INTERMITTENT HYPOXIA ELICITS A UNIQUE PHYSIOLOGICAL COPING STRATEGY IN *FUNDULUS* KILLIFISH

INTERMITTENT HYPOXIA ELICITS A UNIQUE PHYSIOLOGICAL COPING STRATEGY IN *FUNDULUS* KILLIFISH

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LAY ABSTRACT

Oxygen levels in the aquatic environment are dynamic. Many fishes routinely encounter changes in oxygen content in their environment. However, we have very little understanding of how cycles between periods of low oxygen (hypoxia) and periods of high oxygen (normoxia) affect the physiology of fish. This thesis investigated how *Fundulus* killifish cope with daily cycles between hypoxia and normoxia (intermittent hypoxia) by modifying oxygen transport, metabolism, and oxidative stress defense systems. I found that killifish rely on a unique and effective physiological strategy to cope with intermittent hypoxia, and that this strategy is distinct from how they respond to a single bout of hypoxia (followed by normoxia) and to a constant pattern of only hypoxia. This is the most extensive investigation to date on how fish respond to the challenges of intermittent hypoxia, an understudied but ecologically important type of aquatic hypoxia.

ABSTRACT

Fish encounter daily cycles of hypoxia in the wild, but the physiological strategies for coping with repeated cycles of normoxia and hypoxia (intermittent hypoxia) are poorly understood. Contrastingly, the physiological strategies for coping with continuous (constant) exposure to hypoxia have been studied extensively in fish. The main objective of this thesis was to understand how *Fundulus* killifish cope with a diurnal cycle of intermittent hypoxia, an ecologically relevant pattern of aquatic hypoxia in the natural environment. To do this, I characterized the effects of intermittent hypoxia on hypoxia tolerance, oxygen transport, metabolism, and the oxidative stress defense system of killifish, and compared these effects to fish exposed to normoxia, a single cycle of hypoxia-normoxia, and constant hypoxia.

Specifically, I studied the following topics: (i) how acclimation to intermittent hypoxia modifies hypoxia tolerance, and the hypoxia acclimation response of *Fundulus heteroclitus* (Chapter 2), (ii) metabolic adjustments occurring during a hypoxia-reoxygenation cycle (Chapter 3), (iii) how acclimation to intermittent hypoxia alters O_2 transport capacity and maximal aerobic metabolic rate (Chapter 4), (iv) the effects of hypoxia and reoxygenation on reactive oxygen species and oxidative stress (Chapter 5), and (v) variation in hypoxia tolerance and in the hypoxia acclimation responses across *Fundulus* fishes (Chapter 6).

Killifish rely on a unique and effective physiological strategy to cope with intermittent hypoxia, and that this strategy is distinct from both the response to a single bout of acute hypoxia-reoxygenation (12 h hypoxia followed by 6 h reoxygenation) and to chronic exposure to constant hypoxia (24 h hypoxia per day for 28 d). Key features of the acclimation response to intermittent hypoxia include (i) maintenance of resting O_2 consumption rate in hypoxia followed by a substantial increase in O_2 consumption rate during recovery in normoxia, (ii) reversible increases in blood O_2 carrying capacity during hypoxia bouts, (iii) minimal recruitment of anaerobic metabolism during hypoxia bouts, and (iv) protection of tissues from oxidative damage despite alterations in the homeostasis of reactive oxygen species and cellular redox status. Of these features, (i) is unique to intermittent hypoxia, (ii) also occurs in fish exposed to acute hypoxia-reoxygenation, and (iii) and (iv) are observed in both fish acclimated to intermittent hypoxia as well as those acclimated to constant hypoxia.

This is the most extensive investigation to date on how fish cope with the energetic and oxidative stress challenges of intermittent hypoxia, and how these responses differ from constant hypoxia. This thesis adds substantial insight into the general mechanisms by which animals can respond to an ecologically important but poorly understood feature of the aquatic environment.

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TABLES OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS & SYMBOLS

ABAP	2,2'-azobis(2-amidinopropane) dihydrochloride
CAT	Catalase
COX	Cytochrome c oxidase
CS	Citrate synthase
GPX	Glutathione peroxidase
GSH	Reduced glutathione
GSH + GSSG	Total glutathione
GSSG	Oxidized glutathione
GTP	Guanosine triphosphate
HIF	Hypoxia inducible factor
HOAD	3-hydroxyacyl-CoA dehydrogenase
LDH	Lactate dehydrogenase
LOE	Loss of equilibrium
MO_2	Rate of oxygen consumption
$MO_{2,max}$	Maximal rate of oxygen consumption
MRC	Mitochondrion-rich cell
PEPCK	Phosphoenolpyruvate carboxykinase
P_{crit}	Critical oxygen tension

P_{LOE}	PO_2 at loss of equilibrium
PO_2	Partial pressure of oxygen
PO_2 at LOE	PO_2 at loss of equilibrium
PK	Pyruvate kinase
PVC	Pavement cell
RI	Regulation index
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDH	Succinate dehydrogenase
TOSC	Total oxidant scavenging capacity
t_{LOE}	Time to LOE

THESIS ORGANIZATION & FORMAT

This thesis is organized in a "sandwich thesis" format, as recommended and approved by members of my supervisory committee, and consists of seven chapters. Chapter 1 provides a review of relevant background material, discusses the justification for and potential implications of the work, and provides an overview of thesis objectives and subsequent chapters. Chapter 2 through 6 are manuscripts that are published (Chapters 2 and 3), in review (Chapter 6), or ready to be submitted for publication (Chapter 5) in a peer-reviewed scientific journals. Chapter 4 describes a study in progress at the time of the submission of this thesis that will also be prepared for submission to a peer-reviewed scientific journal. Chapter 7 is a general discussion that summarizes the major findings of this thesis that places these findings in the context of current knowledge and indicates potential future directions for the line of research described in this thesis. Chapter 2 is referred to as Borowiec et al. (2015), Chapter 3 is referred to as Borowiec et al. (2018), Chapter 4 is referred to as Borowiec and Scott, in prep., Chapter 5 is referred to as Borowiec and Scott, in prep., Chapter 6 is referred to as Borowiec et al., in review.

CHAPTER 1: General introduction

Author: Brittney G. Borowiec

CHAPTER 2: Distinct physiological strategies are used to cope with constant hypoxia and intermittent hypoxia in killifish (*Fundulus heteroclitus*)

Authors: Brittney G. Borowiec, Kimberly L. Darcy, Danielle M. Gillette, and Graham R. Scott

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CHAPTER 3: Distinct metabolic adjustments arise from acclimation to constant hypoxia and intermittent hypoxia in estuarine killifish (*Fundulus heteroclitus*)

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CHAPTER 4: Reversible modulation of blood O_2 carrying capacity in estuarine killifish (*Fundulus heteroclitus*) acclimated to intermittent hypoxia

Authors: Brittney G. Borowiec and Graham R. Scott

Status: Study in progress (in preparation)

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CHAPTER 5: Hypoxia acclimation alters reactive oxygen species homeostasis and oxidative status in estuarine killifish (*Fundulus heteroclitus*)

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CHAPTER 6: Evolutionary variation in hypoxia tolerance and hypoxia acclimation responses in killifish from the family Fundulidae

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CHAPTER 7: General discussion

Authors: Brittney G. Borowiec

CHAPTER 1

General introduction

B.G. Borowiec

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1.1 Hypoxia in the environment

Oxygen availability varies across space and time in the aquatic environment (Breitburg et al., 2009; Burnett, 1997; Graham, 1990). Dissolved oxygen content can be <1 mg O_2 l^{-1} (~10% air saturation) at the bottom of ice-covered lakes by late winter (Barica and Mathias, 1979; Hasler et al., 2009). O_2 levels can range from hyperoxic (>18 mg O_2 l^{-1}, 200 to 400% air saturation) to near-anoxic (<0.5 mg O_2 l^{-1}) in estuaries and tidal pools during daily or hourly cycles (Richards, 2011; Routley et al., 2002; Truchot and Duhamel-Jouve, 1980; Tyler et al., 2009). Such variations in the availability of oxygen, a vital resource for all aerobic organisms, constrains the quantity and quality of habitat available for aquatic organisms (Chapman and McKenzie, 2009).

Aquatic hypoxia develops naturally under a number of circumstances, including limited atmospheric diffusion (e.g. ice cover, stratification), as a consequence of nutrient loading or pollution, or due to cellular respiration at night in aquatic plants (Breitburg, 1992; Diaz, 2001; Sollid et al., 2003; Stierhoff et al., 2003; Tyler et al., 2009). The severity and length of hypoxia exposure differs considerably between systems, and is influenced by the temperature, total insolation, tidal movements, salinity, and other physical properties of a system (Tyler et al., 2009). Overall, the incidence of aquatic hypoxia is expected to rise due to global climate change, urbanization, pollution, and other anthropogenic influences (Diaz, 2001; Ficke et al., 2007).

Though the existence of different patterns of hypoxia has been known for decades (Breitburg et al., 2018; Diaz, 2001), we have relatively little understanding how *variations* in the pattern of aquatic hypoxia affects water-breathing aquatic organisms. Stable, continuous patterns of hypoxia can occur in stratified environments where oxygen-poor and oxygen-rich waters are separated, or in areas where atmospheric diffusion is limited (e.g. extensive surface vegetation or ice cover) (Barica and Mathias, 1979; Childress and Seibel, 1998; Hasler et al., 2009; Sollid et al., 2003). Such episodes of constant hypoxia typically develop slowly (e.g. as animal respiration slowly depletes O_2 in an ice-covered lake) and can persist for weeks, months, or even years in highly stratified or stable cycles. Contrastingly, tidal cycles and/or interactions between photosynthesis and cellular respiration can produce a diel nocturnal (intermittent) pattern of hypoxia (Richards, 2011; Routley et al., 2002; Truchot and Duhamel-Jouve, 1980; Tyler et al., 2009) that occurs on a shorter time scale compared to most patterns of constant hypoxia. Traditionally, most work on the physiological responses of fish to environmental hypoxia has focused on constant patterns of hypoxia. However, cycles of intermittent hypoxia may also represent a significant physiological challenge for fish with widespread ecological and evolutionary implications.

1.2 The challenges of hypoxia for aerobic organisms

One physiological challenge for aerobic organisms encountering all patterns of hypoxia is the potential development of an ATP supply-demand imbalance (Boutilier, 2001;

Hochachka et al., 1996). This imbalance arises from the limitation of aerobic respiration by the mitochondria in hypoxia, and a corresponding inability to provide enough ATP to meet routine energetic demands (Boutilier, 2001; Hochachka et al., 1996; Richards, 2009). Coping with hypoxia effectively means avoiding, delaying, or correcting this imbalance in some way (Boutilier, 2001; Richards, 2009). In order to do this, animals can rely on a variety of physiological responses that augment ATP supply pathways (increasing O_2 transport capacity or O_2-independent ATP production) and/or minimize cellular ATP demands (metabolic depression) (Boutilier, 2001; Hochachka et al., 1996). The relative success of these strategies in balancing ATP supplies and ATP demands determines the functional and survival range of O_2 tensions of an organism (Farrell and Richards, 2009; Richards, 2009), and therefore plays a key role in determining the hypoxia tolerance of aquatic animals.

Another potential physiological challenge that may be induced by hypoxia directly and/or by emergence from hypoxia into well-oxygenated conditions is oxidative stress. Alterations in the mitochondrial electron transport system or other cellular processes may lead to increased reactive oxygen species (ROS) production during hypoxia exposure (Clanton, 2007; Giraud-Billoud et al., 2019; Hermes-Lima et al., 2015; Hermes-Lima and Zenteno-Savín, 2002; Murphy, 2009), and this can lead to oxidative damage if ROS production exceeds oxyradical scavenging capacity and/or if increased levels of ROS alters redox status or signalling (Betteridge, 2000; Clanton, 2007; Costantini, 2019; Jones, 2006). Reoxygenation events and recovery from hypoxia may also be associated with

increased ROS production due to rapid changes in metabolism and tissue perfusion (Giraud-Billoud et al., 2019; Hermes-Lima et al., 2015; Moreira et al., 2016). Hypoxia tolerance and the oxidative stress induced by hypoxia and/or cycles of hypoxia and reoxygenation may interact (e.g. the ability to cope with oxidative stress may also limit hypoxia tolerance), though this possibility has received relatively little attention in fish (Leveelahti et al., 2014), but may have implications for how animals successfully cope with hypoxia, particularly for animals that routinely cope with variation in O_2 availability as a part of their life history.

1.2.1 Measuring whole-animal hypoxia tolerance

In fishes and other aquatic ectotherms, hypoxia tolerance is typically evaluated using one of several whole-animal indices that reflect a disruption of physiological function such as O_2 consumption rate (MO_2) or the ability to maintain an upright posture in the water column. Hypoxia tolerant organisms typically maintain function in a broader range of O_2 tensions than hypoxia intolerant organisms, meaning that the loss of physiological function occurs at a lower PO_2, or is delayed at a given level of hypoxia, relative to less hypoxia tolerant organisms (Farrell and Richards, 2009; Richards, 2009) (see Fig. 6.2 of a schematic of several methods of measuring hypoxia tolerance).

Perhaps the most prevalent index of hypoxia tolerance in aquatic organisms is the critical oxygen tension (P_{crit}), the PO_2 where aerobic metabolism becomes dependent upon

external O_2 content, and the animal transitions from oxyregulation to oxyconformation (Rogers et al., 2016). P_{crit} is hypothesized to be ecologically relevant as it correlates with the environmental PO_2 at which a species is found, such that animals from more hypoxic environments typically have a lower P_{crit} than animals from less hypoxic environments (Childress and Seibel, 1998; Mandic et al., 2009; Regan et al., 2019; Wishner et al., 2018). Mechanistically, P_{crit} is related to the O_2 supply capacity of an animal, as it often correlates with total gill surface area, the onset of hypoxic bradycardia, and both haemoglobin and mitochondrial affinities for O_2 (Childress and Seibel, 1998; Lau et al., 2017; Mandic et al., 2009; Regan et al., 2019; Speers-Roesch et al., 2012a; Wishner et al., 2018). P_{crit} also indicates the PO_2 where the aerobic scope, an important fitness-related trait, is zero (Claireaux and Chabot, 2016; Clark et al., 2013; Fry, 1947; Fry, 1971; Pörtner, 2010). However, not all species exhibit the classic two-segmented relationship assumed in the calculation of P_{crit} (Fig. 6.2), where resting MO_2 is maintained at higher PO_2, and then rapidly declines as PO_2 decreases below P_{crit} (Urbina et al., 2012; Wood, 2018), and the calculation of P_{crit} varies considerably between studies (Wood, 2018). For this and other reasons, the implementation and value of P_{crit} as an index of hypoxia tolerance is under debate (Regan et al., 2019; Wood, 2018).

Other indices of hypoxia tolerance such at PO_2 at or time to loss of equilibrium (LOE) quantify the ability of fish to maintain an upright position in the water column (i.e. a proxy for acute survival) instead of the maintenance on MO_2 in acute hypoxia. Hypoxia tolerant animals have a lower PO_2 at which LOE occurs (PO_2 at LOE) or maintain

equilibrium for longer at a common severe level of hypoxia (time to LOE) (Mandic et al., 2009; Mathers et al., 2014; Speers-Roesch et al., 2012b). The mechanisms by which LOE occurs in fish, and whether they differ between PO_2 at LOE and time to LOE protocols are unclear. However, a longer time to LOE is associated with better protection of ATP levels and a greater capacity for anaerobic metabolism in the brain (Mandic et al., 2013; Speers-Roesch et al., 2013).

The various indices of hypoxia tolerance have sometimes been found to co-vary across species (Dan et al., 2014; Mandic et al., 2013; Yang et al., 2013), but this is often not the case (Crans et al., 2015; Dhillon et al., 2013; Fu et al., 2014; Mathers et al., 2014; Speers-Roesch et al., 2013). This suggests that the reductions in MO_2 described by P_{crit} and the inability to maintain body posture, described by PO_2 at LOE and time to LOE, may represent distinct aspects of an animal's physiology (Borowiec et al., 2016; Nilsson and Renshaw, 2004). Exactly which measures of hypoxia tolerance are most suitable for a given biological question remains a matter of debate (e.g. Regan et al., 2019 and Wood, 2018). P_{crit} is useful because it presumably provides mechanistic information about MO_2 respond to increasingly severe hypoxia and may provide information about O_2 transport capacity. Contrastingly, while the mechanisms behind PO_2 at LOE and time to LOE are not well-established, they both represent ecologically relevant endpoints for fishes under acute hypoxia stress. There are also considerations to be made depending on the species examined – for example, P_{crit} is likely to be highly ecologically relevant for migrating salmonids that operate near maximal MO_2 for sustained periods. For these reasons, there

is no single superior index of hypoxia tolerance, and experimental designs should carefully consider which index or indices would provide the most useful information.

Hypoxia tolerant animals with a low P_{crit} may be expected to also have a delayed time to LOE or reduced PO_2 at LOE compared to animals with a higher P_{crit} due to differences in relative hypoxia stress and the corresponding depletion of fuel reserves, though a high P_{crit} does not exclude the possibility of a longer time to LOE or lower PO_2 at LOE, particularly in taxa with high anaerobic capacity or large glycogen stores like some cyprinids. However, there have been relatively few previous attempts to examine the relationships between different indices of hypoxia tolerance across species (Crans et al., 2014; Dhillon et al., 2014; Fu et al., 2014), and how these relationships may be altered by environmentally induced plasticity.

1.3 How water-breathing fishes cope with aquatic hypoxia

Greater hypoxia tolerance is generally associated with high O_2 transport capacity and low metabolic rate, and this is supported by data from a wide range of species (Bickler and Buck, 2007; Mandic et al., 2009; Perry et al., 2009; Richards, 2009). Oxidative damage to lipids, proteins, and DNA could foreseeably affect how organisms to respond to the energetic challenge of hypoxia by impacting key components of energy supply such as the activity of redox-sensitive metabolic enzymes like aconitase (Bulteau et al., 2003; Bulteau et al., 2005; Ivanina and Sokolova, 2016), or by altering or inducing some

cellular signalling cascades such as HIF-1α (Hermes-Lima et al., 2015; Prabhakar et al., 2007). While some hypoxia tolerant animals have also been shown to be relatively resistant to oxidative stress or have an enhanced antioxidant defence system when exposed to hypoxia (Giraud-Billoud et al., 2019; Hermes-Lima and Zenteno-Savín, 2002), how the prevention and/or management of hypoxia-induced oxidative stress interacts with and influences variation in hypoxia tolerance has been rarely formally investigated.

Adult phenotypic plasticity, developmental plasticity, and/or natural selection acting on a variety of underlying physiological traits can lead to variation in hypoxia tolerance amongst populations. Often these mechanisms act on traits involved in oxygen transport and metabolism, and thus improve hypoxia tolerance by facilitating either the maintenance of ATP supply and/or minimizing cellular ATP demands in adults exposed to chronic hypoxia (Fu et al., 2011; Greaney et al., 1980; Martinez et al., 2006; Perry et al., 2009; Sollid et al., 2003; Sollid and Nilsson, 2006). For example, encounters with hypoxia during early development can lead to alterations in hypoxia tolerance other phenotypic traits that persist into adulthood (Barrionuevo et al., 2010; Blank and Burggren, 2014; Crocker et al., 2013; Robertson et al., 2014). Fixed improvements in hypoxia tolerance can also evolve among populations and species, and species specialized to hypoxia prone environments tend to exhibit a number of traits that may facilitate enhanced survival in hypoxia such as low resting O_2 consumption rate, increased haemoglobin-O_2 binding affinity, and increased gill surface area (Hopkins and Powell,

2001; Mandic et al., 2009; Regan et al., 2017; Richards, 2011). There may also be interesting interactions between plasticity and evolutionary processes (e.g. through evolution of enhanced plasticity) (Garland and Kelly, 2006). For example, the magnitude of the acclimation response to chronic hypoxia may vary between species, such that hypoxia adapted taxa may be able to respond more effectively to the same hypoxia signal and/or minimize maladaptive plasticity compared to other taxa (McClelland and Scott, 2018; Morris and Rogers, 2013; Velotta et al., 2018).

1.3.1 Increasing O_2 transport capacity to maintain aerobic ATP supply

The transport of O_2 from the external environment to the mitochondria within tissues involves several steps, and physiological adjustments can occur along the O_2 transport cascade to protect O_2 movement under low oxygen conditions (Storz et al., 2010). These adjustments fall into two broad categories: maintenance of O_2 uptake from the environment through respiratory organs and into the blood, and increases in the capacity to move O_2 to the tissues *via* the circulatory system. Both strategies are common in fish exposed to hypoxia (Perry et al., 2009), and these strategies often co-occur and support each other.

Fish from hypoxia-prone habitats typically have greater gill surface area than fishes from well-oxygenated habitats (Chapman et al., 2002; Childress and Seibel, 1998; Mandic et al., 2009), and therefore a greater total area for oxygen uptake. Fish gills are highly

plastic (Nilsson, 2007; Wilson and Laurent, 2002), and prolonged hypoxia exposure can also lead to an increase in gill surface area through gill remodelling, often involving a reduction in interlamellar cell mass (Blank and Burggren, 2014; Dhillon et al., 2013; Fu et al., 2011; Matey et al., 2008; Nilsson et al., 2012; Sollid et al., 2003).

Exposure to hypoxia rapidly increases total gill ventilation *via* increased stroke volume and/or frequency (Gamperl and Driedzic, 2009; Perry et al., 2009; Porteus et al., 2011) in water-breathing fishes. This hypoxic ventilatory response is initiated by neuroepithelial cells, the putative O_2 chemoreceptors of the fish gill, though whether these cells respond to external PO_2, arterial O_2 content, and/or the rate of tissue O_2 delivery is still unclear (Coolidge et al., 2008; Perry and Gilmour, 2002; Perry et al., 2009). By increasing water flow over the gills and thus increasing the O_2 concentration gradient that drives O_2 diffusion into the blood, arterial PO_2 and haemoglobin O_2 loading are protected, and tissue O_2 delivery is maintained (Perry et al., 2009; Randall and Daxboeck, 1984). Changes in gill blood flow can also increase lamellar perfusion and improve diffusive O_2 uptake (Booth, 1979). Secondarily, the hyperventilation associated with the hypoxic ventilatory response induces slight respiratory alkalosis, leading to an increase in the affinity for O_2 and thus O_2 carrying capacity of haemoglobin at the gill *via* the Root and Bohr effects (de Souza and Bonilla-Rodriguez, 2007; Gilmour, 2001; Porteus et al., 2011; Rummer and Brauner, 2015; Rummer et al., 2013). However, as fish have little scope to reduce arterial CO_2 levels, this small change in acid-base status on O_2 transport capacity

is likely of limited significance in most species (Gilmour, 2001; Porteus et al., 2011; Qin et al., 2010).

With prolonged exposure to hypoxia, the initial increase in ventilation is slowly abolished, reflecting greater reliance on other respiratory responses such as changes in blood O_2 carrying capacity (Florindo et al., 2006; Porteus et al., 2011; Powell et al., 1998; Stecyk and Farrell, 2002). This ventilatory decline with sustained hypoxia is thought to be advantageous to water-breathers due to their relatively high routine cost of ventilation (Cameron and Cech, 1970; Edwards, 1971; Perry et al., 2009; Porteus et al., 2011; Steffensen, 1985), which can exceed 70% of standard oxygen consumption rate according to some estimates, though values in the range of 5 to 15% are more widely accepted (Holeton, 1980; Steffensen and Lomholt, 1983). Mechanistically, changes in ventilation during prolonged hypoxia could be facilitated by changes in neuroepithelial cell sensitivity or distribution (Gilmour, 2001; Jonz et al., 2004; Perry et al., 2009; Vulesevic et al., 2006).

Like improving branchial O_2 uptake, increasing the O_2 carrying capacity of blood can also minimize the effects of hypoxia on aerobic metabolism by maximizing tissue O_2 supply in hypoxia. Increases in both the proportion of erythrocytes in the blood (haematocrit) and/or the concentration of haemoglobin within individual cells (mean corpuscular haemoglobin concentration) are common in hypoxia exposed fishes (Affonso et al., 2002; Bose et al., 2019; Hughes, 1973; Nikinmaa and Soivio, 1982; Wells, 2009). Increases in

haematocrit can occur rapidly in hypoxia *via* splenic contraction induced by an adrenergic signalling cascade (Lai et al., 2006) and these changes can persist for weeks (Greaney et al., 1980). Longer term elevations in blood haemoglobin can also involve increased red blood cell production (erythropoiesis) by the kidneys induced by the renal hormone erythropoietin (Lai et al., 2006). How this presumed increase in blood viscosity and cardiac workload (Fletcher and Haedrich, 1987; Rand et al., 1964) interacts with the effectiveness of the hypoxia coping response in fishes is rarely examined, but work in rainbow trout (*Oncorhynchus mykiss*) (Gallaugher et al., 1995; Wells and Weber, 1991) suggests that while an extremely high haematocrit (~55%) can limit oxygen transport capacity in some situations, very high haematocrit generally has little negative effect on aerobic swimming performance and aerobic capacity (Gallaugher and Farrell, 1998).

In addition to the total number of O_2 binding sites offered by haemoglobin, haemoglobin-O_2 binding affinity is sensitive to several conditions that can be exploited by hypoxic fishes, notably the concentration of allosteric modifiers (such as ATP and GTP) and intracellular pH in erythrocytes (Nikinmaa, 1990; Nikinmaa and Salama, 1998). Hypoxia exposure can lead to a reduction in GTP and/or ATP concentration, and this leads to an increase in haemoglobin-O_2 binding affinity (Greany and Powers, 1977; Nikinmaa and Soivio, 1982; Weber and Lykkeboe, 1978; Wells, 2009; Wood and Johansen, 1973). When both ATP and GTP are present, GTP is often the primary allosteric modulator, as reflected by larger decreases in GTP than ATP concentration during hypoxia, and a greater allosteric effect of GTP compared to ATP (Jensen, 2004; Lykkeboe and Weber,

1978; Weber and Jensen, 1988). GTP also typically predominates in fishes that regularly encounter environmental hypoxia (Wells, 2009). The mechanisms by which GTP content can be regulated in red blood cells are unclear, but like ATP, it is produced by aerobic pathways, and therefore is likely sensitive to energy limitation in red blood cells (Wells, 2009). In salmonids, carp, and some characids, one key mechanism of modulating haemoglobin-O_2 binding affinity under environmental hypoxia (Tetens and Christensen, 1987) or other challenges to aerobic metabolism (e.g. exercise) (Nikinmaa, 1983; Primmett et al., 1986) is catecholaminergic activation of Na^+-H^+ exchanger in the erythrocyte membrane (Boutilier and Ferguson, 1989; Fievet et al., 1987; Nikinmaa, 1983; Nikinmaa and Salama, 1998; Salama and Nikinmaa, 1988; Val, 2000; Val et al., 1998; Wells, 2009), which leads to a reduction in ATP concentration through cellular swelling, as well as an increase intracellular pH of the red blood cell. Unlike ATP, red blood cell GTP concentrations seem to be insensitive to catecholamines, suggesting some functional distinction in how these modifiers are regulated (Nikinmaa and Salama, 1998).

In addition to the direct effects of ATP and/or GTP, the affinity of most fish haemoglobins shows considerable pH dependence through the Root effect (reduced carrying capacity at lower pH) and Bohr effect (reduced binding affinity with lower pH) (de Souza and Bonilla-Rodriguez, 2007; Regan and Brauner, 2010; Wells, 2009), such that haemoglobin-O_2 binding affinity is reduced in acidic and hypercapnic conditions. The presence of ATP or GTP can further modulate these pH-dependent effects, and in several species the binding of organic phosphates to haemoglobin is essential for

expression of the full Root effect (Brauner and Weber, 1998; Pelster and Weber, 1990; Wells, 2009). The Root and Bohr effects can be exploited during hypoxia to drive O_2 unloading, particularly in the relatively hypoxic and hypercapnic capillary beds around metabolically-active tissues (de Souza and Bonilla-Rodriguez, 2007; Regan and Brauner, 2010; Rummer and Brauner, 2015; Rummer et al., 2013; Wells, 2009).

Beyond gill O_2 uptake and blood O_2 content, O_2 delivery is finely tuned at the level of tissue perfusion by capillary bed, and can be adjusted by sympathetic activation or local vasoactive factors like nitric oxide or adenosine (Nilsson et al., 1994; Nilsson and Renshaw, 2004; Renshaw et al., 2002; Sandblom and Axelsson, 2011). Hypoxia-sensitive and essential tissues (e.g. the heart and brain) tend to be preferentially perfused under oxygen-limited conditions, while vasoconstriction occurs in more hypoxia tolerant and metabolically inactive tissues (e.g. the skeletal muscle), or tissues not critical to immediately survival (e.g. the gut of fasted fish), though this does not occur in all species (Axelsson et al., 2002; Axelsson and Fritsche, 1991). Unlike mammals, fish display reflexive bradycardia when encountering hypoxia, triggered by external chemoreceptors (Farrell, 2007; Sandblom and Axelsson, 2011). Since this bradycardia can be met with increased stroke volume to maintain cardiac output, its functional significance is unclear, but may involve increased oxygenation of the heart, improved contractility, and/or decreased cardiac O_2 demands (Farrell, 2007).

1.3.2 Reliance on O_2-independent ATP production

Above P_{crit}, tissue O_2 supply is sufficient to meet resting metabolic demands, and support some aerobic scope for activity (Lefrançois et al., 2005; Richards, 2009; Richards, 2011). Below this threshold PO_2, there is increasing reliance on O_2-independent pathways of ATP production to meet energetic demands (Richards, 2009). This depends largely on ATP provisioning from anaerobic glycolysis, a series of reactions that lead to the production of lactate (*via* the enzyme lactate dehydrogenase) from pyruvate, rather than the entrance of pyruvate into the tricarboxylic acid cycle (Hochachka and Somero, 1984). Heavy reliance on anaerobic glycolysis is a classic signal of acute hypoxia exposure on fishes, and this is often associated with accumulation of lactate in plasma and tissues, decreases in intracellular pH, and depletion of tissue glycogen stores (Chippari-Gomes et al., 2005; Richards, 2009; Richards et al., 2008; Richards et al., 2007; Speers-Roesch et al., 2012a).

As anaerobic glycolysis is far less efficient in producing ATP per unit of fuel than aerobic metabolism (yielding ~ 5% of glucose's energy potential in normoxic conditions) (Hochachka and Somero, 1984), glycogen levels are generally indicative of tissue capacity to support ATP turnover through anaerobic glycolysis. Accordingly, hypoxia tolerant fishes tend to have higher levels of glycogen compared to intolerant fish (Bickler and Buck, 2007; Fangue et al., 2008; Hochachka, 1980; Richards, 2009; Richards et al., 2008; Vornanen et al., 2009), and higher activities of enzymes involved in this metabolic

pathway, including lactate dehydrogenase (Abbaraju and Rees, 2012; Almeida-Val et al., 2000; Chippari-Gomes et al., 2005; Farwell et al., 2007; Greaney et al., 1980; Kraemer and Schulte, 2004; Mandic et al., 2013; Nilsson and Ostlund-Nilsson, 2008).

Hypoxia acclimation often augments the glycolytic capacity in tissues that are metabolically flexible enough to subsist on anaerobic metabolism. In these situations, flux through anaerobic glycolysis can be favoured by increasing the activity and gene expression of glycolytic enzymes (e.g. lactate dehydrogenase), by reducing carbohydrate flux into the tricarboxylic acid cycle (e.g. through reversible modulation of pyruvate dehydrogenase activity), and/or by decreasing aerobic gene expression or enzyme activity (e.g. cytochrome c oxidase, citrate synthase) (Abbaraju and Rees, 2012; Almeida-Val et al., 1995; Martinez et al., 2006; Richards, 2009; Richards et al., 2008). For example, in *Fundulus grandis* acclimated to chronic constant hypoxia, the activities of enzymes involved in anaerobic glycolysis and carbohydrate metabolism were strongly increased in the liver, moderately increased in the heart and brain, and strongly suppressed in the skeletal muscle (Martinez et al., 2006). Variation between tissues is likely reflective of differences in their metabolic role during hypoxia. The relatively hypoxia-sensitive heart and brain likely benefit from preferential perfusion and a redistribution of blood flow that buffers their exposure to hypoxia (Axelsson and Fritsche, 1991; Soengas and Aldegunde, 2002), whereas the liver, as a major mediator of carbohydrate metabolism, is likely much more metabolically active than the muscle during chronic hypoxia (Martinez et al., 2006).

While allowing for the continued production of ATP during periods of O_2 limitation, an increased rate of anaerobic metabolism is not a sustainable strategy for prolonged survival in hypoxia. This is due to both its inefficiency (anaerobic glycolysis cannot sustain pre-existing, normoxic energy demands without rapid depletion of fermentable substrate) (Hochachka et al., 1996; Hochachka and Dunn, 1983; Hochachka and Somero, 1984), and the accumulation of toxic end-products (i.e. H^+) that introduce further physiological strain (e.g. development of metabolic acidosis) on organisms (Boutilier, 2001; Hochachka et al., 1996). Correction of these deleterious side-effects through the replenishment of glycogen and phosphocreatine stores, lactate metabolism, and correction of acidosis, plus any transcriptomic or proteomic responses induced by reoxygenation, are traditionally associated with the post-hypoxic O_2 debt observed in many fish recovering from hypoxia exposure (Dowd et al., 2010; Lewis et al., 2007; Rytkönen et al., 2012; van den Thillart and Verbeek, 1991). Classically, O_2 debt is an elevated post-hypoxic O_2 consumption beyond resting rates that is maintained for some time after return to normoxia (Johansson et al., 1995; Lewis et al., 2007; Plambech et al., 2013; van den Thillart and Verbeek, 1991). However, there is sometimes a poor relationship between post-hypoxic MO_2 and the common indicators of anaerobic metabolism and acidosis (i.e. lactate production during hypoxia) (Genz et al., 2013; Lewis et al., 2007), suggesting that perhaps other physiological processes like correcting ionic and/or osmotic disturbances (Iftikar et al., 2010; Robertson et al., 2015), variation in digestive processes (López-Olmeda et al., 2012; Montoya et al., 2010), or alterations in circadian rhythms of metabolism (Egg et al.,

2013; Mortola, 2007; Pelster and Egg, 2015) may contribute to the post-hypoxic increase in O_2 consumption.

1.3.3 Reducing ATP demands via *metabolic depression*

Long-term survival of severe hypoxia or anoxia in highly tolerant organisms (e.g. "facultative vertebrate anaerobes" like crucian carp, *Cyprinus carpio*, and the freshwater turtle, *Trachemys scripta*) requires metabolic depression (Corkum and Gamperl, 2009; Hochachka et al., 1996; Hochachka and Dunn, 1983; Johansson et al., 1995; Nilsson and Renshaw, 2004; Richards, 2009; Vornanen et al., 2009). In this scenario, energetic demands are reduced to the point that even the meager O_2 uptake and aerobic metabolism afforded by severe hypoxia and/or low levels of anaerobic metabolism in severe hypoxia or anoxia can meet the need of reduced rates of energy turnover (and usually activity). Mechanistically, metabolic depression is associated with reductions in energetically costly processes, such as ion transport and regulation, protein turnover, growth, and mitochondrial proton leak (Bickler and Buck, 2007; Boutilier, 2001; De Boeck et al., 2013; Richards et al., 2008; Speers-Roesch et al., 2010; Wood et al., 2009; Wood et al., 2007), though the biochemical signalling cascades that induce metabolic rate depression remain elusive (Griko and Regan, 2018; Storey, 1988; Storey and Storey, 2004).

Effective, active reduction of cellular ATP demands to match the limited capacity of anaerobic metabolism is thought to be the major adaptive strategy for prolonged survival

in hypoxia in vertebrates (Boutilier, 2001; Corkum and Gamperl, 2009; Hochachka et al., 1996; Hochachka and Dunn, 1983; Richards et al., 2008; van Waversveld et al., 1989). Low O_2 consumption rates (which are presumably reflective of low ATP demands) have also been correlated with a low P_{crit} in some species (Mandic et al., 2009; Richards, 2011; Speers-Roesch et al., 2010), suggesting low metabolic rates may be adaptive for hypoxia tolerance, even if it is not directly associated with a co-ordinated metabolic depression.

1.3.4 Coping with the potential oxidative stress challenges of chronic hypoxia

In addition to the energetic crisis induced by O_2 lack, hypoxia may cause oxidative stress for aerobic organisms by a disturbing the delicate balance between ROS production and ROS scavenging in the cellular environment (Betteridge, 2000; Clanton, 2007). In order to defend against this oxidative stress, fishes exposed to hypoxic conditions routinely increase antioxidant capacity, presumably in an effort to defend against elevated ROS (Birnie-Gauvin et al., 2017; Lushchak et al., 2005; Lushchak et al., 2001a) and oxidative stress (Lushchak and Bagnyukova, 2007). Fish that are adapted to environments that undergo natural periods of hypoxia or hypoxia-reoxygenation might be expected to mitigate these effects by better managing ROS levels and protecting tissues against oxidative stress. In support of this, chronic exposure to either constant or intermittent patterns of hypoxia has been shown to increase antioxidant enzyme activities and reduce rates of mitochondrial ROS emission in some fish (Du et al., 2016; Lushchak, 2011; Lushchak and Bagnyukova, 2007), and our own work has found that wild pumpkinseed

and bluegill sunfish (which may routinely encounter hypoxic conditions) generally have higher antioxidant enzyme activities than sunfish acclimated to well-oxygenated conditions in captivity, though interspecific variation in antioxidant capacity showed little relationship with the variation in hypoxia tolerance (Borowiec et al., 2016). Moreover, hypoxia adapted animals are sometimes found to have reduced mitochondrial ROS emission in hypoxia (Hickey et al., 2012) compared to hypoxia intolerant animals, though this pattern is not universal (Brown et al., 2012; Lau and Richards, 2019).

The "preparation of oxidative stress hypothesis" argues that the antioxidant responses observed in animals under chronic hypoxic conditions are a pre-emptive response to oxidative stress during reoxygenation, and not a direct response to oxidative stress caused by hypoxia (Giraud-Billoud et al., 2019; Hermes-Lima et al., 2015; Moreira et al., 2016). In this scenario, rapid changes in tissue perfusion and metabolic rate during recovery from metabolic depression leads to an influx of oxygen, rapid oxidation of accumulated succinate that leads to reverse electron transfer to mitochondrial complex I, and a subsequent burst in ROS production (Chouchani et al., 2014; Giraud-Billoud et al., 2019; Hermes-Lima et al., 2015; Murphy, 2009). Animals exposed to predictable periods of hypoxia may therefore maintain elevated antioxidant scavenging capacity through the glutathione system and antioxidant enzymes like catalase and superoxide dismutase to deal with the future overproduction of ROS during reoxygenation, and this has been recorded in a variety of taxa (Giraud-Billoud et al., 2019; Hermes-Lima et al., 2015; Hermes-Lima et al., 1998; Hermes-Lima and Storey, 1993; Moreira et al., 2016; Welker

et al., 2013). However, there have been few detailed examinations of the effects of chronic hypoxia and/or hypoxia-reoxygenation cycles on ROS and redox homeostasis, scavenging capacity, and oxidative stress in hypoxia-tolerant fish.

1.4 Unique effects of intermittent hypoxia

Like constant hypoxia, intermittent cycles of hypoxia can also result in an ATP supply-demand imbalance that could theoretically be dealt with by similar mechanisms as constant hypoxia. On the other hand, the unique features of intermittent hypoxia may favour divergent coping mechanisms from constant hypoxia. For example, as hypoxia bouts during intermittent patterns of hypoxia are typically shorter than bouts of constant hypoxia (typically minutes or hours, compared to hours, days, or sometimes months), slow-onset responses like metabolic depression may be disfavoured compared to responses that allow for animals to rapidly respond to changes in their environment such as an increased use of anaerobic glycolysis or modulation of O_2 uptake and transport. Moreover, the existence of regular recovery periods may allow animals to repeatedly rely on traditionally unsustainable coping strategies, like anaerobic glycolysis, for short periods and thus shift the cost-benefit trade-off of different coping strategies compared to other patterns of hypoxia.

While the regular periods of normoxia or hyperoxia characteristic of intermittent hypoxia allow opportunities for recovery, they may also increase the production of ROS, which

have been implicated in both cellular damage and signal transduction (Finkel, 2011; Lesser, 2006; Lushchak, 2011), and this may affect the mechanisms of acclimation to intermittent hypoxia. In support of this notion, a large body of literature in mammals, primarily in the context of obstructive sleep apnea and ischemia-reperfusion injuries, demonstrates that intermittent hypoxia has widespread physiological, genomic, and developmental consequences that are distinct from constant hypoxia (Bailey et al., 2000; Clanton and Klawitter, 2001; Douglas et al., 2007; Farahani et al., 2008; Fletcher, 2001; Garcia et al., 2000; Kang et al., 2016; Neubauer, 2001; Prabhakar and Semenza, 2012; Semenza, 2006).

The features that distinguish intermittent hypoxia from constant hypoxia in mammals – relatively fast, repeated transitions between normoxia and hypoxia, and recovery periods with non-hypoxic oxygen levels – are also present in the diurnal patterns of hypoxia encountered by many fish species. However, unlike mammals, the effects of intermittent hypoxia on fish and how they compare to the effects of constant hypoxia has received only sporadic attention. There are substantial physiological differences between fish and mammals, such as the physical features of the respiratory media (water is much denser and less O_2-rich than air), O_2 and CO_2 sensing mechanisms and chemoreceptor sensitivities, and haemoglobin function (e.g. the Root effect vs. the Bohr effect) that may prevent direct comparisons between taxa. Moreover, the context of intermittent hypoxia for fish, which is typically relatively slow changes in O_2 availability occurring over hours, often mediated by the photoperiod or tidal cycles, also differs considerably from the

episodic hypoxia used in most mammalian models in both severity (often <5% air saturation in mammals, and less severe in fish) and time course (repeated bouts of seconds or minutes in mammals, compared cycles occurred over hours in fish) (Neubauer, 2001; Porteus et al., 2011; Prabhakar, 2001). Taken together, this means that the general applicability of the vast biomedical literature on the physiological effects of intermittent hypoxia in mammals to fish may be limited (as discussed below). This may be especially true for biological systems that differ substantially between fish and mammals (e.g. O_2 transport), though on the other hand, work focusing on mechanisms of hypoxia tolerance that are convergent between mammals and fish (e.g. many cellular signalling mechanisms, such as HIF-1) may also provide useful insight into the responses of fishes to intermittent patterns of hypoxia.

1.4.1 Cardiorespiratory effects

In mammals, intermittent hypoxia generally induces cardiorespiratory responses that are distinct from constant hypoxia. Whereas the latter tends to reduce the hypoxic ventilatory response *via* a reduction in the initial increase in ventilation and a greater ventilatory decline, episodic hypoxia abolishes the ventilatory decline and increases normoxic ventilation rates (Cao et al., 1992; Dwinell et al., 1997; Easton et al., 1986; Garcia et al., 2000; Powell and Garcia, 2000; Prabhakar and Kline, 2002; Turner and Mitchell, 1997). Some of these effects are linked to altered carotid body function under intermittent hypoxia due to increased ROS and subsequent altered ROS signalling (Del Rio et al.,

2010; MacFarlane and Mitchell, 2008; MacFarlane et al., 2008; Peng et al., 2006; Pialoux et al., 2009; Prabhakar, 2001; Prabhakar et al., 2001; Prabhakar et al., 2007).

Cyclic hypoxia exposure leads to reversible, hypoxia-induced splenic contraction and increased whole blood haemoglobin content in rats, and this may protect O_2 supply during hypoxia without the cardiovascular burden of polycythemia during normoxia (Kuwahira et al., 1999). Intermittent hypoxia as well as constant hypoxia also increases blood pressure, sympathetic activity, and the amount of and sensitivity to plasma catecholamines in mammalian models (Bao et al., 1997; Brooks et al., 1997; Fletcher, 2001; Fletcher et al., 1992; Kumar et al., 2006; Prabhakar et al., 2001), though in the case of constant hypoxia, some of these effects are blunted with chronic exposure (Banchero, 1987; Prabhakar and Kou, 1994). Finally, while short-term exposure to intermittent hypoxia seems to precondition the heart against ischemia/reperfusion injury and improve cardiac function (increased O_2 supply and gene expression of antioxidant enzymes), chronic exposure seems to induce cardiac dysfunction (Neubauer, 2001; Yin et al., 2012).

Exposure to intermittent or diurnal hypoxia also seems to alter the respiratory physiology of fishes, though these investigations are much more limited than in mammals. Prolonged exposure to diel O_2 cycles improved aerobic swimming performance in hypoxia in southern catfish (*Silurus meridionalis*) (Yang et al., 2013) and increases the resting metabolic rate of summer flounder (*Paralichthys dentatus*) (Taylor and Miller, 2001), though the underlying physiological mechanisms of these changes remain to be

investigated. One mechanism that may be involved is improvement in O_2 transport capacity at either the level of the gills or blood, allowing for a corresponding increase in resting oxygen consumption rate. Indeed, two repeated cycles of diurnal hypoxia have been shown to increase the O_2 affinity of arterial blood in carp (*C. carpio*) chiefly through action on the negative allosteric modifier GTP, and to increase haematocrit of this species (Lykkeboe and Weber, 1978). Intertidal epaulette sharks (*Hemiscyllium ocellatum*) also show an increase in haematocrit following a single bout of anoxia and within 2 h of reoxygenation event (Chapman and Renshaw, 2009). Chronic oscillating (nocturnal) hypoxia for 28 d also induces as similar nighttime-specific elevation in haematocrit in southern flounder (*Paralichthys lethostigma)* (Taylor and Miller, 2001).

