus, thermal fluctuations can elicit physiological adjustments that are challenging to predict from those exhibited by fish in constant thermal environments. With the predicted effects of future climate change of increased mean and variability of environmental temperature and the increased incidence and severity of aquatic hypoxia, appreciating the impacts of thermal fluctuations and how they interact with other stressors associated with climate change will be vital for predicting how aquatic organisms will cope with environmental change in the future.

The results of my MSc project emphasize the critical importance of investigating the effects of fluctuating environments on aquatic organisms. Within the field of temperature acclimation and its capacity to modify tolerance to other abiotic factors, the focus has remained mostly on the effects of constant temperature acclimation. These studies are incredibly valuable for elucidating the distinct mechanisms involved in thermal acclimation, however they limit environmental relevance to wild organisms. To predict how aquatic organisms such as fish will respond to future global change, future studies should incorporate environmentally relevant patterns of abiotic factors that are expected to be modified by climate change (e.g. pH and salinity). In addition, this study highlights the response to diurnal temperature fluctuations, however more complex patterns of temperature (e.g. seasonal effects and tidal effects) are important to consider as well. Finally, the predictability or stability of environmental variation is believed to have significant effects on the responses of fish, however this is largely unknown (da Silva et al., 2019; Seebacher et al., 2015). Environmental variability and stochasticity may present a unique stress to aquatic organisms and considering the predicted increase in thermal

variability with climate change, it is crucial to investigate how more complex patterns of environmental variability may impact the response of an organism to other environmental stressors such as hypoxia. Overall, further investigation into environmentally relevant fluctuations of commonly co-occurring stressors (e.g. temperature and hypoxia) may improve our understanding of how climate change may impact the physiology and health of aquatic organisms in the future.

3.5 Figures

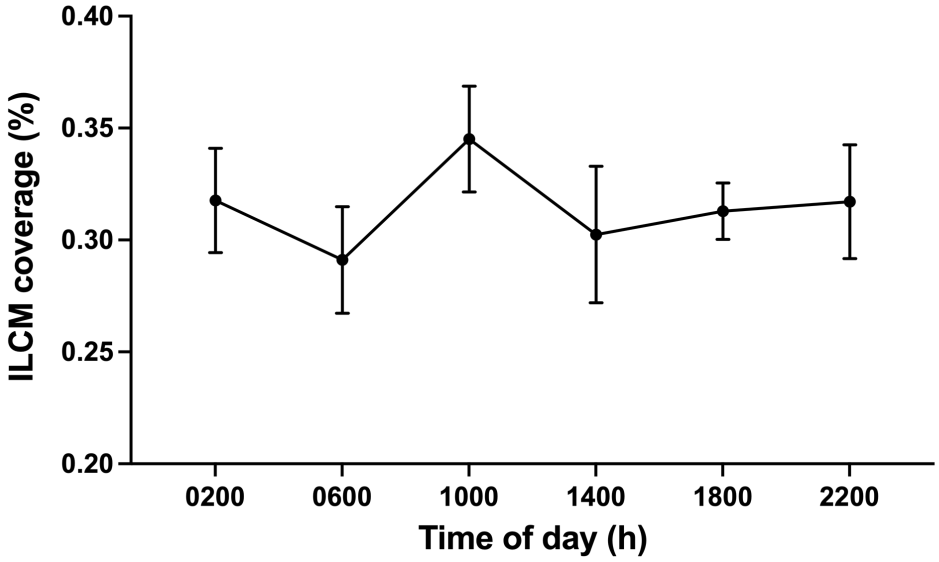
0.40
0.35
0.30
0.25
0.20
ILCM coverage (%)
0200
0600
1000
1400
1800
2200
Time of day (h)

Fig. 3.1. Gill structure across a diel temperature cycle. The effects of time of day on ILCM coverage (%) were compared using one-way ANOVA (main effect of time, P=0.39). Data are represented as means ± SEM. Fish were acclimated to a diel cycle of fluctuating temperature as in Section 2.3.2. Individuals were sampled, and their gills were dissected as described in Section 2.3.4 at 0200h (n=8), 0600h (n=7), 1000h (n=8), 1400h (n=7), 1800h (n=8) and 2200h (n=8). Gill histology as per Section 2.3.5 was carried out before morphology was then quantified as described in Section 2.4.3.

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