1.4.2 Oxidative stress and cellular signalling

Rapid, biomedically relevant patterns of intermittent hypoxia (bouts of severe hypoxia over seconds or minutes) are associated with increased production of reactive oxygen species and/or oxidative stress in mammals (Chen et al., 2005; Friedman et al., 2014; Pialoux et al., 2009; Prabhakar and Kumar, 2004; Row et al., 2003; Semenza and Prabhakar, 2007; Troncoso Brindeiro et al., 2007; Xu et al., 2004). Accordingly, elevated ROS or oxidative damage has been linked to many of the unique effects of intermittent hypoxia, such as altered carotid body function, including increased sensitivity to hypoxia and an altered hypoxic ventilatory response (Del Rio et al., 2010; Peng et al., 2003; Pialoux et al., 2009; Prabhakar, 2001; Prabhakar et al., 2001; Prabhakar et al., 2007), as

well as increased systematic blood pressure and arteriosclerosis linked to increased circulating plasma catecholamines, oxidative stress, and inflammation (Chen et al., 2005; Fletcher, 2001; Fletcher et al., 1992; Friedman et al., 2014; Gozal and Kheirandish-Gozal, 2008; Kumar et al., 2006; Prabhakar et al., 2001; Savransky et al., 2007; Troncoso Brindeiro et al., 2007). Exposure to these biomedically relevant patterns of intermittent hypoxia also leads to altered metabolism, including dyslipidemia, insulin resistance, and mitochondria dysfunction (Drager et al., 2010; Peng et al., 2003). Finally, these patterns of intermittent hypoxia alter the expression of a number of redox sensitive genes involved in many of the systems described above (Prabhakar et al., 2007; Suzuki et al., 2006), compounding the direct effects of ROS and oxidative stress.

One well-studied redox and oxygen sensitive transcription factor is hypoxia inducible factor 1α (HIF-1α), a master regulator of oxygen homeostasis. HIF signalling regulates several processes key to the vertebrate hypoxia response, including angiogenesis, energy metabolism, and the hypoxic chemoreflex (Nikinmaa et al., 2004; Robertson et al., 2014; Semenza, 2000; Semenza, 2006; Semenza and Prabhakar, 2007). HIF-1α is indirectly stabilized by ROS through at least two signal transduction pathways (Semenza and Prabhakar, 2007) and HIF-1α protein abundance is typically elevated in mammals by intermittent hypoxia (Prabhakar and Semenza, 2012; Semenza, 2009). Altered HIF-1α signalling has been implicated in the effects of intermittent hypoxia on the carotid body and cardiovascular system, and mice heterozygous for a HIF-1α knockout exposed to intermittent hypoxia do not develop elevated circulating catecholamines or ROS, and lack

a carotid body response to hypoxia (Peng et al., 2006; Semenza and Prabhakar, 2007). Differences in key cell signalling events like HIF-1α expression may play an important mechanistic role in the development of the intermittent hypoxia acclimation response.

The implications of intermittent hypoxia for oxidative stress and redox or oxygen-sensitive cellular signalling pathways is much less understood in fish. Transcript levels of genes regulating oxygen and energy homeostasis are amplified after repeated hypoxia bouts relative to a single hypoxia bout in the epaulette shark (*Hemiscyllium ocellatum*), including some genes involved in energy metabolism, suggesting there is a pre-conditioning or acclimation component in the response to intermittent hypoxia (Rytkönen et al., 2012). Beyond studies detailing the effects of single bouts of hypoxia and reoxygenation on fishes (Lushchak et al., 2005; Lushchak et al., 2001b; Welker et al., 2012), there is relatively little work on how repeated or chronic exposure to cycles of intermittent hypoxia alter oxidative stress defense systems and cellular signalling, including reactive oxygen species, and how these responses may differ rom acute exposure or other patterns of hypoxia.

1.5 Fundulidae as a model for understanding intermittent hypoxia in fishes

The Fundulidae family (Teleostei, Cyprinodontiformes) occupies a diverse range of habitats in coastal and inland systems of North and Central America, with limited distribution in the Caribbean (Burnett et al., 2007; Whitehead, 2010). The habitats of even

closely related species vary considerably in salinity, dissolved oxygen content, temperature, pH, and other conditions (e.g. the sister taxa *Luciana parva* and *L. goodei* inhabit salt marshes and freshwater systems, respectively) (Burnett et al., 2007; Griffith, 1974; Whitehead, 2010). The large variation in physiological tolerance amongst closely related Fundulids as well as the extensive physiology data available for some members (e.g. *F. heteroclitus* and *F. grandis*) (Whitehead, 2010) mean that this group of fish is an excellent potential model for testing hypotheses about the evolution of hypoxia tolerance.

The hypoxia-tolerant mummichog (*Fundulus heteroclitus*) is a popular model in comparative and evolutionary physiology and toxicology (Burnett et al., 2007). *F. heteroclitus* naturally occurs in salt marshes and estuaries along the east coast of North America, with a range extending from the northern shore of the St. Lawrence River to southern Florida. *F. heteroclitus* is non-migratory (Skinner et al., 2005), and populations may experience variations in oxygen content on both a seasonal (e.g. ice cover in the northern area of its range) and daily (e.g. tidal movements) basis. Accordingly, these animals are an excellent model for elucidating the physiological mechanisms involved in coping with different patterns of hypoxia.

Differences in both the geophysical characteristics in the habitats of different Fundulids (Fuller, 2008; Fuller et al., 2007) and in osmotic tolerance ranges (Nordlie, 2006; Whitehead, 2010) have been suggested to contribute to species distributions in Fundulid fishes. Speciation within *Fundulus* appears to have been associated with a contraction of

physiological plasticity in response to salinity, as more narrowly-distributed freshwater specialists have generally been derived from broadly-distributed generalist types (Whitehead, 2010). Whether hypoxia, another environment stressor commonly encountered by *Fundulus* fishes in the wild, shows a similar pattern is unclear.

1.6 Justification for thesis work

1.6.1 Intermittent hypoxia is ecologically relevant for wild fishes

Though well-documented in the aquatic environment (Richards, 2011; Tyler et al., 2009), and known in mammalian models to have widespread and distinct physiological, genomic and developmental consequences from constant hypoxia (Clanton and Klawitter, 2001; Douglas et al., 2007; Farahani et al., 2008; Fletcher, 2001; Neubauer, 2001; Prabhakar, 2001), the effects of intermittent hypoxia on fish have received little attention. It is poorly understood how the physiological effects of intermittent hypoxia compare to those of constant hypoxia, and the extent to which hypoxia pattern influences the acclimation response to hypoxia. As the occurrence of all patterns of hypoxia increases worldwide, it is important to appreciate the differences between them, and their potential impacts on aquatic organisms (Diaz, 2001; Ficke et al., 2007).

1.6.2 Intermittent hypoxia and aquaculture

Though cycles of intermittent hypoxia are well-documented in natural environments (Richards, 2011; Tyler et al., 2009), they are also common in aquaculture settings, especially at high stocking densities and in shallow water pens, and such hypoxia cycles are known to negatively impact fish growth (Burt et al., 2013; Green et al., 2012; Remen et al., 2014; Remen et al., 2012), immune function (Burt et al., 2013; Wang et al., 2018), and sometimes the feeding behaviour of economically important fishes (Burt et al., 2013; Green et al., 2012; Remen et al., 2014; Remen et al., 2012). However, this is not always the case, as mild intermittent hypoxia improved growth rate and feeding rate in juvenile qingbo (Dan et al., 2014) and had very mild effects on sea bass (Thetmeyer et al., 1999). Encounters with intermittent hypoxia also alter the reproductive success of fishes, and this can occur through direct effects on the reproductive capacity of adult fish (Bera et al., 2017; Cheek et al., 2009) and/or increased offspring mortality (Bose et al., 2019). Understanding how intermittent hypoxia alters fish growth and reproduction could have major implications for animal husbandry practices.

1.6.3 Intermittent hypoxia may have novel physiological effects on fishes

In addition to the physiological effects common to oxygen limitation in general, the periods of reoxygenation characteristic of intermittent hypoxia may present some unique challenges to fish, and thus induce a different acclimation response than a constant pattern

of hypoxia. While allowing an opportunity to recover from hypoxic bouts, and perhaps altering the cost-benefit trade-off between certain energy strategies over others, these reoxygenation periods may also increase the production of ROS, which have been implicated in both cellular damage and signal transduction, and this may have downstream effects on the hypoxia acclimation response (Lesser, 2006; Prabhakar, 2001; Prabhakar et al., 2001; Prabhakar et al., 2007; Semenza, 2006; Semenza and Prabhakar, 2007).

1.7 General objectives & objectives of subsequent chapters

The general objective of this thesis was to determine how fish cope with intermittent (diurnal) hypoxia. More specifically, this thesis investigated if, and how, the physiological coping strategy for intermittent hypoxia differs from the coping strategy for constant (continuous) hypoxia in *Fundulus* killifish. I used an integrative approach to test the general hypothesis that acclimation to intermittent hypoxia induces unique physiological coping responses (compared to constant hypoxia) at the whole animal level and in underlying traits putatively related to hypoxia tolerance and/or defense against oxidative stress.

In the subsequent chapters, four manuscripts (two published, two in preparation for submission) that explicitly tested this hypothesis are presented. A fifth manuscript (in review for publication) that indirectly tested this hypothesis by examining the broader

variation in the plasticity of hypoxia tolerance across Fundulid killifishes is also presented. The general outcome of this thesis is an integrated understanding of how fish cope with intermittent hypoxia, and how hypoxia pattern influences the hypoxia coping response of fishes. This thesis contributes substantial insight into an ecologically and economically important part of natural encounters with hypoxia for fishes, and the general mechanisms by which animals can respond to the contemporary environmental challenges of the Anthropocene.

*1.7.1 Chapter 2: Distinct physiological strategies are used to cope with constant hypoxia and intermittent hypoxia in killifish (*Fundulus heteroclitus*).*

1.7.1.1 Objectives

How does acclimation to intermittent (diurnal) hypoxia impact the physiology of killifish, and how does it differ from constant (continuous) hypoxia? The objectives of the study described in Chapter 2 were to (i) compare how acclimation these patterns of hypoxia affected the hypoxia tolerance of *Fundulus heteroclitus*, and (ii) investigate how the hypoxia acclimation response differed between fish acclimated to intermittent hypoxia and constant hypoxia at multiple levels of biological organization. To achieve these objectives, we combined whole animal respirometry with measurements of several underlying physiological traits putatively related to hypoxia tolerance, including gill

morphology, haematology, capillarity and the fibre composition of skeletal muscle, and the activities of metabolic enzymes in the muscle, liver, heart, and brain.

*1.7.2 Chapter 3: Distinct metabolic adjustments arise from acclimation to constant hypoxia and intermittent hypoxia in estuarine killifish (*Fundulus heteroclitus*).*

1.7.2.1 Objectives

The results from Chapter 2 suggested that nocturnal intermittent hypoxia and constant hypoxia were distinct stressors for killifish. Constant hypoxia and intermittent hypoxia lead to distinct changes in O_2 transport and metabolism. However, the implications of these distinct responses for animal performance, particularly in terms of metabolism and balancing cellular ATP supplies, were unclear. Furthermore, Chapter 2 only examined animals during the day, and did not address how the physiology of killifish acclimated to intermittent hypoxia, changed during the 24 h hypoxia-reoxygenation cycle.

The objective of the study described in Chapter 3 was (i) to better characterize the whole-animal and tissue-level metabolic consequences of acclimation to constant hypoxia and intermittent hypoxia during both the day and nighttime phases of a hypoxia-reoxygenation cycle. To do this, we conducted a time course study of O_2 consumption rate and tissue metabolites in normoxia-acclimated fish exposed acute hypoxia and to fish acclimated to either pattern of chronic hypoxia.

*1.7.3 Chapter 4: Reversible modulation of blood O_2 carrying capacity in estuarine killifish (*Fundulus heteroclitus*) acclimated to intermittent hypoxia*

1.7.3.1 Objectives

Chapters 2 and 3 demonstrate that nocturnal intermittent hypoxia and constant hypoxia were distinct stressors for killifish, and this involved changes in metabolism and potentially O_2 transport capacity. However, how the latter changes over the hypoxia-reoxygenation cycle (e.g. during nighttime hypoxia) in intermittent hypoxia has yet to be examined in detail. This is especially interesting as many indices of O_2 transport capacity, such as blood haemoglobin content and haemoglobin-O_2 binding affinity, can change rapidly and reversibly with hypoxia exposure, and therefore may be especially well-suited for coping with repeated hypoxia-normoxia cycles. Moreover, our previous work suggested that fish acclimated to intermittent hypoxia seemed to have elevated O_2 transport capacity, being able to maintain high MO_2 in hypoxia and reach very high MO_2 during reoxygenation (Borowiec et al., 2018, see Chapter 3 of this volume).

The objective of Chapter 4 was to (i) characterize the variation of O_2 transport capacity in fish acclimated to constant hypoxia and intermittent hypoxia in a time-sensitive manner. To do this, we sampled normoxia-acclimated fish exposed acute hypoxia and fish acclimated to either pattern of chronic hypoxia during the day (analogous to the 18 h time

point in Chapter 3 and 5) and night (analogous to the 6 h time point in Chapters 3 and 5). We measured indicators of blood O_2 carrying capacity, including haematocrit, haemoglobin content, and red blood cell ATP and GTP concentrations.

*1.7.4 Chapter 5: Hypoxia acclimation alters reactive oxygen species homeostasis and oxidative status in estuarine killifish (*Fundulus heteroclitus*)*

1.7.4.1 Objectives

Beyond the potential development of an ATP supply-demand balance, hypoxia and/or hypoxia-reoxygenation cycles may also challenge animals by inducing oxidative stress. Results from Chapters 2, 3, and 4 demonstrated that intermittent hypoxia and constant hypoxia are different stressors that induce substantial alterations in a number of physiological systems, including some directly or indirectly related to oxidative stress such as O_2 consumption rate (Borowiec et al., 2015, see Chapter 2 of this volume; Borowiec et al., 2018, see Chapter 3 of this volume), as well as mitochondrial ROS emission and oxidative damage (Du et al., 2016).

Our objectives for Chapter 5 were to investigate (i) how acute hypoxia and reoxygenation affected oxidative stress in killifish, and (ii) how acclimation to constant hypoxia or repeated hypoxia-normoxia cycles (intermittent hypoxia) modulated these responses. To do this, we used a similar approach to Chapter 3, and tracked the levels of reactive

oxygen species and the responses of oxidative stress defense system in killifish exposed to a cycle of acute hypoxia and reoxygenation in normoxia.

1.7.5 Chapter 6: Evolutionary variation in hypoxia tolerance and hypoxia acclimation responses in killifish from the family Fundulidae

1.7.5.1 Objectives

Chapters 2, 3, 4, and 5 suggest that strategies for coping with hypoxia vary substantially according to the pattern of hypoxia exposure in *Fundulus heteroclitus*. How broadly applicable these effects are is unclear, as we know relatively little about how the magnitude of plasticity in hypoxia tolerance varies across closely related species. Our objectives for Chapter 6 were to better understand (i) the interspecific variation in hypoxia tolerance across Fundulidae, and (ii) whether there is interspecific variation in the plasticity of hypoxia tolerance in response to chronic hypoxia. We also (iii) examined the relationships between different indices of hypoxia tolerance across a variety of taxa.

1.7.6 Chapter 7: General Discussion

This chapter presents an integrated overview of the results, their caveats, and their contribution to the wider body of literature on hypoxia in fishes and other vertebrates. Potential future directions are also discussed.

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CHAPTER 2

Distinct physiological strategies are used to cope with constant hypoxia and intermittent hypoxia in killifish (*Fundulus heteroclitus*)

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2.1 Abstract

Many fish encounter hypoxia on a daily cycle, but the physiological effects of intermittent hypoxia are poorly understood. We investigated whether acclimation to constant (sustained) hypoxia or to intermittent diel cycles of nocturnal hypoxia (12 h normoxia: 12 h hypoxia) had distinct effects on hypoxia tolerance or on several determinants of O_2 transport and O_2 utilization in estuarine killifish. Adult killifish were acclimated to normoxia, constant hypoxia, or intermittent hypoxia for 7 or 28 days in brackish water (4 ppt). Acclimation to both hypoxia patterns led to comparable reductions in critical O_2 tension and resting O_2 consumption rate, but only constant hypoxia reduced the O_2 tension at loss of equilibrium. Constant (but not intermittent) hypoxia decreased filament length and the proportion of seawater-type mitochondrion-rich cells in the gills (which may reduce ion loss and the associated costs of active ion uptake), increased blood haemoglobin content, and reduced the abundance of oxidative fibres in the swimming muscle. In contrast, only intermittent hypoxia augmented the oxidative and gluconeogenic enzyme activities in the liver and increased the capillarity of glycolytic muscle, each of which should facilitate recovery between hypoxia bouts. Neither exposure pattern affected muscle myoglobin content or the activities of metabolic enzymes in the brain or heart, but intermittent hypoxia increased brain mass. We conclude that the pattern of hypoxia exposure has an important influence on the mechanisms of acclimation, and that the optimal strategies used to cope with intermittent hypoxia may be distinct from those for coping with constant hypoxia.

2.2 List of symbols & abbreviations

COX	Cytochrome c oxidase
CS	Citrate synthase
SEM	Scanning electron micrograph
HOAD	3-hydroxyacyl-CoA dehydrogenase
LDH	Lactate dehydrogenase
LOE	Loss of equilibrium
MO_2	Rate of oxygen consumption
MRC	Mitochondrion-rich cell
PEPCK	Phosphoenolpyruvate carboxykinase
P_{crit}	Critical oxygen tension
PO_2	Partial pressure of oxygen
PK	Pyruvate kinase
PVC	Pavement cell
ROS	Reactive oxygen species
SDH	Succinate dehydrogenase

2.3 Introduction

Variations in oxygen availability influence the quantity and quality of habitat available for fish (Graham, 1990; Burnett, 1997; Breitburg et al., 2009). Relatively stable, constant hypoxia can develop in ice-covered or stratified lakes, or following eutrophication events (Diaz, 2001; Diaz and Rosenberg, 2008). Intermittent patterns of hypoxia exposure are common in tide pools and estuaries due to a variety of factors, including daily cycles of respiration and photosynthesis (Breitburg, 1992; Diaz, 2001; Tyler at al., 2009). Overall, the incidence of aquatic hypoxia is expected to rise due to global climate change, urbanization, pollution, and other anthropogenic causes (Diaz, 2001; Ficke et al., 2007).

The major challenge presented by hypoxia is the potential development of a cellular ATP supply-demand imbalance (Hochachka et al., 1996; Boutilier, 2001). Fish encountering hypoxia often attempt to maintain cellular ATP supply either by increasing O_2 transport to support aerobic respiration, or by increasing the use of anaerobic energy metabolism. Tissue O_2 supply can be improved by increasing branchial O_2 uptake (e.g., increasing ventilation, lamellar perfusion) or the rate of circulatory O_2 transport (e.g., increasing haemoglobin content or blood flow, changes in the concentration of allosteric modifiers) to counteract the effects of O_2 limitation on aerobic metabolism in hypoxia (Holeton and Randall, 1967; Hughes, 1973; Greaney and Powers, 1977, 1978; Nikinmaa and Soivio, 1982; Claireaux et al., 1988; Weber and Jensen, 1988; Perry et al., 2009). At any oxygen tension (PO_2) above the critical oxygen tension (P_{crit}), tissue O_2 supply is sufficient to

meet metabolic demands, and support some aerobic scope for activity (Burton and Heath, 1980; Pörtner and Grieshaber, 1993; Lefrancois et al., 2005; Pörtner, 2010). Below P_{crit}, aerobic metabolism becomes dependent upon and decreases with environmental PO_2, and anaerobic metabolism is often used to help supplement ATP supply (Dunn and Hochachka, 1986; Pörtner and Grieshaber, 1993; Scott et al., 2008; Richards, 2009). Anaerobic metabolism can be favoured by increasing the activity and gene expression of glycolytic enzymes (e.g., lactate dehydrogenase), or by reducing carbohydrate flux into the tricarboxylic acid cycle and decreasing aerobic enzyme activity (e.g., cytochrome c oxidase, citrate synthase) (van den Thillart et al., 1980; van den Thillart et al., 1994; Almeida-Val et al., 1995; Martinez et al., 2006; Richards et al., 2008). In addition to mechanisms that help maintain ATP supply, some tolerant organisms can also reduce ATP demands through active depression of resting metabolic rate (van Waversveld et al., 1989; van Ginneken et al., 1997). This is associated with reductions in energetically costly processes, such as ion transport, protein synthesis, and mitochondrial proton leak (Bickler and Buck, 2007; Wood et al., 2007, 2009; De Boeck et al., 2013). The reliance on each of these strategies to balance ATP supply and demand varies between species, and could foreseeably be altered by the pattern or severity of hypoxia exposure.

The effects of intermittent hypoxia on fish physiology are poorly understood. This is starkly contrasted by the extensive literature on intermittent hypoxia in mammals, which has uncovered widespread physiological, developmental, and genomic consequences that are distinct from continuous hypoxia exposure (Neubauer, 2001; Douglas et al., 2007;

Farahani et al., 2008). There is evidence that exposure to repeated bouts of hypoxia compromises growth in some fish species (Atlantic salmon, *Salmo salar*, and southern catfish, *Silurus meridionalis*) but not others (spot, *Leiostomus xanthurus*, and killifish, *Fundulus heteroclitus*) (Stierhoff et al., 2003; McNatt and Rice, 2004; Yang et al., 2013; Burt et al., 2014). Exposure to daily oxygen cycles has been observed to increase hypoxia tolerance and aerobic swimming performance in hypoxia in southern catfish (Yang et al., 2013), to increase resting metabolism measured in normoxia in summer flounder (*Paralichthys dentatus*) (Taylor and Miller, 2001), and to reduce red blood cell GTP concentration and increase plasma bicarbonate concentration in carp (*Cyprinus carpio*) (Lykkeboe and Weber, 1978). Repeated 2 h bouts of hypoxia has also been shown to amplify some of the transcriptional responses to hypoxia compared to a single bout in the epaulette shark (*Hemiscyllium ocellatum*), including some genes in oxygen and energy homeostasis pathways (Rytkönen et al., 2012). However, the extent to which the physiological effects of intermittent hypoxia differ from those of constant hypoxia is unclear. Intermittent hypoxia differs from constant hypoxia as a stressor because it potentiates the production of reactive oxygen species (ROS), which may cause oxidative stress, but it also provides opportunities for recovery during the oxygenated periods between hypoxia bouts – distinctions that could favour divergent coping mechanisms between these two patterns of hypoxia exposure.

The objectives of this study were (i) to compare the effects on hypoxia tolerance of acclimation to constant hypoxia *versus* intermittent diel cycles of nocturnal hypoxia and

(ii) to investigate the physiological mechanisms underlying the acclimation response to each pattern of hypoxia exposure. We examined the killifish *Fundulus heteroclitus*, an estuarine species that copes with both seasonal and daily fluctuations in dissolved oxygen content in its native habitat (Stierhoff et al., 2003; Burnett et al., 2007; Tyler et al., 2009). These fluctuations can be sudden, severe, and are mediated by factors such as the daily interplay between photosynthesis and cellular respiration, tidal movements, temperature, and wind patterns (Tyler et al., 2009). We integrated whole-animal respirometry with measurements of subordinate physiological traits dictating oxygen transport and utilization, including gill morphology, haematology, capillarity and fibre composition of skeletal muscle, and the activities of metabolic enzymes in the skeletal muscle, liver, heart, and brain.

2.4 Materials & methods

2.4.1 Study animals and experimental hypoxia acclimations

Adult, wild-caught *Fundulus heteroclitus* of both sexes were purchased from a commercial supplier (Aquatic Research Organisms, NH, USA), shipped to McMaster University, and held for at least one month in brackish (4 ppt) water at room temperature (~21^{o}C) before experimentation. Water quality (pH, nitrates, nitrites and ammonia) was maintained with regular water changes. Fish were fed commercial flakes (Big Al's

Aquarium Supercentres, Mississauga, ON, Canada) six days weekly, and were not fed for 24 hours prior to respirometry or sampling.

Exposures were carried out in 35 L glass aquaria with the same water chemistry as described above, in which normoxia, constant hypoxia, or nocturnal (intermittent) hypoxia was sustained for 7 or 28 days (Table 2.5). Constant normoxia (20 kPa, 8 mg O_2 L^{-1}) was maintained by continuously bubbling the water with air. Constant hypoxia was maintained by bubbling the water with nitrogen gas, and the appropriate PO_2 was maintained by a feedback loop using a galvanic oxygen sensor that automatically controlled the flow of nitrogen with a solenoid valve (Loligo Systems, Tjele, Denmark). Nocturnal ('intermittent') hypoxia was maintained using the same O_2 controller as for constant hypoxia, but gas flow was alternated between air (8 am to 8 pm local time) and nitrogen (8 pm to 8 am) with an additional solenoid valve controlled by a photoperiod timer that was synchronized with the light cycle (12 h light: 12 h dark). During the hypoxic periods, the set-point (i.e. 5 or 2 kPa) was tightly regulated with a hysteresis of 0.02 kPa. Larger deviations from set-point were infrequent and never exceeded 0.4 kPa. Two levels of hypoxia were used for 7 d exposures, either moderate (5 kPa, 2 mg O_2 L^{-1}) or severe (2 kPa, 0.8 mg O_2 L^{-1}), but only moderate hypoxia was used for 28 d exposures. Fish were prevented from respiring at the water surface with a plastic grid barrier. The body masses and standard body lengths of fish used for each series of experiments are shown in Table 2.5.

2.4.2 Respirometry experiments

Stop-flow respirometry was used to determine MO_2, P_{crit}, and the PO_2 at LOE. Fish were held overnight in normoxia in a respirometry chamber (90 mL cylindrical glass) that was situated in a darkened buffer tank and continuously flushed with normoxic water (flushing circuit). The chamber was also connected to a recirculating circuit that flowed passed a fibre-optic oxygen sensor (PreSens, Regensburg, Germany). Both circuits were driven by pumps, controlled by AutoResp software (Loligo systems, Tjele, Denmark).

Oxygen consumption measurements began the following morning in normoxia, with two sequential flush and measurement periods. During flush periods, both the flush and recirculating pumps were active, and the chamber received a steady flow of water from the buffer tank (i.e. the chamber and buffer tank were equilibrated). During measurement periods, the flush pump was turned off, isolating the chamber from the buffer tank, and the change in oxygen concentration due to fish respiration was measured. Fish were then subjected to a progressive hypoxia protocol, in which buffer tank PO_2 was reduced in 2 kPa increments using the O_2 control system described above. Each level of hypoxia was maintained for 10 min, and oxygen consumption rate was measured as in normoxia. After measurement at an ambient PO_2 of 2 kPa, the chamber was closed such that the fish consumed the remaining oxygen until it lost equilibrium. Oxygen consumption was calculated from the change in chamber oxygen concentration over time as previously

recommended (Clark et al., 2013). P_{crit} was calculated using a program developed by Yeager and Ultsch (Yeager and Ultsch, 1989).

Immediately after losing equilibrium, fish were euthanized by a blow to the head followed by pithing. The tail was bisected at the base of the anal fin, and blood was collected from the caudal blood vessels in a heparinized capillary tube. Whole blood was used to measure haemoglobin content (using Drabkin's Reagent following manufacturer instructions; Sigma-Aldrich, Oakville, ON, Canada) or was frozen in liquid nitrogen and stored at -80^{o}C for later determination of lactate concentration. Some blood was also centrifuged for 5 minutes at 14,000 rpm to measure haematocrit.

2.4.3 Sampling

A separate set of fish from those used for respirometry were acclimated and sampled at rest (rather than at LOE). Sampling was done at a consistent time of day (between 1 pm and 4 pm) to minimize the effect of circadian rhythms and other diurnal variations on our results. Sampling was therefore during the normoxic period for the intermittent hypoxia acclimation groups. Fish were euthanized and blood was collected and analyzed as described above. A transverse steak of the trunk was cut at the anterior base of the anal fin, coated in embedding medium (Fisher Scientific Company, Ottawa, ON, Canada), frozen in liquid N_2-cooled isopentane, and stored at -80^{o}C until use for muscle histology. An adjacent hemi-section of the axial muscle (containing the entirety of the red and white

fibres) and the entire intact liver, heart, and brain were removed, frozen immediately in liquid nitrogen, and stored at -80°C for later measurement of enzyme activities. The gill baskets were removed intact and fixed (2% paraformaldehyde, 2% glutaraldehyde) at 4°C.

2.4.4 Gill morphometrics

After fixing, the four arches on one side of the gill basket were isolated and cleaned of excess tissue such that individual filaments and lamellae were visible. Images at 10x magnification were taken on each side of the arches using a stereomicroscope to determine the length and number of filaments. Total gill filament length for an entire fish was calculated by doubling the sum of all individual filament lengths measured, and then multiplying the result by 1.15 to account for the approximate effects of curling, as recommended previously (Hughes, 1966).

Six fish from each 28 d treatment group that had a similar body mass (3 to 5 g) were selected for scanning electron microscopy. Filaments from the middle portion of one side of the second gill arch were removed to enable viewing of both the leading and trailing edges of the remaining filaments. The gills were postfixed in 1% OsO_4 for one hour and then dehydrated in progressively higher concentrations of ethanol (from 50% to 100%). After critical point drying with liquid CO_2, samples were sputter-coated and viewed in an ESEM 2020 scanning electron microscope (Electroscan Corporation, Wilmington, MA,

USA). Images were taken at 350x and 500x magnification to determine gill filament depth (parallel to water flow), maximum lamellar height (perpendicular to the filament and to water flow), and lamellar depth (parallel to filament depth). High-magnification images were taken at 2000x and 5000x to quantify MRC density, MRC and PVC size, and MRC phenotype (seawater-type apical crypts or freshwater, transitional-type MRCs for which the cell surface is visible) (Scott et al., 2004; Laurent et al., 2006). PVC and MRC measurements were restricted to the trailing edge. All gill morphometric images were analyzed using ImageJ software (Rasband, 2014).

2.4.5 Muscle histology

Muscle blocks were cut into 10 μm sections at -20°C with a cryostat (Leica Microsystems GmbH, Wetzler, Germany), mounted on Superfrost Plus slides (Fisher Scientific Company, Ottawa, ON, Canada), air dried, and stored at -80°C until staining. Staining of sections was performed using standard methods that have been previously described (Egginton, 1990; Scott and Johnston, 2012). Sections were stained for succinate dehydrogenase (SDH) activity to identify oxidative muscle fibres, using the following assay conditions: 41.7 mM Na_2HPO_4, 8.3 mM NaH_2PO_4, 80 mM sodium succinate, 0.1% NBT, pH 7.6. Alkaline phosphatase activity was used to stain for capillaries, using the following assay conditions (in mM): 28 $NaBO_2$, 7 $MgSO_4$, 1 NBT, 0.5 BCIP, pH 9.3. Sections were stained for 1 hour at room temperature in each protocol, after which slides were mounted with Aquamount (Fisher Scientific Company, Ottawa, ON, Canada) and

stored at 2°C until they were imaged with a Nikon Eclipse E800 light microscope (Nikon Instruments Inc., Melville, NY, USA).

The total transverse area and number of SDH-positive (oxidative) muscle fibres were determined for each fish from the SDH activity stains. Average oxidative fibre size was calculated by dividing the area by the number of fibres within that area. We observed two distinct patterns of SDH-positive staining intensity – one indicative of high oxidative capacity (HOx; dark SDH staining throughout the fibre) and one indicative of modest oxidative capacity (MOx; less intense but still positive SDH staining, usually near the fibre periphery) (Fig. 2.7) – so we also quantified the total number of fibres exhibiting each staining pattern. We quantified from only one lateral side of the fish, but we multiplied the results by two to calculate the total oxidative fibre area and number of fibres in the entire trunk. Based on their location in the axial muscle, the fibres we characterized as having a high oxidative capacity included both slow oxidative and a subset of fast oxidative fibre types (Scott and Johnston, 2012; McClelland and Scott, 2014).

Average glycolytic fibre size, capillary density, and capillary-to-fibre ratios were determined from the alkaline phosphatase stains. Average glycolytic (SDH-negative) fibre size was determined from 6-8 images taken throughout the white muscle of each fish, by dividing the known area of the image by the unbiased number of fibres within the image (Egginton, 1990). Capillary density (number of capillaries counted per unit of area)

and capillary-to-fibre ratio (the number of capillaries relative to the number of fibres counted in sections) were quantified in the entirety of the highly oxidative region on one lateral side of the fish. The same capillarity indices were determined from 6-8 images taken throughout the glycolytic region. Preliminary assessments verified that a sufficient number of images was analyzed to account for heterogeneity across the axial musculature (determined by the number of replicates necessary to yield a stable mean value). For all histological measurements, the average value for each fish was calculated and used for statistical comparison between treatment groups. All muscle histology images were analyzed using ImageJ by an observer that was blind to treatment group.

2.4.6 Assays

The maximal activities of several enzymes were assayed using standard methods (Bears et al., 2006; Schnurr et al., 2014). The frozen muscle, liver, heart, and brain samples were weighed and homogenized on ice in 20 volumes of homogenization buffer (20 mM hepes, 1 mM sodium EDTA, and 0.1% triton X-100) at pH 7.0. Assays were performed to determine the maximal activity of each enzyme in the tissue homogenate at 25^{o}C, by measuring the rate of change in absorbance at 550 nm (COX), 340 nm (HOAD, LDH, PEPCK, PK), or 412 nm (CS) for at least 5 minutes. The COX assay was performed immediately following homogenization, after which the homogenate was stored at -80°C. Other enzymes were assayed after a consistent number of freeze-thaw cycles for each enzyme. Assay conditions were as follows: COX, 100 μM (brain, liver, and heart) or 50

μM (muscle) of fully reduced cytochrome c in 50 mM tris containing 0.5% tween-20 at pH 8.0; CS, 1.0 mM oxaloacetate and 0.15 mM acetyl-CoA (brain), or 0.5 mM oxaloacetate and 0.3 mM acetyl-CoA (other tissues), each in 50 mM tris containing 0.1 mM DTNB at pH 8.0; HOAD, 0.1 mM acetoacetyl-CoA and 0.3 mM NADH in 50 mM imidazole at pH 7.2; LDH, 0.3 mM NADH and 0.5 mM pyruvate (heart, liver, and muscle) or 0.15 mM NADH and 1 mM pyruvate (brain), each in 50 mM hepes at pH 7.4; PEPCK, 1.1 mM PEP, 0.15 mM NADH, 0.5 mM dGDP, 20 mM $NaHCO_3$, 1 mM $MnCl_2 \cdot 4H_2O$, and excess coupling enzyme (malate dehydrogenase) in 50 mM imidazole at pH 7.4; PK, 10 mM PEP, 0.15 mM NADH, 5 mM ADP, 100 mM KCl, 10 mM $MgCl_2 \cdot 6H_2O$, 10 μM fructose-1,6-bisphosphate, and excess coupling enzyme (LDH) in 50 mM mops at pH 7.4. Preliminary assays determined the lowest possible substrate concentrations that would stimulate maximal activity. All enzyme assays were run in triplicate in a 96-well microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) with temperature control. Activities were determined by subtracting the background reaction rate without a key substrate (COX, cytochrome c; CS, oxaloacetate; HOAD, acetoacetyl-CoA; LDH, pyruvate; PK, phosphoenolpyruvate; PEPCK, dGDP) from the rates measured in the presence of all substrates. We used extinction coefficients (ε) of 28.5 and 13.6 optical density (mmol l^{-1}) cm^{-1} for COX and CS assays, respectively. We calculated ε empirically for the remaining assays by constructing standard curves of absorbance versus NADH concentration in the buffers appropriate for each assay.

Undiluted skeletal muscle homogenate was assayed for myoglobin concentration using a modification of the method described by Reynafarje (Reynafarje, 1963). Homogenates were centrifuged at 11,000 rpm for 100 minutes at 4^{o}C (Eppendorf, Hamburg, Germany). The supernatant was completely reduced by rotating for 8 minutes in a tonometer containing pure carbon monoxide gas, followed by addition of sodium dithionite and a further 2 minutes of rotation in CO. Reduced samples were diluted and absorbance was read at 538 and 568 nm and tissue myoglobin content was calculated as described (Reynafarje, 1963).

Whole-blood lactate concentrations were measured by thawing the frozen samples and acidifying them using an excess of 8% $HClO_4$ solution. Acidified extracts were incubated for 40 min at 37^{o}C in assay buffer (0.6 M glycine, 0.5 M hydrazine sulphate with excess β-NAD^+ and LDH), and then absorbance was read at 340 nm in duplicate.

2.4.7 Calculations and statistics

A residual approach accounted for the influence of body mass on O_2 consumption rate (Fig. 2.1), critical oxygen tension and oxygen tension at loss of equilibrium (Fig. 2.2) and total gill filament length (Fig. 2.4). Data were first regressed to body mass (M) using the general allometric equation $Y=aM^b$ (where a and b are constants), and residuals from the regression were then calculated for each individual. The calculated residuals were used for statistical comparisons (see below). Data are reported graphically as both residuals

and as the sum of the residual and the expected value for an average-sized killifish (see Figs. 2.1, 2.2 and 2.4 for details).

Data are reported as means ± standard error (except where data for individual fish are shown). For most data, hypoxic acclimation treatments were compared to normoxic controls with a one-way ANOVA. A two-way ANOVA was used to assess the effects of hypoxic acclimation treatment, sampling point (i.e., rest *versus* LOE), and their interaction on blood [lactate]. Bonferroni multiple comparisons post-tests were used for paired comparisons. A significance level of $p<0.05$ was used throughout.

2.5 Results

2.5.1 Effects of hypoxia acclimation on hypoxia tolerance

We exposed killifish to constant hypoxia or diel cycles of nocturnal hypoxia (12 h hypoxia at night, 12 h normoxia during the day) for 7 or 28 days at a moderate O_2 tension (PO_2=5 kPa) or for 7 d at a severe PO_2 (2 kPa). Hypoxia acclimation tended to reduce resting rates of oxygen consumption (MO_2). Because MO_2 did not scale isometrically ($MO_2=12.061M^{0.491}$, where M is body mass), as previously observed in the closely-related *F. grandis* (Everett and Crawford, 2010), we corrected MO_2 for body mass using the residuals from an allometric regression (Fig. 2.1A). Exposure to 7 d of either pattern of severe hypoxia substantially reduced MO_2 compared to normoxic controls (Fig. 2.1B).

Though not significant, 7 d of moderate hypoxia also tended to reduce MO_2 (Fig. 2.1B). There was no effect of moderate hypoxia on MO_2 after 28 d of exposure (Fig. 2.1C), but the MO_2 across all 28 d groups appeared to be lower on average than the same 7 d treatment groups.

Hypoxia acclimation increased hypoxia tolerance, indicated by a lower P_{crit} and/or a lower PO_2 at loss of equilibrium (LOE) during progressive hypoxia, relative to normoxia-acclimated controls (Fig. 2.2). Exposure severity, but not pattern, influenced the magnitude of the effect of 7 d of hypoxia acclimation on P_{crit}, which was lowest after acclimation to severe hypoxia but was also reduced by moderate hypoxia (Fig. 2.2C). In contrast, only constant hypoxia significantly reduced PO_2 at LOE, after acclimation to either 7 d of severe or 28 d of moderate hypoxia (Fig. 2.2D, F). We corrected P_{crit} and PO_2 at LOE for body mass using the same residual approach that we used for MO_2 (Figs. 2.2A, B), but very similar results were obtained without using this mass correction (data not shown).

Hypoxia acclimation also affected blood lactate concentration (Fig. 2.3). Unlike other treatment groups, resting blood [lactate] was higher after acclimation to severe intermittent hypoxia, relative to normoxic controls. Blood [lactate] rose substantially at LOE across all treatments compared to levels in each group at rest in their acclimation condition ($p<0.001$ for both the 7 and 28 d data for the main effect of sampling point, i.e. rest *versus* LOE, in two-factor ANOVA) (Fig. 2.3). Furthermore, there was a significant

interaction between sampling point and severity of hypoxia during acclimation in the 7 d group ($p=0.042$), and a similar trend was seen in the 28 d groups ($p=0.090$). This implies that acclimation to severe hypoxia for 7 d, whether constant or intermittent, blunts the rise in blood [lactate] at LOE. Indeed, the fold-increase in blood [lactate] at LOE was lower on average in fish acclimated to severe hypoxia (approximately 6.6- and 4.6-fold above resting levels in the constant and intermittent groups, respectively) as compared to that in normoxic controls (~11.1-fold). Considering the concurrent differences in the PO_2 at LOE, it suggests that hypoxia acclimation changed the relationship between PO_2 and blood lactate accumulation.

2.5.2 Effects of hypoxia acclimation on gill morphology

Constant hypoxia had numerous effects on gill morphology. Because body mass has a significant effect on overall gill size, we again used the residuals from an allometric regression of gill morphometric measurements to compare these variables statistically (Fig. 2.4). Constant but not intermittent hypoxia acclimation led to a minor (<10%) but significant decrease in the total length of gill filaments relative to normoxia, after both 7 d of severe hypoxia or 28 d of moderate hypoxia (Fig. 2.4). Both 28 d hypoxia acclimations also decreased the average length of the gill filaments slightly (<8%) (Table 2.1). Clearly, hypoxia acclimation does not lead to morphometric changes that increase the surface area for branchial O_2 diffusion in this species.

The cell composition of the gill epithelium also changed following 28 d acclimation to moderate constant hypoxia (Fig. 2.5). In the 4 ppt brackish water in which we held the killifish, most of the mitochondrion-rich cells (MRCs) of normoxia-acclimated animals exhibited a typical seawater morphology with deep apical crypts. The proportion of freshwater-type MRCs increased after acclimation to constant hypoxia, as many more MRCs exhibited a transitional (wide and shallow apical crypts) or typical freshwater (convex surface) morphology (Fig. 2.5D). There were no differences in pavement cell (PVC) surface area or MRC density on the trailing edge of the filaments (Fig. 2.5C; Table 2.1).

2.5.3 Effects of hypoxia acclimation on haematology

There were substantial differences in the O_2 carrying capacity of the blood between fish acclimated to constant and intermittent hypoxia. Fish acclimated to either duration of constant hypoxia significantly increased haematocrit and whole-blood haemoglobin concentration, whereas fish acclimated to intermittent hypoxia did not differ from normoxic controls (Fig. 2.6). The increases in constant hypoxia acclimation were greater with severe hypoxia (~1.9-fold) than with moderate hypoxia (~1.4-fold). There were no changes in mean-cellular haemoglobin concentration ($p=0.876$ for 7 d groups, and $p=0.371$ for 28 d groups; data not shown).

2.5.4 Effects of hypoxia acclimation on the swimming muscle

Hypoxia acclimation influenced the oxidative phenotype of the axial (swimming) muscle. Relative to normoxia, killifish acclimated to 28 d of constant hypoxia had significantly less total oxidative muscle area (as reflected by succinate dehydrogenase (SDH) positive staining), fewer oxidative fibres, and a 27% lower density of oxidative muscle as a proportion of the entire axial musculature (Fig. 2.7; Table 2.2). The muscle phenotype of fish acclimated to intermittent hypoxia did not differ significantly from normoxia-acclimated animals. Oxidative muscle area declined exclusively due to a decrease in the number of modestly oxidative fibres (MOx) at the interface between the oxidative and glycolytic regions (Table 2.2), with no change in the number of highly oxidative (HOx) fibres or in the average size of either fibre type (see Materials and Methods for details). There were no significant differences in the myoglobin content of the entire axial musculature, but the non-significant pattern of variation after 28 d of acclimation mirrored the variation in muscle oxidative phenotype (Tables 2.3, 2.4). There was also a significant 15% decrease in cytochrome c oxidase (COX) activity in the entire axial musculature (sampled to include all fibre types) following 28 d acclimation to both hypoxia patterns. However, there were no other differences in the activities of metabolic enzymes in the muscle (Tables 2.3, 2.4).

Only intermittent hypoxia significantly altered muscle capillarity. Capillary density and capillary-to-fibre ratio in the glycolytic (SDH-negative) muscle increased 30% following

acclimation to 28 d of intermittent hypoxia (Fig. 2.7, Table 2.2). In contrast, there were no changes in the capillarity of the oxidative muscle with intermittent hypoxia, or in capillarity for either muscle fibre type in fish acclimated to constant hypoxia.

2.5.5 Effect of hypoxia acclimation on enzyme activities in the liver, heart, and brain

Intermittent hypoxia, but not constant hypoxia, increased the biochemical capacities for oxidative energy metabolism and gluconeogenesis in the liver. Acclimation to severe intermittent hypoxia for 7 d increased the maximal activities of COX, citrate synthase (CS), lactate dehydrogenase (LDH), and phosphoenolpyruvate carboxykinase (PEPCK), without affecting pyruvate kinase (PK) or hydroxyacyl-coA dehydrogenase (HOAD) activities (Fig. 2.8). In contrast, acclimation to severe constant hypoxia for 7 d increased only LDH activity. There was no significant variation in liver mass or protein contents in the 7 d groups. Acclimation to moderate intermittent hypoxia for 28 d also increased liver COX activity and liver protein content, and acclimation to either of the hypoxia patterns for 28 d reduced liver mass (Table 2.4).

Capacities for oxidative phosphorylation and substrate oxidation appeared to remain unchanged by hypoxia acclimation in the heart and brain, as there were no differences in the activities of COX, CS, LDH, PK, or HOAD (the latter two measured in heart only) (Tables 2.3, 2.4). PEPCK activity was not detected in muscle, heart, or brain.

Interestingly, brain mass was larger in fish acclimated to 7 d of severe intermittent hypoxia, but heart mass did not vary between treatments (Tables 2.3, 2.4).

2.6 Discussion

Killifish are routinely exposed to fluctuating conditions in their native estuarine environment. Our results show that the responses of killifish to intermittent hypoxia during the course of acclimation are distinct from those to constant hypoxia. Although acclimation to both patterns of hypoxia exposure reduced resting MO_2 and P_{crit}, there were considerable differences between patterns in several physiological traits that dictate oxygen transport and utilization. Constant hypoxia reduced gill surface area, increased blood haemoglobin content, and led to greater reductions in muscle oxidative capacity. These changes did not occur in response to intermittent hypoxia, which was alone in amplifying the oxidative and gluconeogenic capacities of the liver and in increasing the capillarity of glycolytic fibres in the swimming muscle, both of which should improve recovery and lactate clearance between hypoxia bouts. Our results suggest that there are different mechanisms of acclimation that depend upon the pattern of exposure, and that the strategies for coping with constant and intermittent hypoxia may differ.

2.6.1 Responses that occur for both patterns of hypoxia exposure

Acclimation to the same duration and magnitude of constant or intermittent hypoxia had similar effects on P_{crit} (Fig. 2.2C, E), suggesting that both patterns of exposure somehow improve the extraction and transport of O_2 in hypoxia, and thus broaden the functional PO_2 range for sustaining resting metabolism (Chapman et al., 2002; Søllid et al., 2003; Fu et al., 2011). This was at least partly explained by reductions in the resting MO_2 measured in normoxia (Fig. 2.1), which has been shown to be related to P_{crit} in many previous studies (van Ginneken et al., 1997; Mandic et al., 2009; Speers-Roesch et al., 2010). This supports the notion that low routine O_2 demands are associated with hypoxia tolerance, but there are clearly other changes in physiology with hypoxia acclimation that are also important (including those observed here).

Although constant and intermittent hypoxia led to comparable reductions in P_{crit} and resting MO_2, there is only modest evidence that this is caused by similar underlying physiological mechanisms. Increases in plasma lactate concentration with acute hypoxia are well documented in several fish species (Dunn and Hochachka, 1986; Cochran and Burnett, 1996; Virani and Rees, 2000; Scott et al., 2008). However, severe hypoxia acclimation, whether constant or intermittent, partially blunted this rise in blood lactate concentration at LOE (Fig. 2.3). This suggests that severe hypoxia acclimation reduces lactate accumulation at a given level of hypoxia, in association with lower rates of metabolism and ATP demand. Nevertheless, the capacity for lactate production by LDH

is either similarly unaffected (heart, brain, and muscle) or elevated (liver) (Fig. 2.8) by acclimation to both patterns of hypoxia. The general absence of any changes in metabolic capacity in the heart or brain with hypoxia acclimation, and the increases in LDH in the liver, are similar to previous observations in killifish (Greaney et al., 1980; Martinez et al., 2006). As the organs that are most sensitive to oxygen limitation, the heart and brain are likely protected from hypoxia by a preferential re-distribution of blood flow (Axelsson and Fritsche, 1991; Gamperl et al., 1995; Soengas and Aldegunde, 2002). Otherwise, the physiological responses to constant and intermittent hypoxia were largely distinct.

2.6.2 Unique responses to constant hypoxia

Acclimation to constant hypoxia leads to the greatest reduction in the absolute lower PO_2 limit for acute survival, as reflected by a significant decrease in PO_2 at LOE, relative to normoxic controls (Fig. 2.2D, F). The potential causes of LOE in hypoxia are numerous, and could include metabolic acidosis or the cascade of cellular events that result from ATP supply-demand imbalance (Boutilier, 2001; Bickler and Buck, 2007). Variation in the time to or PO_2 at LOE has been suggested to arise from differences in total glycogen stores, the capacity for anaerobic metabolism, tolerance of metabolic acidosis, and the capacity for metabolic depression (Almedia-Val et al., 2000; Nilsson and Östlund-Nilsson, 2008; Mandic et al., 2013). As discussed above, anaerobic capacity probably did not distinguish constant hypoxia from intermittent hypoxia because LDH activities were

generally similar between groups (Fig. 2.8; Tables 2.3, 2.4). However, it is possible that fish acclimated to constant hypoxia have a greater capacity for depressing metabolism or tolerating metabolic acidosis in acute hypoxia than those acclimated to normoxia or intermittent hypoxia.

The reductions in gill filament length and the changes in cell composition on the gill epithelium in response to constant hypoxia may be mechanisms for depressing the metabolic costs of ion transport. As the first step of the O_2 transport cascade, the gills are crucial for O_2 uptake, and gill surface area often increases in water-breathing fishes with hypoxia acclimation (Hughes, 1966; Chapman et al., 1999; Søllid et al., 2003; Evans, 2005). However, the large gas exchange area of the gills facilitates passive ion loss in a hyposmotic environment, which necessitates active ion pumping to maintain ionic homeostasis. This underlies the "osmorespiratory compromise" that leads to trade-offs between respiratory gas exchange and osmoregulation (Randall et al., 1972; Nilsson, 2007). Correspondingly, some species reduce gill surface area and/or ion permeability in response to hypoxia exposure, presumably to minimize ionic disruption rather than facilitate O_2 uptake (McDonald and McMahon, 1977; Wood et al., 2007, 2009; Matey et al., 2011; De Boeck et al., 2013). The killifish acclimated to constant hypoxia in this study decreased gill surface area and shifted their gill epithelium towards a freshwater morphology (Figs. 2.4, 2.5; Table 2.1). Because the freshwater gill is less permeable to ions than the seawater gill due to the presence of deep tight junctions between cells (Chasiotis et al., 2012), this transition should have reduced passive ion loss to the

surrounding hyposmotic brackish water (Sardet et al., 1979; Scott et al., 2004; Chasiotis et al., 2012). The structural changes in the gills with constant hypoxia acclimation may then act to minimize the costs of ion transport and facilitate metabolic depression. This likely reduced O_2 diffusion capacity across the gill epithelium, and may have limited the ability of killifish acclimated to constant hypoxia to support high metabolic rates (e.g. exercise). However, as the fish in this study were inactive and their oxygen demands were quite low (Fig. 2.1), there may have been excess gill surface area and uptake capacity that could be done without in hypoxic fish.

Fish acclimated to constant hypoxia increased whole-blood haemoglobin content and haematocrit (Fig. 2.6). A similar response has been observed in many species following hypoxia acclimation, and is often accompanied by increases in haemoglobin-O_2 affinity that are mediated by changes in allosteric effectors (Wood and Johansen, 1972; Greaney and Powers, 1977, 1978; Claireaux et al., 1988; Weber and Jensen, 1988; Chapman et al., 2002; Silkin and Silkina, 2005). Circulatory O_2 carrying capacity would have been enhanced in killifish acclimated to constant hypoxia if the measured increase in haemoglobin content was reflective of the entire blood volume. It is also possible that changes in peripheral vasoconstriction and blood flow, which are known to occur in some species in hypoxia (Axelsson and Fritsche, 1991), reduced the entrance of erythrocytes into the capillaries, thus reducing capillary haematocrit and concentrating the erythrocytes in the major vessels (from which haemoglobin content and haematocrit were measured). Interestingly, we and others (Lykkeboe and Weber, 1978; Taylor and Miller, 2001) have

shown that the same haemoglobin and haematocrit responses do not occur with intermittent hypoxia acclimation. This suggests that cumulative exposure duration, and not only absolute PO_2 during each hypoxia bout, may control haemoglobin content by promoting erythropoiesis and erythropoietin release by the kidneys, or by changing blood volume and/or the proportion of plasma and erythrocytes in the capillaries and secondary circulation (Lai et al. 2006; Rummer et al., 2014).

Constant hypoxia reduced the abundance of oxidative fibres in the muscle, due to a reduction in the number of modestly oxidative fibres at the interface between the oxidative and glycolytic regions, where fast oxidative muscle fibres are normally situated (Fig. 2.7; Table 2.2) (Scott and Johnston, 2012; McClelland and Scott, 2014). Because muscle recruitment proceeds from slow oxidative, to fast oxidative, to fast glycolytic as swimming intensity increases (Rome et al., 1984), a general reduction in swimming activity with hypoxia could have reduced the neural stimulation of fast oxidative fibres. Reduced neural activation is a key stimulus initiating the transition of fast fibres from an oxidative to a glycolytic phenotype (Bassel-Duby and Olson, 2006), so it is foreseeable that this process was induced in the fast oxidative muscle region due to a reduction in activity levels during hypoxia acclimation. Hypoxia could also regulate mitochondrial abundance within individual fibres, as hypoxia inducible factor stimulates mitochondrial autophagy in mammals (Zhang et al., 2008), and thus reduce overall muscle oxidative capacity even further. However, there is some interspecific variability in the effects of hypoxia on muscle phenotype, as tench (*Tinca tinca*), but not crucian carp (*Carassius*

carassius), have been observed to reduce muscle oxidative capacity by decreasing mitochondrial content with hypoxia acclimation (Johnston and Bernard, 1982; Johnston and Bernard, 1984).

2.6.3 Unique responses to intermittent hypoxia

Acclimation to intermittent hypoxia appears to improve the capacity to recover from each hypoxia bout during the intervening periods of normoxia. The use of anaerobic metabolism during hypoxia, reflected by increases in lactate production (Fig. 2.3) and metabolic acidosis (Johnston, 1975; Dunn and Hochachka, 1986; Scott et al., 2008), incurs an oxygen debt that must later be repaid (Heath and Pritchard, 1965). Increases in the activity of several enzymes occurred in the liver in response to intermittent hypoxia acclimation, including COX, CS, LDH, and PEPCK (Fig. 2.8). These changes should have augmented the capacity for glucogeneogenesis and lactate oxidation in the liver, possibly to increase this organ's capacity for metabolizing the lactate produced during each hypoxia bout. Constant hypoxia did not affect PEPCK activity (Fig. 2.8), consistent with previous observations (Martinez et al., 2006).

The capillarity of the glycolytic (but not oxidative) muscle also increased in fish acclimated to intermittent hypoxia (Fig. 2.7), which should increase the capacity for lactate clearance from the muscle (the largest tissue in the body) during the daily normoxic periods between hypoxia bouts. Capillarity did not increase in response to

constant hypoxia in this study (Fig. 2.7) or in previous studies of other fish species (Johnston and Bernard, 1982; Jaspers et al., 2014). As fibre size and number were unaffected by intermittent hypoxia, the increased capillarity appears to be caused by angiogenesis, and not a reduction in fibre size due to muscle atrophy. Angiogenesis could have been stimulated by an increase in muscle lactate (Constant et al., 2000; Gladden, 2004) or by high blood flows during recovery from hypoxia that may be needed to clear a lactate load (Egginton, 2011). Angiogenesis could have also occurred as a response to a decline in intracellular PO_2 (Mathieu-Costello, 1993; Hoppeler and Vogt, 2001). However, we did not observe any significant variation in the myoglobin content of the muscle, which would have increased cellular O_2 supply and has been observed to occur in response to hypoxia (Fraser et al., 2006). Regardless of the cause of this increase in capillarity, it is possible that it represents part of a general strategy to enhance the overall capacity for lactate turnover. This could even involve an increased use of the Cori cycle, in which the liver re-synthesizes glucose from lactate through gluconeogenesis and then returns glucose to the muscle via the circulation, although the existence of the Cori cycle in fish is uncertain (Milligan and Girard, 1993).

Acclimation to intermittent hypoxia increases hypoxia tolerance in killifish, and the mechanisms involved appear to be distinct from those for constant hypoxia. Intermittent hypoxia is also known to have different effects than constant hypoxia on the control of ventilation and the circulation in mammals (MacFarlane et al., 2008; Prabhakar and Semenza, 2012). On the one hand, it is possible that comparable changes occur in fish and

mammals in response to intermittent hypoxia. On the other hand, fish that are routinely exposed to intermittent hypoxia in their native environment, such as estuarine killifish, might employ uniquely evolved strategies for coping with intermittent hypoxia. As the occurrence of hypoxia increases worldwide, it will be important to better appreciate how the pattern of exposure influences the impacts of hypoxia on aquatic organisms.

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2.8 Competing interests

The authors declare no competing interests.

2.9 Author contributions

B.G.B. wrote the paper and led the majority of the experimentation, data collection, and analysis. K.L.D., D.M.G., and G.R.S. contributed to data collection and analysis. G.R.S.

designed and supervised the experiments. All authors contributed to the interpretation of data and to revising the manuscript. All authors approve the manuscript.

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2.11 Figures & figure legends

Figure 2.1

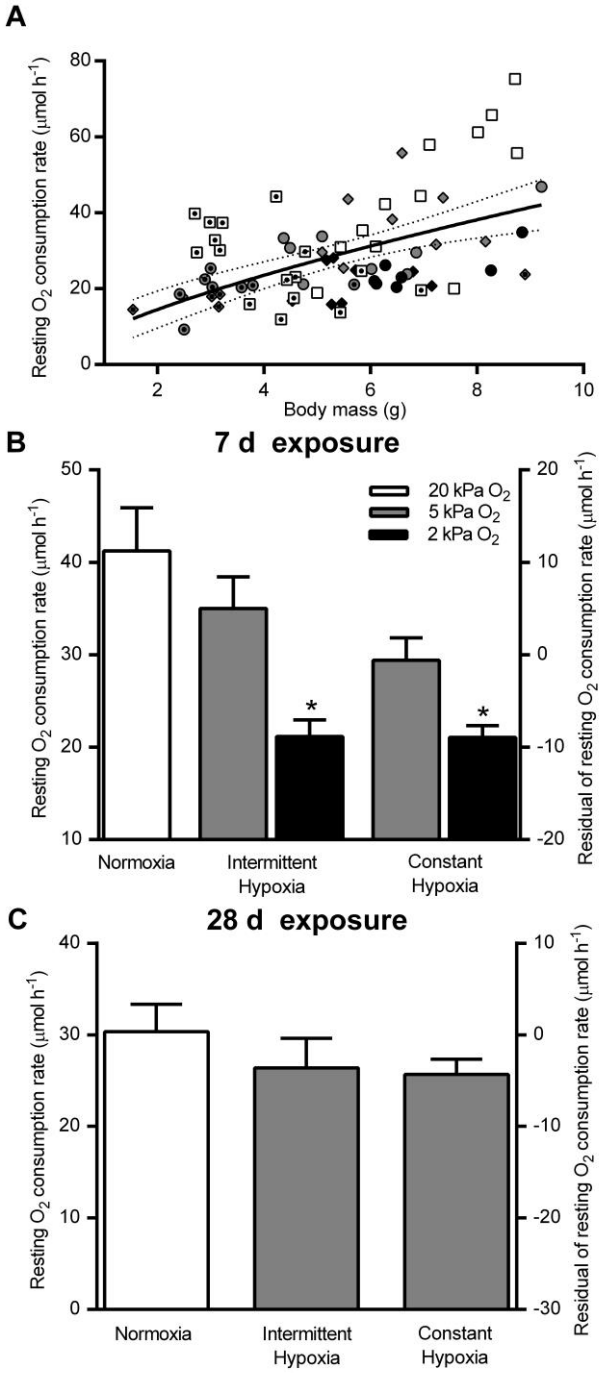

Fig. 2.1 The effects of hypoxia acclimation on resting metabolic rate. (A) The resting rate of oxygen consumption (MO_2) measured in normoxia was regressed to body mass (M) with an allometric equation generated using all data ($MO_2=12.061M^{0.491}$). Dashed lines represent the 95% confidence intervals of the regression. Symbols are as follows: white squares, 7 d normoxia acclimation at 20 kPa; grey diamonds, 7 d intermittent hypoxia acclimation at 5 kPa; black diamonds, 7 d intermittent hypoxia acclimation at 2 kPa; grey circles, 7 d constant hypoxia acclimation at 5 kPa; black circles, 7 d constant hypoxia acclimation at 2 kPa; dotted white squares, 28 d normoxia acclimation; dotted grey diamonds, 28 d intermittent hypoxia acclimation at 5 kPa; dotted grey circles, 28 d constant hypoxia acclimation at 5 kPa. Acclimation to 7 d of severe (2 kPa) intermittent or constant hypoxia reduced the resting MO_2 measured in normoxia (B), but there were no significant differences between the 28 d acclimation groups (C). The right axes in (B) and (C) represent the residuals from the regression in (A) for each treatment group. The left axes in (B) and (C) are the MO_2 calculated for average-sized 5.39 g killifish, determined by adding the residual to the MO_2 value predicted at 5.39 g by the regression. * indicates a significant difference from normoxia ($p<0.05$). Sample sizes were as follows: n=8 for 7 d groups, except normoxia (n=12) and 2 kPa constant hypoxia (n=7). For the 28 d groups, n=16 for normoxia, n=7 for 5 kPa intermittent hypoxia and n=8 for 5 kPa constant hypoxia.

Figure 2.2

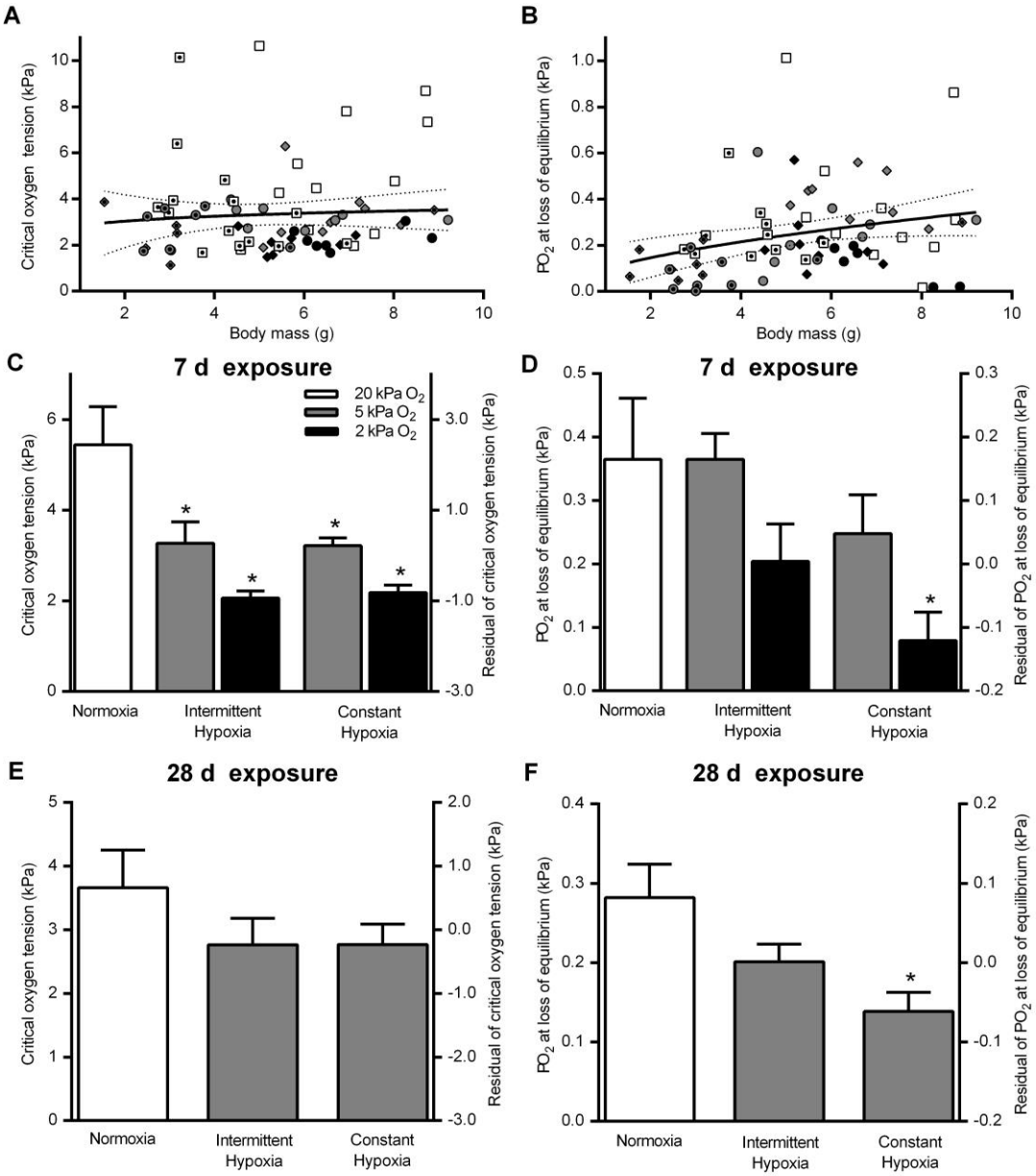
A
Critical oxygen tension (kPa)
Body mass (g)
B
PO_2 at loss of equilibrium (kPa)
Body mass (g)
C
7 d exposure
20 kPa O_2
5 kPa O_2
2 kPa O_2
Critical oxygen tension (kPa)
Residual of critical oxygen tension (kPa)
Normoxia
Intermittent Hypoxia
Constant Hypoxia
D
7 d exposure
PO_2 at loss of equilibrium (kPa)
Residual of PO_2 at loss of equilibrium (kPa)
Normoxia
Intermittent Hypoxia
Constant Hypoxia
E
28 d exposure
Critical oxygen tension (kPa)
Residual of critical oxygen tension (kPa)
Normoxia
Intermittent Hypoxia
Constant Hypoxia
F
28 d exposure
PO_2 at loss of equilibrium (kPa)
Residual of PO_2 at loss of equilibrium (kPa)
Normoxia
Intermittent Hypoxia
Constant Hypoxia

Fig. 2.2 The effects of hypoxia acclimation on critical oxygen tension (P_{crit}) and the oxygen tension (PO_2) at loss of equilibrium (LOE). (A) Critical oxygen tension (P_{crit}) was regressed to body mass (M) with an allometric equation ($P_{crit}=2.527M^{0.096}$). (B) Oxygen tension (PO_2) at loss of equilibrium (LOE) was also regressed to M with an allometric equation (PO_2 at LOE$=0.039M^{0.930}$). The 95% confidence intervals of each regression are represented by dashed lines. Symbols are as described in Fig. 1. The right axes in (C - F) represent the residuals from the regression using all data for P_{crit} (C, E) or PO_2 at LOE (D, F). The left axes are the P_{crit} (C, E) or PO_2 at LOE (D, F) calculated for average-sized 5.31 g killifish, determined by adding the residual to the P_{crit} or PO_2 at LOE value predicted at 5.31 g by the regression. * indicates a significant difference from normoxia-acclimated controls ($p<0.05$). Sample sizes for 7 d exposures (C, D) were as follows: n=11 for normoxia, n=7 for 2 kPa constant hypoxia, n=8 for all other groups. Sample sizes for 28 d exposures (E, F) were as follows: n=15 for normoxia, n=6 for 5 kPa intermittent hypoxia, n=8 for 2 kPa constant hypoxia.

Figure 2.3

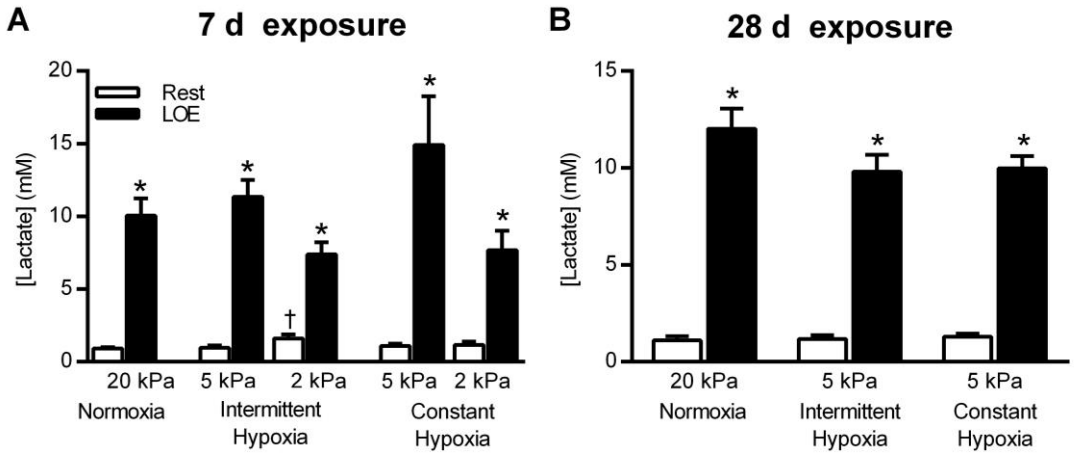
A
7 d exposure
Rest
LOE
[Lactate] (mM)
20
15
10
5
0
*
*
*
†
*
*
20 kPa
Normoxia
5 kPa
2 kPa
Intermittent
Hypoxia
5 kPa
2 kPa
Constant
Hypoxia
B
28 d exposure
[Lactate] (mM)
15
10
5
0
*
*
*
20 kPa
Normoxia
5 kPa
Intermittent
Hypoxia
5 kPa
Constant
Hypoxia

Fig. 2.3 Hypoxia acclimation reduced the blood lactate concentration at loss of equilibrium (LOE). Plasma lactate measured at loss of equilibrium was significantly higher than lactate measured in whole-blood at rest in both the 7 d (A) and 28 d (B) acclimation conditions. There was also a significant effect of hypoxia acclimation on the rise in blood lactate (see text for details). * indicates a significant difference from the resting fish within each acclimation group ($p<0.05$). † indicates a significant difference from resting normoxic controls ($p<0.05$). Sample sizes for fish at rest were as follows: n=10 for 7 d normoxia; n=9 for 7 d 5 kPa intermittent hypoxia, 7 d 2 kPa constant hypoxia, and 28 d 5 kPa constant hypoxia; n=8 for 28 d normoxia and 28 d 5 kPa intermittent hypoxia; n=7 for 7 d 5 kPa constant hypoxia; n=6 for 7 d 2 kPa intermittent hypoxia. Sample sizes for fish at LOE were as follows: n=12 for 7 d normoxia; n=8 for 7 d 5 kPa constant hypoxia; n=7 for 7 d 5 kPa intermittent hypoxia, 7 d 2 kPa constant hypoxia, 28 d normoxia and 28 d 5 kPa constant hypoxia; n=6 for 7 d 2 kPa intermittent hypoxia and 28 d 5 kPa intermittent hypoxia.

Figure 2.4

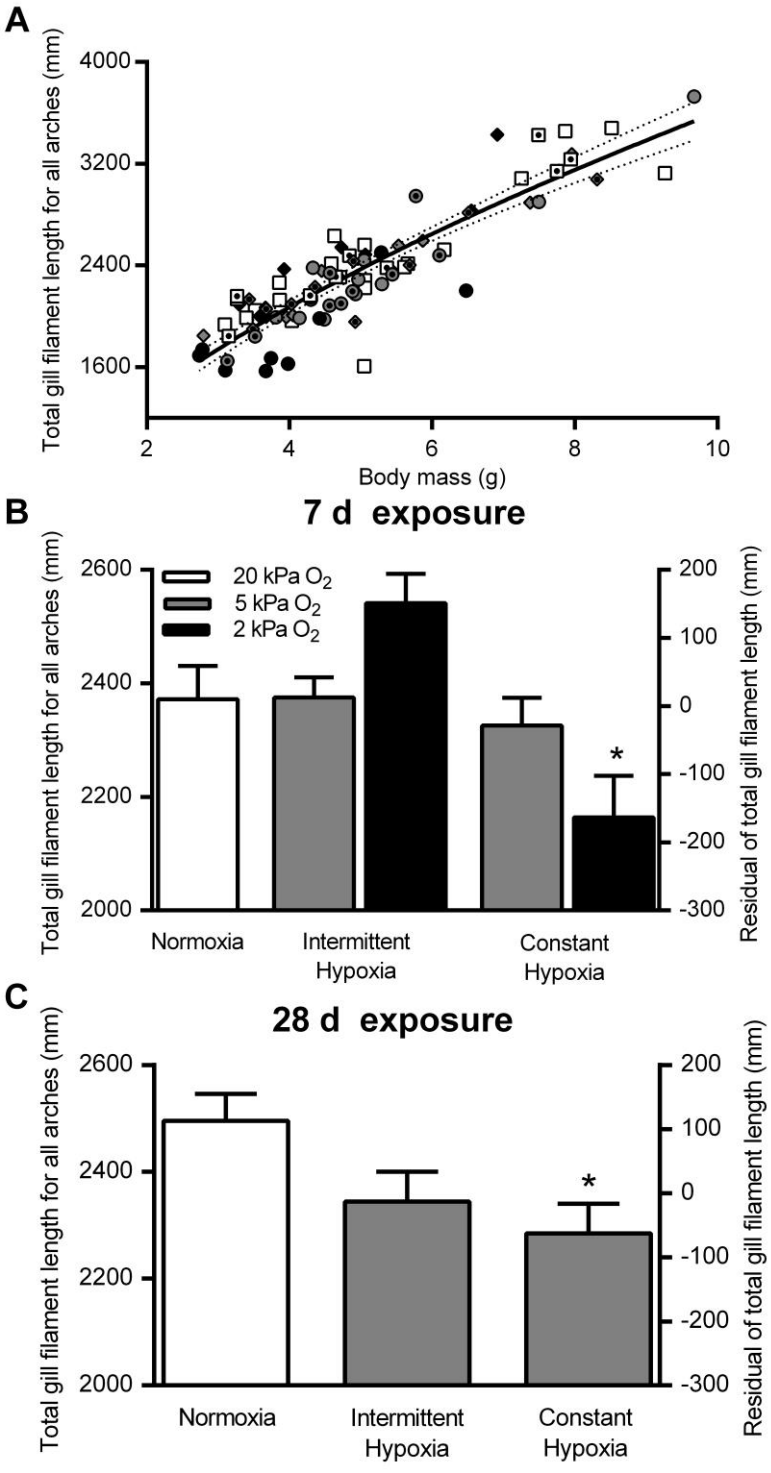
A
Total gill filament length for all arches (mm)
4000
3200
2400
1600
2
4
6
8
10
Body mass (g)
B
7 d exposure
20 kPa O2
5 kPa O2
2 kPa O2
Total gill filament length for all arches (mm)
Residual of total gill filament length (mm)
2600
2400
2200
2000
200
100
0
-100
-200
-300
*
Normoxia
Intermittent Hypoxia
Constant Hypoxia
C
28 d exposure
Total gill filament length for all arches (mm)
Residual of total gill filament length (mm)
2600
2400
2200
2000
200
100
0
-100
-200
-300
*
Normoxia
Intermittent Hypoxia
Constant Hypoxia

Fig. 2.4 Hypoxia acclimation reduced the total length of gill filaments. (A) The total length (L) of gill filaments across all four pairs of gill arches was regressed to body mass (M) with an allometric equation generated using all data ($L=930.79M^{0.5785}$). Dashed lines represent the 95% confidence intervals of the regression, and symbols are as described in Fig. 1. Acclimation to 7 d of severe (2 kPa) constant hypoxia (B) or 28 d of moderate (5 kPa) constant hypoxia (C) reduced total gill filament length. The right axes in (B) and (C) represent the residuals from the regression in (A) for each treatment group. The left axes in (B) and (C) are the total gill filament lengths calculated for average-sized 4.97 g killifish, determined by adding the residual to the value predicted at 4.97 g by the regression. * indicates a significant difference from normoxia ($p<0.05$). Sample size was n=10 for each group, except 7 d normoxia (n=20).

Figure 2.5

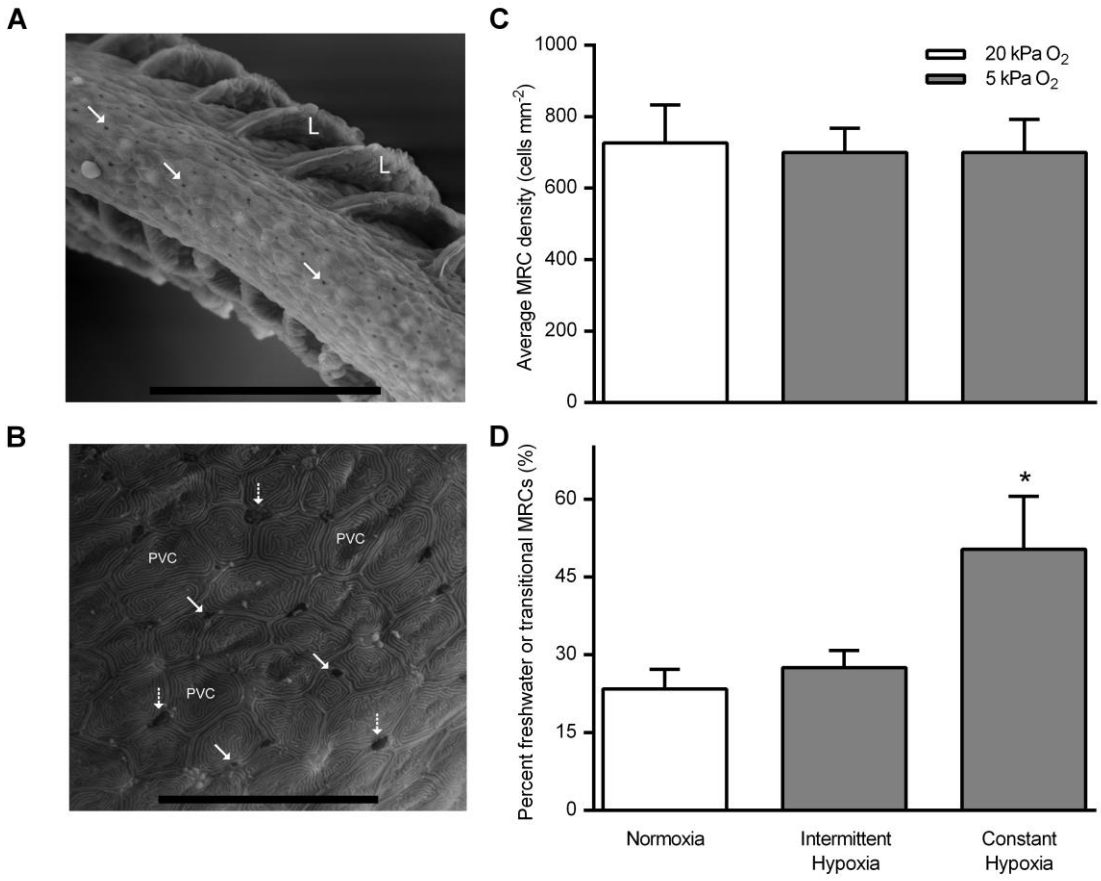
A
L
L
B
PVC
PVC
PVC
C
20 kPa O2
5 kPa O2
Average MRC density (cells mm-2)
1000
800
600
400
200
0
D
Percent freshwater or transitional MRCs (%)
60
45
30
15
0
*
Normoxia
Intermittent Hypoxia
Constant Hypoxia

Fig. 2.5 Acclimation to 28 d constant hypoxia affected mitochondrion-rich cell (MRC) morphology on the trailing edge of the gill filament. (A) Scanning electron micrograph (SEM) at 500x magnification of the gill filament, with lamellae (L) and apical crypts (arrows) visible. Scale bar is 100 μm. (B) SEM of the trailing edge of the gill filament at 2000x magnification with pavement cells (PVC) and two morphologies of MRCs. Freshwater- or transitional-type MRCs have wide and shallow or absent apical crypts and are indicated by dashed, vertical arrows. Seawater-type MRCs have the typical narrow and deep apical crypts and are indicated by solid, diagonal arrows. Scale bar is 25 μm. There was no significant effect of hypoxia acclimation on the total MRC density on the trailing edge of the gill filament (C), but acclimation to constant hypoxia for 28 d increased the proportion of MRCs with a freshwater- or transitional-type morphology compared to normoxic controls (D). * indicates a significant difference from normoxia ($p<0.05$). Sample size of n=6 for each group.

Figure 2.6

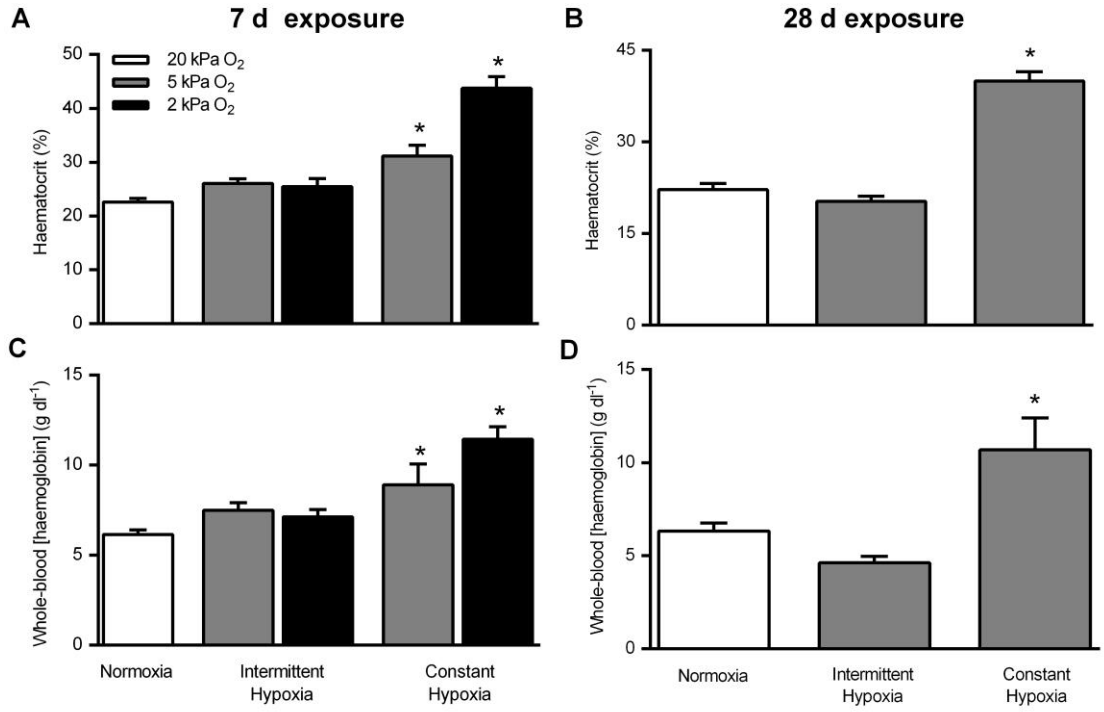
A
7 d exposure
20 kPa O2
5 kPa O2
2 kPa O2
Haematocrit (%)
B
28 d exposure
Haematocrit (%)
C
Whole-blood [haemoglobin] (g dl-1)
D
Whole-blood [haemoglobin] (g dl-1)
Normoxia
Intermittent Hypoxia
Constant Hypoxia

Fig. 2.6 Acclimation to constant hypoxia increased the haematocrit (A, B) and haemoglobin concentration (C, D) of the blood relative to normoxia. There were no significant differences in mean-cell haemoglobin concentration (data not shown). * indicates a significant difference from normoxia ($p<0.05$). Sample sizes for 7 d exposures (A, C) were as follows: n=18 for normoxia, n=8 (A) or n=10 (C) for 5 kPa intermittent hypoxia, n=10 for 2 kPa intermittent hypoxia, n=10 (A) or n=8 (C) for 5 kPa constant hypoxia, n=10 (A) or n=7 (C) for 2 kPa constant hypoxia. Sample sizes for 28 d exposures (B, D) were as follows: n=10 for normoxia and 5 kPa constant hypoxia, n=9 (B) or n=6 (D) for 5 kPa intermittent hypoxia.

Figure 2.7

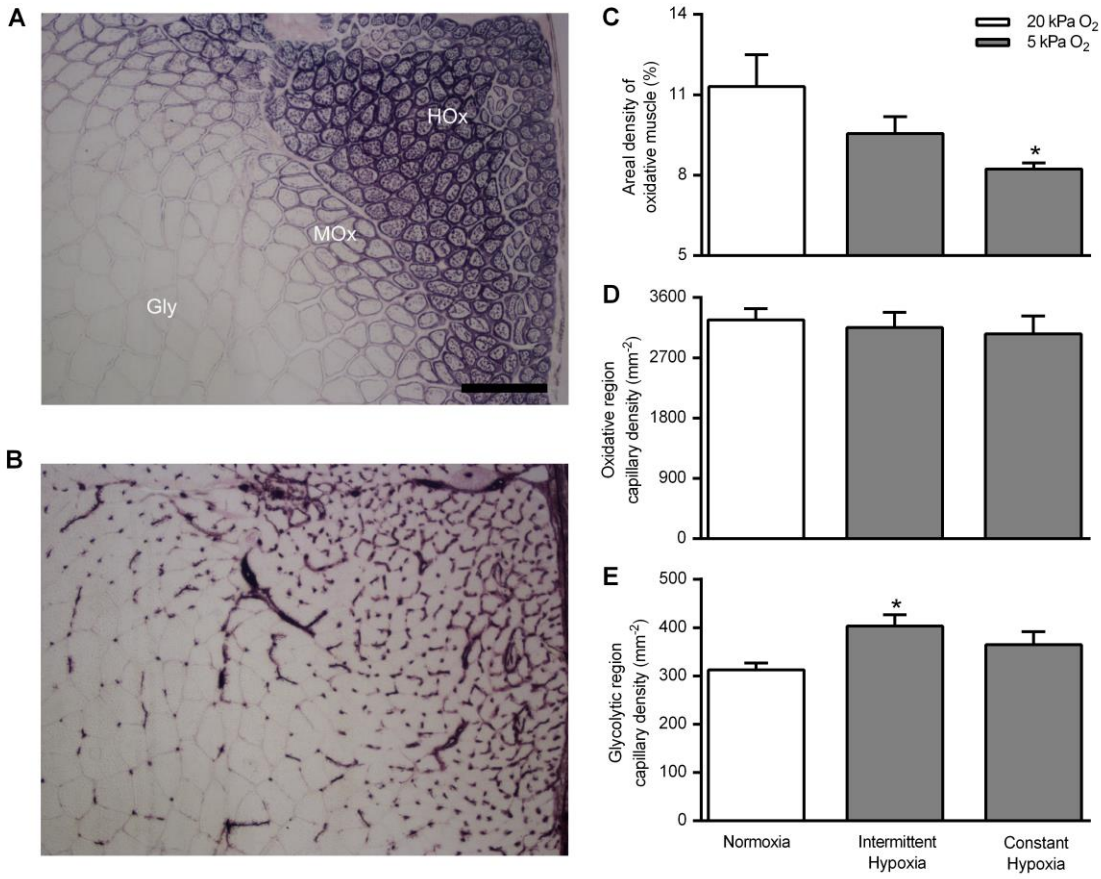
A
HOx
MOx
Gly
B
C
20 kPa O_2
5 kPa O_2
Areal density of oxidative muscle (%)
14
11
8
5
*
D
Oxidative region capillary density (mm^{-2})
3600
2700
1800
900
0
E
Glycolytic region capillary density (mm^{-2})
500
400
300
200
100
0
*
Normoxia
Intermittent Hypoxia
Constant Hypoxia

Fig. 2.7 Hypoxia acclimation for 28 d affected the phenotype of the axial swimming muscle. (A) Representative image of a transverse muscle section of a fish acclimated to 5 kPa intermittent hypoxia, stained for succinate dehydrogenase activity to indicate oxidative capacity. Highly oxidative (HOx), modestly oxidative (MOx), and glycolytic (Gly) fibres are indicated. Scale bar is 0.1 mm. (B) A serial muscle section from the same individual as in (A), stained for alkaline phosphatase activity to identify capillaries. (C) Acclimation to constant hypoxia reduced the areal density of oxidative muscle as a percentage of total muscle area. (D) There were no significant differences in capillary density in the oxidative muscle after hypoxia acclimation, but (E) intermittent hypoxia increased capillary density in the glycolytic (SDH-negative) muscle. * indicates a significant difference from normoxia ($p<0.05$). Sample sizes were as follows: (A) n=10 for normoxia and intermittent hypoxia, n=7 for constant hypoxia; (B) n=9 for normoxia, n=8 for intermittent hypoxia, and n=4 for constant hypoxia; (C) n=10 for normoxia, n=8 for intermittent hypoxia, and n=6 for constant hypoxia.

Figure 2.8

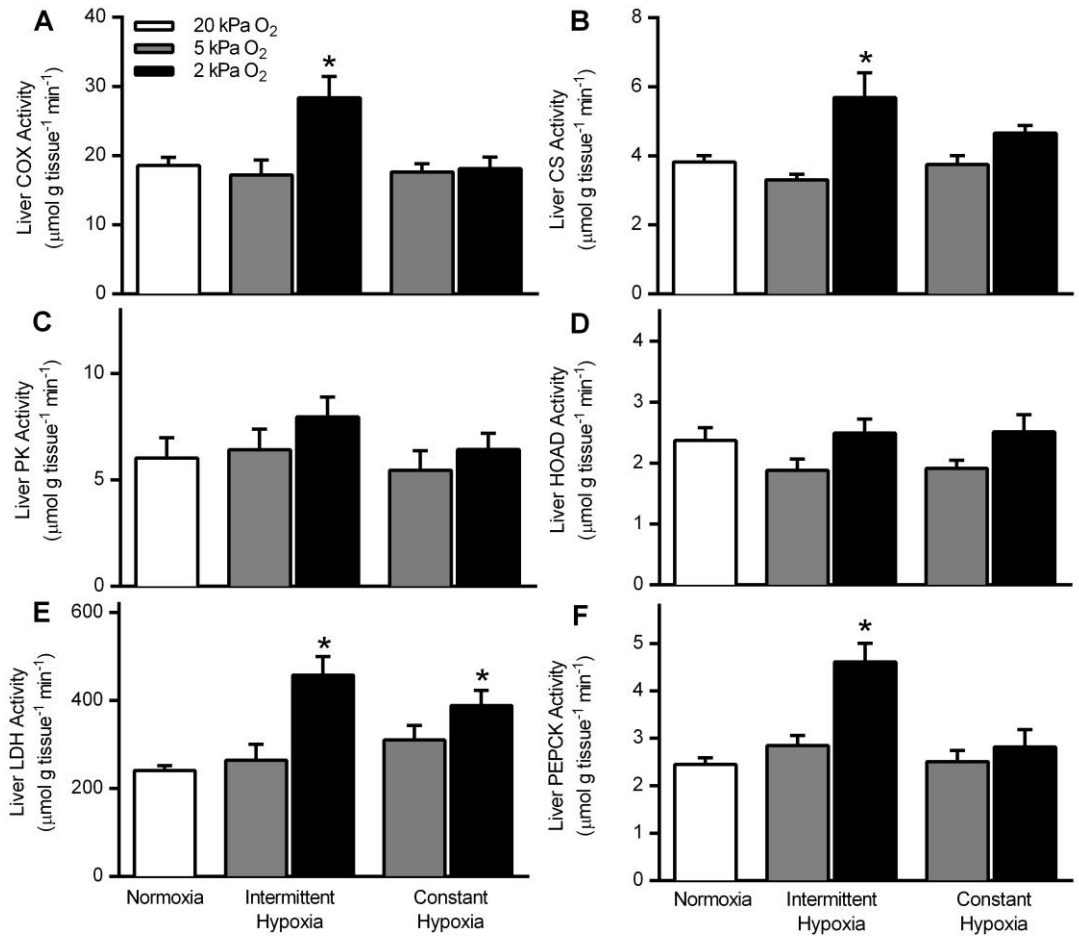
A
B
C
D
E
F
20 kPa O2
5 kPa O2
2 kPa O2
Liver COX Activity (μmol g tissue-1 min-1)
Liver CS Activity (μmol g tissue-1 min-1)
Liver PK Activity (μmol g tissue-1 min-1)
Liver HOAD Activity (μmol g tissue-1 min-1)
Liver LDH Activity (μmol g tissue-1 min-1)
Liver PEPCK Activity (μmol g tissue-1 min-1)
Normoxia
Intermittent Hypoxia
Constant Hypoxia

Fig. 2.8 Acclimation to severe intermittent hypoxia for 7 d increased the oxidative and gluconeogenic capacities of the liver. Cytochrome c oxidase (COX), citrate synthase (CS), lactate dehydrogenase (LDH), and phosphoenolpyruvate carboxykinase (PEPCK) activities were elevated after 7 d of severe intermittent hypoxia, without any change in pyruvate kinase (PK) or hydroxyacyl-coA dehydrogenase (HOAD). Neither liver masses (relative to body mass, in mg g^{-1}: normoxia, 17.3 ± 1.5; 5 kPa intermittent hypoxia, 17.2 ± 2.1; 2 kPa intermittent hypoxia, 15.6 ± 1.2; 5 kPa constant hypoxia, 13.7 ± 1.9 and 2 kPa constant hypoxia, 22.6 ± 2.1), nor liver protein contents (relative to tissue mass, in mg g^{-1}: normoxia, 117 ± 6; 5 kPa intermittent hypoxia, 128 ± 9; 2 kPa intermittent hypoxia, 99.2 ± 4.5; 5 kPa constant hypoxia, 110 ± 14 and 2 kPa constant hypoxia, 121 ± 12) varied between 7 d acclimation groups. * indicates a significant difference from normoxia ($p<0.05$). Sample sizes were n=10, except for the normoxia-acclimated group (n=20).

2.12 Tables

Table 2.1 Gill morphometrics

	Normoxia	Intermittent Hypoxia		Constant Hypoxia	
	20 kPa O_2	5 kPa O_2	2 kPa O_2	5 kPa O_2	2 kPa O_2
7 d exposure duration					
Avg. filament length (mm)	3.15 ± 0.10	3.07 ± 0.15	3.08 ± 0.09	3.12 ± 0.14	2.57 ± 0.11*
Total number of filaments	769 ± 14	789 ± 21	779 ± 23	767 ± 18	727 ± 18
28 d exposure duration					
Avg. filament length (mm)	3.21 ± 0.14	2.99 ± 0.11*		2.94 ± 0.09*	
Total number of filaments	781 ± 23	771 ± 15		751 ± 21*	
Depth of filaments (μm)	163 ± 15	176 ± 10		190 ± 7	
Depth of lamellae (μm)	122 ± 7	127 ± 3		126 ± 7	
Height of lamellae (μm)	25.1 ± 1.1	24.9 ± 1.4		26.5 ± 2.5	
Lamellar area (μm^2)	3066 ± 216	3140 ± 163		3391 ± 426	
PVC surface area (μm^2)	27.2 ± 1.8	31.5 ± 2.1		30.2 ± 2.2	
MRC surface area (μm^2)	1.21 ± 0.13	1.69 ± 0.38		2.43 ± 0.34*	

Avg., average; PVC, pavement cell; MRC, mitochondrion-rich cell Data are reported means ± standard error. * indicates a significant difference from normoxia ($p<0.05$). Sample size of n=20 for normoxia, n=10 for other groups for average filament length and total number of gill filament measurements. Sample size n=6 per group for all other measurements.

Table 2.2 Muscle phenotype after 28 d of hypoxia acclimation

	Normoxia 20 kPa O_2	Intermittent Hypoxia 5 kPa O_2	Constant Hypoxia 5 kPa O_2
Oxidative fibres			
Total stained area (mm^2)	3.07 ± 0.23	2.53 ± 0.19	2.25 ± 0.17*
Total number of oxidative fibres	3762 ± 139	3371 ± 156	3200 ± 154*
Number of highly oxidative fibres	1926 ± 84	1805 ± 78	1806 ± 89
Number of modestly oxidative fibres	1836 ± 76	1566 ± 129	1394 ± 85*
Fibre size (μm^2)	816 ± 47	752 ± 51	708 ± 55
Capillary-fibre ratio	2.83 ± 0.21	2.39 ± 0.19	2.44 ± 0.23
Glycolytic fibres			
Fibre size (μm^2)	1737 ± 122	1756 ± 89	1687 ± 90
Capillary-fibre ratio	0.54 ± 0.03	0.70 ± 0.04*	0.61 ± 0.03

Oxidative fibres were identified based upon relative succinate dehydrogenase activity. Capillaries were identified by staining for alkaline phosphatase activity. Data are reported means ± standard error. * indicates a significant difference from normoxia ($p<0.05$). Sample size n=9 for normoxia and intermittent hypoxia, n=7 for constant hypoxia, except for fibre size and capillary-fibre ratio measurements, where n=10 for normoxia, n=8 for intermittent hypoxia, n=4 (oxidative fibre size) or n=6 (glycolytic fibre size) for constant hypoxia.

Table 2.3 Metabolic enzyme activities in the muscle, heart, and brain for 7 d acclimation groups

	Normoxia	Intermittent Hypoxia		Constant Hypoxia	
	20 kPa O_2	5 kPa O_2	2 kPa O_2	5 kPa O_2	2 kPa O_2
Skeletal muscle					
Protein cont.	72.0 ± 4.7	64.8 ± 2.7	69.5 ± 2.5	76.7 ± 7.0	65.9 ± 2.8
[Mb]	1.85 ± 0.22	1.77 ± 0.39	1.76 ± 0.22	1.78 ± 0.38	2.09 ± 0.28
COX	11.1 ± 0.8	12.5 ± 1.3	11.7 ± 0.7	10.9 ± 1.3	11.1 ± 1.1
CS	5.27 ± 0.28	4.59 ± 0.34	5.45 ± 0.30	4.96 ± 0.36	6.11 ± 0.65
PK	234 ± 13	201 ± 15	265 ± 22	252 ± 24	299 ± 51
HOAD	0.82 ± 0.08	0.67 ± 0.11	0.90 ± 0.11	0.84 ± 0.10	0.87 ± 0.11
LDH	500 ± 21	533 ± 44	598 ± 56	572 ± 47	575 ± 46
Heart					
Organ mass	1.51 ± 0.15	1.56 ± 0.07	1.52 ± 0.06	1.48 ± 0.10	1.54 ± 0.13
Protein cont.	54.1 ± 2.8	57.4 ± 5.2	46.2 ± 3.7	50.5 ± 3.3	50.8 ± 5.1
COX	10.2 ± 1.7	12.0 ± 2.2	13.0 ± 1.3	10.7 ± 2.2	12.6 ± 2.7
CS	10.8 ± 0.6	9.35 ± 0.41	9.75 ± 0.95	9.53 ± 0.33	10.8 ± 0.6
PK	89.9 ± 8.3	88.9 ± 3.8	114 ± 13	93.9 ± 9.5	90.1 ± 6.7
HOAD	2.99 ± 0.25	2.93 ± 0.25	2.10 ± 0.27	3.09 ± 0.42	3.10 ± 0.34
LDH	338 ± 26	328 ± 31	328 ± 32	376 ± 28	359 ± 50
Brain					
Organ mass	5.44 ± 0.39	6.97 ± 0.35	7.62 ± 0.48*	5.94 ± 0.88	5.97 ± 0.62
Protein cont.	105 ± 7.4	117 ± 5.6	103 ± 4.5	117 ± 3.9	98.2 ± 3.9
COX	4.32 ± 0.45	3.17 ± 0.27	3.75 ± 0.22	4.11 ± 0.30	3.87 ± 0.33
CS	5.11 ± 0.13	5.10 ± 0.13	5.08 ± 0.17	4.97 ± 0.35	5.13 ± 0.17
LDH	300 ± 13	315 ± 27	302 ± 22	342 ± 8	280 ± 19

[Mb], myoglobin content; cont., content; COX, cytochrome c oxidase; CS, citrate synthase; PK, pyruvate kinase; HOAD, hydroxyacyl-coA dehydrogenase; LDH, lactate dehydrogenase. Organ masses are expressed relative to body mass (mg g^{-1}). Tissue protein and whole-muscle myoglobin contents are reported per unit tissue mass (mg g^{-1}). Enzyme activities are expressed relative to tissue mass (μmol g $tissue^{-1}$ min^{-1}). Data are reported means ± standard error. * indicates a significant difference from normoxia ($p<0.05$). For heart, and muscle enzyme activities, n=20 for normoxia, and n=10 for all other groups, except for myoglobin content (n=17 for normoxia, n=9 for 5 kPa intermittent hypoxia and n=10 for all other groups). For brain enzyme activities, n=12 for normoxia and n=8 for all other groups, except for CS (n=11 for normoxia) and LDH (n=7 for 2 kPa intermittent hypoxia and 5 kPa constant hypoxia).

Table 2.4 Metabolic enzyme activities in the muscle, liver, heart, and brain for 28 d acclimation groups

	Normoxia 20 kPa O_2	Intermittent Hypoxia 5 kPa O_2	Constant Hypoxia 5 kPa O_2
Skeletal muscle			
Protein content	60.7 ± 2.7	60.6 ± 2.2	60.9 ± 2.0
[Mb]	1.90 ± 0.36	1.70 ± 0.37	1.64 ± 0.32
COX	15.9 ± 0.7	13.6 ± 0.6*	13.4 ± 0.6*
CS	5.36 ± 0.54	5.61 ± 0.53	5.61 ± 0.30
PK	278 ± 22	285 ± 27	285 ± 21
HOAD	2.41 ± 0.23	2.17 ± 0.37	2.20 ± 0.27
LDH	410 ± 19	435 ± 23	446 ± 20
Liver			
Organ mass	21.8 ± 1.5	16.1 ± 1.3*	13.9 ± 1.1*
Protein content	82.5 ± 2.5	99.3 ± 7.1*	83.7 ± 3.4
COX	27.3 ± 2.1	41.3 ± 5.4*	37.1 ± 3.3
CS	4.25 ± 0.19	5.78 ± 0.53	5.17 ± 0.77
PK	8.39 ± 1.82	8.54 ± 1.45	8.50 ± 0.56
HOAD	2.41 ± 0.23	2.17 ± 0.37	2.20 ± 0.27
LDH	393 ± 22	465 ± 43	476 ± 22
PEPCK	3.59 ± 0.22	2.49 ± 0.34	3.12 ± 0.34
Heart			
Organ mass	1.44 ± 0.10	1.59 ± 0.22	1.74 ± 0.18
Protein content	44.0 ± 2.9	44.9 ± 2.0	42.7 ± 1.0
COX	10.2 ± 1.3	10.5 ± 1.5	8.6 ± 0.8
CS	10.2 ± 0.7	9.17 ± 1.11	8.26 ± 0.55
PK	155 ± 13	156 ± 13	150 ± 7
HOAD	2.56 ± 0.17	2.10 ± 0.35	1.82 ± 0.24
LDH	340 ± 31	395 ± 47	325 ± 20
Brain			
Organ mass	7.45 ± 0.62	9.60 ± 0.64	8.96 ± 0.64
Protein content	90.7 ± 8.3	74.0 ± 16	76.1 ± 9.6
COX	6.12 ± 1.10	7.75 ± 0.97	6.63 ± 0.84
CS	5.12 ± 0.24	4.82 ± 0.09	4.54 ± 0.28
LDH	323 ± 21	311 ± 25	288 ± 23

[Mb], myoglobin content; COX, cytochrome c oxidase; CS, citrate synthase; PK, pyruvate kinase; HOAD, hydroxyacyl-coA dehydrogenase; LDH, lactate dehydrogenase; PEPCK, phosphoenolpyruvate carboxykinase. Organ masses are expressed relative to body mass (mg g^{-1}). Tissue protein and whole-muscle myoglobin contents are reported per unit tissue mass (mg g^{-1}). Enzyme activities are expressed

Table 2.4 continued
relative to tissue mass (μmol g tissue^{-1} min^{-1}). Data are reported means ± standard error. * indicates a significant difference from normoxia ($p<0.05$). For heart, and muscle enzyme activities, n=10 per group, except for myoglobin content (n=9 for normoxia and 5 kPa intermittent hypoxia, n=8 for 5 kPa constant hypoxia). For brain enzyme activities, n=15 for normoxia and n=8 for all other groups, except for COX (n=16 for normoxia).

Table 2.5 Body masses and lengths of fish used in each series of experiments

	Normoxia	Intermittent Hypoxia		Constant Hypoxia	
	20 kPa O_2	5 kPa O_2	2 kPa O_2	5 kPa O_2	2 kPa O_2
Respirometry experiments					
7 d WW	7.00 ± 0.37	6.49 ± 0.38	5.68 ± 0.31	5.93 ± 0.58	6.80 ± 0.40
28 d WW	4.17 ± 0.30	3.33 ± 0.83		3.36 ± 0.37	
Sampling experiments					
7 d WW	5.31 ± 0.39	5.21 ± 0.50	4.60 ± 0.40	5.36 ± 0.58	4.05 ± 0.37
7 d SL	61.0 ± 1.3	61.7 ± 2.2	60.4 ± 1.9	62.1 ± 2.2	56.1 ± 1.5
28 d WW	5.2 ± 0.60	4.93 ± 0.49		4.76 ± 0.29	
28 d SL	60.9 ± 2.2	61.7 ± 1.9		61.1 ± 1.3	

WW, wet weight (in g); SL, standard length (in mm). Data are means ± standard error, and there were no significant differences between treatments within an exposure duration for each experiment. Fish were measured at the time of sampling either after loss of equilibrium in the respirometry experiments, or at rest in the acclimation condition in the sampling experiments (standard body length was only measured in the latter experiments)

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CHAPTER 3

Distinct metabolic adjustments arise from acclimation to constant hypoxia and intermittent hypoxia in estuarine killifish (*Fundulus heteroclitus*)

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3.1 Abstract

Many fish experience daily cycles of hypoxia in the wild, but the physiological strategies for coping with intermittent hypoxia are poorly understood. We examined how killifish adjust O_2 supply and demand during acute hypoxia, and how these responses are altered after prolonged acclimation to constant or intermittent patterns of hypoxia exposure. We acclimated killifish to normoxia (~20 kPa O_2), constant hypoxia (2 kPa), or intermittent cycles of nocturnal hypoxia (12 h normoxia: 12 h hypoxia) for 28 days, and then compared whole-animal O_2 consumption rates (MO_2) and tissue metabolites during exposure to 12 h of hypoxia followed by reoxygenation in normoxia. Normoxia-acclimated fish experienced a pronounced 27% drop in MO_2 during acute hypoxia, and modestly increased MO_2 upon reoxygenation. They strongly recruited anaerobic metabolism during acute hypoxia, indicated by lactate accumulation in plasma, muscle, liver, brain, heart, and digestive tract, as well as a transient drop in intracellular pH, and they increased hypoxia inducible factor (HIF)-1α protein abundance in muscle. Glycogen, glucose, and glucose-6-phosphate levels suggested that glycogen supported brain metabolism in hypoxia, while the muscle used circulating glucose. Acclimation to constant hypoxia caused a stable ~50% decrease in MO_2 that persisted after reoxygenation, with minimal recruitment of anaerobic metabolism, suggestive of metabolic depression. Contrastingly, fish acclimated to intermittent hypoxia maintained sufficient O_2 transport to support normoxic MO_2, modestly recruited lactate metabolism, and increased MO_2 dramatically upon reoxygenation. Both groups of hypoxia-acclimated

fish had similar glycogen, ATP, intracellular pH, and HIF-1α levels as normoxic controls. We conclude that different patterns of hypoxia exposure favour distinct strategies for matching O_2 supply and O_2 demand.

3.2 Introduction

Hypoxia (low O_2 availability) is widespread in aquatic environments (Breitburg et al., 2018; Diaz and Breitburg, 2009; Ficke et al., 2007). Animals can be exposed to low O_2 for prolonged periods in ice-covered ponds or in deep and/or stratified waters, particularly when hypoxic zones are difficult to avoid or escape (e.g. ponds isolated from other waterways, large dead zones stretching hundreds of kilometres) (Diaz, 2001; Diaz and Rosenberg, 2008). Animals can also be exposed to intermittent cycles of hypoxia, in which bouts of low O_2 are separated by periods of normoxia (or even hyperoxia), as is common in tide pools, estuaries, coral reefs, and heavily vegetated systems (Andersen et al., 2017; Breitburg, 1992; Diaz, 2001; Nilsson and Renshaw, 2004; Richards, 2011; Tyler et al., 2009). Instances of both constant hypoxia and intermittent hypoxia are well documented in aquatic environments, but the biological effects of the latter have received comparably little attention. Studies that have investigated these effects in fish suggest that intermittent hypoxia can affect whole-animal O_2 consumption rates (Taylor and Miller, 2001), growth rates (Cheek, 2011; Stierhoff et al., 2009), behaviour (Brady and Targett, 2010; Brady et al., 2009), gene expression and protein abundance in tissues (Dowd et al., 2010; Rytkönen et al., 2012), and some other aspects of physiology that underlie hypoxia tolerance (Borowiec et al., 2015, see Chapter 2 of this volume; Du et al., 2016; Routley et al., 2002; Taylor and Miller, 2001; Yang et al., 2013). With only sporadic investigation of the physiological effects of intermittent hypoxia, it is unclear whether these two patterns of hypoxia exposure represent similar or distinct stressors to aquatic animals.

Hypoxia challenges animals by disrupting the balance between O_2 supply and O_2 demand, and thus constraining cellular ATP supply (Bickler and Buck, 2007; Boutilier, 2001; Hochachka et al., 1996). Animals encountering environmental hypoxia may compensate for decreased O_2 supply to tissues through an assortment of cardiorespiratory responses, including amplified gill ventilation and greater gill surface area, increased haematocrit, and enhanced haemoglobin-O_2 binding affinity (Hughes, 1973; Nikinmaa and Soivio, 1982; Perry et al., 2009; Scott et al., 2008; Sollid et al., 2003), to help sustain routine aerobic metabolism. Alternatively, a decrease in aerobic ATP production can be offset by relying more heavily on O_2-independent (anaerobic) energy pathways or by decreasing ATP demands (i.e. metabolic depression). Both strategies can lengthen survival in hypoxia, but they have different underlying mechanisms, costs, and benefits (Bickler and Buck, 2007; Gorr et al., 2010; Hopkins and Powell, 2001; Richards, 2009). Increased use of anaerobic glycolysis is a common response of fish to hypoxia (Bickler and Buck, 2007; Richards, 2009; Routley et al., 2002; Virani and Rees, 2000; Vornanen et al., 2009). Increases in flux through anaerobic pathways can occur rapidly (Richards et al., 2008; van Ginneken et al., 1995; Wallimann et al., 1992), and can then be reinforced over time by modifications in gene expression and/or enzyme activities (Almeida-Val et al., 2011; Greaney et al., 1980; Martinez et al., 2006). However, chronic use of anaerobic metabolism is unsustainable in most species due to the depletion of carbohydrate fuel stores, the accumulation of end products (e.g. lactate, protons), and other detrimental repercussions such as the development of metabolic acidosis (Dunn and Hochachka,

1986; Richards, 2009; Richards et al., 2007; Speers-Roesch et al., 2012; Speers-Roesch et al., 2013; Thomas et al., 1986). During normoxic recovery, repayment of the 'O_2 debt' accrued during hypoxia exposure requires that glucose/glycogen be resynthesized and anaerobic end-products be cleared, potentially associated with various transcriptomic and proteomic adjustments (Dowd et al., 2010; Rytkönen et al., 2012), all of which require additional energy and may contribute to the excess post-hypoxic oxygen consumption (Lewis et al., 2007; Plambech et al., 2013; van den Thillart and Verbeek, 1991).

The use of metabolic depression when O_2 supply is limited is a less common response to hypoxia that is only exhibited in some hypoxia-tolerant species (Guppy and Withers, 1999; Hochachka et al., 1996; Lutz and Nilsson, 2004; Nilsson and Renshaw, 2004). In these species, ATP demands are greatly decreased by regulated cutbacks in many energetically costly processes (Bickler and Buck, 2007; Guppy and Withers, 1999; Hochachka et al., 1996; Richards, 2009). While this approach avoids the pitfalls of accelerated anaerobic metabolism, the so-called "turning down the pilot light" (Hochachka et al., 1996) has other physiological and ecological costs. Metabolically depressed animals may have little energy to invest in important fitness-relevant functions beyond those that maintain survival (e.g., locomotion, reproduction, growth, immune defence, etc.), they may have diminished cognitive ability (Johansson et al., 1997), and they may be more susceptible to disease and predation (Burton and Reichman, 1999; Nilsson et al., 1993; Prendergast et al., 2002). It is therefore likely that the use of

metabolic depression is restricted to bouts of hypoxia that are especially prolonged or severe (Regan et al., 2017a).

Intermittent cycles of hypoxia are likely to pose challenges that require physiological coping responses that are distinct from the responses to constant hypoxia. Routine hypoxia-reoxygenation cycles may lessen the costs of prolonged reliance on anaerobic metabolism by providing regular periods of recovery in normoxia. Moreover, if the timing of the hypoxic episodes is rapid, then physiological responses to hypoxia that are relatively slow to induce or reverse may not be possible. For example, gradual induction of hypoxia leads to significantly lower critical O_2 tension in goldfish (*Carassius auratus*), suggesting that a minimum amount of time is needed for some hypoxia responses to take place (Regan and Richards, 2017). Intermittent hypoxia may also introduce further, unique challenges associated with recurrent reoxygenation or the costs of repeated metabolic transitions (Ivanina et al., 2016; Prabhakar et al., 2001; Semenza and Prabhakar, 2007). The responses to such challenges of cyclical hypoxia may contribute a natural hypoxic preconditioning that improves tolerance of subsequent hypoxia exposure (Nilsson and Renshaw, 2004).

Our recent work on the mummichog (*Fundulus heteroclitus*), a well-studied estuarine fish (Burnett et al., 2007), suggests that nocturnal intermittent hypoxia and constant hypoxia are indeed distinct stressors (Borowiec et al., 2015, see Chapter 2 of this volume; Du et al., 2016). Fish exhibited different acclimation responses to each pattern of hypoxia

exposure to improve hypoxia tolerance, including changes in routine daytime O_2 consumption rate (MO_2), the activity of metabolic enzymes involved in glycolysis and recovery, the O_2 carrying capacity of the blood, gill morphology, and muscle phenotype. However, the implications of these distinct acclimation responses to the strategy used by fishes to balance cellular O_2 supply and O_2 demand, and to thus cope with hypoxia, remain unclear. For example, it is unclear how MO_2 may vary across a hypoxia-normoxia cycle in fish acclimated to intermittent hypoxia, especially during the night-time hypoxia exposure, and how this might compare to fish acclimated to constant hypoxia. Our earlier work suggested that there are considerable differences in metabolic enzyme activities in the liver between fish acclimated to intermittent and constant patterns of hypoxia exposure (Borowiec et al., 2015, see Chapter 2 of this volume; Du et al., 2016), but it remains unclear how these changes affect tissue energy metabolism. Our objective here was to investigate this issue by contrasting the whole-animal and tissue-level metabolic responses to hypoxia in fish acclimated to constant hypoxia versus intermittent diel cycles of nocturnal hypoxia. To do this, we determined the responses to acute hypoxia in normoxia-acclimated killifish, and then examined how these responses are altered by chronic exposure to intermittent hypoxia or constant hypoxia.

3.3 Materials & methods

3.3.1 Study animals

Adult, wild caught *Fundulus heteroclitus* of mixed sex (~2 to 5 g) were purchased and shipped from a commercial supplier (Aquatic Research Organisms, NH, USA) to McMaster University. Fish were held at room temperature (~22°C) in well-aerated brackish (4 ppt) water on a photoperiod of 12 h light (0700 to 1900 local time) to 12 h dark. Fish were fed by hand between ~0900 to 1100 local time to satiation with commercial flakes (Big Al's Aquarium Supercentres, Mississauga, ON, Canada) 5 days per week. Water quality (ammonia, pH, nitrates, and nitrites) was maintained by cycling water through a charcoal filter, and with routine water changes. Fish were held in well-aerated normoxic conditions for at least 4 weeks before acclimation treatments (see below). All animal protocols followed guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board.

3.3.2 Hypoxia acclimations

Fish were subjected to one of three 28 d acclimation treatments: (i) normoxia (~20 kPa O_2, ~8 mg O_2 l^{-1}), (ii) constant hypoxia (2 kPa O_2, ~0.8 O_2 l^{-1}), or (iii) nocturnal ('intermittent') hypoxia (12 h normoxia during the daytime light phase: 12 h of hypoxia

during the night-time dark phase). We chose a 12 h:12 h pattern of intermittent hypoxia, in which hypoxia occurred during the dark phase of the daily cycle, because it represents a reasonable representation of the diel patterns of hypoxia experienced in estuaries along the east coast of North America (Tyler et al., 2009). Acclimations were carried out in 35 l glass aquaria under the same salinity, photoperiod, temperature, and feeding conditions as are described above. Normoxia was achieved by continuously bubbling aquarium water with air. Hypoxia exposure was achieved by regulating the O_2 tension in the aquaria using a galvanic O_2 sensor that automatically controlled the flow of nitrogen with a solenoid valve (Loligo Systems, Tjele, Denmark), as described previously (Borowiec et al., 2015, see Chapter 2 of this volume). Fish were prevented from respiring at the water surface during hypoxic periods with a plastic grid barrier and bubble wrap.

3.3.3 Respirometry measurements

We used stop-flow respirometry to measure the changes in oxygen consumption rate (MO_2) during a 24 h cycle of normoxia-hypoxia-reoxygenation for each acclimation group (Fig. 3.1). As a normoxic control, we also measured MO_2 during a 24 h cycle of normoxia in an additional set of fish from the normoxia acclimation group, so that diurnal changes in MO_2 could be measured across the same times of day (Fig. 3.1). Measurements were made in respirometry chambers (90 ml cylindrical glass) that were situated in a buffer tank (with its sides covered in dark plastic to minimize visual disturbance to the fish) and connected to two water circulation circuits. One of these

circuits flushed the respirometry chamber with water from the surrounding buffer tank ('flushing circuit'). The second circuit continuously pumped water from the respirometry chamber across a fibre-optic O_2 sensor (PreSens, Regensburg, Germany) in a closed loop ('recirculation circuit'). The O_2 level in the respirometry chambers could thus be set by controlling the O_2 levels in the buffer tank, which was accomplished by bubbling with air or by using the same galvanic O_2 sensor system that was used for acclimations (see above). Water temperature and salinity were matched to the acclimation conditions.

To obtain respirometry measurements, fish were transferred into a respirometry chamber immediately following their 28 d acclimation at approximately 0900 local time. During this period of habituation to the chambers, O_2 levels were maintained at those that the fish would normally experience at that time of day in acclimation (i.e., hypoxia for the constant hypoxia group, normoxia for all other acclimation groups). MO_2 was then measured every ~14 min by alternating between 10 min flush periods, when the flushing circuit was on and the respirometry chamber was maintained at the conditions in the buffer tank, and 3 min measurement periods, when the flushing circuit was off, and the chamber was thus a closed system, allowing MO_2 to be measured as the rate of change in oxygen concentration in the water. The effects of handling stress subsided and fish achieved a stable resting MO_2 within 8 h of being placed in the chamber. Animals were held at their acclimation O_2 level until shortly before 1900 local time. Fish in the normoxia and intermittent hypoxia acclimation groups were then transitioned to hypoxia (2 kPa O_2, which took ~25 min), while fish acclimated to constant hypoxia were

maintained at the 2 kPa hypoxia in which they were held during acclimation, and MO_2 was measured in hypoxia for 12 h for all three acclimation groups. Each of these groups (including the constant hypoxia group) was then transitioned to normoxia (which again took ~25 min), and MO_2 was measured for an additional ~6 h. The normoxic controls were treated similarly, except that were held in normoxia for the full duration of respirometry measurements. The changes in MO_2 within each treatment group were analyzed using one-way, repeated measures ANOVA (see *Statistical analysis* below).

We compared the MO_2 data, processed into five distinct measurements, between each treatment group using one-way ANOVA (see *Statistical analysis* below). Resting daytime MO_2 in normoxia was determined as the average of the last 10 stable measurements before lights off at 1900 local time. The minimum MO_2 during the night time (the single lowest value recorded between 1900 and 0700 local time, 0 h and 12 h in our study) was also determined for each individual fish, as was the average MO_2 over the entire 12 h night-time dark phase. Excess post-hypoxic O_2 consumption (EPHOC) was determined during reoxygenation in normoxia, by calculating the area under the curve of measured MO_2 above resting MO_2 over time. EPHOC was calculated for the initial 90 min of normoxia (12 h to 13.5 h) and for the entire 6 h recovery period (12 h to 18 h). For comparison purposes, measurements analogous to EPHOC (referred to as daytime excess O_2 consumption) were also made for the constant normoxia group, though these animals were not exposed to hypoxia during the experiment.

3.3.4 Metabolite measurements

We investigated the tissue-level metabolic changes that occurred over the hypoxia-reoxygenation cycle by sampling fish in each treatment group over a time course of the daily cycle (Fig. 3.1). The day before sampling, at ~1000 local time following the 28 d acclimation, fish were transferred into a series of small plastic enclosures (up to 4 fish per enclosure) held within the same glass aquaria as was used for acclimations (and weighed down to sit at the bottom of the tank with large air stones), to facilitate rapid euthanasia and freeze-clamping of tissues – an approach that has previously been used with success in this species (Richards et al. 2008). Enclosures were 2.1 l in volume and were constructed with a mesh lid and sides to allow water to flow through the submerged enclosure. The galvanic O_2 sensor system that is described above (Loligo Systems) continued to control O_2 levels in the water to those appropriate for each acclimation group. A submersible filter and water pumps ensured adequate water circulation and uniform O_2 tensions within the enclosures and throughout the aquarium. Fish were held in these enclosures in their acclimation condition until the following day, and were then sampled at times during the daily cycle depicted by arrows in Fig. 3.1. One set of fish in the normoxia acclimation group were sampled in normoxia at 1800 (0 h), after 1 h, 6 h, or 12 h of acute hypoxia (2 kPa O_2) during the night-time dark phase (at 2000, 0100, or 0700, respectively), or after 1 h or 6 h of reoxygenation in normoxia during the daytime light phase (at 0800 or 1300, respectively). A second set of fish in the normoxia acclimation group acted as time-matched normoxia controls, held in normoxia and

sampled at the same times of day but not exposed to low oxygen. Fish in the intermittent hypoxia acclimation group were also sampled at the same times of day, during the normal hypoxia-reoxygenation cycle that they experienced in acclimation (i.e., in normoxia at 1800, after 1 h, 6 h, or 12 h of hypoxia, or after 1 h or 6 h of reoxygenation in normoxia). Fish acclimated to constant hypoxia were sampled in hypoxia (2 kPa O_2) at 1800, 2000, 0100, or 0700, or were transitioned to normoxia at 0700 and sampled after 1 h (0800) or 6 h (1300) of daytime reoxygenation. A two-way ANOVA was used to assess the effects of treatment group, sampling time, and their interaction on metabolite levels (see *Statistical analysis* below).

To sample fish at each time point, the mesh-covered enclosures were gently removed from the aquaria, and excess water was allowed to drain out of the mesh sides, leaving the fish in a ~1 l reservoir of water. Fish were quickly anaesthetized by adding a concentrated solution of benzocaine (Sigma-Aldrich, Oakville, ON) dissolved in 95% ethanol into the enclosure (final concentration 1 g l^{-1}) to the reservoir. Because the water in the reservoir was from the aquarium, fish were held in the PO_2 of their current treatment during euthanasia, such that fish sampled from hypoxic water were not exposed to normoxia prior to sampling. Fish were removed in pairs, weighed, and quickly dissected. A transverse section of the axial muscle (containing both red and white muscle fibres) was cut at the anterior base of the anal fin and immediately freeze clamped between two aluminum blocks pre-cooled in liquid nitrogen. Blood was collected from the caudal blood vessels in a heparinized capillary tube, spun at 14,000 rpm for 3 min, and the

plasma was isolated and frozen in liquid nitrogen. The liver and the whole brain were dissected and freeze clamped. Freeze clamping occurred rapidly (within 1 min of euthanasia) to minimize changes in metabolite levels. The heart and the full length of the digestive tract (cleared of its contents) were then dissected and frozen in liquid nitrogen. All samples were stored at -80°C until analyzed.

3.3.5 Intracellular pH and tissue metabolite analysis

For all analyses, frozen samples of muscle, brain, liver, and digestive tract were first ground into a fine powder using an insulated mortar and pestle that was pre-cooled with liquid nitrogen, and then returned to storage at -80°C until the subsequent homogenization and analysis of metabolites (see *Statistical analysis* below). The only exception was the heart, which was not ground into a powder, and instead homogenized whole for analysis.

ATP, phosphocreatine, and carbohydrate metabolites were measured in approximately 20 to 30 mg of powdered muscle, liver, and brain samples. Tissue was homogenized for 20 s in 300 µl of ice-cold 6% $HClO_4$ using the highest setting of a PowerGen 125 electric homogenizer (Fisher Scientific, Whitby, ON, Canada). Homogenates were vortexed, and 100 µl of the acidified extract was immediately frozen in liquid nitrogen for later analysis of glycogen, glucose, and glucose-6-phosphate content. The remaining homogenate was centrifuged at 4°C for 10 min at 10,000 g. The supernatant was transferred to a new

microcentrifuge tube, and neutralized ($6.8 \leq pH \leq 7.2$) with 3 mol l^{-1} K_2CO_3. Neutralized extracts were centrifuged at 10,000 g for 10 min at 4°C, and the resulting supernatant was immediately used for quantification of ATP, phosphocreatine, and lactate content by standard methods adapted for a 96-well plate format (Bergmeyer, 1983). Initial assay conditions were as follows: ATP, 5 mmol l^{-1} glucose, 2 mmol l^{-1} $NADP^+$, 5 mmol l^{-1} $MgCl_2$, and excess coupling enzyme (1 U ml^{-1} glucose-6-phosphate dehydrogenase) in 20 mmol l^{-1} Tris (pH 8.0); lactate, 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); phosphocreatine, conditions as listed for ATP, plus 1 mmol l^{-1} ADP. Assays were then conducted by adding an excess amount (5 U ml^{-1}) of the appropriate coupling enzyme (hexokinase for ATP, lactate dehydrogenase for lactate, and creatine kinase for phosphocreatine). All assays were coupled to a change in NADH or NADPH concentration in the well, such that the absolute change in absorbance at 340 nm following the addition of the final coupling enzyme was indicative of the metabolite content of the extract. All metabolite assays were run in duplicate at 37°C on a SpectraMax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Lactate content was also assayed in the digestive tract (~10 mg of powdered tissue) and heart (the whole heart, typically ~5 mg) using the same methods that are described above for lactate.

Glycogen, glucose, and glucose-6-phosphate content were measured in the acidified homogenates of muscle, liver, and brain tissues. Aliquots of the acidified homogenate were thawed on ice, and 50 µl of 1 mol l^{-1} K_2HCO_3 and 100 µl of 400 mmol l^{-1} acetate

buffer (pH=4.8) were added. Half of this solution was used to determine total glycogen content, by digesting the glycogen in the solution with 7 µl of amyloglucosidase (4 U l^{-1}, suspended in 300 mmol l^{-1} Tris-HCl, 4.05 mmol l^{-1} $MgSO_4$, pH=7.5) for 2 h at 40°C. The other half of this solution was used to determine free glucose and glucose-6-phosphate, by simply incubating the solution without amyloglucosidase at 4°C for 2 h. All samples were neutralized with 1 mol l^{-1} K_2HCO_3 following the 2 h incubation. Glucose-6-phosphate content was first determined in undigested samples by measuring the difference in absorbance after the addition of excess of the coupling enzyme glucose-6-phosphate dehydrogenase (3 U ml^{-1}) under the following conditions: 1 mmol l^{-1} ATP, 0.5 mmol l^{-1} $NADP^+$, and 5 mmol l^{-1} $MgCl_2$ in 20 mmol l^{-1} imidazole (pH=7.4). Glucose content was then assayed in both undigested samples (containing only endogenous free glucose) and digested samples (containing both endogenous free glucose and glucose originating from the enzymatic breakdown of glycogen) by measuring the change in absorbance with the addition of excess of the coupling enzyme hexokinase (5 U ml^{-1}) under the same conditions as for glucose-6-phospate plus excess coupling enzyme (glucose-6-phosphate dehydrogenase). The difference in glucose content detected between the digested and undigested samples was used to calculate sample glycogen content.

Intracellular pH (pH_i) was measured in a separate portion of powdered muscle and liver (~10 to 30 mg) samples, using a method similar to those that have been described previously (Baker et al., 2009; Pörtner, 1990; Pörtner et al., 1990). Tissue was briefly homogenized in 0.5 ml Pellet Pestle Microtubes (Fisher Scientific) containing five

volumes of ice-cold 150 mmol l^{-1} KCl and 8 mmol l^{-1} of the metabolic inhibitor nitrilotriacetic acid. The microtube was quickly capped to minimize loss of CO_2, and incubated on ice for 10 min. The homogenate was then vortexed, and its pH was measured within 15-20 s with a glass microelectrode (Sartorius, Bohemia, NY, USA) that was preconditioned to the appropriate temperature in ice-cold homogenization solution.

3.3.6 Protein abundance of hypoxia inducible factor (HIF) 1α

We measured HIF-1*α* protein abundance in skeletal muscle tissue by immunoprecipitation followed by western blotting. Powdered muscle tissue (~50 mg) was homogenized on ice with 30 passes of a glass Tenbroeck tissue grinder in 20 volumes of immunoprecipitation (IP) buffer, which was composed of 1% IGEPAL CA 630 (octylphenol ethoxylate), 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mmol l^{-1} EDTA, 1 mmol l^{-1} orthovanadate, 1% protease inhibitor cocktail (Sigma P-8340), and 50 ug ml^{-1} MG 132 (Z-Leu-Leu-Leu-aldehyde) in phosphate buffered saline (137 mmol l^{-1} NaCl, 2.7 mmol l^{-1} KCl, 10 mmol l^{-1} Na_2HPO_4, 1.76 mmol l^{-1} KH_2PO_4, pH=7.6). Homogenates were centrifuged at 10,000 g for 10 min at 4°C, and supernatant protein content was determined using the bicinchoninic acid assay (ThermoFisher Scientific), in which interfering substances were first removed by precipitation with trichloroacetic acid and deoxycholate (Brown et al., 1989). Tissue supernatants were diluted to 2 mg protein ml^{-1} in IP buffer and HIF-1*α* was immunoprecipitated from 1 ml samples using a chicken polyclonal antibody generated against a recombinant fragment of

HIF-1α from *Fundulus heteroclitus* (Townley et al., 2017). We first eliminated non-specific binding by pre-incubating the samples with 20 µl of secondary antibody (goat anti-chicken IgY bound to agarose beads; Aves Labs, Tigard, OR, USA) for 30 min at 4°C, followed by centrifugation at 1,500 g for 10 min at 4°C, and then reserving the supernatant for use in the following steps. Primary antibody (4 µl of 0.92 mg ml^{-1} solution) was added to each 1 ml of supernatant, which was then incubated for 1 h on ice with gentle rocking. This was followed by addition of 20 µl of secondary antibody and incubation overnight at 4°C with gentle rocking. The following morning, samples were centrifuged for 5 min at 1,500 g at 4°C, and the supernatant was discarded. The agarose beads (bound to HIF-1α) were washed by resuspension in TBST (20 mmol l^{-1} Tris, 150 mmol l^{-1} NaCl, 0.05% Tween-20, pH=7.5), incubated for 5 min on ice with gentle rocking, centrifuged as above, and the supernatant was discarded. The agarose beads were washed once more in TBST, followed by two washes in TBS (10 mmol l^{-1} Tris, 150 mmol l^{-1} NaCl, 0.02% NaN_3, pH=8.0) and two washes in 50 mmol l^{-1} Tris (pH=6.8). The final pellet (containing the washed agarose beads bound to HIF-1α) was re-suspended in 26 µl of distilled H_2O, 10 µl of LDS 4X sample buffer (Invitrogen), and 4 µl of 0.5 mol l^{-1} dithiothreitol (DTT). The solution was briefly centrifuged (~15 s) and heated at 70°C for 10 min to denature the protein and to release HIF-1α from the immunoprecipitation beads. The solution was cooled to room temperature, spun at 1,500 g for 5 min, and the supernatant was transferred to a new microcentrifuge tube. The pellet was then centrifuged again at 15,000 g for 5 min to crush the immunoprecipitation beads, and the small volume of resulting supernatant was combined with the first supernatant.

The final solution from the above immunoprecipitation of HIF-1α protein was used for gel electrophoresis. Solutions (~25 µl total) were loaded into a SDS-polyacrylamide gel (pre-cast 4-12% gradient NuPage Bis-tris gels; Invitrogen), and run at 150 V for 65 min in MOPS-SDS running buffer (50 mmol l^{-1} MOPS, 50 mmol l^{-1} Tris Base, 0.1% SDS, 1 mmol l^{-1} EDTA, pH=7.7). Proteins were wet transferred at 10°C onto a PVDF membrane for 2 h at 100 V using Invitrogen NuPage Transfer Buffer (25 mmol l^{-1} Bicine, 25 mmol l^{-1} Bis-Tris, 1 mmol l^{-1} EDTA, pH= 7.2; Fisher Scientific) containing 20% methanol and 0.05% SDS. After transfer, blots were blocked in 5% skim milk powder in TBST for 1 h at room temperature. The blot was then incubated in primary HIF-1α antibody, diluted 1:500 in blocking buffer, for 1 h at room temperature and then overnight at 4°C. The blot was washed 3 times for 5 min each in TBST, and then incubated for 1 h at room temperature with a donkey anti-chicken horseradish peroxidase secondary antibody (2 µl of 32 µg ml^{-1} solution), diluted 1:5000 in blocking buffer. Blots were washed 5 times for 5 min each in TBST. Bound secondary antibody was detected by incubating the blot in ECL reagent (1.3 mmol l^{-1} luminol, 0.2 mmol l^{-1} p-coumaric acid, 0.01% H_2O_2 in 100 mmol l^{-1} Tris, pH=8.5) and imaging the resulting bands by chemiluminescence on a ChemiDoc XRS imager. The band intensity of each sample, relative to the intensity of the IgY band as a loading control, was analyzed using Image Lab software (Bio-Rad) as an indication of protein abundance. We only quantified HIF-1α protein in the skeletal muscle due to the large quantity of tissue required to perform the immunoprecipitation (at 12 h, 0700 sampling time only), and we compared each treatment group using one-way

ANOVA (see *Statistical analysis* below). HIF-1α protein abundance is reported in arbitrary units (band intensity of HIF-1α / band intensity of IgY) and is expressed relative to the abundance of the constant normoxia group.

3.3.7 Statistical analysis

One-way or two-way ANOVA accompanied by Bonferroni post-tests were used to examine the effects of hypoxia exposure and treatment group. A significance level of $P<0.05$ was used for all statistical analyses. All data are reported as means ± standard error.

3.4 Results

3.4.1 Whole-animal metabolism during the hypoxia-reoxygenation cycle

We examined the changes in MO_2 during exposure to hypoxia followed by reoxygenation in normoxia, in killifish that were acclimated for 28 d to normoxia, intermittent hypoxia, or constant hypoxia (Fig. 3.1). Control fish held in normoxia ("constant normoxia" treatment group) had a relatively stable MO_2, but exhibited some circadian variation in MO_2, primarily reflected by modest increases in MO_2 during the daytime from ~1.5-3 h after the lights turned on, the typical time of day at which the fish were fed (Fig 3.2A). Normoxia acclimated fish that were exposed to their first bout of hypoxia ("acute

hypoxia" treatment group) exhibited an initial ~50% decrease in MO_2 after ~20 min of exposure (Fig 3.2B). This decrease in MO_2 recovered slightly by ~45 min of exposure, but MO_2 remained low throughout much of the night-time hypoxia exposure. Reoxygenation in normoxia led to a moderate increase in MO_2 that lasted ~5 h during recovery in normoxia. The changes in MO_2 during hypoxia-reoxygenation were similar in fish that had been acclimated to this hypoxia-reoxygenation cycle on a daily basis for several weeks ("intermittent hypoxia" treatment group) (Fig. 3.2C). However, the decrease in MO_2 in hypoxia was smaller and was completely recovered within ~2 h of exposure, and the increase in MO_2 during reoxygenation appeared to be greater and to last longer relative to fish experiencing their first cycle of acute hypoxia. By contrast, fish acclimated to constant hypoxia ("constant hypoxia" treatment group) exhibited a low and stable MO_2, which increased modestly and transiently during reoxygenation in normoxia (Fig. 3.2D).

Hypoxia acclimation altered metabolic rate during hypoxia-reoxygenation compared to normoxia acclimated fish that were experiencing their first bout of hypoxia (Fig. 3.3). Acclimation to constant hypoxia decreased MO_2, both during hypoxia and after reoxygenation in normoxia. This appeared to be caused by a general, active decreases in O_2 demands, because fish acclimated to constant hypoxia showed minimal excess post-hypoxic O_2 consumption (EPHOC) during reoxygenation in normoxia beyond a small increase in MO_2 shortly after reoxygenation (Fig. 3.3B, 12 to 13.5 h of the daily cycle). The initial 90 min (12 to 13.5 h) of excess O_2 consumption of fish acclimated to constant

hypoxia represented ~50% of the total excess O_2 consumption over the 6 h reoxygenation period, which far exceed that of the constant normoxia (~18%), acute hypoxia (~36%), and intermittent hypoxia (~30%) groups (Fig. 3.3B). In contrast, fish acclimated to intermittent hypoxia maintained MO_2 in hypoxia at levels that were statistically indistinguishable from fish held in normoxia (Fig. 3.3A), and exhibited much greater EPHOC during reoxygenation than other acclimation groups (Fig. 3.3B). This increase in EPHOC appeared to be due to increases in both the peak MO_2 reached during reoxygenation as well as in total duration of the increase in MO_2 (Fig. 3.2B).

Interestingly, the timing and pattern of the excess O_2 consumption also appeared to change with hypoxia acclimation. The increase in MO_2 during the day in the constant normoxia group did not occur until ~1.5-3 h after lights on and peaked at ~14 h of the daily cycle. Contrastingly, normoxia acclimated fish that were exposed to acute hypoxia exhibited a biphasic EPHOC response, with separate peaks in MO_2 apparent at approximately 12.5 h and 14 h (Fig. 3.2B). The pattern of these day-time increases in O_2 consumption rate in both normoxia acclimated groups differed from fish acclimated to intermittent hypoxia and constant hypoxia, which showed single, early peaks at ~12.5 h that declined thereafter with no obvious second peak (Fig 3.2C, 3.2D).

3.4.2 Tissue metabolites during a hypoxia-reoxygenation cycle

We tracked changes in the concentration of lactate, the end product of anaerobic glycolysis, in a variety of tissues during hypoxia-reoxygenation. There was a significant time×treatment interaction on the lactate content of a number of tissues (Fig. 3.4, Table 3.1), because lactate changed appreciably over time in the acute hypoxia group but not in the other groups. Fish experiencing their first cycle of hypoxia showed substantial 3.5, 4.4, 3.1, 2.7, and 2.8 fold increases in lactate in the plasma, muscle, liver, brain, and digestive tract, respectively, that peaked at 6 h to 12 h of hypoxia, and remained elevated after 1 h of recovery (13 h) in normoxia. Lactate content in the heart also increased at 6 h of hypoxia in the acute hypoxia group, but recovered to levels typical of normoxia before the end of the hypoxia exposure. These large increases in lactate content in the acute hypoxia group drove the significant main effects of time and treatment group in muscle, brain, digestive tract, and heart (Table 3.1), because there were no statistically significant changes in lactate content in these tissues in fish acclimated to constant hypoxia or to intermittent hypoxia. Lactate levels were also stable in the plasma and liver of fish acclimated to constant hypoxia (Fig. 3.4). However, fish acclimated to intermittent hypoxia accumulated a small amount of lactate in the liver at 6 h hypoxia (Fig. 3.4C), and released some lactate into the plasma at 1 h of recovery in normoxia (Fig. 3.4A).

We also examined intracellular pH (pH_i) in muscle and liver during hypoxia-reoxygenation. There was a significant time×treatment interaction on pH_i in both tissues

(Fig. 3.5, Table 3.1), again caused by changes in only the acute hypoxia group, which also drove the significant main effects of time and treatment (Table 3.1). In the muscle, these effects were mainly attributed to a pronounced but transient decline of 0.32 pH units in the acute hypoxia group at 6 h of hypoxia that was absent in all other groups (Fig. 3.5A). For normoxic acclimated fish exposed to acute hypoxia, there was a less severe but still significant decrease in pH_i of 0.17 units at 6 h of hypoxia in liver, as well as a 0.04 unit rise in pH_i at 1 h of recovery in normoxia (Fig. 3.5B). Despite our observation that fish acclimated to intermittent hypoxia accumulated lactate in the liver during hypoxia exposure (Fig. 3.4C), they maintained a stable pH_i in this tissue and in the muscle throughout the time course.

The concentrations of glycogen, free glucose, and glucose-6-phosphate were also examined in muscle and liver during hypoxia-reoxygenation. There appeared to be some diel variation in muscle glycogen levels, reflected by a significant main effect of time without significant effects of treatment group of the time×treatment interaction, though these statistical results seemed to be driven largely by lower glycogen levels in the late afternoon (0 h) in most groups (Fig 3.6A, Table 3.1). However, there were significant time×treatment interactions for free glucose and glucose-6-phosphate content in the muscle that resulted from changes in only the acute hypoxia group, and that also drove significant or marginally significant main effects of treatment and time (Table 3.1). These effects were primarily driven by large 2.6 fold and 14.0 fold increases at 6 h in free glucose and glucose-6-phosphate content, respectively, in the acute hypoxia treatment

group, and these increased levels continued after reoxygenation in normoxia (Fig 3.6B, C). There were also significant time×treatment interaction and main effect of time for glycogen content of the liver (Table 3.1), which seemed to be primarily driven by decreased liver glycogen at 12 h of hypoxia and after 1 h of reoxygenation in fish from the acute hypoxia treatment group (although the post-tests did not reach statistical significance) (Fig. 3.6B). This variation in the liver of the acute hypoxia group was associated with a 3.0 fold increase in free glucose content at 12 h, which was associated with a significant time×treatment interaction and a significant main effect of treatment (Fig. 3.6C, Table 3.1). Glucose-6-phosphate levels were somewhat variable in the liver, but there was a significant time×treatment interaction resulting from noticeable variation across time between acclimation groups (e.g., glucose-6-phosphate levels in the acute hypoxia group tended to be very low after 12 h of hypoxia) (Fig. 3.6F, Table 3.1). Overall, there appeared to be much more variation in these metabolites in fish from the acute hypoxia group that were experiencing their first cycle of hypoxia, than in fish acclimated to constant hypoxia or intermittent hypoxia.

There was a pronounced depletion of brain glycogen stores in normoxia-acclimated fish experiencing acute hypoxia (Fig. 3.7). This was reflected by a strong decrease in glycogen content in the acute hypoxia group at 6 h of hypoxia, which did not worsen by 12 h but that persisted into the reoxygenation period in normoxia. Glycogen depletion did not occur during hypoxia in other treatment groups, such that there was a significant time×treatment interaction on brain glycogen levels along with the significant main

effects of time and of treatment (Table 3.1). The decreases in brain glycogen in the acute hypoxia group occurred in conjunction with increases in free glucose content, for which there was also a significant time×treatment interaction and a significant main effect of treatment (Table 3.1). In contrast, fish acclimated to intermittent hypoxia were able to protect brain glycogen content and experienced no significant variation in free glucose throughout the hypoxia-normoxia cycle. Fish acclimated to constant hypoxia also had stable brain glycogen levels during hypoxia, but they experienced significantly lower brain glycogen content during reoxygenation compared to the constant normoxia group. Glucose-6-phosphate content in the brain was variable, and showed a significant main effect of time, but was not affected by treatment or a time×treatment interaction (Table 3.1).

There was diel variation in the ATP content of all tissues, as reflected by significant main effects of time (Tables 3.1 and 3.2). This variation appeared to be most strongly attributed to decreases in ATP levels in the liver and brain, and to decreases in phosphocreatine content in the muscle in fish exposed to their first bout of hypoxia (and in some cases to some more modest variation in ATP in the intermittent hypoxia group). There was a significant main effect of treatment and of the time×treatment interaction on phosphocreatine in the muscle, but neither the main effects of treatment nor the time×treatment interactions were significant for ATP levels in any tissue (Table 3.1).

3.4.3 Expression of HIF1a

Hypoxia acclimation attenuated the increase in HIF-1α protein levels in the muscle in both the constant hypoxia and intermittent hypoxia groups (Fig. 3.8). Fish experiencing their first bout of hypoxia (acute hypoxia group) showed a ~3 fold increase in HIF-1α protein abundance in the muscle after 12 h of hypoxia exposure. In contrast, HIF-1α protein levels were similar between fish acclimated to either intermittent hypoxia or constant hypoxia and those held in normoxia.

3.5 Discussion

Hypoxia in the aquatic environment comes in many forms and results from various biotic and abiotic causes. Here we show that chronic exposure of the estuarine killifish to different patterns of hypoxia exposure can lead to divergent changes in physiology, each of which improve metabolic homeostasis compared to normoxic fish encountering their first bout of acute hypoxia. Acclimation to constant hypoxia appeared to induce a pronounced metabolic depression that helped decrease O_2 demands, avoid the recruitment of anaerobic metabolism, and thus safeguard intracellular pH, carbohydrate stores, and cellular ATP levels. In contrast, acclimation to intermittent diel cycles of nocturnal hypoxia helped fish maintain sufficient O_2 transport to support routine O_2 demands, and may have modestly recruited lactate metabolism, but without any apparent metabolic acidosis or glycogen depletion. Therefore, our results suggest that both intermittent

hypoxia and constant hypoxia induced robust acclimation responses that differed considerably from the response to acute hypoxia, but that these different patterns of exposure can result in distinct strategies for coping with hypoxia.

3.5.1 Responses of killifish to acute hypoxia

Killifish exposed to their first bout of acute hypoxia were unable to maintain MO_2 at resting normoxic levels (Fig. 3.2, 3.3) and appeared to recruit anaerobic metabolism (Fig. 3.4, 3.5). Increased use of anaerobic glycolysis is common in fishes exposed to acute hypoxia, particularly when they are near or below their critical O_2 tension as they likely were during this experiment (Borowiec et al., 2015, see Chapter 2 of this volume), and this is often associated with accumulation of lactate in plasma and tissues, decreases in intracellular pH, and depletion of glycogen stores (Chippari-Gomes et al., 2005; Richards, 2009; Richards et al., 2007; Speers-Roesch et al., 2012). Lactate production in hypoxia generally results from feedback inactivation of pyruvate dehydrogenase, which is partly caused by accumulation and signalling by HIF-1α (Kim et al., 2006; Richards et al., 2008; Seagroves et al., 2001). This may have occurred in killifish, based on our observation that HIF-1*α* protein levels were elevated in the muscle after 12 h of acute hypoxia (Fig. 3.8). This recruitment of anaerobic metabolism likely augmented cellular ATP supply during hypoxia, and may have helped avoid any statistically significant changes in tissue ATP levels over the 12 h exposure to acute hypoxia (Table 3.2). These results are consistent

with a previous study on *F. heteroclitus*, in which ATP was stable in the white muscle over 15 h of severe hypoxia (Richards et al. 2008).

Killifish exposed to acute hypoxia showed some evidence that they accrued an 'O_2 debt' that was repaid upon reoxygenation (Fig. 3.2, 3.3). The task of correcting tissue homeostasis during recovery from hypoxia exposure – which can involve lactate oxidation, glycogen synthesis, gluconeogenesis, acid-base regulation, etc. – is considered to be energetically expensive and to contribute to the increase in metabolic rate that is often observed upon return to well-oxygenated conditions (EPHOC) (Johansson et al., 1995; Nonnotte et al., 1993; Plambech et al., 2013; Svendsen et al., 2011; van den Thillart and Verbeek, 1991). The total increase in O_2 consumption observed in the acute hypoxia group was similar in magnitude, but occurred earlier in the day than the circadian cycling of MO_2 in normoxic controls (Figs. 3.2, 3.3), suggesting that the diurnal changes in MO_2 in these groups may have had different underlying causes. For example, the biphasic nature of the change in O_2 consumption in the acute hypoxia group may represent a combination of (i) the effects of recovering from hypoxia, largely represented by the peak at 12.5 h and (ii) circadian cycling in MO_2, largely represented by the peak at 14 h. The lack of a further increase in MO_2 at ~14 h in the hypoxia acclimation groups may be due to either the exaggerated effects of hypoxia on MO_2 that masked normal circadian oscillations (such as with intermittent hypoxia) and/or a blunting of circadian oscillations in MO_2, as observed previously (Egg et al., 2013; Mortola, 2007; Pelster and Egg, 2015; Pelster and Egg, 2018). Related to the potential dampening effects of hypoxia on

circadian rhythms, our anecdotal observations suggest that fish are less active in the early period of reoxygenation after acute hypoxia, suggesting that increases in MO_2 to repay a hypoxic O_2 debt may have also been offset by decreases in the O_2 demands of activity, highlighting the complex nature of MO_2 as a metric of whole-animal metabolism.

Different patterns of variation in metabolite concentrations suggested that acute hypoxia had distinct effects between tissues. The decrease in brain glycogen stores in acute hypoxia suggested that this tissue relied at least partly on endogenous carbohydrate stores to fuel energy metabolism (Fig. 3.7). In contrast, the muscle experienced no decline in glycogen stores in hypoxia, but there were significant increases in free glucose and glucose-6-phosphate levels (Fig. 3.6), perhaps to support lactate production. Increases in glycogen breakdown products without a significant decrease in glycogen levels may be indicative of glucose entering the muscle from the circulation and/or of an accumulation of metabolites upstream of the potential downstream regulatory sites of glycolysis (e.g., phosphofructokinase) (Nascimben et al., 2004; Özand and Narahara, 1964). It is possible that this glucose, if imported from the blood, originated from glycogenolysis in the liver, at least after ~12 h of hypoxia, based on the declines in glycogen and glucose-6-phosphate levels and the increase in free glucose at this time (Fig. 3.6). It is intriguing to consider whether the Cori cycle – the shuttling of muscle lactate to the liver to support gluconeogenesis and the return of glucose to the muscle (Cori and Cori, 1929; Milligan and McDonald, 1988) – might have been active in killifish during hypoxia. However, this possibility is uncertain, given that in some fish species most produced lactate tends to be

retained in the muscle rather than entering the circulation, and Cori cycle and liver gluconeogenesis activities after exercise are low (Walsh, 1989; Weber et al., 1986; Wood, 1991).

3.5.2 Acclimation to constant hypoxia

Fish acclimated to constant hypoxia appeared to rely on metabolic depression to decrease O_2 demands, as indicated by a ~50% decrease in resting MO_2 compared to other groups that was not recovered within several hours of reoxygenation (Figs. 3.2, 3.3). This presumed decrease in routine energy demands likely helped these animals to maintain stable ATP levels (Table 3.1, 3.2), and to avoid the recruitment of anaerobic metabolism (Fig. 3.4), metabolic acidosis (Fig. 3.5), and depletion of glycogen stores (Fig. 3.6, 3.7). Metabolic depression may be a response that only some hypoxia-tolerant fish can use to match O_2 supply and demand and thus cope with oxygen limitation (Nilsson and Renshaw, 2004; Regan et al., 2017b; Richards, 2009; Scott et al., 2008; Vornanen et al., 2009), particularly in response to severe or prolonged bouts of hypoxia (Regan et al., 2017a), and our results suggest that killifish are also capable of a similar regulated depression of aerobic metabolism. This appears to be associated with morphological changes in the gills that limit the costs of ionoregulation, and transition from an oxidative to a glycolytic phenotype in the swimming muscle (Borowiec et al., 2015, see Chapter 2 of this volume).

Unlike fish exposed to their first bout of acute hypoxia, fish acclimated to constant hypoxia showed no increase in HIF-1α protein abundance in the muscle compared to the control levels typical of fish in normoxia (Fig 3.8). HIF-1α is a critical regular of the cellular and systems-level responses to hypoxia (Iyer et al., 1998; Nikinmaa and Rees, 2005; Richards, 2009; Semenza, 2000; Wang et al., 1995), and HIF signalling regulates many processes that influence O_2 supply and demand, such as angiogenesis, energy metabolism, and the hypoxic chemoreflex (Nikinmaa et al., 2004; Robertson et al., 2014; Semenza, 2000; Semenza, 2006; Semenza and Prabhakar, 2007). This decrease in HIF-1α abundance with prolonged acclimation could have resulted from an improvement in tissue O_2 supply or a change in the O_2 sensitivity of HIF degradation (e.g., changes in prolyl hydroxylase activity or O_2 kinetics) (Kopp et al., 2011; Wenger, 2002). Whatever the mechanism, a decrease in HIF-1α protein abundance in chronic hypoxia has been suggested to decrease lactate production by alleviating the inactivation of pyruvate dehydrogenase (PDH) via PDH kinase and thus restoring pyruvate oxidation potential (Le Moine et al., 2011), which may have contributed to the low lactate loads in killifish acclimated to hypoxia.

There was an unexpected decrease in brain glycogen stores during reoxygenation in fish that had been acclimated to constant hypoxia (Fig. 3.7). The reason for this decrease is unclear, but perhaps the energetic demands of the brain increased upon reoxygenation to an extent that was not fully supported by circulatory fuel supplies. Such an increase in energetic demands may have contributed to the small rise in MO_2 upon reoxygenation

(Figs. 3.2, 3.3). Considering the extreme and rapid increase in PO_2 that these animals experienced after a prolonged 28 d period of continuous hypoxia, it is possible that brain glycogen depletion occurred as animals fuelled the metabolic costs of repairing damage, activating transcription and translation, or reversing some neuroplasticity that was accrued during hypoxia exposure (Rytkönen et al., 2012). For example, prolonged anoxia is known to decrease cognitive ability in crucian carp (Johansson et al., 1997). Alternatively, it might have been associated with energetic stresses of reoxygenation, which can in some cases induce signs of oxidative stress, which has been suggested to be a serious challenge for animals emerging from prolonged periods of metabolic depression (Hermes-Lima et al., 2015; Hermes-Lima et al., 1998; Hermes-Lima and Zenteno-Savín, 2002; Lushchak et al., 2001).

3.5.3 Acclimation to intermittent hypoxia

Fish acclimated to intermittent hypoxia were uniquely able to maintain routine MO_2 throughout most of the night-time hypoxia exposure (Fig. 3.2). This may have resulted from an augmentation of O_2 transport capacity in hypoxia relative to other treatment groups. Acclimation to moderate levels of constant hypoxia has been shown to increase gill surface area, haematocrit, and blood haemoglobin content (Claireaux et al., 1988; Hughes, 1973; Richards, 2011; Sollid et al., 2003; Wells, 2009). These responses did not occur in killifish after acclimation to intermittent hypoxia at a higher PO_2 of ~5 kPa (Borowiec et al., 2015, see Chapter 2 of this volume), but could have occurred in the fish

acclimated to the more severe level of intermittent hypoxia used here. Two daily cycles of hypoxia-reoxygenation have been shown to decrease erythrocyte GTP content and thus increase haemoglobin-O_2 affinity (without any effect on blood haemoglobin content) in carp (*Cyprinus carpio*) (Lykkeboe and Weber, 1978), and if a similar change occurred in killifish, it might have helped sustain metabolic rate by increasing arterial O_2 saturation and tissue O_2 delivery. In some mammalian species, chronic exposure to intermittent hypoxia enhances the hypoxic ventilatory response (Garcia et al., 2000; MacFarlane and Mitchell, 2008; Prabhakar and Kline, 2002), and if a similar response exists in killifish, it could have helped further augment branchial gas exchange in hypoxia.

Fish acclimated to intermittent hypoxia also expressed an appreciable increase in MO_2 during reoxygenation (Fig. 3.2, 3.3). One possible explanation for this finding is that these fish accrued an exaggerated O_2 debt during hypoxia due to a significant recruitment of anaerobic metabolism. Consistent with this possibility, we have shown previously that acclimation to intermittent hypoxia increases the activities of lactate dehydrogenase, the gluconeogenic enzyme phosphoenolpyruvate carboxykinase, and the mitochondrial enzymes citrate synthase and cytochrome c oxidase in the liver (Borowiec et al., 2015, see Chapter 2 of this volume), which could have increased the capacity of this tissue to use and recover from anaerobic metabolism. Furthermore, lactate content increased in the liver during hypoxia and appeared to be released into the plasma upon reoxygenation (Fig. 3.4). However, the magnitude of these changes in lactate levels were relatively modest compared to those in normoxia-acclimated fish exposed to acute hypoxia, and fish

acclimated to intermittent hypoxia otherwise avoided metabolic acidosis (Fig. 3.5) and did not accumulated lactate in a number of other tissues (Fig. 3.4). This may reflect the sometimes poor relationship between post-hypoxia increases in MO_2 and lactate production during hypoxia (Genz et al., 2013; Lewis et al., 2007). It is possible that there were increases in lactate production in some tissues that did not result in lactate accumulation, if that lactate entered the circulation and was oxidized or otherwise disposed of in other tissues (Milligan and Wood, 1986). Alternatively, post-hypoxic increases in MO_2 may reflect the cost of correcting ionic and/or osmotic disturbances that arise during hypoxia exposure. For example, in some freshwater species, hypoxia exposure increases passive ion losses across the gills due to the well-known trade-off between the requirements for respiration and ionoregulation at the gills, termed the osmorespiratory compromise (Iftikar et al., 2010; Robertson et al., 2015). This does not appear to occur in normoxic killifish exposed to acute hypoxia (Robertson et al., 2015), but could arise after acclimation to intermittent hypoxia if other physiological adjustments (e.g., increases in ventilation or in gill surface area or permeability) intensify the osmo-respiratory compromise, and thus increase the metabolic demands of re-establishing ionic homeostasis upon reoxygenation. Reoxygenation-induced increases in transcription and translation could also contribute to the post-hypoxic increases in MO_2 (Dowd et al., 2010; Rytkönen et al., 2012).

An alternative and intriguing potential explanation for the large post-hypoxic increases in MO_2 in killifish acclimated to intermittent hypoxia is that they amplified and/or hastened

some normal circadian processes so they could be completed faster during the normoxic period in the daytime. We synchronized the photoperiod and oxygen cycles in this study, such that night-time hypoxia was predictable in fish that were acclimated to intermittent hypoxia. Furthermore, feeding occurred predictably ~2 h after lights on and the onset of normoxia. Therefore, fish could have anticipated feeding (though they were not fed for ~8 h before onset of the experiment, nor during the sampling period), potentially ramping up energetically expensive digestive processes before the predicted feeding time (López-Olmeda et al., 2012; Montoya et al., 2010; Vera et al., 2007). Fish acclimated to intermittent hypoxia may have exaggerated such anticipatory digestive processes to ensure that ingested food could be rapidly digested before the next bout of night-time hypoxia. Whatever the cause of this distinct metabolic phenotype, it is clear that killifish acclimate to intermittent hypoxia in a very different way than they acclimate to constant hypoxia, and thus acquire a distinct strategy for coping with O_2 limitation.

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3.7 Competing interests

The authors declare no competing or financial interests.

3.8 Author contributions

Conceptualization: B.G.B., G.R.S.; Methodology: B.G.B., G.B.M., B.B.R., G.R.S.; Validation: B.G.B.; Formal analysis: B.G.B., G.B.M., B.B.R., G.R.S.; Investigation: B.G.B.; Resources: G.B.M., B.B.R., G.R.S.; Writing - original draft: B.G.B.; Writing - review & editing: B.G.B., G.B.M., B.B.R., G.R.S.; Visualization: B.G.B., G.R.S.; Supervision: G.R.S.; Funding acquisition: G.R.S.

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3.10 Figures & figure legends

Figure 3.1

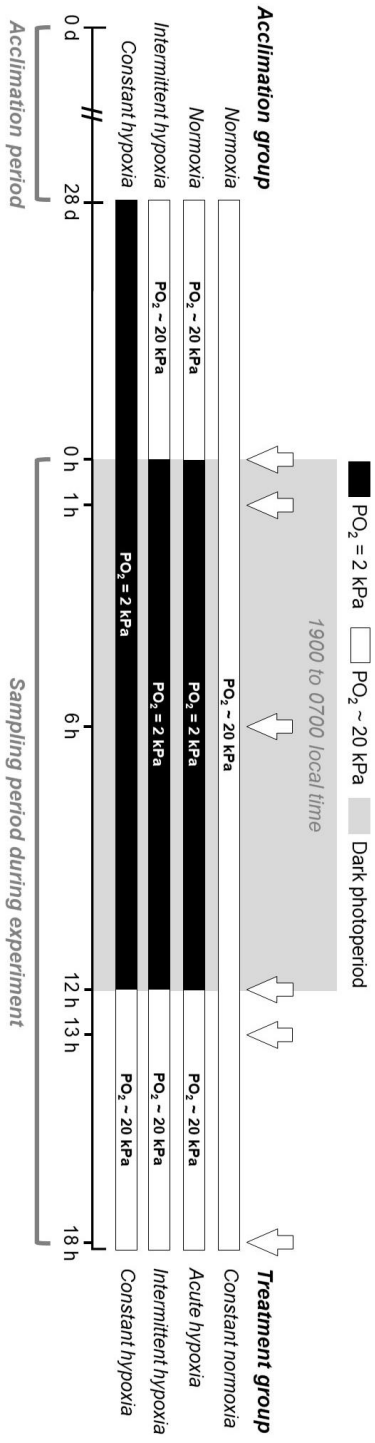

Fig. 3.1 Experimental groups used in this study. Killifish were first acclimated for 28 d to normoxia (~20 kPa), intermittent cycles of nocturnal hypoxia (12 h normoxia during the daytime light phase: 12 h of 2 kPa O_2 during the night-time dark phase), or constant hypoxia at 2 kPa O_2. Whole-animal O_2 consumption was measured throughout a normoxia-hypoxia-reoxygenation cycle in each acclimation group, with the exception that fish acclimated to constant hypoxia were held in hypoxia until transitioning to normoxia at the same time as the acute hypoxia and intermittent hypoxia groups. A second treatment group of normoxia-acclimated fish were held in constant normoxia throughout to act as time-matched normoxia controls. The grey shading represents the dark phase of the daily cycle, which lasted from 0700 to 1900 local time in all groups, with the light phase making up the remainder of the daily cycle. In a different set of fish, each treatment group was sampled for measurements of tissue metabolites at the times indicated by white arrows (fish at the 0 h and 12 h time points were sampled immediately *before* the change in PO_2 and/or light phase took effect).

Figure 3.2

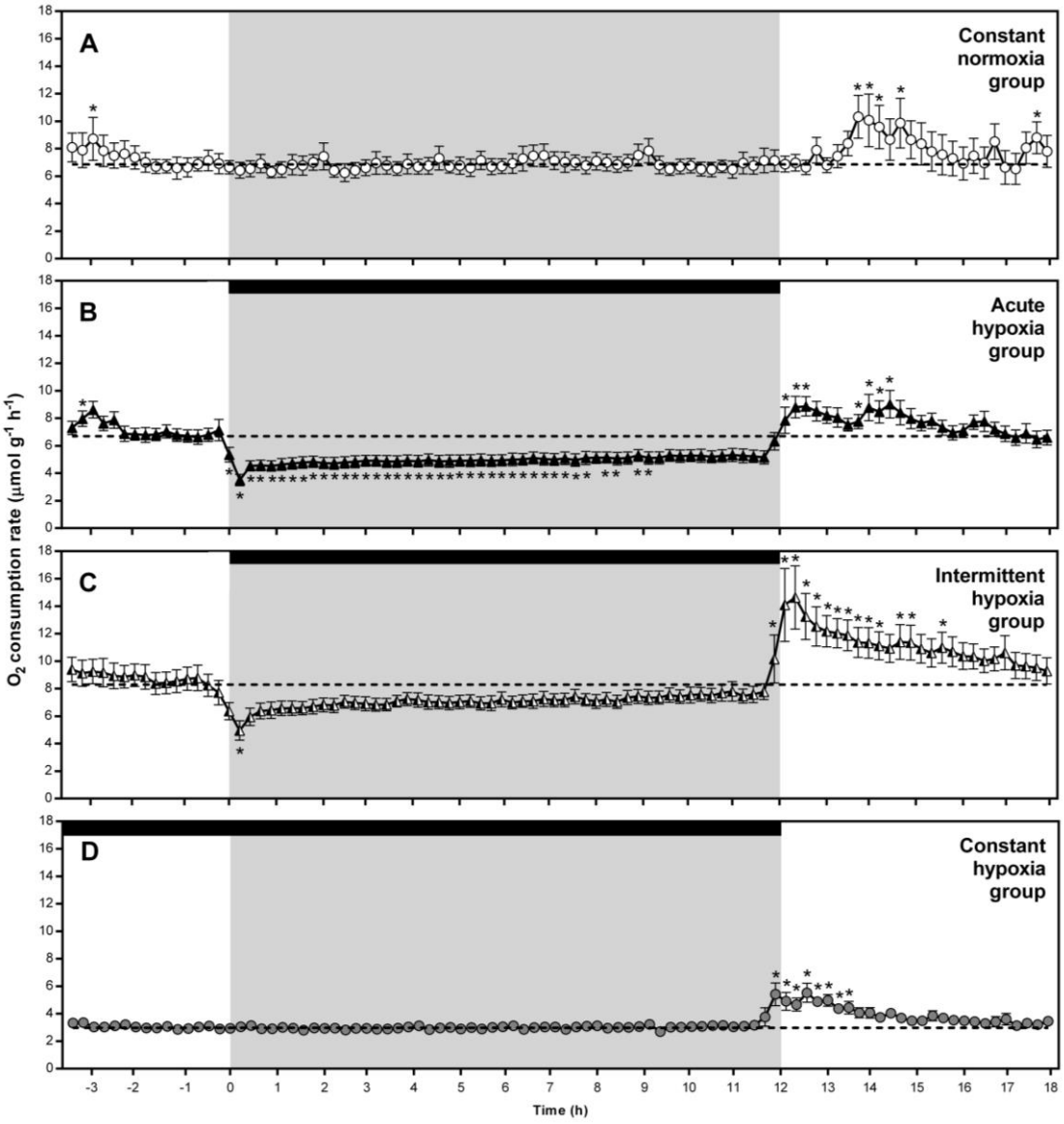
A
Constant normoxia group
B
Acute hypoxia group
C
Intermittent hypoxia group
D
Constant hypoxia group
O_2 consumption rate (μmol g^{-1} h^{-1})
Time (h)

Fig. 3.2 Whole-animal oxygen consumption rate (MO_2) of killifish acclimated to normoxia, intermittent hypoxia, and constant hypoxia. (A) Fish acclimated to and held in constant normoxia (main effect of time on MO_2: $F_{[94,658]}= 2.543$, $p<0.0001$). (B) Fish acclimated to constant normoxia, but exposed to 12 h of acute hypoxia at 2 kPa O_2 during the night (0 h to 12 h) followed by 6 h of reoxygenation in normoxia during the day ($F_{[94,1222]}= 19.59$, $p<0.0001$). (C) Fish acclimated to intermittent cycles of nocturnal hypoxia (12 h normoxia: 12 h hypoxia at 2 kPa O_2) and measured during the same cycle ($F_{[94,658]}= 10.75$, $p<0.0001$). (D) Fish acclimated to and measured in constant hypoxia at 2 kPa O_2 until 12 h, followed by reoxygenation in normoxia ($F_{[98,686]}= 5.982$, $p<0.0001$). The dark phase of the photoperiod (1900 to 0700 local time) is indicated by the grey section of the background, and the timing of hypoxia exposures are indicated by black bars along the top of each panel. Dashed lines represent average resting MO_2. * indicates a significant pairwise difference from resting MO_2. Sample sizes were as follows: constant normoxia and constant hypoxia, 8; acute hypoxia, 14; intermittent hypoxia, 8.

Figure 3.3

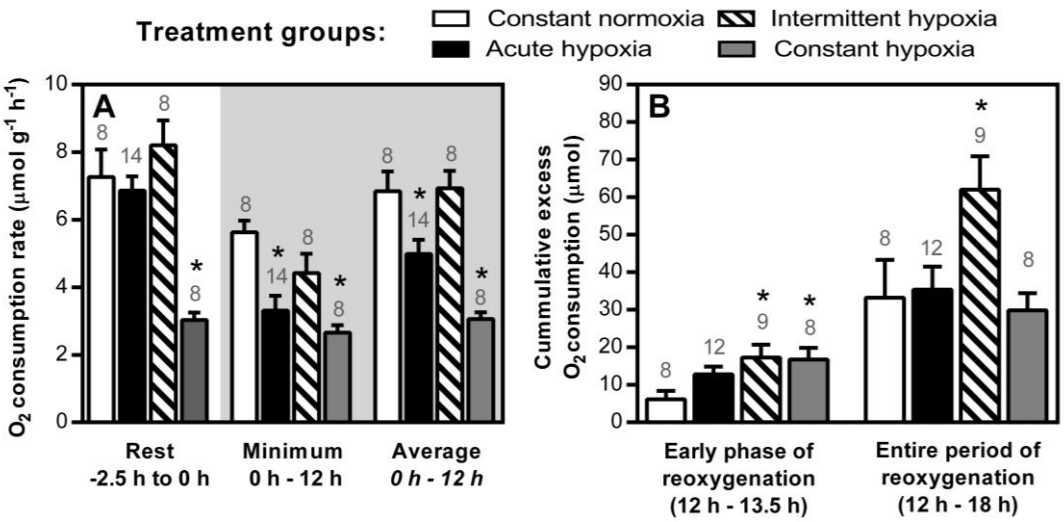
Treatment groups:
Constant normoxia
Intermittent hypoxia
Acute hypoxia
Constant hypoxia
A
B
O_2 consumption rate (μmol g^{-1} h^{-1})
Cummulative excess O_2 consumption (μmol)
Rest
-2.5 h to 0 h
Minimum
0 h - 12 h
Average
0 h - 12 h
Early phase of reoxygenation
(12 h - 13.5 h)
Entire period of reoxygenation
(12 h - 18 h)

Fig. 3.3 Effects of hypoxia acclimation on O_2 consumption rates during a hypoxia-reoxygenation cycle. (A) Resting MO_2 (measured from -2.5 h to 0 h of the daily cycle, which was in normoxia for all groups except the constant hypoxia group; main effect of treatment group, $F_{[3,34]}$=13.96, p<0.0001), minimum overnight MO_2 (measured between 0 h to 12 h of the daily cycle, which was in hypoxia for all groups except normoxic controls; $F_{[3,34]}$= 7.771, p=0.0004), and average overnight MO_2 (also measured from 0 h to 12 h of the daily cycle; $F_{[3,34]}$=13.62, p<0.0001). (B) Cumulative increase in O_2 consumption above resting MO_2 in the early phase of reoxygenation in normoxia (12 h to 13.5 h of the daily cycle) ($F_{[3,32]}$= 3.200, p=0.0359) and the entire normoxic reoxygenation period (12 h to 18 h of the daily cycle) ($F_{[3,33]}$= 3.653, p=0.0223). *Significant pairwise difference from constant normoxia group (P<0.05). Sample sizes for each group for each measurement are indicated directly above the bar.

Figure 3.4

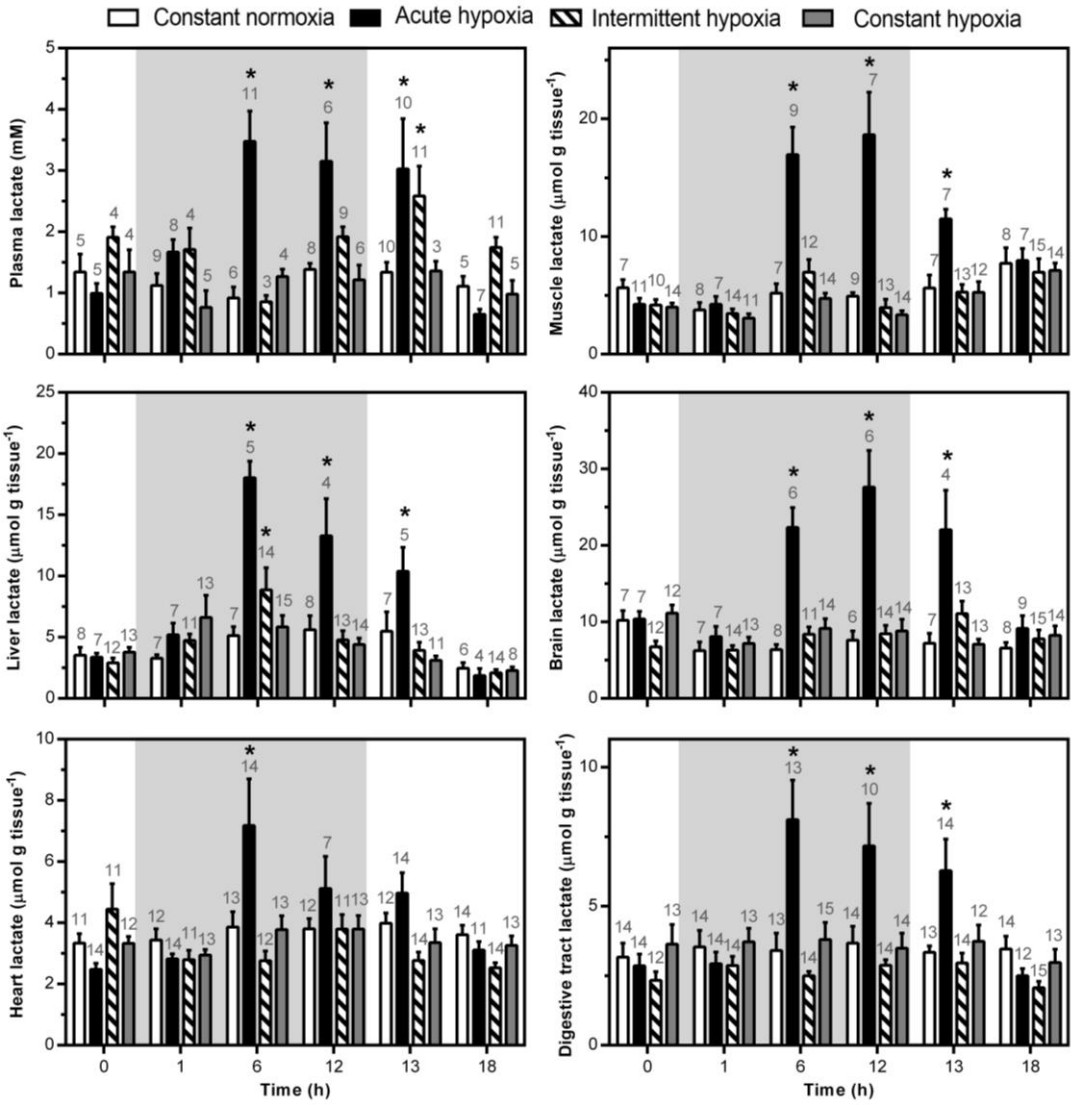
Constant normoxia
Acute hypoxia
Intermittent hypoxia
Constant hypoxia
Plasma lactate (mM)
Muscle lactate (µmol g tissue-1)
Liver lactate (µmol g tissue-1)
Brain lactate (µmol g tissue-1)
Heart lactate (µmol g tissue-1)
Digestive tract lactate (µmol g tissue-1)
Time (h)
0
1
6
12
13
18

Fig. 3.4 Fish exposed to acute hypoxia accumulated lactate in several tissues during hypoxia. Dark phase of the photoperiod (1900 to 0700 local time) is indicated by the grey section of the background. *Significant pairwise difference from constant normoxia group at the same time point ($P<0.05$). Sample sizes for each group at each time point are indicated directly above the bar.

Figure 3.5

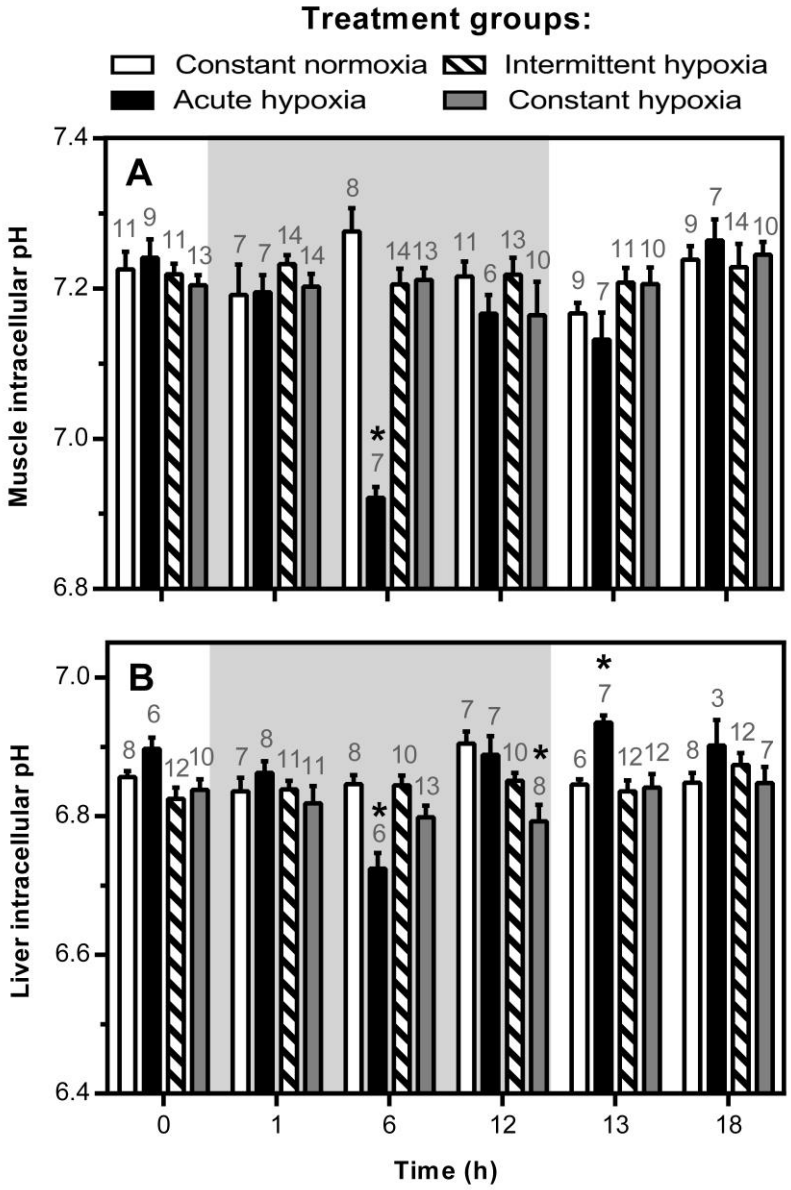
Treatment groups:
Constant normoxia
Intermittent hypoxia
Acute hypoxia
Constant hypoxia
A
Muscle intracellular pH
7.4
7.2
7.0
6.8
B
Liver intracellular pH
7.0
6.8
6.6
6.4
0
1
6
12
13
18
Time (h)

Fig. 3.5 Fish exposed to acute hypoxia experienced a transient acidosis in the muscle and liver during hypoxia. Dark phase of the photoperiod (1900 to 0700 local time) is indicated by the grey section of the background. *Significant pairwise difference from constant normoxia group at the same time point ($P<0.05$). Sample sizes for each group at each time point are indicated directly above the bar.

Figure 3.6

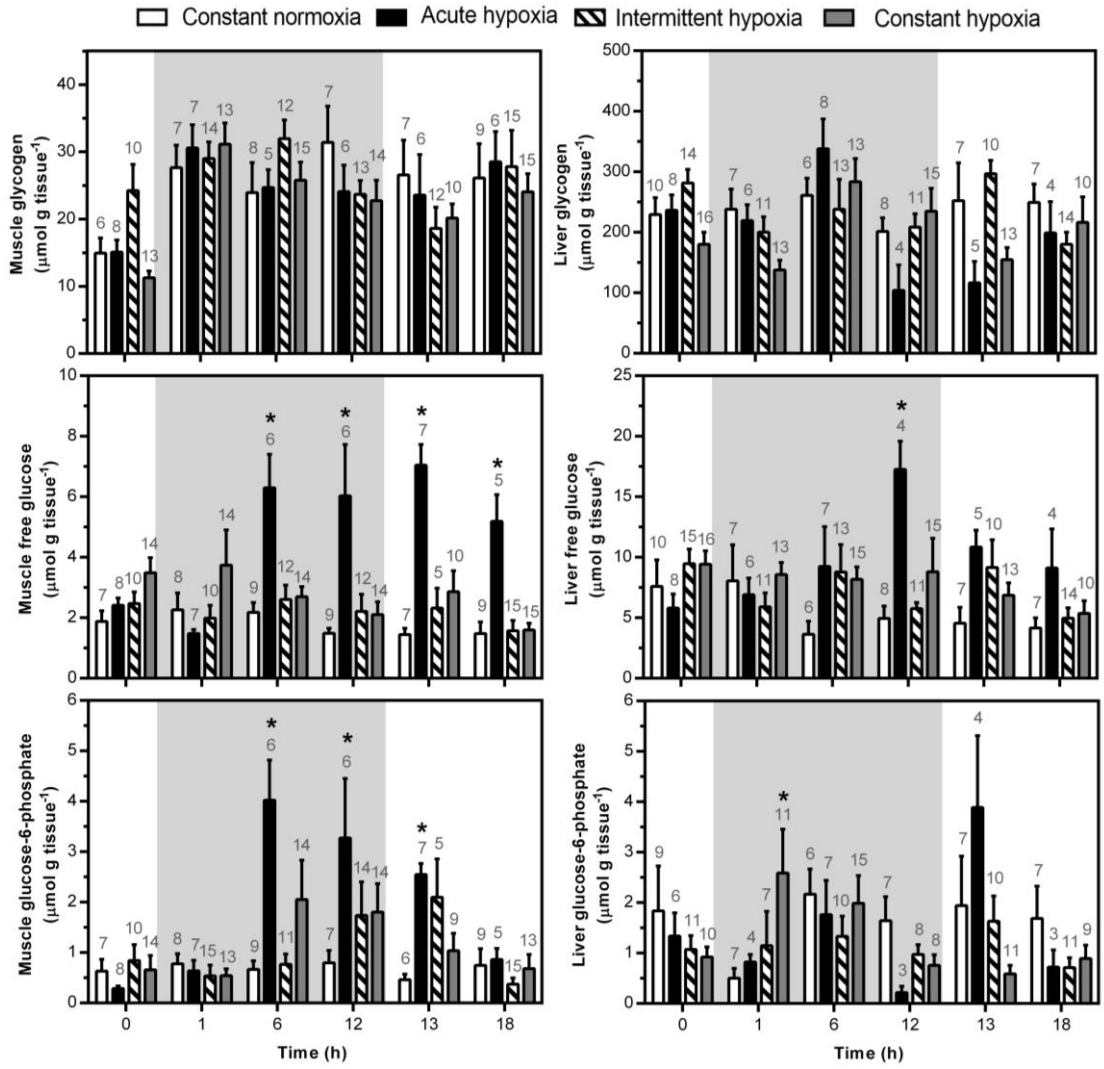
Constant normoxia
Acute hypoxia
Intermittent hypoxia
Constant hypoxia
Muscle glycogen (µmol g tissue-1)
Liver glycogen (µmol g tissue-1)
Muscle free glucose (µmol g tissue-1)
Liver free glucose (µmol g tissue-1)
Muscle glucose-6-phosphate (µmol g tissue-1)
Liver glucose-6-phosphate (µmol g tissue-1)
Time (h)
0
1
6
12
13
18

Fig. 3.6 Glycogen, glucose, and glucose-6-phosphate content of skeletal muscle and liver during hypoxia-reoxygenation. Dark phase of the photoperiod (1900 to 0700 local time) is indicated by the grey section of the background. *Significant pairwise difference from constant normoxia group at the same time point ($P<0.05$). Sample sizes for each group at each time point are indicated directly above the bar.

Figure 3.7

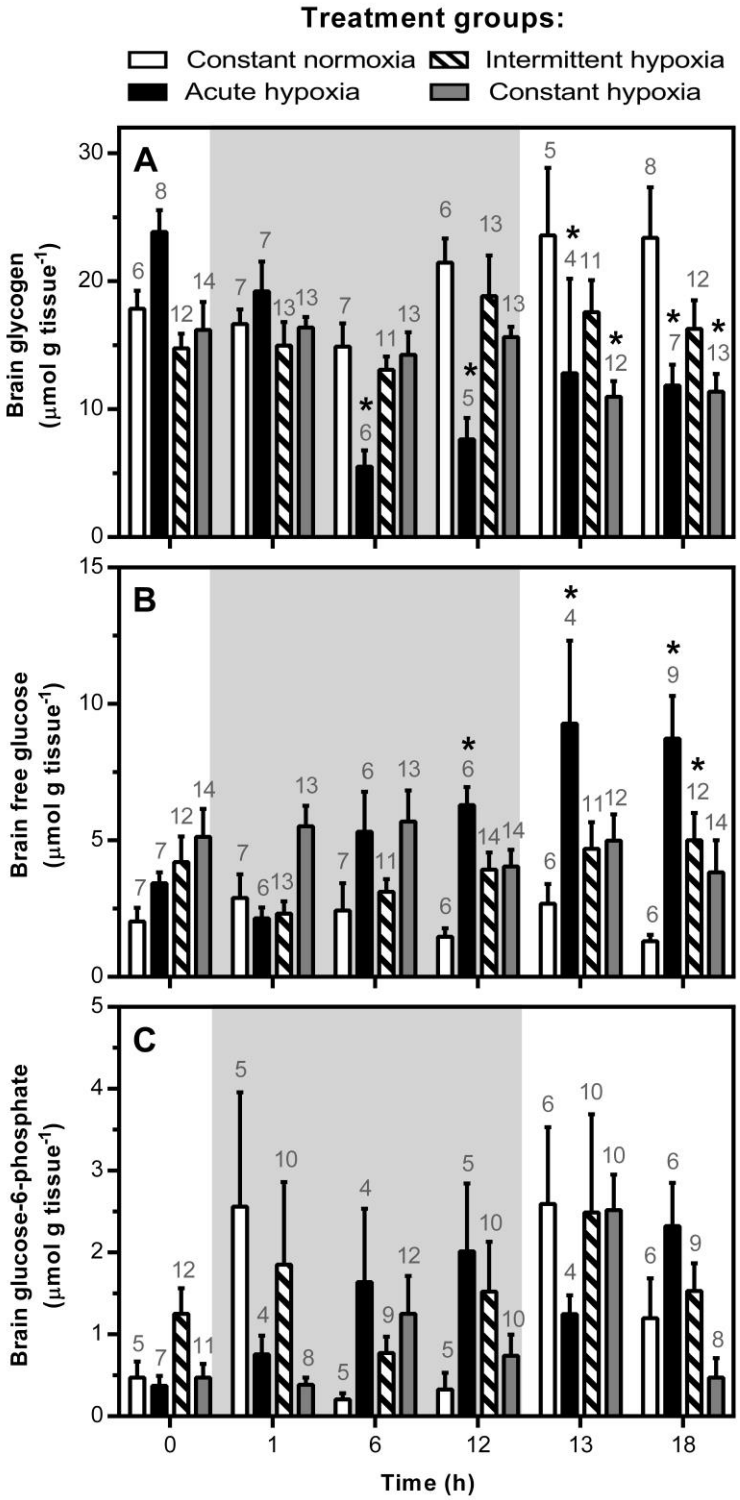
Treatment groups:
Constant normoxia
Intermittent hypoxia
Acute hypoxia
Constant hypoxia
A
B
C
Brain glycogen (μmol g tissue-1)
Brain free glucose (μmol g tissue-1)
Brain glucose-6-phosphate (μmol g tissue-1)
Time (h)
0
1
6
12
13
18

Fig. 3.7 Glycogen, glucose, and glucose-6-phosphate content of the brain during hypoxia-normoxia. Dark phase of the photoperiod (1900 to 0700 local time) is indicated by the grey section of the background. *Significant pairwise difference from constant normoxia group at the same time point ($P<0.05$). Sample sizes for each group at each time point are indicated directly above the bar.

Figure 3.8

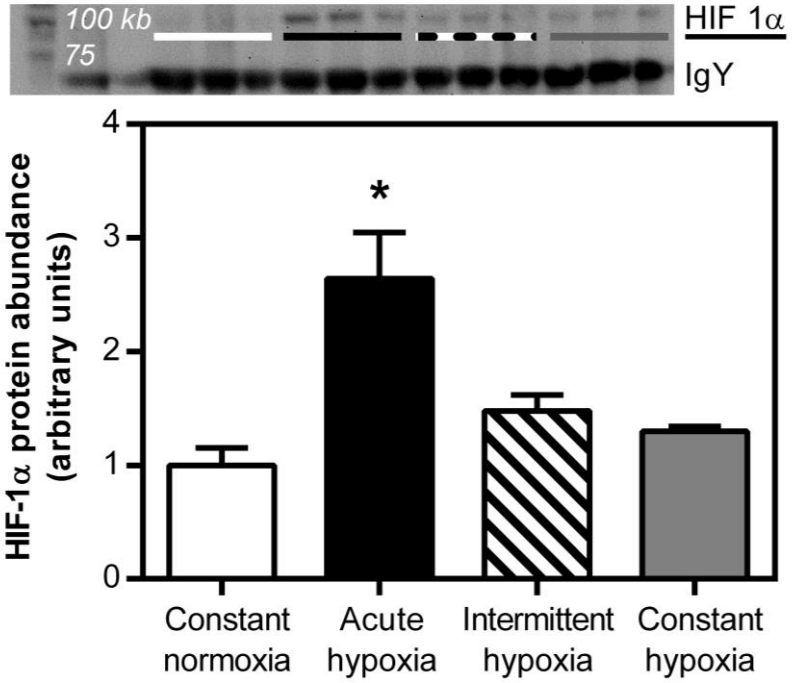
100 kb
75
HIF 1α
IgY
HIF-1α protein abundance (arbitrary units)
0
1
2
3
4
*
Constant normoxia
Acute hypoxia
Intermittent hypoxia
Constant hypoxia

Fig. 3.8 Fish exposed to acute hypoxia had increased HIF-1*α* protein in skeletal muscle at 12 h of the hypoxia-reoxygenation cycle. There was a significant main effect of treatment group ($F_{[3,8]}$=9.818, p=0.0047; n=3 for each acclimation group). *Significant pairwise difference from constant normoxia group (P<0.05).

3.11 Tables

Table 3.1: Statistical analysis of data presented in Figs. 3.4 – 3.7 and Table 3.2

	Main effect of acclimation		Main effect of time		Main effect of interaction	
	F	p	F	p	F	p
Brain						
ATP	1.331 $_{(3, 194)}$	0.2654	3.103 $_{(5, 194)}$	0.0102	1.697 $_{(15, 194)}$	0.0541
Glu	11.63 $_{(3,208)}$	<0.0001	2.100 $_{(5, 208)}$	0.0667	2.257 $_{(15, 208)}$	0.0058
G6P	1.282 $_{(3, 157)}$	0.2826	2.721 $_{(5, 157)}$	0.0218	1.136 $_{(15, 157)}$	0.3284
Gly	7.255 $_{(3, 202)}$	0.0001	3.400 $_{(5, 202)}$	0.0057	3.236 $_{(15, 202)}$	<0.0001
Lac	37.59 $_{(3, 217)}$	<0.0001	10.42 $_{(5, 217)}$	<0.0001	6.741 $_{(15, 217)}$	<0.0001
Heart						
Lac	4.574 $_{(3, 274)}$	0.0038	4.426 $_{(5, 274)}$	0.0007	3.452 $_{(15, 274)}$	<0.0001
Digestive tract						
Lac	14.8 $_{(3, 298)}$	<0.0001	5.491 $_{(5, 298)}$	<0.0001	3.669 $_{(15, 298)}$	<0.0001
Liver						
ATP	2.177 $_{(3, 199)}$	0.0919	4.798 $_{(5, 199)}$	0.0004	1.309 $_{(15, 199)}$	0.1992
Glu	4.103 $_{(3, 211)}$	0.0072	1.166 $_{(5, 211)}$	0.0327	1.163 $_{(15, 211)}$	0.0722
G6P	0.823 $_{(3, 167)}$	0.4832	2.099 $_{(5, 167)}$	0.0679	1.794 $_{(15, 167)}$	0.0391
Gly	2.076 $_{(3, 209)}$	0.1044	3.558 $_{(5, 209)}$	0.0048	2.344 $_{(15, 209)}$	0.0040
pH_i	6.043 $_{(3, 185)}$	0.0006	6.594 $_{(5, 185)}$	<0.0001	3.821 $_{(15, 185)}$	<0.0001
Lac	16.19 $_{(3, 207)}$	<0.0001	20.90 $_{(5, 207)}$	<0.0001	5.123 $_{(15, 207)}$	<0.0001
Muscle						
ATP	0.2029 $_{(3, 226)}$	0.8943	8.169 $_{(5, 226)}$	<0.0001	1.465 $_{(15, 226)}$	0.1199
Glu	19.65 $_{(3, 212)}$	<0.0001	2.218 $_{(5, 212)}$	0.0537	3.815 $_{(15, 212)}$	<0.0001
G6P	5.772 $_{(3, 208)}$	0.0008	7.372 $_{(5, 208)}$	<0.0001	2.114 $_{(15, 208)}$	0.0105
Gly	1.196 $_{(3, 214)}$	0.3121	5.796 $_{(5, 214)}$	<0.0001	0.985 $_{(15, 214)}$	0.4721
pH_i	8.181 $_{(3, 221)}$	<0.0001	7.072 $_{(5, 221)}$	<0.0001	6.159 $_{(15, 221)}$	<0.0001
Lac	41.62 $_{(3, 226)}$	<0.0001	14.71 $_{(5, 226)}$	<0.0001	8.933 $_{(15, 226)}$	<0.0001
PCr	4.184 $_{(3, 230)}$	0.0066	3.217 $_{(5, 230)}$	0.0079	1.443 $_{(15, 230)}$	0.1285
Plasma						
Lac	7.830 $_{(3, 135)}$	0.0001	3.070 $_{(5, 135)}$	0.0117	2.511 $_{(15, 135)}$	0.0027

G6P, glucose-6-phosphate; Glu, glucose; Gly, glycogen; Lac, lactate; pH_i, intracellular pH; PCr, phosphocreatine. The degrees of freedom for the numerator and the denominator, respectively, for each ANOVA are reported in parentheses.

Table 3.2: ATP and Phosphocreatine in the muscle, liver, and brain

		Treatment group			
	Time	Constant normoxia 20 kPa O_2	Acute hypoxia 2 kPa O_2	Intermittent hypoxia 2 kPa O_2	Constant hypoxia 2 kPa O_2
Muscle					
ATP	0 h	5.96 ± 1.17 (7)	3.97 ± 0.34 (10)	4.54 ± 0.60 (10)	4.37 ± 0.43 (14)
	1 h	6.58 ± 0.91 (8)	6.82 ± 0.91 (7)	5.52 ± 0.42 (12)	6.45 ± 0.53 (14)
	6 h	4.15 ± 0.72 (7)	2.84 ± 0.40 (10)	3.62 ± 0.48 (13)	4.65 ± 0.74 (14)
	12 h	3.77 ± 0.63 (8)	4.42 ± 0.63 (10)	4.16 ± 0.56 (11)	4.40 ± 0.53 (13)
	13 h	4.09 ± 0.42 (7)	3.56 ± 0.43 (7)	4.52 ± 0.83 (13)	2.94 ± 0.35 (12)
	18 h	3.97 ± 0.57 (8)	6.46 ± 1.07 (7)	4.56 ± 0.67 (14)	4.35 ± 0.67 (14)
PCr	0 h	12.48 ± 1.00 (6)	10.98 ± 0.85 (11)	17.47 ± 2.44 (10)	13.54 ± 1.50 (14)
	1 h	17.10 ± 4.76 (8)	11.58 ± 1.56 (7)	11.60 ± 1.74 (14)	13.24 ± 1.25 (14)
	6 h	9.99 ± 1.03 (7)	5.53 ± 0.94 (10)	10.75 ± 0.82 (13)	11.30 ± 0.94 (14)
	12 h	7.21 ± 1.03 (9)	7.39 ± 0.93 (10)	16.80 ± 4.27 (12)*	14.44 ± 3.34 (13)*
	13 h	12.38 ± 0.71 (7)	14.94 ± 1.27 (7)	17.53 ± 3.26 (13)	15.09 ± 1.02 (12)
	18 h	9.52 ± 1.16 (8)	12.04 ± 1.65 (7)	14.03 ± 2.48 (14)	8.51 ± 1.49 (14)
Liver					
ATP	0 h	2.73 ± 0.54 (7)	3.98 ± 0.87 (6)	2.35 ± 0.47 (11)	2.35 ± 0.44 (10)
	1 h	3.80 ± 0.41 (8)	2.51 ± 0.45 (7)	3.24 ± 0.46 (11)	3.21 ± 0.48 (13)
	6 h	2.17 ± 0.52 (8)	1.25 ± 0.26 (6)	1.42 ± 0.17 (13)	1.91± 0.23 (14)
	12 h	2.11 ± 0.47 (8)	1.11 ± 0.22 (6)	2.56 ± 0.39 (11)	2.96 ± 0.73 (12)
	13 h	3.75 ± 0.86 (7)	1.84 ± 0.45 (7)	3.12 ± 0.63 (12)	2.44 ± 0.28 (13)

Table 3.2 continued

	18 h	3.30 ± 0.50 (8)	2.02 ± 0.66 (4)	3.05 ± 0.42 (12)	2.86 ± 0.34 (9)
Brain					
ATP	0 h	0.93 ±0.27 (6)	1.15 ± 0.10 (7)	0.92 ± 0.34 (10)	0.54 ± 0.11 (12)
	1 h	0.71 ± 0.16 (6)	0.13 ± 0.04 (6)	0.30 ± 0.05 (12)	0.60 ± 0.17 (12)
	6 h	0.88 ± 0.07 (7)	0.34 ± 0.09 (5)	0.80 ± 0.12 (11)	0.83 ± 0.06 (12)
	12 h	0.77 ± 0.01 (6)	0.33 ± 0.09 (5)	0.81 ± 0.13 (14)	0.82 ± 0.08 (13)
	13 h	0.62 ± 0.12 (7)	1.07 ± 0.60 (4)	0.66 ± 0.26 (12)	0.75 ± 0.11 (13)
	18 h	0.90 ± 0.14 (8)	0.41 ± 0.12 (9)	0.56 ± 0.05 (11)	0.59 ± 0.14 (10)

PCr, phosphocreatine. Metabolite contents are expressed in μmol g tissue^{-1}. Data are reported as means ± standard error, with the same size in brackets. *Significant pairwise difference from constant normoxia at the same time point ($P<0.05$), tested via a two-way ANOVA followed by a Bonferroni post-hoc comparison.

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CHAPTER 4

Reversible modulation of blood O_2 carrying capacity in estuarine killifish (*Fundulus heteroclitus*) acclimated to intermittent hypoxia

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4.1 Introduction

Periods of low O_2 availability are common in the aquatic environment, and originate from both natural and anthropogenic sources (Breitburg et al., 2018; Diaz, 2001; Diaz and Breitburg, 2009; Hasler et al., 2009). Aquatic hypoxia can take on several different patterns of exposure, such as periods of stable constant hypoxia or repeated periods of hypoxia followed by reoxygenation to normoxia or even hyperoxia (Breitburg et al., 2018; Breitburg, 1992; Diaz and Breitburg, 2009; Hasler et al., 2009; Tyler et al., 2009). Global climate change and extensive human interactions with aquatic ecosystems (e.g. fertilizer run-off, pollution, etc.) are expected to increase the incidence of aquatic hypoxia worldwide (Breitburg et al., 2018; Diaz, 2001; Diaz and Rosenberg, 2008).

To successfully cope with hypoxia, aerobic organisms must mitigate the decrease in aerobic ATP production induced by hypoxia, and thus avoid the development of an ATP supply-demand imbalance (Boutilier, 2001; Hochachka et al., 1996; Richards, 2009). One ubiquitous strategy is to activate several cardiorespiratory responses that help protect O_2 transport from the environment to the mitochondria within tissues (Perry et al., 2009; Storz et al., 2010). Generally, these cardiorespiratory responses work by extending the PO_2 range where animals can maintain resting O_2 consumption rate, avoid unsustainable use of anaerobic metabolism (e.g. glycolysis), and support some aerobic scope for activity (Chabot and Claireaux, 2008; Lefrançois et al., 2005; Pörtner and Grieshaber, 1993; Portner and Peck, 2010; Richards, 2009).

Fish from hypoxia-prone habitats typically have greater gill surface area than fishes from well-oxygenated habitats (Chapman et al., 2002; Childress and Seibel, 1998; Mandic et al., 2009), and therefore a greater total area available for O_2 uptake. Fish gills are highly plastic (Nilsson, 2007; Wilson and Laurent, 2002), and prolonged hypoxia exposure can also lead to an increase in gill surface area through gill remodelling, often involving a reduction in interlamellar cell mass (Blank and Burggren, 2014; Dhillon et al., 2013; Fu et al., 2011; Matey et al., 2008; Nilsson et al., 2012; Søllid et al., 2003). Even without alterations in total gill surface area, fish can improve diffusive O_2 uptake through increases in total gill ventilation (Gamperl and Driedzic, 2009; Perry et al., 2009; Porteus et al., 2011) and/or lamellar perfusion (Booth, 1979; Perry et al., 2009; Randall and Daxboeck, 1984) during acute hypoxia. With prolonged exposure to hypoxia, the initial increase in ventilation is slowly abolished, reflecting greater reliance on other respiratory responses such as changes in blood O_2 carrying capacity (Florindo et al., 2006; Porteus et al., 2011; Powell et al., 1998; Stecyk and Farrell, 2002).

The O_2 carrying capacity of the blood can be modulated by increasing blood haemoglobin content through the proportion of erythrocytes in the blood (haematocrit) and/or the concentration of haemoglobin within erythrocytes (mean corpuscular haemoglobin concentration) (Affonso et al., 2002; Bose et al., 2019; Hughes, 1973; Nikinmaa and Soivio, 1982; Wells, 2009), and these responses can be maintained for weeks during chronic exposure to hypoxia (Borowiec et al., 2015, see Chapter 2 of this volume;

Greaney et al., 1980). The affinity of haemoglobin for O_2 can also be increased in hypoxia *via* decreases in the concentration of negative allosteric modifiers of haemoglobin-O_2 binding like ATP and GTP (Nikinmaa, 2001; Val, 2000; Val et al., 1998; Wells, 2009) or by expressing different haemoglobin isoforms (Pan et al., 2017; Rutjes et al., 2007; van den Thillart et al., 2018) to improve O_2 loading at the gills. Haemoglobin-O_2 binding affinity is also highly sensitive to the intracellular pH and chloride levels within red blood cells, and the former can be finely tuned to facilitate O_2 unloading at the tissue (and loading at the gills) (Jensen, 2004; Nikinmaa, 2001).

Our recent work suggests that acclimation to intermittent cycles of hypoxia may lead to substantial alterations in the O_2 transport cascade of *Fundulus* killifish (Borowiec et al., 2015, see Chapter 2 of this volume; Borowiec et al., 2018, see Chapter 3 of this volume). Unlike fish exposed to acute hypoxia or acclimated to constant hypoxia, fish acclimated for 28 d to intermittent maintain O_2 consumption rates comparable to normoxic fish at rest during bouts of severe hypoxia (Borowiec et al., 2018, see Chapter 3 of this volume). Measurements during daytime normoxia suggested that this ability was not associated with an expansion of gill surface area, increases in haematocrit and whole-blood haemoglobin content, or with alterations in heart mass (Borowiec et al., 2015, see Chapter 2 of this volume), but it is possible that fish acclimated to intermittent hypoxia are capable of rapid modifications of O_2 transport physiology such that differences may only be apparent during night-time bouts of hypoxia. Such dynamic and effective modulation of O_2 transport capacity could be a key component of the acclimation response to rapid

cycles of hypoxia and reoxygenation in killifish. While alterations in gill and cardiac morphology typically occur over days or weeks, alterations in blood O_2 carrying capacity can occur much more quickly through modulation of haematocrit by splenic contraction or modulation of haemoglobin-O_2 binding affinity through decreases in the levels of negative allosteric modulators or intracellular pH within red blood cells. Therefore, comparatively rapid alterations in blood O_2 carrying capacity may be key in the response to intermittent hypoxia in fishes and were therefore the focus of the present investigation.

Our objective was to investigate how killifish may modulate O_2 transport capacity during nighttime hypoxia bouts, and how these responses contribute to the distinct coping strategies observed in fish acclimated to intermittent and constant patterns of hypoxia. To do this, we characterized how maximal oxygen consumption rate, aerobic scope, and blood O_2 carrying capacity responded to acute hypoxia in normoxia-acclimated killifish, and then examined how these responses were altered by chronic exposure to intermittent or constant patterns of hypoxia.

4.2 Materials and methods

4.2.1 Study animals

Adult, wild-caught killifish (*Fundulus heteroclitus*) of mixed sex (average body mass of 5.62 g ± 0.22) were shipped from a commercial supplier (Aquatic Research Organisms,

NH, USA) to McMaster University, Ontario, Canada. Prior to acclimation (see below), killifish were held in large ~300 L fibreglass tanks filled with brackish (4 ppt), room temperature (~22°C) water that was continuously aerated with an air stone. Fish were fed commercial feed (EWOS Canada, Ltd.) at least four days a week. The photoperiod was maintained as 12 h light to 12 h dark, with the daylight portion occurring from 0700 to 1900 h local time. Water quality (ammonia, pH, nitrates and nitrites) was maintained by cycling water through a charcoal filter, and by routine water changes. Normoxia and hypoxia acclimation treatments began at least four weeks after fish arrived at McMaster University. All animal protocols followed guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board.

4.2.2 Hypoxia acclimations

Fish were subjected to 28 day acclimation to normoxia, constant hypoxia (2 kPa O_2, ~0.8 O_2 l^{-1}), or nocturnal ('intermittent') hypoxia (12 h normoxia during the daytime light phase:12 h at 2 kPa O_2 during the night-time dark phase) carried out in a custom 88 l multi-stressor exposure system that automatically monitors and controls water PO_2, salinity, and temperature (Aquabiotech, Coaticook, QC, Canada). Water quality was regularly monitored, and maintained by daily water changes, totalling 20% of system volume. Normoxia (20 kPa O_2, 8 mg O_2 l^{-1}) or hypoxia (2 kPa O_2, 0.8 mg O_2 l^{-1}) exposures were achieved by bubbling aquarium water with oxygen or nitrogen gas based

on a feedback loop from a galvanic oxygen probe. Transitions between normoxia and hypoxia during acclimation to intermittent hypoxia occurred over ~1 h at 0700 or 1900 local time, such that these animals were exposed to fully air saturated normoxic water from 0800 to 1900, and exposed to the nominal PO_2 of hypoxic water from 2000 to 0700. Fish acclimated to constant hypoxia were held in hypoxic water for 24 h a day. Tanks were covered with a hard plastic lid and had minimal headspace (~2 cm) to limit diffusion from the atmosphere.

4.2.3 Sampling

We sampled killifish acclimated to normoxia, intermittent hypoxia, and constant hypoxia in resting conditions at 0100 and 1300 local time (i.e. at the 6 h midpoint of the normoxia and hypoxia periods for the intermittent treatment group). We also sampled a group of normoxia acclimated fish after 6 h of night-time exposure to acute hypoxia at 2 kPa O_2 ("acute hypoxia" group).

Fish were netted and euthanized in a small volume of aquarium water containing an overdose of benzocaine (final concentration ~1 g l^{-1}, dissolved in 95% ethanol). The tail was immediately bisected at the base of the anal fin, and blood was collected from the caudal blood vessels in a heparinized capillary tube. The blood in the capillary tube was immediately spun in a chilled centrifuge for 3 min at 12,700 g to measure haematocrit. Separated plasma and red bloods cells were then snap frozen in liquid nitrogen. A small

sample (6 µl) of whole blood was also used to measure haemoglobin content (using Drabkin's reagent following manufacturer's instructions; Sigma-Aldrich, Oakville, ON, Canada). Fish mass as well as the masses of the whole spleen and whole heart were measured. Red blood cells were stored at −80°C until measurement of ATP and GTP levels.

4.2.4 Red blood cell ATP and GTP

Frozen red blood cells were lysed in 5 volumes of a hypotonic solution of 10 mmol l^{-1} Tris-HCl (pH 7.4), vortexed, and spun for 10 min at 15,000 g at 4°C. A portion of this supernatant (20 µl) was added to 80 µl of 3% $HClO_3$. This acidified sample was spun for 10 min at 10,000 g at 4°C before being neutralized with 16 µl of 3 mol l^{-1} Tris (pH 12.0). Neutralized extracts were immediately used for quantification of red blood cell ATP and then GTP content using a 96-well plate format assay. Initial assays conditions were as follows: 65 mmol l^{-1} $MgCl_2$, 7.2 mmol l^{-1} glucose, 7.4 mmol l^{-1} EDTA, 6.67 mmol l^{-1} β-mercaptoethanol, 12.7 mmol l^{-1} β-$NADP^+$, and excess of the coupling enzyme glucose-6-phosphate dehydrogenase (G6PDH; 2.5 U ml^{-1}) in 0.67 mol^{-1} Tris (pH 8.0). Excess hexokinase (HK; 15 U ml^{-1}) was added to initiate the conversion of ATP to ADP, measured as the production of β-NADPH via the coupled G6PDH reaction. ADP (11.8 mmol l^{-1}) and then excess nucleoside diphosphate kinase (15 U ml^{-1}) was then added to begin the conversion of GTP to ATP, measured as the production of β-NADPH via the coupled HK and G6PDH reactions. Standard curves were constructed with known

concentrations of ATP and GTP to relate the absolute change in absorbance at 340 nm to the total ATP/GTP concentration of the extract. Assays were run in triplicate at 37°C on a BioTek Synergy H1 microplate reader (BioTek Instruments, Inc., VT, USA).

Red blood cell ATP and GTP concentrations were expressed relative to the concentration of haemoglobin within the lysed red blood cell sample (measured as described above).

4.2.5 Respirometry experiments

We measured the maximal O_2 consumption rate ($MO_{2,max}$) elicited by exhaustive exercise at 0100 local time at the typical acclimation PO_2 for each treatment (e.g. 20 kPa O_2 for normoxia-acclimated fish, and 2 kPa O_2 for intermittent hypoxia and constant hypoxia acclimated fish). We also determined $MO_{2,max}$ in a group of normoxia acclimated fish after 6 h of night-time exposure to 2 kPa O_2 hypoxia ("acute hypoxia" group).

Killifish were introduced to 160 ml respirometry chambers at ~0900 local time, which was ~15 h prior to the start of the exhaustive chase (see below), and were held at the same PO_2 as they would have been exposed to during their acclimation treatment (i.e., normoxia for all groups except for constant hypoxia, which were held at 2 kPa O_2). At 1900, the acute hypoxia and intermittent hypoxia groups were exposed to hypoxia at 2 kPa O_2, while the normoxic controls remained in normoxia. Resting rates of O_2 consumption (MO_2) were then measured continuously using stop-flow respirometry

(Borowiec et al., 2015, see Chapter 2 of this volume; Borowiec et al., 2018, see Chapter 3 of this volume) until just before 0100 local time. Beginning at 0100 local time, killifish were individually removed from the respirometry chambers and chased in a bucket with a net for 2 min, and were then removed from water and exposed to air for 30 s (this combined chase and air exposure approach has been shown to induce higher O_2 consumption rates than chasing alone) (Roche et al., 2013). Fish were noticeably sluggish at the end of the chase period and did not respond to gentle tapping with the net. Fish were returned to their respirometry chambers and MO_2 was measured continuously in normoxia (normoxia group only) or hypoxia (acute hypoxia, intermittent hypoxia, and constant hypoxia groups) using stop-flow respirometry for 6 h. $MO_{2,max}$ was the highest MO_2 recorded immediately after exhaustion and resting MO_2 was the average of the last 10 measurements of MO_2 prior to the start of the exhaustive chase (between ~2320 and 0100 local time). Both $MO_{2,\ max}$ and resting MO_2 are expressed relative to body mass.

4.2.6 Statistics

Data are reported as means ± standard error. Data were first checked for normality using a Shapiro-Wilk test (data not shown). We used one-way ANOVA (on ranks in cases when normality was not confirmed) followed by Sidak's multiple comparisons test (for normal data) or a Dunn's test (for non-normal data) between hypoxia treatments against their relevant time-matched normoxic controls. A significance level of $P<0.05$ was used throughout.

4.3 Results

4.3.1 Blood O_2 carrying capacity

Whole-blood haemoglobin content varied dynamically in response to daily cycles of nocturnal hypoxia ($F_{[6,67]} = 8.689$, $p < 0.0001$) (Fig. 4.1A). Haemoglobin content did not vary between day and night in normoxic controls. However, exposure to acute hypoxia for 6 h lead to a ~60% increase in whole-blood haemoglobin content relative to normoxic controls. Fish acclimated to intermittent hypoxia exhibited a remarkably similar pattern of variation, in which haemoglobin content was similar to normoxic controls during daytime normoxia but was similar to the acute hypoxia group during night-time hypoxia (Fig. 4.1A). Fish acclimated to constant hypoxia maintained high levels of haemoglobin throughout the night and day, at levels that were statistically similar but slightly less on average than the other hypoxia-exposed groups. This variation in whole-blood haemoglobin was mirrored by changes in haematocrit (Fig. 4.1B), as reflected by a significant main effect of treatment group ($F_{[6,67]} = 9.610$, $p < 0.0001$), such that mean cell haemoglobin concentration did not differ across groups ($F_{[6,67]} = 0.8048$, $p = 0.5698$) (Fig. 4.1C).

This variation in whole-blood haemoglobin content and haematocrit appeared to be largely mediated by storage and release of red blood cells by the spleen (Fig. 4.2).

Hypoxia-exposed fish tended to have smaller spleens relative to fish mass ($H_{[7]} = 19.81$, $p = 0.0030$), and this was most apparent in the acute hypoxia group. (Fig. 4.2A). Mass-specific spleen mass also correlated with whole-blood haemoglobin content, such that treatment groups with high whole-blood haemoglobin content tended to also have smaller spleens relative to fish mass (Fig. 4.2B). The pattern of variation in spleen mass between groups was similar after correcting for body mass using a residual approach using an allometric regression (data not shown). Mass-specific spleen mass also correlated with whole-blood haemoglobin content, such that treatment groups with high whole-blood haemoglobin content tended to also have smaller spleens relative to fish mass (Fig. 4.2B).

In contrast in the substantial variation in haematocrit and spleen mass, heart mass did not vary significantly across treatment groups ($H_{[7]} = 8.631$, $p = 0.1954$) (Fig. 4.S1). Like spleen mass, the pattern of variation in heart mass between groups was similar after correcting for body mass using a residual approach using an allometric regression (data not shown).

Treatment groups did not differ in the concentrations of the nucleoside triphosphates ATP ($H_{[7]} = 7.399$, $p = 0.2855$) or GTP ($H_{[7]} = 3.431$, $p = 0.7531$) relative to haemoglobin content within red blood cells (Fig. 4.3), nor did they differ in the total amount of GTP + ATP relative to haemoglobin content ($F_{[6,67]} = 0.5658$, $p = 0.7560$) or in the ATP, GTP, or ATP + GTP concentrations per volume of red blood cells (data not shown). These data

suggest haemoglobin-O_2 binding affinity is not differentially modulated via changes in the concentration of these key allosteric modifiers between hypoxia exposure groups.

4.3.2 Resting and maximal O_2 consumption rates and aerobic scope

Given the dynamic nature of blood O_2 carrying capacity in fish exposed to acute or chronic hypoxia, we examined how resting MO_2 and maximal MO_2 varied across treatment groups (Fig. 4.4). Acute hypoxia exposure led to a significant ~50% reduction in resting MO_2 in normoxia-acclimated fish, and a low resting MO_2 was also observed in fish acclimated to constant hypoxia ($H_{[7]} = 18.60$, $p = 0.0003$). Contrastingly, fish acclimated to intermittent hypoxia had a much higher resting MO_2 that was comparable to normoxic controls (Fig. 4.4). Hypoxia exposure also lead to strong reductions in $MO_{2,max}$ ($F_{[3,30]} = 16.79$, $p < 0.0001$) in fish acclimated to normoxia (MO_2 was 49% of normoxia-acclimated controls in normoxia), intermittent hypoxia (54%), or constant hypoxia (33%) (Fig. 4.4).

The relatively high resting MO_2 of fish acclimated to intermittent hypoxia constituted a significantly higher proportion of $MO_{2,max}$ compared to the other acclimation groups (Fig. 4.5A), and this was reflected by a significant main effect of treatment group ($F_{[3,30]} = 5.515$, $p = 0.0039$). Similarly, fish acclimated to intermittent hypoxia showed a significant reduction in factorial aerobic scope (the ratio of $MO_{2,max}$ and resting MO_2) compared to time-matched normoxic controls ($F_{[3,30]} = 3.569$, $p = 0.0255$) (Fig. 4.5B). Absolute

aerobic scope (the absolute difference between $MO_{2,max}$ and resting MO_2) was also much lower in hypoxia-exposed fish ($F_{[3,30]} = 14.34$, $p < 0.0001$) compared to normoxic controls, but similar between fish acclimated to normoxia, intermittent hypoxia, and constant hypoxia (Fig. 4.5C).

4.4 Discussion

Intermittent hypoxia is an ecologically relevant but understudied stressor for fishes that leads to substantial alterations in metabolism and several underlying candidate traits important for hypoxia tolerance (Borowiec et al., 2015, see Chapter 2 in the volume; Borowiec et al., 2018, see Chapter 3 in this volume). Here we show that chronic exposure of estuarine killifish to an intermittent (12 h: 12 h) pattern of hypoxia exposure leads to substantial alterations in blood O_2 carrying capacity that work to protect aerobic metabolic rate during the hypoxia-reoxygenation cycle. Fish acclimated to intermittent hypoxia show rapid reversible modulation of whole-blood haemoglobin content, chiefly through the release and storage of red blood cells in their spleen. This allows fish acclimated to intermittent hypoxia to maintain a relatively high MO_2 during hypoxic bouts (unlike fish exposed to an acute bout of hypoxia or fish acclimated to constant hypoxia), though this comes at the cost of reduced aerobic scope due to the suppressive effect of hypoxia in $MO_{2,max}$. Intermittent hypoxia therefore induces substantial alterations in O_2 transport in killifish that work to counteract the effects of hypoxia on aerobic metabolism rate.

4.4.1 Intermittent hypoxia leads to rapid reversible modulation of blood O_2 carrying capacity

Hypoxia exposure led to significant increases in blood haemoglobin concentration and haematocrit (Fig. 4.1), and these modulations in blood O_2 carrying capacity likely helped maintain tissue O_2 delivery during low oxygen conditions by allowing more O_2 to be transported per unit of blood (Gallaugher and Farrell, 1998; Wells, 2009). The changes in blood O_2 carrying capacity seemed to arise largely from rapid and dynamic storage and release of erythrocytes from the spleen, as fish under hypoxic conditions generally had smaller spleens that normoxic animals (Fig. 4.2). Splenic contraction mediated by an adrenergic signalling cascade is the primary mechanism by which fishes increase blood haemoglobin content when challenged with acute hypoxia, and erythropoiesis by the kidneys may become important after longer periods of hypoxia exposure (Lai et al., 2006; Randall and Perry, 1992; Yamamoto, 1987). However, the importance of the spleen did not appear to diminish after chronic exposure to intermittent hypoxia and the changes in spleen size appeared to be rapidly reversible: fish acclimated to intermittent hypoxia had comparable spleen sizes during the day as normoxia-acclimated fish and presumably this occurs through controlled release followed by re-storage of erythrocytes across the daily cycle. Future work investigating the histology of the red pulp of the spleen would help investigate this possibility. Unlike previous work (Lai et al., 2006), fish acclimated to

constant hypoxia retained relatively small spleens and did not appear to re-build the splenic store of erythrocytes even after 28 d of hypoxia exposure (Fig 4.2).

The reversible increase in haematocrit observed in fish acclimated to intermittent hypoxia may be a strategy to avoid the cardiovascular burden of prolonged polycythemia and a corresponding increase in blood viscosity when tissues are well-supplied with O_2 in normoxia. A similar response of increased haematocit and haemoglobin concentration, mediated by adrenergic stimulation of the spleen, during hypoxia but not normoxia is seen in mice exposed to 10% O_2 for 1 h/day for 5 weeks, and this may help mitigate the increased cardiac workload and hypertension associated with intermittent hypoxia (Kuwahira et al., 1993). This sensitive modulation of haematocrit (and presumably blood viscosity) in killifish may help mitigate the development of cardiac hypertrophy (Fig. 4.S1) and/or other alterations in cardiac morphology and physiology (e.g. compact layer thickness, collagen content). Aquatic hypoxia is known induce alterations in cardiac physiology that enhance cardiac function under oxygen limited conditions (e.g. protection of stroke volume) in some species, and these alterations are not always accompanied by a change in heart mass with hypoxia acclimation (Gamperl and Farrell, 2004; Petersen and Gamperl, 2010). Future investigation of the cardiac morphology in killifish exposed to different hypoxia patterns will provide more information about how cardiac function is affected by hypoxia exposure (e.g. whether the variation in haematocrit and presumably blood viscosity lead to cardiac hypertrophy, or if hypoxia acclimation alters the extent of the spongy or company myocardial layers).

4.4.2 Control of haemoglobin-O_2 binding affinity via modulation of organic phosphates

Some fish have been observed to reduce the concentration of ATP and/or GTP in their red blood cells under hypoxic conditions, which should act to increase haemoglobin-O_2 binding affinity and thus help protect O_2 loading during hypoxia (Greany and Powers, 1977; Nikinmaa and Soivio, 1982; Weber and Lykkeboe, 1978; Wells, 2009; Wood and Johansen, 1973). However, increases in haemoglobin-O_2 affinity could impair peripheral O_2 unloading at the tissues (Powers et al., 1979), and this potential trade-off between O_2 loading and unloading could influence the ability of fish acclimated to intermittent hypoxia to maintain high O_2 consumption rates during both hypoxia and recovery in normoxia (Borowiec et al., 2018, see Chapter 3 of this volume). We saw no evidence that fish acclimated to intermittent hypoxia modulated haemoglobin-O_2 affinity dynamically by altering the ATP or GTP content of the red blood cells, as observed in some other species (Lykkeboe and Weber, 1978). However, it remains possible that killifish rely on other modulators of haemoglobin-O_2 binding affinity, such as intracellular pH or chloride content. Moreover, it is also possible that killifish instead alter the expression of haemoglobin isoforms to modulate haemoglobin-O_2 affinity during chronic hypoxia (Pan et al. 2017). Isoform switching is known to occur in response to chronic hypoxia in both marine fishes (Pan et al., 2017) and hypoxia-adapted cichlids (Rutjes et al., 2007; van den Thillart et al., 2018). While it may be a component of the response to acclimation to constant hypoxia response in killifish, it is unlikely to be utilized by fish acclimated to

intermittent hypoxia, as executing a substantial change in haemoglobin isoform composition would be difficult within a single hypoxia bout due to the time (and energy) required to express, synthesize, and incorporate new haemoglobin isoforms into red blood cells.

4.4.3 Reduced aerobic scope during hypoxic phase of intermittent cycles of hypoxia

Fish exposed to acute hypoxia and fish acclimated to intermittent hypoxia showed comparable changes in blood O_2 carrying capacity (Fig. 4.1, 4.3) and exhibited similar $MO_{2,max}$ in hypoxia (Fig. 4.4). However, killifish acclimated to intermittent hypoxia maintained much higher metabolic rates than fish exposed to acute hypoxia through most of the nighttime hypoxic period (Fig. 4.4), as previously observed (Borowiec et al., 2018, see Chapter 3 of this volume). During this time, killifish acclimated to intermittent hypoxia show no signs of anaerobic glycolysis, unlike the substantial use in fish exposed to acute hypoxia (Borowiec et al., 2018, see Chapter 3 of this volume). Therefore, killifish acclimated to intermittent hypoxia have an augmented ability to maintain MO_2 at rates that more closely approach $MO_{2,max}$ than killifish acclimated to normoxia or constant hypoxia. Other components of the oxygen transport cascade (e.g., hypoxic ventilatory response, lamellar perfusion, the extent of interlamellar cell mass, etc.) could potentially be modified by acclimation to intermittent hypoxia to improve O_2 transport during prolonged periods of hypoxia and help killifish maintain routine MO_2 at a higher proportion of $MO_{2,max}$ (Fig. 4.5). Alternatively, metabolic regulation may be altered after

acclimation to intermittent hypoxia such that cells are not impeded from operating chronically at a high proportion of $MO_{2,max}$.

Field metabolic rates are challenging to estimate in fishes due to the inherent difficulties in using techniques like doubly labelled water in aquatic organisms (Butler et al., 2004; Speakman and Hambly, 2016). However, work using a combination of respirometry and telemetry suggests that field metabolic rates are typically 40 to 60% of metabolic scope in tropical bonefish (Murchie et al., 2011), and 20 to 40% in rainbow trout (Briggs and Post, 1997), which generally agrees with an expected factorial aerobic scope for maximal O_2 consumption rates being 2 to 3-fold greater than resting MO_2. Given these data, killifish acclimated to intermittent hypoxia are operating at a relatively high proportion of their maximal O_2 consumption rate (~64%), especially for resting fish in controlled laboratory conditions (Fig 4.6). The processes underlying the relatively high resting MO_2 (as a proportion of $MO_{2,max}$) in fish acclimated to intermittent hypoxia are unclear, but may include the reestablishment of non-survival processes such as growth, reproduction, and immunity that are disrupted by exposure to acute hypoxia or reduced during the metabolic depression associated with constant hypoxia (Bickler and Buck, 2007; Egg et al., 2013; Guppy and Withers, 1999). The trade-off between maintenance of aerobic scope and maintenance of normoxic rates of O_2 consumption in hypoxia in fish acclimated to intermittent hypoxia may have important ecological implications (e.g. maintenance of reproductive output, increased susceptibility to nocturnal predators), especially if it also occurs during the normoxic phase of the hypoxia-reoxygenation cycle.

4.5 Acknowledgements

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4.6 Author contributions

B.G.B. wrote the paper and conducted the experimentation and analysis of results. B.G.B. and G.R.S. designed the experiments and contributed to the interpretation of data. G.R.S. supervised the experiments.

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4.8 Figures & figure legends

Figure 4.1

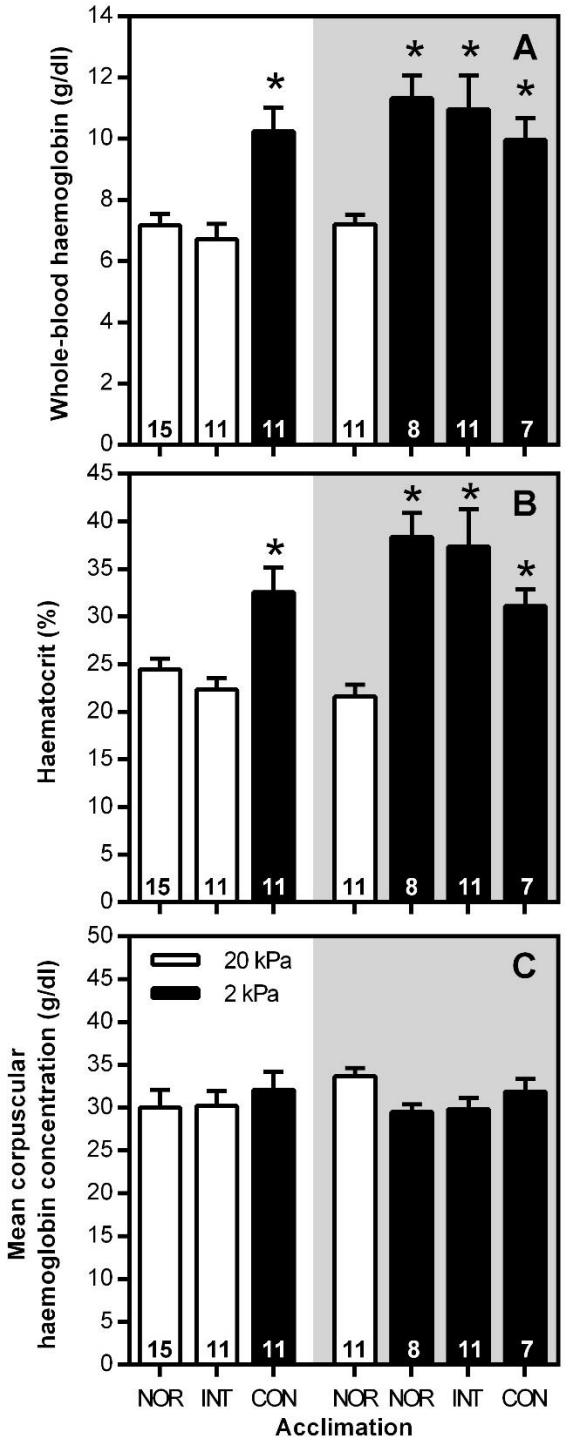

Fig. 4.1 Variation in blood O_2 carrying capacity during exposure to normoxia or hypoxia. Whole blood haemoglobin content (A) and haematocrit (B) increased during hypoxia exposure, but mean cell haemoglobin content (C) did not. Fish were sampled at 1300 (white section of the background) or 0100 (grey section of the background) local time, in either normoxia (white bars) or hypoxia at 2 kPa O_2 (black bars). *Significant pairwise difference from the normoxic control group at the same time point ($P<0.05$). Sample sizes are indicated on each bar directly above the x-axis. Further statistical details are included in the text.

Figure 4.2

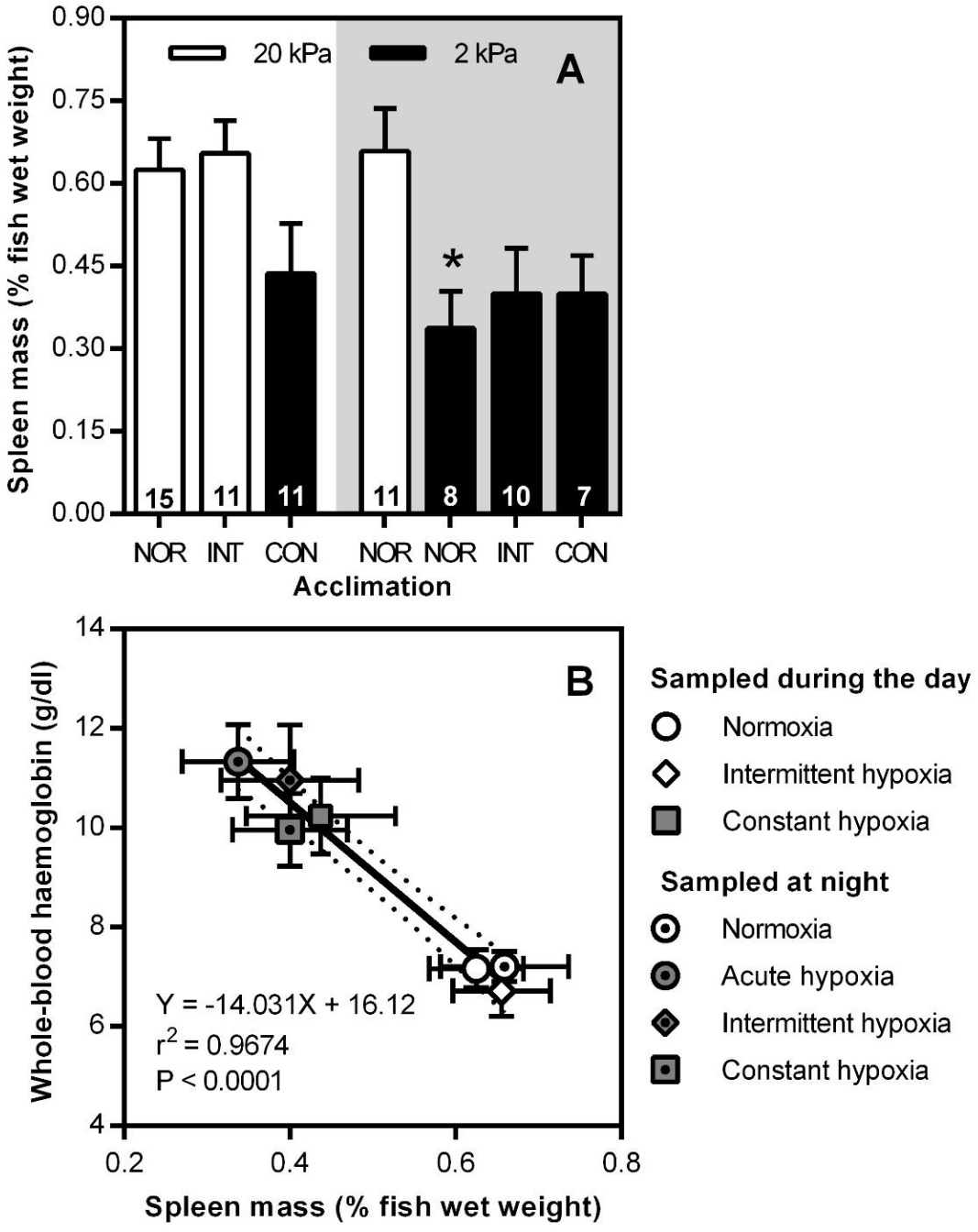
A
20 kPa
2 kPa
Spleen mass (% fish wet weight)
0.90
0.75
0.60
0.45
0.30
0.15
0.00
15
11
11
11
8
10
7
*
NOR
INT
CON
NOR
NOR
INT
CON
Acclimation
B
Whole-blood haemoglobin (g/dl)
14
12
10
8
6
4
Y = -14.031X + 16.12
r2 = 0.9674
P < 0.0001
0.2
0.4
0.6
0.8
Spleen mass (% fish wet weight)
Sampled during the day
Normoxia
Intermittent hypoxia
Constant hypoxia
Sampled at night
Normoxia
Acute hypoxia
Intermittent hypoxia
Constant hypoxia

Fig. 4.2 Reduction in spleen size with hypoxia exposure (A) and correlation between relative spleen size and whole-blood haemoglobin concentration amongst all fish (B). Fish were sampled at 1300 (white section of the background) or 0100 (grey section of the background) local time, in either normoxia (black bars) or hypoxia at 2 kPa O_2 (grey bars). *Significant pairwise difference from the normoxic control group at the same time point ($P<0.05$). Sample sizes are indicated on each bar directly above the x-axis. Further statistical details are included in the text. The 95% confidence bands of the linear regression in (B) are shown.

Figure 4.3

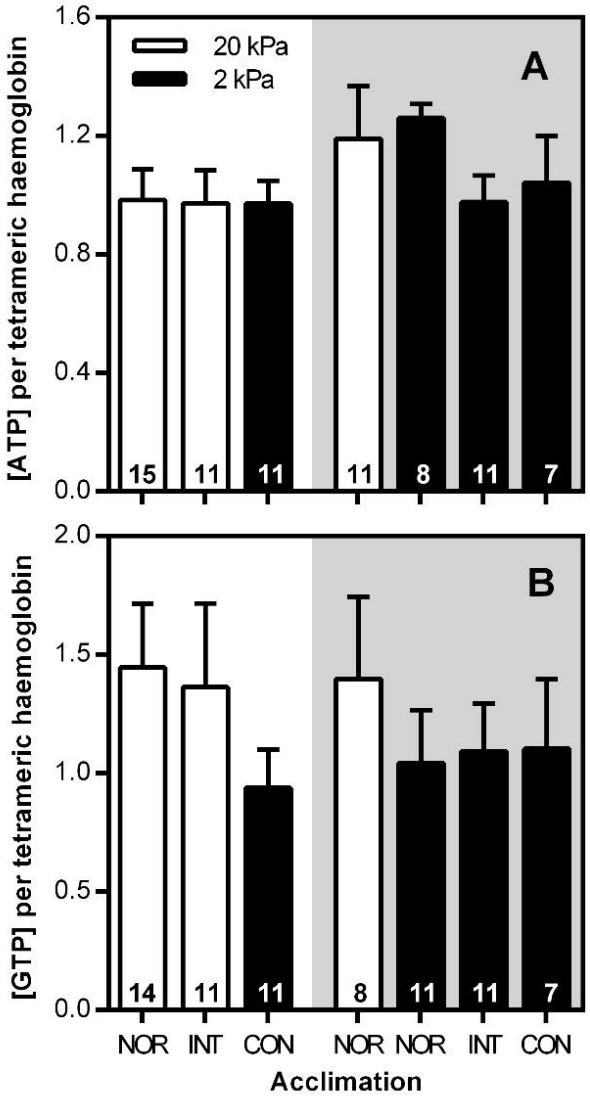
20 kPa
2 kPa
A
B
[ATP] per tetrameric haemoglobin
[GTP] per tetrameric haemoglobin
1.6
1.2
0.8
0.4
0.0
2.0
1.5
1.0
0.5
0.0
15 11 11 11 8 11 7
14 11 11 8 11 11 7
NOR INT CON NOR NOR INT CON
Acclimation

Fig. 4.3 Red blood cell organic phosphate content as reflected by ATP (A) and GTP (B) was similar between treatment groups. Fish were sampled at 1300 (white section of the background) or 0100 (grey section of the background) local time, in either normoxia (white bars) or hypoxia at 2 kPa O_2 (black bars). *Significant pairwise difference from the normoxic control group at the same time point ($P<0.05$). Sample sizes are indicated on each bar directly above the x-axis. Further statistical details are included in the text.

Figure 4.4

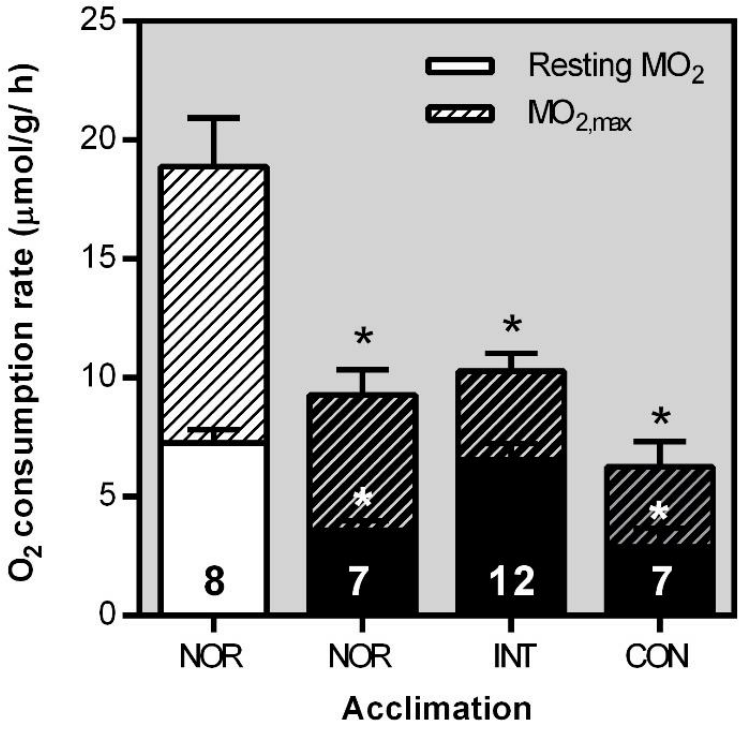
25
20
15
10
5
0
O_2 consumption rate (μmol/g/ h)
Resting MO_2
$MO_{2,max}$
*
*
*
*
8
7
12
7
NOR
NOR
INT
CON
Acclimation

Fig. 4.4 Variation in rates of O_2 consumption (MO_2) at rest and in maximal MO_2 ($MO_{2,max}$) as a result of acute and chronic hypoxia exposure. $MO_{2,max}$ was induced by an exhaustive chase following by air exposure at 0100 local time in either normoxia (white bars) or hypoxia at 2 kPa O_2 (black bars). *Significant pairwise difference from the constant normoxia group ($P<0.05$). Sample sizes are indicated on each bar directly above the x-axis. Resting MO_2 and $MO_{2,max}$ are overlaid and graphed on the same scale.

Figure 4.5

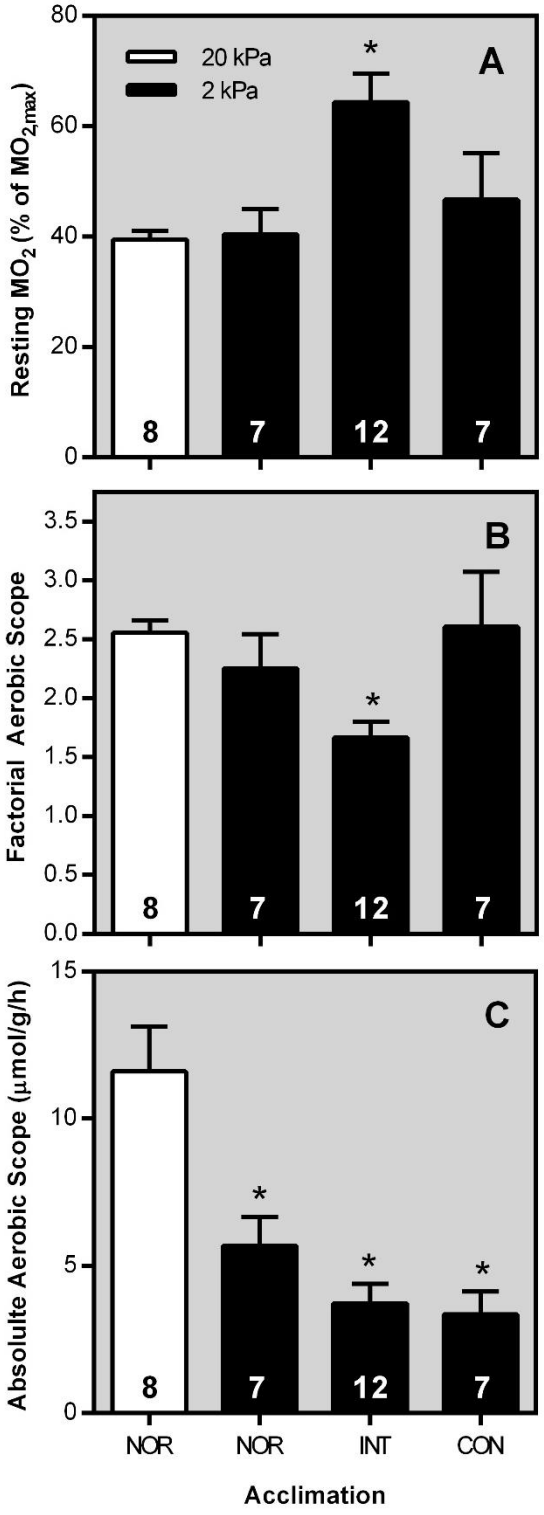
20 kPa
2 kPa
A
B
C
Resting MO_2 (% of $MO_{2,max}$)
Factorial Aerobic Scope
Absolulte Aerobic Scope (µmol/g/h)
80
60
40
20
0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
15
10
5
0
*
8
7
12
7
NOR
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Fig. 4.5 Variation in aerobic scope as a result of acute and chronic hypoxia exposure. Fish acclimated to intermittent hypoxia maintained resting MO_2 at a greater proportion of $MO_{2,max}$ (A), and had reduced factorial aerobic scope (B), but not absolute aerobic scope (C). *Significant pairwise difference from the constant normoxia group ($P<0.05$). Sample sizes are indicated on each bar directly above the x-axis.

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4.10 Supplementary data

Figure 4.S1

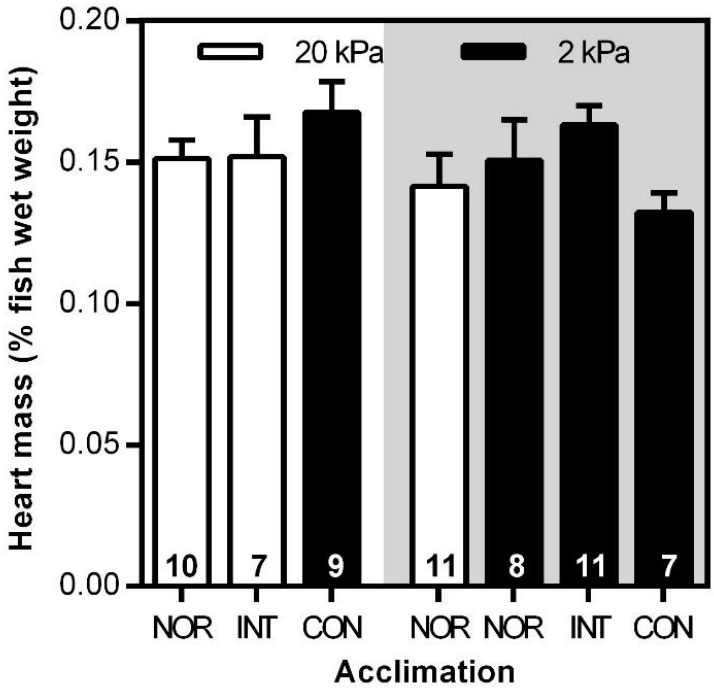

Fig. 4.S1 Heart mass was similar between treatment groups. Fish were sampled at 1300 (white section of the background) or 0100 (grey section of the background) local time, in either normoxia (white bars) or hypoxia at 2 kPa O_2 (black bars). *Significant pairwise difference from the normoxic control group at the same time point ($P<0.05$). Sample sizes are indicated on each bar directly above the x-axis. Further statistical details are included in the text.

CHAPTER 5

Hypoxia acclimation alters reactive oxygen species homeostasis and oxidative status in estuarine killifish (*Fundulus heteroclitus*)

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5.1 Abstract

Hypoxia is common in many aquatic environments and exposure to hypoxia followed by reoxygenation is often believed to induce oxidative stress. However, there have been relatively few studies of reactive oxygen species (ROS) homeostasis and oxidative status in fish that experience natural hypoxia-reoxygenation cycles. We examined how exposure to acute hypoxia (2 kPa O_2) and subsequent reoxygenation affects ROS homeostasis, oxidative damage, and antioxidant defenses in estuarine killifish (*Fundulus heteroclitus*), and whether these effects were ameliorated or potentiated by prolonged (28 day) acclimation to either constant hypoxia or intermittent cycles of nocturnal hypoxia (12 h normoxia: 12 h hypoxia). Acute hypoxia and reoxygenation led to some modest and transient increases in ROS and oxidized glutathione levels, depletion of scavenging capacity, and oxidative damage to lipids in the skeletal muscle. The liver had greater scavenging capacity, total glutathione concentrations, and activities of antioxidant enzymes (catalase, glutathione peroxidase) than the muscle, and generally experienced less variation in glutathiones and lipid peroxidation. Unexpectedly, acclimation to constant hypoxia or intermittent hypoxia led to substantial elevations in ROS (muscle and liver) and oxidized glutathione (muscle). However, hypoxia acclimated fish exhibited little to no oxidative damage (as reflected by lipid peroxidation and aconitase activity), in association with improvements in scavenging capacity and catalase activity in muscle. We conclude that hypoxia acclimation leads to adjustments in ROS homeostasis and

oxidative status that do not reflect oxidative stress but may instead be part of changes in the homeostasis of the signalling cascade that killifish use to cope with chronic hypoxia.

5.2 List of abbreviations

GSSG	Oxidized glutathione
GSH	Reduced glutathione
GSH + GSSG	Total glutathione
PO_2	Partial pressure of O_2
ROS	Reactive oxygen species

5.3 Introduction

Hypoxia and hypoxia-reoxygenation cycles are common in the aquatic environment (Breitburg et al., 2018; Diaz, 2001; Diaz and Breitburg, 2009). In order to effectively cope with such variations in environmental oxygen availability, aerobic organisms must overcome the challenges of energy shortfall (Boutilier, 2001; Richards, 2009) and the potential for oxidative stress (Hermes-Lima and Zenteno-Savín, 2002; Lushchak, 2011; Lushchak and Bagnyukova, 2006). Hypoxia-tolerant fish exhibit a range of well-described mechanisms for avoiding energy shortfall by maintained the balance between cellular ATP supply and ATP demand (Bickler and Buck, 2007; Richards, 2009), but the potential oxidative stress challenge posed by hypoxia to fish is not as well understood.

Acute hypoxia and reoxygenation could induced oxidative stress by upsetting the balance between reactive oxygen species (ROS) production and ROS scavenging by antioxidants, and thereby producing excess ROS that damages critical cellular components and/or disrupts redox signalling (Costantini, 2019; Hermes-Lima and Zenteno-Savín, 2002; Jones, 2006). For example, ROS generation is well known to increase in response to hypoxia-reoxygenation in cells in culture (Chandel et al., 2000; Giraud-Billoud et al., 2019; Guzy et al., 2005; Korde et al., 2011), and in response to ischemia-reperfusion in mammals (Bhogal et al., 2010; Chouchani et al., 2014; Kalogeris et al., 2014; Sanderson et al., 2013; Zhang et al., 2007). In fish, exposure to acute hypoxia causes oxidative damage (Lushchak and Bagnyukova, 2007) and to increase antioxidant capacity (Birnie-

Gauvin et al., 2017; Lushchak et al., 2005; Lushchak et al., 2001). Fish have also been shown to experience oxidative stress during reoxygenation periods that follow acute hypoxia exposure, and some species also increase the activity of antioxidant enzymes (Hermes-Lima and Zenteno-Savín, 2002; Lushchak et al., 2001). However, there are also many situations in which acute hypoxia and/or reoxygenation have not been observed to cause oxidative stress, potentially because of interspecific or inter-tissue variation in how redox status is managed across time. Additionally, the biological significance (e.g. level or timing of damage) of changes in specific markers is not always clear (Costantini, 2019; Halliwell and Whiteman, 2004; Jones, 2006). Understanding discrepancies in the interaction between hypoxia, reoxygenation, and oxidative damage assessed with different markers in a variety of tissues are key to understanding how the management of oxidative stress may influence animal physiology, and perhaps fitness and life history traits (Costantini, 2019; Monaghan et al., 2009; Smith et al., 2016; Speakman et al., 2015).

The effects of prolonged periods of sustained hypoxia or of repeated hypoxia-reoxygenation cycles on oxidative stress in fish are poorly understood. In mammals (e.g., obstructive sleep apnea), repeated cycles of hypoxia-reoxygenation increase ROS production, and thereby lead to pathological changes in respiratory and cardiovascular system function, metabolism, and redox-sensitive gene expression (Lavie, 2003; Neubauer, 2001; Prabhakar et al., 2007; Semenza and Prabhakar, 2007). Fish that are adapted to environments that undergo natural periods of hypoxia or hypoxia-

reoxygenation might be expected to mitigate these deleterious effects by better managing ROS levels and protecting tissues against oxidative stress. Indeed, chronic exposure to either constant or intermittent patterns of hypoxia has been shown to increase antioxidant enzyme activities and reduce rates of mitochondrial ROS emission in some fish (Du et al., 2016; Lushchak, 2011; Lushchak and Bagnyukova, 2007). Some animals have also been shown to elevate antioxidant capacity during periods of metabolic depression, which is believed to be an anticipatory strategy to defend against future overproduction of ROS during the surge in O_2 supply that occurs upon resumption of normal metabolic rates (Giraud-Billoud et al., 2019; Hermes-Lima et al., 2015; Hermes-Lima et al., 1998; Hermes-Lima and Storey, 1993; Moreira et al., 2016; Welker et al., 2013). However, there have been relatively few detailed examinations of the effects of chronic hypoxia and/or hypoxia-reoxygenation cycles on ROS and redox homeostasis, scavenging capacity and antioxidant enzymes, and oxidative stress in hypoxia-tolerant fish.

The mummichog killifish *Fundulus heteroclitus* is a good species in which to examine the effects of prolonged exposure to constant hypoxia or to cycles of hypoxia-reoxygenation on ROS homeostasis and oxidative status. This species lives in estuaries on the east coast of North America, where they experience dynamic changes in O_2 levels and other environmental variables (Tyler et al., 2009). Killifish can tolerate prolonged periods of low O_2 (e.g., only ~10% of normoxic air saturation), in association with substantial alterations in respiratory physiology and metabolism that appear to help maintain energy homeostasis and improve hypoxia tolerance (Borowiec et al., 2015, see Chapter 2 of this

volume; Borowiec et al., 2018, see Chapter 3 of this volume). Chronic hypoxia also reduces mitochondrial ROS emission in the liver of killifish (Du et al., 2016), but we otherwise have a poor understanding of how ROS homeostasis and oxidative status in various tissues are affected by hypoxia and/or reoxygenation. Our objectives were to investigate the interactions between hypoxia and hypoxia-reoxygenation and oxidative stress by examining how killifish respond to (i) acute exposure to hypoxia followed by reoxygenation in normoxia and (ii) chronic exposure to constant hypoxia or to repeated hypoxia-normoxia cycles, in tissues with low and high endogenous antioxidant capacity (skeletal muscle and liver, respectively).

5.4 Materials & methods

5.4.1 Study animals

Adult, wild-caught killifish (*Fundulus heteroclitus*, Linnaeus 1766) of mixed sex (~2–5 g) were shipped from a commercial supplier (Aquatic Research Organisms, NH, USA) to McMaster University, Ontario, Canada. Prior to acclimation treatments (see below), killifish were held in large ~300 L fibreglass tanks filled with brackish (4 ppt) water that was maintained at room temperature (~22°C) and continuously aerated with an air stone. Fish were fed commercial flakes (Big Al's Aquarium Supercentres, Mississauga, ON, Canada) at least five days a week. The photoperiod was maintained at 12 h light to 12 h dark, with the daylight portion occurring from 0700 to 1900 h local time. Water quality

(ammonia, pH, nitrates and nitrites) was maintained by cycling water through a charcoal filter, and by routine water changes. Fish were maintained in these conditions for at least four weeks before acclimation treatments began. The acclimation treatments and hypoxia-reoxygenation exposures that are described below were equivalent and were conducted alongside another experiment examining the effects of hypoxia acclimation on energy metabolism in killifish (Borowiec et al., 2018, see Chapter 3 of this volume). All animal use protocols followed guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board.

5.4.2 Acclimation Treatments

Fish were subjected to 28 day acclimation to either normoxia, constant hypoxia (2 kPa O_2, ~0.8 O_2 l^{-1}), or nocturnal ('intermittent') hypoxia (12 h normoxia during the light phase: 12 h hypoxia during the dark phase), all of which were carried out in 35 l glass aquaria. We selected this standardized pattern of intermittent hypoxia because it is a reasonable approximation of the diel patterns of hypoxia that occur naturally in estuaries along the east coast of North America (Tyler et al., 2009). Normoxia (~20 kPa O_2, ~8 mg O_2 l^{-1}) was achieved by continuously bubbling aquarium water with air. Hypoxia (2 kPa O_2, 0.8 mg O_2 l^{-1}) exposure was achieved by regulating the O_2 tension in the aquaria using a feedback system controlled by a galvanic O_2 sensor (Loligo Systems, Tjele, Denmark), as described previously (Borowiec et al., 2015, see Chapter 2 of this volume; Borowiec et al., 2018, see Chapter 3 of this volume). Transitions between normoxia and

hypoxia during acclimation to intermittent hypoxia were typically completed within 45 minutes. Because killifish sometimes respond to hypoxia with aquatic surface respiration (Stierhoff et al., 2003), we prevented fish from respiring at the water surface during hypoxic periods by placing a plastic grid barrier just below the surface of the water and overlaying it with bubble wrap.

5.4.3 Hypoxia-reoxygenation exposures

Fish were sampled during exposure to a hypoxia-reoxygenation cycle, as described previously (Borowiec et al., 2018, see Chapter 3 of this volume). Briefly, fish were transferred at ~1000 h local time in groups of 3-4 fish into custom-built, mesh-sided 2.1 l enclosures that were weighed down to sit at the bottom of a 35 l glass aquarium. Animals recovered from handling for ~8 h, during which they were exposed to the PO_2 appropriate to their acclimation condition at that time of day.

Animals were sampled at the times indicated in Fig. 5.1 under normoxic or hypoxic conditions beginning with an initial (‘0 h’) sampling period between 1830-1900 h local time (i.e., shortly before lights off at 1900). Subsequent sampling points, relative to 1900 h local time, were as follows: 1 h (2000 h), 6 h (0100 h), 12 h (0700 h), 13 h (0800 h) or 18 h (1300 h). Fish acclimated to normoxia were either (i) sampled in normoxia for all six time points (‘normoxia control’ treatment group), or (i) sampled in normoxia at 0 h, and then sampled after 1, 6, or 12 h of acute hypoxia (2 kPa O_2) during the dark phase of the

photoperiod, and then after 1 h (13 h) and 6 h (18 h) of reoxygenation in normoxia during the light phase of the photoperiod ('acute hypoxia-reoxygenation' treatment group). Fish acclimated to intermittent hypoxia were sampled following their typical acclimation PO_2 pattern (i.e. in normoxia at 0 h, after 1, 6 or 12 h of hypoxia, or after 1 or 6 h of reoxygenation in normoxia). For fish acclimated to constant hypoxia, the first four sampling points were in hypoxia at the same times of day as the other groups (corresponding to 0, 1, 6, or 12 h time points). Fish in this acclimation group were also subjected to reoxygenation in normoxia (starting at 0700) and were sampled after 1 h and 6 h (corresponding to 13 h and 18 h time points, respectively).

At each sampling time point, two mesh-covered enclosures were gently removed from the aquarium, and excess water was drained out of the mesh sides. Fish in the remaining ~1 l reservoir of water were quickly euthanized by adding a concentrated solution of benzocaine (Sigma-Aldrich, Oakville, ON, Canada; dissolved in 95% ethanol to a final concentration 1 g l^{-1}). Fish were removed and weighed. Two transverse sections of the axial muscle were cut from the anterior base of the anal fin. The anterior section was immediately freeze-clamped between two aluminium blocks pre-cooled in liquid nitrogen and was then stored in liquid N_2. White muscle was punched out of the posterior section using a tissue biopsy punch, and was then transferred unfrozen to ice-cold Tris buffer (100 mmol l^{-1} Tris-HCl, 2 mmol l^{-1} EDTA, 5 mmol l^{-1} $MgCl_2$, pH 7.75) and placed on ice for ROS assays (see below). Most of the liver was also freeze-clamped, except for a small piece (~20-50 mg) that was placed in ice-cold Tris buffer for ROS assays. All freeze-

clamped samples were stored at −80°C, and were ground into a fine powder under liquid nitrogen before use in glutathione, lipid peroxidation, and enzyme activity assays (see below). Sampling was completed for all fish within a few minutes of removing the enclosure from the aquarium, such that fish sampled from hypoxic water were not exposed to normoxia prior to sampling.

5.4.4 ROS and total oxidant scavenging capacity (TOSC) assays

Muscle and liver portions that had been placed in ice-cold Tris buffer during sampling were processed immediately for measurement of ROS levels using a fluorometric assay described previously (Amado et al., 2009). This assay detects hydrogen peroxide and various one-electron-oxidizing species (e.g., hydroxyl radical, nitrogen dioxide, etc.) (Kalyanaraman et al. 2012), which are collectively referred to here as 'ROS' for simplicity. Tissues were homogenized in a glass Tenbroeck tissue grinder containing 10 volumes of ice-cold Tris buffer, and were then centrifuged at 10,000 g for 20 min at 4^{o}C. The supernatant was retained, its protein content was measured using a standard Bradford assay (according to instructions from the manufacturer; Bio-Rad, Mississauga, ON), and it was then diluted to 1 mg protein per ml with Tris buffer. This solution (10 µl) was added to 135 µl of ROS assay buffer (200 mmol l^{-1} KCl and 1 mmol l^{-1} $MgCl_2$ in 30 mmol l^{-1} Hepes, pH 7.2) containing 16 µmol l^{-1} of 2',7'-dichlorodihydrofluorescein diacetate (Sigma Aldrich) (from a stock solution in 95% ethanol). ROS signal was determined in triplicate by tracking the accumulated fluorescence signal over 60 min at

37^0C from the oxidation of 2',7'-dichlorodihydrofluorescein diacetate into the fluorescent product 2′,7′-dichlorofluorescein (excitation: 485 nm, emission: 528 nm). Background conversion was determined using the Tris buffer described above in place of tissue supernatant. Total fluorescence over time was fit to a polynomial function, and the area under this curve was subtracted from the area under the curve representing the background reaction to calculate the total ROS signal. We reported the total ROS signal in arbitrary units (AU) per g tissue, such that samples with higher AU per g tissue had higher ROS levels. Preliminary assays determined that background fluorescence was minimal and there was full dose-recovery for the amount of homogenate protein that was used in the assay, based on manipulations of homogenate protein around this range. The remaining diluted supernatants were frozen in liquid nitrogen and stored at -80^0C for later determination of TOSC.

TOSC was determined by similarly measuring the change in 2',7'-dichlorofluorescein fluorescence in response to the strong oxidant 2,2'-azobis(2-amidinopropane) dihydrochloride (ABAP). Diluted supernatants were thawed on ice and added to the ROS assay buffer described above in sextuplicates. ABAP (final concentration of 20 μmol l^{-1}) was added to half of the wells immediately before the start of the assay. ROS signal was then measured as above as the accumulated fluorescence signal of 2′,7′-dichlorofluorescein over 60 min at 37^0C. The relative area for the wells containing homogenate but not ABAP (background ROS) was subtracted from the area of wells containing homogenate and ABAP (e.g. oxidant-induced and background ROS), and then

divided by the area produced by wells containing homogenate but not ABAP to produce a relative metric of the amount of ROS that was produced by exogenous ABAP in the presence of tissue homogenate. As a result, this relative metric is *larger* for tissue homogenate samples that have *lower* scavenging capacity (e.g. a greater fluorescent signal produced by the inclusion of ABAP), and is expressed in AU per g tissue.

5.4.5 Glutathione assays

Concentrations of oxidized glutathione (GSSG), reduced glutathione (GSH), and total glutathione (GSH+GSSG) were determined in duplicates using established protocols (Baker et al., 1990; Rahman et al., 2006). Frozen and powdered samples of muscle and liver were sonicated at low power in 20 or 50 volumes, respectively, of potassium phosphate extraction buffer (16 mmol l^{-1} KH_2PO_4, 84 mmol l^{-1} K_2HPO_4, 5 mmol l^{-1} EDTA, 0.6% w/v sulfosalicyclic acid, 0.1% Triton X-100, pH 7.5) in two 10 s bursts on ice with a ≥10 s wait between bursts. These sonicated samples were centrifuged for 10 min at 8000 g at 4^0C.The supernatants were then split into two groups: one for measurement of total glutathione, and one for the measurement of GSSG. To prepare the latter samples for measurement of GSSG, 5 µl of 0.35 mol l^{-1} 2-vinylpyridine (which covalently reacts with GSH, but not GSSG, and makes GSH undetectable by the assay) was added to 70 µl of supernatant in a new 0.5 mL Eppendorf tube, which were then incubated for 1 h at room temperature with gentle mixing. Excess 2-vinylpyridine was then neutralized by adding 3 µl of 20% triethanolamine (TEA; diluted from ≥98% stock

in 16 mmol l^{-1} KH_2PO_4, 84 mmol l^{-1} K_2HPO_4), vortexing, and incubating for 10 min. Following this incubation, samples were fully neutralized with TEA, and were centrifuged again for 10 min at 8000 g. Samples for measuring both total glutathione and GSSG were incubated at 25^{o}C for 10 min in 96-well plate under the following initial conditions: 0.15 mmol l^{-1} DTNB, 5 mmol l^{-1} EDTA, glutathione reductase (5.5 U per ml), 16 mmol l^{-1} KH_2PO_4, 84 mmol l^{-1} K_2HPO_4, pH 7.5. The reaction was begun by adding NADPH (final concentration 0.3 mmol l^{-1}) to each well, and the rate of change in absorbance at 412 nm was monitored for at least 2 min. A standard curve produced by known concentrations of GSH and GSSG was used to calculate the total glutathione and GSSG concentrations in each sample. The concentration of GSH was determined by subtracting the measured amount of GSSG from total glutathione.

5.4.6 Lipid peroxidation assays

Lipid peroxidation was determined in triplicate using a xylenol orange assay (Hermes-Lima et al., 1995) that was modified for use in a standard 96-well plate. Powdered samples of frozen liver and muscle were homogenized in a glass Tenbroeck tissue grinder in 20 volumes of ice-cold methanol, and these homogenized samples were centrifuged for 5 min at 1000 g at 4^{0}C. The assay was carried out in solution with the following initial conditions: 0.25 mmol l^{-1} $Fe(II)SO_4$, 25 mmol l^{-1} H_2SO_4 and 0.1 mmol l^{-1} xylenol orange. Absorbance of this solution was first read at 580 nm after a 15 min incubation in the dark at 25^{0}C (“blank reading”), and absorbance at 580 nm was monitored continuously after

the rapid addition of homogenate (or distilled water as a control) to each well until a stable plateau was reached ("plateau reading," ~15 min for liver tissue, ~25 min for muscle tissue). Finally, cumene hydroperoxide (5 µmol l^{-1} final concentration) was added to each well, and the change in absorbance at 580 nm was monitored for another ~20 min until it stabilized ("final reading"). Lipid peroxidation was reported in cumene equivalents per g tissue by relating the change in absorbance caused by the addition of tissue (i.e. plateau reading – blank reading) to the change in absorbance caused by adding a known concentration of cumene hydroperoxide (i.e. final reading – plateau reading).

5.4.7 Assays of antioxidant and metabolic enzyme activities

The maximal activities of the antioxidant enzymes glutathione peroxidase and catalase were assayed using standard protocols in duplicate wells. Frozen, powdered muscle and liver tissues were homogenized on ice in 20 volumes of homogenization buffer (16 mmol l^{-1} KH_2PO_4, 84 mmol l^{-1} K_2HPO_4, 1 mmol l^{-1} EDTA, 0.1% Triton X-100, pH 7.4) using a PowerGen 125 electric homogenizer (Fisher Scientific, Whitby, ON, Canada), with two 10 s bursts at the highest setting. Homogenates were then centrifuged at 10,000 g for 10 min at 4^0C and the supernatant was retained. For catalase only, these supernatants were also then sonicated on ice with three 10 s bursts interspersed by ≥10 s breaks between bursts. These samples were used in assays to determine the maximal activity of glutathione peroxidase (0.2 mmol l^{-1} NADPH, 3 U ml^{-1} glutathione reductase, 1 mmol l^{-1} NaN_3, 1 mmol l^{-1} reduced glutathione, 12 mmol l^{-1} cumene hydroperoxide, 1 mmol l^{-1}

EDTA, 8 mmol l^{-1} KH_2PO_4, 42 mmol l^{-1} K_2HPO_4; pH 7.4) and catalase (20 mmol l^{-1} H_2O_2 and 3.2 mmol l^{-1} KH_2PO_4, 16.8 mmol l^{-1} K_2HPO_4; pH 7.0) by measuring the rate of change in absorbance for at least 2 min at 340 nm or 240 nm, respectively.

The maximal activity of aconitase was assayed as follows: samples were sonicated with ten 1 s bursts in 10 volumes of ice-cold isolation buffer (50 mmol l^{-1} Tris-HCl, 0.6 mmol l^{-1} $MnCl_2$, 2 mmol l^{-1} sodium citrate, 0.1% Triton X-100, pH 7.4), centrifuged at 10,000 g for 10 min at 4^0C, and the supernatant was used in assays in the following conditions: 5 mmol l^{-1} sodium citrate, 0.2 mmol l^{-1} $NADP^+$, 0.6 mmol l^{-1} $MnCl_2$, 0.5 U ml^{-1} isocitrate dehydrogenase, 50 mmol l^{-1} Tris-HCl; pH 7.4. Aconitase activity was measured as the rate of change in absorbance at 340 nm over 10 min.

All enzyme assays were run in duplicate in a 96-well microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 25^0C with temperature control or at room temperature (catalase only). Preliminary assays determined that substrate concentrations stimulated maximal activity. There was full dose-recovery for the amount of homogenate that was used in the assay for catalase, glutathione peroxidase, and aconitase. We used extinction coefficients (ε) of 6.22 (l $mmol^{-1}$ cm^{-1}) for glutathione peroxidase and aconitase (NADPH) and 0.0260 (l $mmol^{-1}$ cm^{-1}) for catalase (H_2O_2).

5.4.8 Statistical analysis

Two-way ANOVA was used to examine the effects of sampling time point, experimental treatment group, and their interaction. Bonferroni post-hoc tests were used to compare each hypoxic treatment group to the time-matched normoxic control group. A significance level of $P<0.05$ was used for all statistical analyses. All data are reported as means ± s.e.m.

5.5 Results

5.5.1 ROS homeostasis and oxidative status in the muscle during hypoxia-reoxygenation

We examined how ROS levels and glutathione redox status varied during exposure to hypoxia followed by reoxygenation in killifish acclimated to normoxia, intermittent hypoxia, or constant hypoxia (Fig. 5.1). Exposure of normoxia acclimated fish to acute hypoxia and reoxygenation did not lead to any significant changes in ROS signal in the muscle (Fig. 5.2A, Table 5.1). There was a significant transient increase in GSSG in normoxia acclimated fish after 1 h of hypoxia as compared to time-matched normoxic controls (Fig. 5.2B, Table 5.1), but this increase in GSSG was rapidly corrected by 6 h, and it was not associated with any change in the ratio of GSH and GSSG concentrations (Fig. 5.2D, Table 5.1). Therefore, exposure of normoxia acclimated killifish to acute

hypoxia-reoxygenation had little effect on ROS levels or glutathione redox status in the muscle.

In contrast, ROS levels and glutathione redox status were appreciably altered in the muscle of killifish that were chronically exposed to diel cycles of hypoxia (intermittent hypoxia) or to constant hypoxia. Fish acclimated to both patterns of chronic hypoxia showed an amplification of the ROS signal, as reflected by a significant main effects of acclimation treatment ($P < 0.0001$) (Table 5.1), which was driven largely by higher ROS in these treatment groups during hypoxia (Fig. 5.2A). There was some variation in the temporal pattern of variation between hypoxia-acclimated treatment groups, based on the significant interaction between acclimation group and time ($P = 0.0060$) (Table 5.1). Specifically, fish acclimated to intermittent hypoxia showed increased ROS early (1 h) and late (12 h) in the night-time hypoxia exposure, whereas fish acclimated to constant hypoxia had generally high ROS levels during hypoxia except for a transient decline at the 12 h time point at the end of the night time (Fig. 5.2A). The hypoxia acclimation groups also tended to have higher GSH + GSSG and GSSG concentrations and lower GSH:GSSG in the muscle (Fig. 5.2B-D), as reflected by a significant main effect of acclimation treatment for each trait ($P < 0.0001$ for each) (Table 5.1). The elevated GSSG concentration and reduced GSH:GSSG persisted throughout the reoxygenation period in normoxia (Fig. 5.2B,D).

We examined whether hypoxia-reoxygenation induced oxidative stress in the muscle by measuring lipid peroxidation and the activity of aconitase (an enzyme in the tricarboxylic acid cycle that is highly sensitive to inactivation by oxidation of its iron-sulfur core). Exposure of normoxia acclimated fish to acute hypoxia-reoxygenation led to significant increases in lipid peroxidation compared to time-matched normoxic controls, which were greatest after 1 h of hypoxia and after 6 h of reoxygenation in normoxia, and there appeared to be a general elevation in lipid peroxidation with acute hypoxia-reoxygenation that drove the significant main effect of treatment group ($P < 0.0001$) (Fig. 5.3A). Lipid peroxidation in the chronic intermittent hypoxia group was similar or less than that in the acute hypoxia-reoxygenation group, with fish acclimated to intermittent hypoxia showing significantly higher levels of lipid peroxidation than normoxic controls at 12 h and 18 h. However, lipid peroxidation in the chronic constant hypoxia group was never elevated above the levels in normoxic controls. In contrast, there was no significant variation in aconitase activity between treatment groups or across time (Fig. 5.3B; Table 5.1). Therefore, chronic hypoxia exposure was associated with similar or lower levels of oxidative stress than experienced in fish exposed to acute hypoxia-reoxygenation.

We measured the total oxidant scavenging capacity of the muscle tissue in response to treatment with the strong oxidant 2,2'-azobis(2-amidinopropane) dihydrochloride (ABAP). Exposure to acute hypoxia-reoxygenation led to an elevation in the ROS signal induced by ABAP, which was significant after 1 h of hypoxia and after 6 h of reoxygenation (Fig. 5.4). In contrast, fish acclimated to both hypoxia acclimation groups

maintained or in some cases improved their scavenging capacity compared to time-matched normoxic controls (Fig. 5.4). These divergent responses appeared to drive the significant main effect of acclimation treatment on scavenging capacity ($P < 0.0001$) (Table 5.1).

The loss of scavenging capacity in normoxia acclimated fish exposed to acute hypoxia-reoxygenation was concurrent with a substantial reduction in the maximal activity of the antioxidant enzyme catalase (Fig. 5.5A). This reduction began after 1 h of hypoxia and persistent throughout reoxygenation, and contributed strongly to the significant main effect of acclimation treatment on this enzyme ($P < 0.0001$) (Table 5.1). Normoxic fish exposed to acute hypoxia-reoxygenation exhibited some modest increases in glutathione peroxidase activity after 12 h of hypoxia and 1 h of reoxygenation (Fig. 5.5B), contributing to a significant main effect of acclimation treatment ($P = 0.002$) (Table 5.1).

Hypoxia acclimation appeared to attenuate the changes in antioxidant enzyme activity during hypoxia and reoxygenation. In particular, fish acclimated to chronic hypoxia exhibited partial (intermittent hypoxia group) or full (constant hypoxia group) restoration of catalase activity (Fig. 5.5A). The intermittent hypoxia group increased glutathione peroxidase activity after 1 h of reoxygenation, but the variation for this enzyme in this acclimation group was less appreciable than the variation in the acute hypoxia-reoxygenation group, and there was no significant variation for this enzyme in the constant hypoxia group (Fig. 5.5B).

5.5.2 ROS homeostasis and oxidative status in the liver during hypoxia-reoxygenation

The patterns of variation in liver ROS levels during hypoxia-reoxygenation were qualitatively similar to or greater than those in the muscle. Exposure of normoxia acclimated fish to hypoxia-reoxygenation led to a significant increase in the ROS signal after 1 h of hypoxia, but the variation at other time points was not significant (Fig. 5.6). Similar to the patterns of variation in the muscle, fish acclimated to intermittent hypoxia and constant hypoxia exhibited a high ROS signal (main effect of acclimation treatment, $P < 0.0001$), which was particularly apparent during hypoxia in the constant hypoxia group, but also extended to 1 h of reoxygenation in the intermittent hypoxia group (significant acclimation×time interaction, $P = 0.0324$) (Fig. 5.6, Table 5.2).

Compared to the muscle, the liver had a much greater total glutathione pool, but generally had similar GSH:GSSG on average (Table 5.2, 5.3). Exposure of normoxic fish to acute hypoxia-reoxygenation led to some increases in total glutathione (after 6 h and 12 h of hypoxia) and GSSG (after 12 h of hypoxia and 1 h of reoxygenation), but GSH:GSSG was generally maintained (Table 5.2, 5.3). Lipid peroxidation also increased transiently in this treatment group after 1 h reoxygenation (Table 5.2, 5.3). However, hypoxia acclimated fish showed very little variation in glutathione redox status or lipid peroxidation during hypoxia or reoxygenation relative to time-matched normoxic controls, with the exception of a rise in GSSG in the constant hypoxia group after 1 h of

reoxygenation and lower lipid peroxidation in the intermittent hypoxia group after 12 h of normoxia (the 0 h time point) (Table 5.2, 5.3). The emergent pattern was that glutathione redox status and lipid peroxidation during hypoxia and reoxygenation varied less in the liver than in the muscle.

TOSC was also much higher in the liver than in the muscle, as reflected by much smaller responses to the oxidant ABAP, and the liver also had much greater maximal activities of antioxidant enzymes (Table 5.2, 5.4). Scavenging capacity in the liver was maintained or elevated (i.e., the effects of ABAP were maintained or reduced), as were the activities of antioxidant enzymes, in all groups exposed to hypoxia-reoxygenation relative to time-matched normoxic controls (Table 5.2, 5.4). However, there were some differences in the hypoxia acclimation groups, as reflected by significant main effects of acclimation treatment (Table 5.2). Specifically, fish acclimated to intermittent hypoxia generally had higher catalase activities (Table 5.2, 5.4).

5.6 Discussion

Prolonged periods of low O_2 and daily cycles of hypoxia-reoxygenation are common in many aquatic environments, but the implications of these challenging environments to ROS homeostasis and oxidative status in fish remain elusive. Here, we show that acute hypoxia and reoxygenation led to some modest transient disturbances in ROS homeostasis and redox status that resulted in oxidative damage in killifish. Chronic

acclimation to constant hypoxia or to diel cycles of nocturnal hypoxia (intermittent hypoxia) led to much greater alterations in ROS homeostasis and glutathione redox status, but with less or even negligible levels of oxidative damage, and this seemed to arise largely from a maintenance or improvement in antioxidant capacity. Therefore, rather than representing a sign of oxidative stress, the adjustments in ROS homeostasis and oxidative status after hypoxia acclimation may be part of the suite of adaptive adjustments to homeostasis that killifish use to cope with chronic hypoxia.

5.6.1 Oxidative status during acute hypoxia and reoxygenation

There are multiple possible sources and mechanisms for increased ROS production during hypoxia and/or reoxygenation, including mitochondrial complexes, NADPH oxidases, and xanthine oxidase (Brand, 2016; Granger and Kvietys, 2015; Murphy, 2009; Smith et al., 2017). Acute hypoxia-reoxygenation had relatively minor effects on ROS signals in the muscle tissue (Fig. 5.2A, Fig. 5.6). It is possible that more dramatic increases in ROS occurred in the very early phases of acute hypoxia (<1 h) and were rapidly reduced thereafter (Chandel et al., 1998; Zuo and Clanton, 2005). This may account for the early transient increase in GSSG and depletion of scavenging capacity in the muscle, which was later associated with modest but persistent increases in lipid damage (Fig. 5.2, 5.3, Table 5.1). Increases in ROS levels were observed in the liver, but the potential resulting changes in other markers of oxidative status were more moderate and delayed until late hypoxia or into reoxygenation (Table 5.2, 5.3).

The skeletal muscle experienced more substantial alterations in glutathione redox state, lipid peroxidation, and scavenging capacity than the liver (Fig. 5.2 - 5.4, 5.6, Table 5.3, 5.4). This observation may originate from the relatively lower scavenging capacity, total glutathione concentrations, and activities of antioxidant enzymes in the muscle compared to the liver, as reported in previous studies (Ansaldo et al., 2000; Cooper et al., 2002; Hegazi et al., 2010; Leggatt et al., 2007; Lushchak et al., 2001; Otto and Moon, 1996). It is also possible that changes in tissue perfusion (and thus tissue O_2 levels) associated with hypoxia are greater in the skeletal muscle than in the liver, because of preferential redistribution of blood flow away from the muscle to critical organs like the brain (Axelsson and Fritsche, 1991; Hylland et al., 1994; Nilsson et al., 1994) and/or maintenance of blood flow through the liver to facilitate metabolic responses to hypoxia (e.g. glucose transport to tissue with minimal glycogen stores).

Killifish in this study generally had antioxidant enzyme activities and total glutathione pools that are comparable to many other fishes (Farombi et al., 2007; Lisser et al., 2017; Lushchak et al., 2001; Sinha et al., 2014). However, acute hypoxia led to a surprising reduction in catalase activity in the muscle that may have contributed to the decline in scavenging capacity and the increase in oxidative damage to lipids (Figs. 5.3 – 5.5). Catalase activity is typically maintained or increased during hypoxia or anoxia, presumably to protect tissues from oxidative stress (Hermes-Lima et al., 2015). Indeed, inhibition of catalase leads to increased oxidative damage in both goldfish and frogs in

normoxia (Bagnyukova et al., 2005; Barja de Quiroga et al., 1989), as well as in nile tilapia during periods of reoxygenation (Welker et al., 2012). Although the killifish in this study showed only modest levels oxidative stress during acute hypoxia-reoxygenation, the associated loss of catalase activity may leave these animals especially vulnerable to greater oxidative stress if hypoxia occurs concurrently with other oxidative stressors, such as aquatic pollution (Birnie-Gauvin et al., 2017).

5.6.2 Hypoxia acclimation leads to substantial changes in ROS homeostasis and oxidative status

Chronic exposure to constant hypoxia or to cycles of hypoxia-reoxygenation amplified the ROS signal in the muscle and liver during hypoxia and increased the levels of oxidized glutathione in the muscle (Fig. 5.2, 5.6). Mitochondria are a significant source of ROS in hypoxia, but it is not clear whether hypoxia increases mitochondrial ROS production as a direct result of effects of low PO_2 on mitochondrial electron transport, or instead as a result of low PO_2 on other factors in the cell (e.g., nitric oxide, hydrogen sulfide, and/or other signalling) that interact with mitochondria to regulate ROS efflux (Murphy, 2009; Smith et al., 2017). The latter possibility is more consistent with some previous mitochondrial studies, which have shown that mitochondrial ROS emission decreases with reductions in PO_2 (Treberg et al., 2018) and that hypoxia acclimation further reduces mitochondrial ROS emission in killifish (Du et al., 2016) and epaulette shark (Hickey et al., 2012). However, the relationship between whole tissue ROS levels

and mitochondrial ROS emission is unclear, and mitochondria may be able to regulate the levels ROS directly (Munro and Treberg, 2018).

Although chronic hypoxia increased cellular ROS levels in the muscle and liver, it did not increase lipid or protein damage (Fig. 5.3, Table 5.3). In the muscle, this avoidance of oxidative damage may have been associated with the restoration of catalase activity and increased oxidant scavenging capacity after hypoxia acclimation (Fig. 5.4, 5.5). Changes in these parameters were less apparent in the liver after hypoxia acclimation, possibly because the liver is already well protected by its relatively high scavenging capacity, total glutathione concentrations, and antioxidant enzyme activities (Table 5.3, 5.4).

Changes in ROS levels after hypoxia acclimation could be involved in important signalling functions that help killifish cope with hypoxia. Changes in ROS levels and/or redox status may have important implications for a number of cellular signalling events, and they can do so in localized regions of the cell where they do not induce oxidative damage (Smith et al., 2017) (Costantini, 2019; D'Autréaux and Toledano, 2007). For example, ROS has been shown to stabilize hypoxia inducible factor (HIF) 1α during short-periods of hypoxia, and thereby contribute to hypoxia signalling (Prabhakar and Semenza, 2012). However, this is unlikely to be a dominant role for the elevated ROS levels in hypoxia acclimated killifish, since we have previously shown that HIF-1α protein levels are low in the muscle of killifish after chronic exposure to either constant hypoxia or intermittent hypoxia (Borowiec et al., 2018, see Chapter 3 of this volume).

ROS could instead be involved in various other signalling pathways during chronic hypoxia, through its modulating effects on the reversible oxidation of cysteine thiol residues and subsequent alterations in protein function (Smith et al., 2017).

One possible source of the elevated ROS signal in chronic hypoxia is nitric oxide and its metabolites. NO can react with superoxide to form peroxynitrite, a potent oxidant that is detected by our fluorometric ROS assay (Kalyanaraman et al., 2012). Under O_2 limited conditions, nitrite can be converted to NO by several proteins – including deoxygenated haemoglobin in the blood, deoxygenated myoglobin in the muscle, xanthine oxidoreductase, and others – and NO is believed to be cytoprotective and to facilitate some adaptive cardiovascular responses to chronic hypoxia that improve hypoxia/anoxia tolerance in fish and other ectotherms (Fago and Jensen, 2015; Jacobsen et al., 2012; Jensen et al., 2014). Other gases such as hydrogen sulfide (H_2S) and carbon monoxide (CO) may also have interesting implications for ROS homeostasis and oxidative stress. H_2S levels increase under hypoxia and reversibly inhibit mitochondrial metabolism (Clanton et al., 2013), and this may lead to downstream effects of ROS homeostasis. CO produced by the catabolism of heme groups may interfere in NO, H_2S, and O_2 signalling, and therefore exert diverse effects on mitochondrial electron transport, HIF-1 signaling, and other key redox pathways, especially in heme-rich tissues like the skeletal muscle (Clanton et al., 2013; Kajimura et al., 2010). Examining the role of NO, H_2S, and CO signalling in the killifish hypoxia acclimation response would be an exciting area for future work. Better understanding of how killifish manage ROS homeostasis, oxidative

status, and signalling by ROS and NO during chronic hypoxia will provide valuable insight into key mechanisms for coping with life in harsh and variable environments.

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5.8 Competing interests

The authors declare no competing or financial interests.

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5.10 Figures & figure legends

Figure 5.1

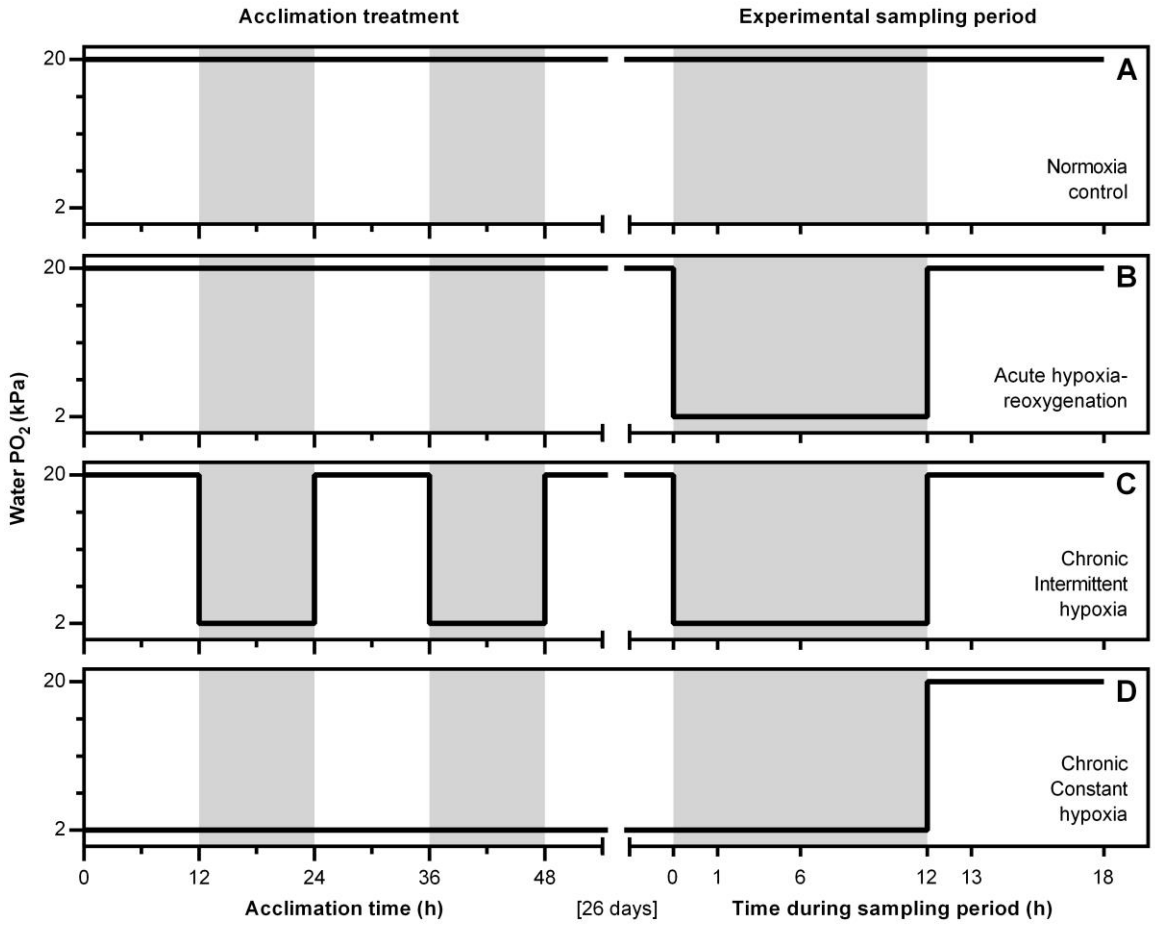

Fig. 5.1 Experimental groups used in this study. Killifish were first acclimated for 28 d to normoxia (~20 kPa), diel cycles of nocturnal hypoxia ('intermittent hypoxia', 12 h normoxia during the daytime light phase [white; from 0700 to 1900 local time] and 12 h of hypoxia at 2 kPa O_2 during the night-time dark phase [gray]), or constant hypoxia at 2 kPa O_2. These acclimation groups are shown to the left of the break in the x-axis. Following these 28 d acclimation periods, fish were divided into one of four treatment groups. (A) One batch of normoxia-acclimated fish were held in constant normoxia throughout to act as time-matched normoxia controls ('normoxia control group'). (B) A second batch of normoxia-acclimated fish were exposed to hypoxia (2 kPa O_2) for 12 h and then subsequent reoxygenation in normoxia ('acute hypoxia-reoxygenation group'). (C) Fish acclimated to intermittent hypoxia were exposed to a hypoxia-reoxygenation cycle identical to their acclimation treatment ('chronic intermittent hypoxia group'). (D) Fish acclimated to constant hypoxia continued to be held in hypoxia until they were exposed to reoxygenation in normoxia ('chronic constant hypoxia group'). Sampling times for fish in each treatment group are indicated right of the break in the x-axis (fish at the 0 h and 12 h time points were sampled just before the change in PO_2 and light/dark phase).

Figure 5.2

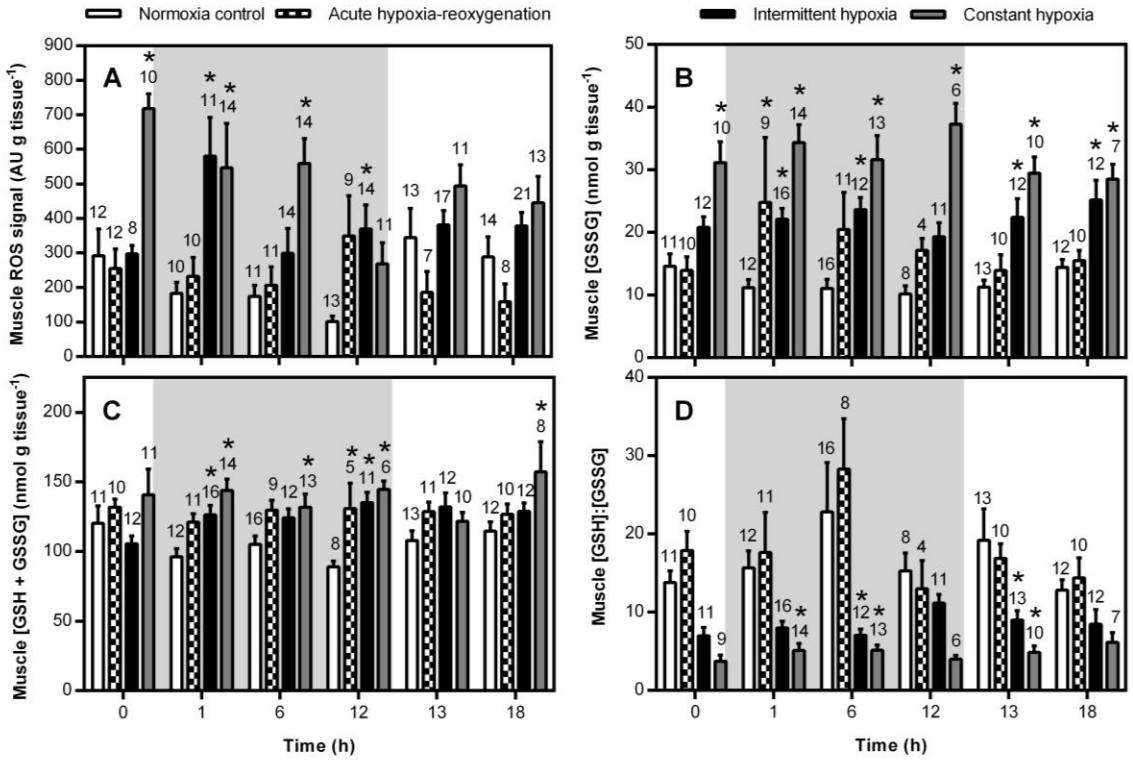
Normoxia control
Acute hypoxia-reoxygenation
Intermittent hypoxia
Constant hypoxia
A
B
C
D
Muscle ROS signal (AU g tissue-1)
Muscle [GSSG] (nmol g tissue-1)
Muscle [GSH + GSSG] (nmol g tissue-1)
Muscle [GSH]:[GSSG]
Time (h)
0
1
6
12
13
18

Fig. 5.2 Hypoxia acclimation altered ROS levels and glutathione redox status in the muscle. The night-time dark phase (1900 to 0700 local time) is indicated by the grey section of the background (see Fig. 1 for definition of treatment groups). *Significant pairwise difference from the normoxic control group within a time point ($P<0.05$). Sample sizes for each group are indicated directly above each bar. AU, arbitrary units (see Materials and Methods); GSH, reduced glutathione; GSSG, oxidized glutathione; GSH + GSSG, total glutathione.

Figure 5.3

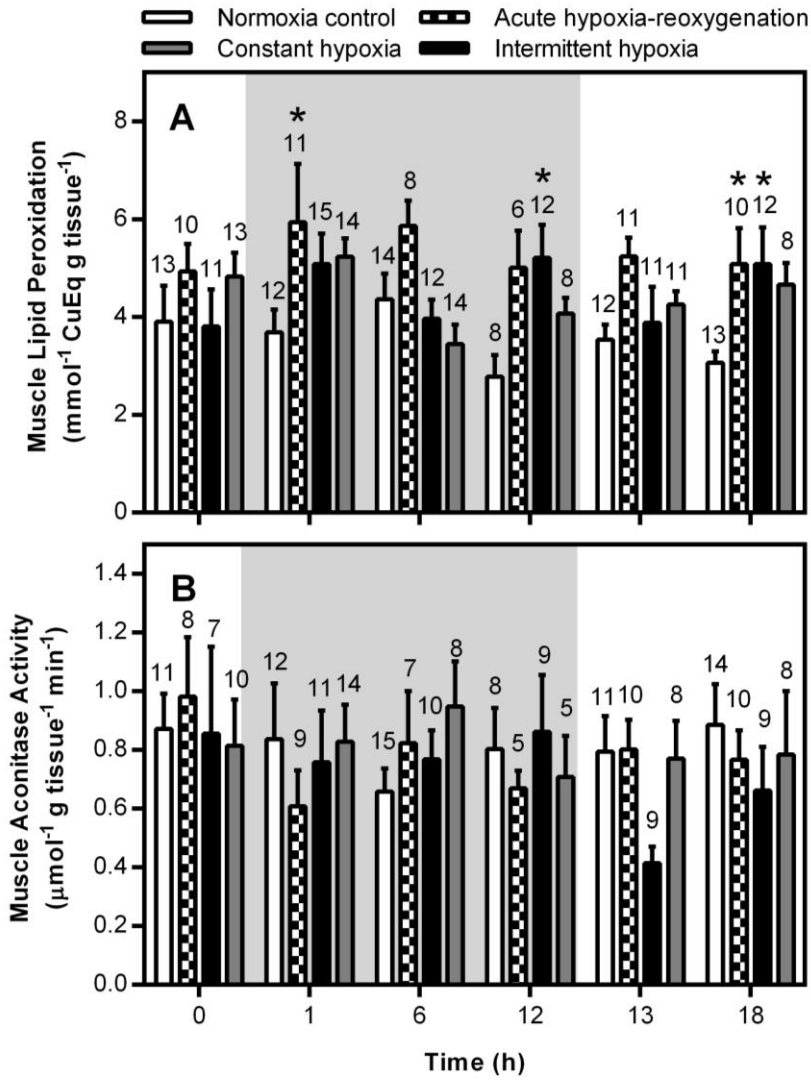
Normoxia control
Acute hypoxia-reoxygenation
Constant hypoxia
Intermittent hypoxia
A
Muscle Lipid Peroxidation (mmol-1 CuEq g tissue-1)
B
Muscle Aconitase Activity (μmol-1 g tissue-1 min-1)
Time (h)
0
1
6
12
13
18

Fig. 5.3 Effects of hypoxia and reoxygenation on lipid peroxidation and aconitase activity in the muscle. The night-time dark phase (1900 to 0700 local time) is indicated by the grey section of the background (see Fig. 1 for definition of treatment groups). *Significant pairwise difference from the normoxic control group within a time point ($P<0.05$). Sample sizes for each group are indicated directly above each bar.

Figure 5.4

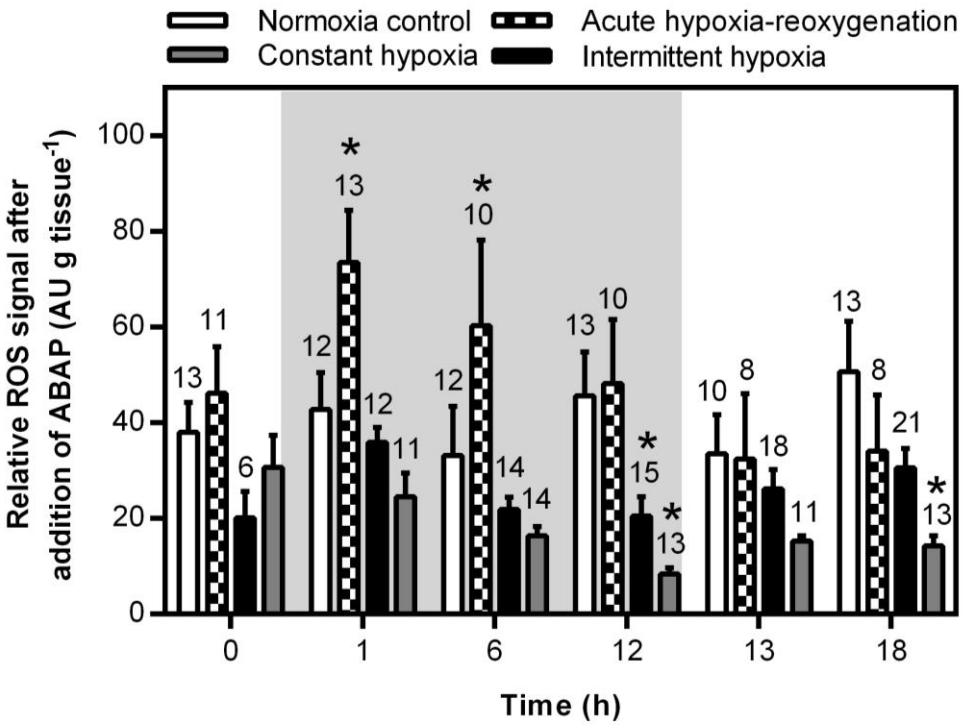
Normoxia control
Acute hypoxia-reoxygenation
Constant hypoxia
Intermittent hypoxia
Relative ROS signal after addition of ABAP (AU g tissue-1)
100
80
60
40
20
0
0
1
6
12
13
18
Time (h)
13
11
6
12
13
12
11
12
10
14
14
13
10
15
13
10
8
18
11
13
8
21
13

Fig. 5.4 Acute hypoxia decreased scavenging capacity in the muscle, but hypoxia acclimation restored or even enhanced scavenging capacity. Total oxidant scavenging capacity was measured as the change in relative ROS signal in response to the strong oxidant 2,2'-azobis(2-amidinopropane) dihydrochloride (ABAP), such that a greater ROS signal is indicative of reduced scavenging capacity (AU, arbitrary units; see Materials and Methods). The night-time dark phase (1900 to 0700 local time) is indicated by the grey section of the background (see Fig. 1 for definition of treatment groups). *Significant pairwise difference from the normoxic control group within a time point ($P<0.05$). Sample sizes for each group are indicated directly above each bar.

Figure 5.5

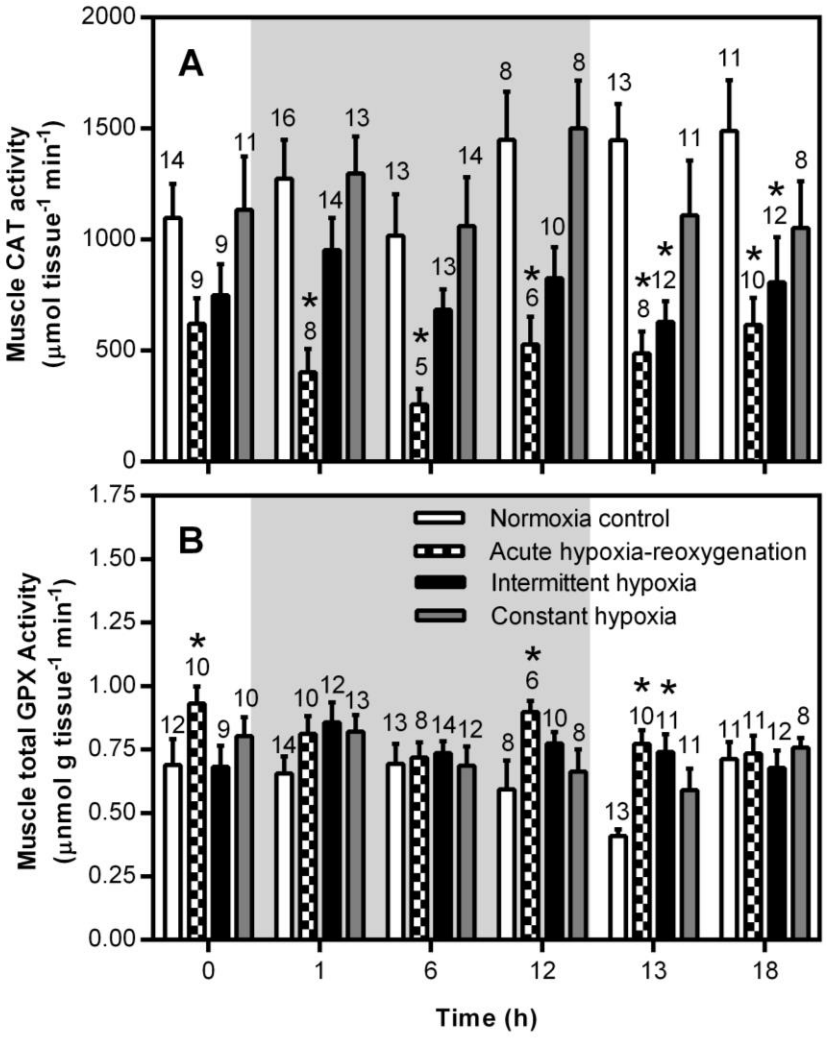
A
B
Muscle CAT activity (μmol tissue-1 min-1)
Muscle total GPX Activity (μmnol g tissue-1 min-1)
Normoxia control
Acute hypoxia-reoxygenation
Intermittent hypoxia
Constant hypoxia
Time (h)
0
1
6
12
13
18

Fig. 5.5 Effects of hypoxia and reoxygenation on the activities of antioxidant enzymes in the muscle. The night-time dark phase (1900 to 0700 local time) is indicated by the grey section of the background (see Fig. 1 for definition of treatment groups). *Significant pairwise difference from the normoxic control group within a time point ($P<0.05$). Sample sizes for each group are indicated directly above each bar.

Figure 5.6

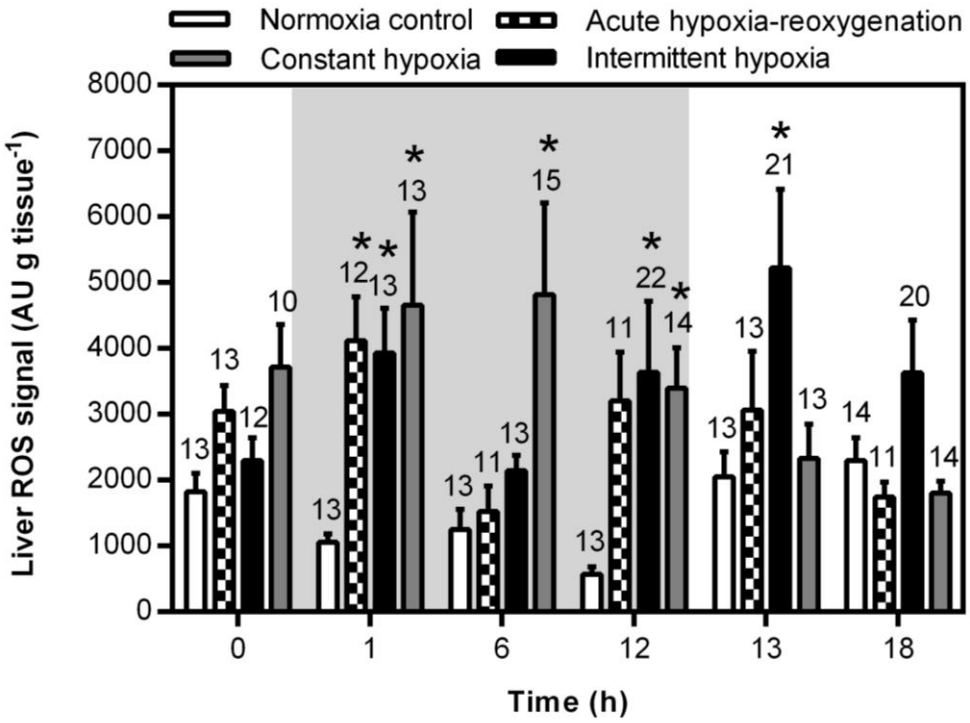
Normoxia control
Acute hypoxia-reoxygenation
Constant hypoxia
Intermittent hypoxia
Liver ROS signal (AU g tissue-1)
8000
7000
6000
5000
4000
3000
2000
1000
0
0
1
6
12
13
18
Time (h)

Fig. 5.6 Hypoxia acclimation altered ROS levels in the liver. The night-time dark phase (1900 to 0700 local time) is indicated by the grey section of the background (see Fig. 1 for definition of treatment groups). *Significant pairwise difference from the normoxic control group within a time point ($P<0.05$). Sample sizes for each group are indicated directly above each bar. AU, arbitrary units (see Materials and Methods).

5.11 Tables

Table 5.1: Statistical results of two-way ANOVA in the muscle

	Main effect of acclimation		Main effect of time		Main effect of interaction	
	F	p	F	p	F	p
ROS signal	20.88 (3,264)	< 0.0001	1.646 (5, 264)	0.1480	2.226 (15, 264)	0.0060
[GSH+GSSG]	14.90 (3, 241)	< 0.0001	0.6568 (5, 241)	0.6566	1.382 (15, 241)	0.1565
[GSSG]	40.36 (3, 237)	< 0.0001	0.7721 (5, 237)	0.5707	0.8935 (15, 237)	0.5721
[GSH]:[GSSG]	24.58 (3, 235)	< 0.0001	1.931 (5, 235)	0.0900	1.013 (15, 235)	0.4419
Lipid peroxidation	8.434 (3, 245)	< 0.0001	0.9328 (5, 245)	0.4603	1.102 (15, 245)	0.3545
TOSC	18.68 (3, 267)	< 0.0001	2.283 (5, 267)	0.0468	1.392 (15, 267)	0.1506
Aconitase	0.4542 (3, 204)	0.7146	0.6243 (5, 204)	0.6814	0.6380 (15, 204)	0.8413
Catalase	22.78 (3, 232)	< 0.0001	1.240 (5, 232)	0.2912	0.5682 (15, 232)	0.8974
GPX	6.696 (3, 232)	0.0002	2.659 (5, 232)	0.0233	1.277 (15, 232)	0.2175

GPX, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; GSH + GSSG, total glutathione; TOSC, total oxidant scavenging capacity. The degrees of freedom for the numerator and the denominator, respectively, for each ANOVA are reported in parentheses.

Table 5.2: Statistical results of two-way ANOVA in the liver

	Main effect of acclimation		Main effect of time		Main effect of interaction	
	F	p	F	p	F	p
ROS signal	7.797		1.054		1.811	
	(3, 306)	< 0.0001	(5, 306)	0.3861	(15, 306)	0.0324
[GSH+GSSG]	8.948		0.8161		0.9758	
	(3, 200)	< 0.0001	(5, 200)	0.5394	(15, 200)	0.4821
[GSSG]	6.238		1.191		1.061	
	(3, 187)	0.0005	(5, 200)	0.3150	(15, 187)	0.3957
[GSH]:[GSSG]	2.896		1.107		0.7136	
	(3, 183)	0.0366	(5, 183)	0.3581	(15, 183)	0.7689
Lipid	4.751		1.819		1.608	
peroxidation	(3, 202)	0.0032	(5, 202)	0.1107	(15, 202)	0.0738
TOSC	14.58		1.269		2.524	
	(3, 311)	< 0.0001	(5, 311)	0.2770	(15, 311)	0.0015
Catalase	15.12		2.274		1.305	
	(3, 176)	< 0.0001	(5, 176)	0.0493	(15, 176)	0.2032
GPX	1.222		1.629		0.8129	
	(3, 173)	0.3033	(5, 173)	0.1546	(15, 173)	0.6623

GPX, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; TOSC, total oxidant scavenging capacity. The degrees of freedom for the numerator and the denominator, respectively, for each ANOVA are reported in parentheses.

Table 5.3: Effects of hypoxia and reoxygenation on glutathione redox status and lipid peroxidation in the liver

		Treatment group			
	Time	Normoxia control	Acute hypoxia-reoxygenation	Chronic intermittent hypoxia	Chronic constant hypoxia
[GSH+ GSSG] (nmol g tissue^{-1})	0 h	894.5 ± 82.7 (13)	902.3 ± 172.7 (7)	764.6 ± 78.1 (16)	679.1 ± 192.5 (4)
	1 h	764.0 ± 89.1 (13)	972.6 ± 71.6 (15)	859.2 ± 88.8 (7)	994.5 ± 98.5 (5)
	6 h	812.7 ± 52.3 (15)	1142 ± 64 (9)*	747.8 ± 126.5 (7)	964.3 ± 70.5 (9)
	12 h	802.0 ± 72.1 (11)	1147 ± 214 (11)*	631.4 ± 124.0 (3)	717.5 ± 121.4 (4)
	13 h	793.4 ± 83.1 (11)	1087 ± 57 (12)	709.5 ± 55.8 (7)	866.6 ± 65.0 (10)
	18 h	775.7 ± 65.4 (12)	894.0 ± 140.9 (6)	567.4 ± 46.2 (9)	966.2 ± 118.2 (8)
[GSSG] (nmol g tissue^{-1})	0 h	91.67 ± 12.53 (13)	103.5 ± 15.8 (7)	94.01 ± 12.69 (11)	86.10 ± 14.31 (4)
	1 h	84.74 ± 9.75 (13)	95.56 ± 9.97 (14)	104.0 ± 16.8 (7)	81.56 ± 19.82 (5)
	6 h	92.00 ± 11.23 (13)	133.4 ± 14.4 (9)	90.33 ± 9.28 (7)	99.40 ± 11.83 (9)
	12 h	65.33 ± 6.82 (11)	126.1 ± 23.3 (10)*	81.90 ± 10.82 (3)	76.50 ± 27.22 (4)
	13 h	59.06 ± 8.14 (11)	126.4 ± 21.2 (11)*	106.7 ± 7.1 (5)	115.5 ± 12.2 (10)*
	18 h	69.79 ± 11.05 (12)	80.14 ± 13.63 (6)	88.32 ± 13.49 (9)	90.07 ± 24.02 (7)
[GSH]: [GSSG]	0 h	18.65 ± 2.97 (13)	21.90 ± 8.81 (6)	11.28 ± 1.76 (11)	21.05 ± 6.37 (3)
	1 h	14.79 ± 1.42(13)	27.19 ± 8.86 (13)	16.11 ± 4.59 (7)	22.95 ± 3.15 (5)
	6 h	18.96 ± 4.33 (13)	15.48 ± 3.06 (9)	12.95 ± 2.26 (7)	18.28 ± 3.22 (9)
	12 h	21.77 ± 2.13 (11)	16.98 ± 3.13 (10)	12.77 ± 5.12 (3)	17.70 ± 7.00 (4)
	13 h	22.96 ± 4.66 (10)	16.57 ± 2.43 (11)	8.97 ± 1.63 (5)	12.72 ± 2.35 (10)

Table 5.3 continued

	18 h	26.68 ± 5.85 (12)	22.45 ± 5.44 (6)	12.76 ± 3.62 (9)	28.42 ± 9.39 (7)
Lipid peroxid. (mmol l^{-1} cumene equivals g $tissue^{-1}$)	0 h	12.71 ± 1.54 (13)	8.50 ± 0.69 (7)	8.04 ± 0.70 (12)*	10.60 ± 1.58 (5)
	1 h	10.75 ± 1.03 (13)	7.81 ± 0.65 (13)	7.16 ± 0.79 (8)	9.64 ± 1.32 (9)
	6 h	10.15 ± 0.63 (15)	7.94 ± 0.77 (10)	8.49 ± 0.86 (7)	11.43 ± 1.18 (10)
	12 h	10.38 ± 1.10 (11)	7.85 ± 1.01 (8)	9.02 ± 0.96 (3)	10.94 ± 1.92 (5)
	13 h	9.03 ± 0.87 (11)	15.00 ± 3.68 (11)*	9.31 ± 1.79 (8)	12.62 ± 0.75 (10)
	18 h	11.14 ± 0.57 (12)	10.05 ± 1.36 (8)	7.31 ± 0.72 (10)	13.16 ± 1.94 (7)

equivals., equivalents; GSH, reduced glutathione; GSSG, oxidized glutathione, GSH + GSSG, total glutathione; peroxid., peroxidation. Data are reported as means ± standard error, with sample sizes in parentheses. *Significant pairwise difference from the normoxic control within a time point ($P<0.05$).

Table 5.4: Effects of hypoxia and reoxygenation on total oxidant scavenging capacity (TOSC) and antioxidant enzyme activities in the liver

		Treatment group			
	Time	Normoxia control	Acute hypoxia-reoxygenation	Chronic intermittent hypoxia	Chronic constant hypoxia
TOSC (AU g tissue^{-1})	0 h	7.16 ± 1.42 (14)	1.47 ± 0.24 (13)	4.70 ± 0.89 (12)	4.50 ± 0.59 (11)
	1 h	2.72 ± 0.23 (13)	2.74 ± 0.76 (12)	5.12 ± 1.15 (15)	2.68 ± 0.43 (13)
	6 h	4.13 ± 0.76 (14)	3.01 ± 0.56 (13)	5.77 ± 1.00 (13)	2.44 ± 0.29 (15)
	12 h	4.26 ± 0.49 (14)	3.50 ± 0.74 (11)	6.00 ± 0.79 (21)	2.69 ± 0.36 (13)
	13 h	6.10 ± 1.18 (13)	2.61 ± 0.63 (13)*	4.30 ± 0.52 (21)	4.31 ± 0.51 (13)
	18 h	5.56 ± 0.65 (14)	2.82 ± 0.67 (11)*	3.56 ± 0.47 (19)	3.03 ± 0.45 (14)*
Catalase (mmol g tissue^{-1})	0 h	12.04 ± 1.65 (11)	16.54 ± 2.84 (8)	19.00 ± 3.56 (10)	3.11 ± 2.39 (4)
	1 h	8.43 ± 1.93 (12)	12.38 ± 1.25 (14)	22.84 ± 3.70 (8)*	14.08 ± 3.47 (5)
	6 h	9.96 ± 1.52 (15)	8.60 ± 1.75 (7)	17.47 ± 2.62 (5)	11.21 ± 3.57 (8)
	12 h	8.05 ± 1.84 (11)	9.75 ± 2.48 (8)	10.72 ± 1.68 (3)	6.30 ± 3.51 (4)
	13 h	7.58 ± 1.76 (11)	17.41 ± 2.12 (10)*	22.42 ± 3.64 (7)*	10.71 ± 3.67 (6)
	18 h	6.82 ± 2.05 (10)	10.38 ± 3.73 (7)	18.78 ± 2.84 (7)*	8.87 ± 2.39 (9)
GPX (µmol g tissue^{-1})	0 h	8.15 ± 1.31 (11)	8.77 ± 1.28 (9)	9.21 ± 2.33 (8)	4.54 ± 1.98 (4)
	1 h	7.88 ± 0.82 (13)	9.11 ± 1.31 (14)	7.32 ± 1.76 (8)	10.52 ± 2.60 (5)
	6 h	7.60 ± 0.96 (14)	7.27 ± 0.90 (8)	9.08 ± 2.17 (5)	8.27 ± 0.98 (8)
	12 h	4.89 ± 1.04 (11)	8.56 ± 1.84 (8)	6.03 ± 2.00 (3)	2.47 ± 1.42 (3)
	13 h	7.92 ± 1.12 (10)	10.47 ± 1.72 (10)	9.50 ± 1.60 (6)	7.34 ± 1.73 (7)

Table 5.4 continued

18 h	9.84 ± 1.61 (10)	7.77 ± 1.27 (7)	8.32 ± 1.57 (7)	7.81 ± 2.42 (8)

GPX, glutathione peroxidase; TOSC, total oxidant scavenging capacity. TOSC is expressed in 10^4 RA g tissue^{-1}. Data are reported as means ± standard error, with the same size in brackets. *Significant pairwise difference from normoxia control at the same time point ($P<0.05$), tested via a two-way ANOVA followed by a Bonferroni post-hoc comparison. Further statistical information is detailed in Table 3. Data are reported as means ± standard error, with sample sizes in parentheses. *Significant pairwise difference from the normoxic control within a time point ($P<0.05$).

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CHAPTER 6

Evolutionary variation in hypoxia tolerance and hypoxia acclimation responses in killifish from the family Fundulidae

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6.1 Abstract

Hypoxia is a pervasive stressor in aquatic environments, and both phenotypic plasticity and evolutionary adaptation could shape the ability to cope with hypoxia. We investigated evolved variation in hypoxia tolerance and the hypoxia acclimation response across fundulid killifishes that naturally experience different patterns of hypoxia exposure. We compared resting O_2 consumption rate (MO_2), and various indices of hypoxia tolerance (critical O_2 tension [P_{crit}], regulation index [RI], O_2 tension [PO_2] at loss of equilibrium [P_{LOE}], and time to LOE [t_{LOE}] at 0.6 kPa O_2) in *Fundulus confluentus*, *F. diaphanus*, *F. heteroclitus*, *F. rathbuni*, *Lucania goodei*, and *L. parva*. We examined the effects of chronic (28 d) exposure to constant hypoxia (2 kPa) or nocturnal intermittent hypoxia (12 h normoxia: 12 h hypoxia) in a subset of species. Some species exhibited a two-breakpoint model in MO_2 caused by early, modest declines in MO_2 in moderate hypoxia. We found that hypoxia tolerance varied appreciably across species: *F. confluentus* was the most tolerant (lowest P_{LOE} and P_{crit}, longest t_{LOE}), whereas *F. rathbuni* and *F. diaphanus* were the least tolerant. However, there was not a consistent pattern of interspecific variation for different indices of hypoxia tolerance, with or without taking phylogenetic relatedness into account, likely because these different indices are underlaid by partially distinct mechanisms. Hypoxia acclimation generally improved hypoxia tolerance, but the magnitude of plasticity and responsiveness to different hypoxia patterns varied interspecifically. Our results therefore suggest that hypoxia tolerance is a complex trait that is best appreciated by considering multiple indices of tolerance.

6.2 List of symbols & abbreviations

LOE	Loss of equilibrium
MO_2	Rate of O_2 consumption
P_{crit}	Critical O_2 tension
P_{LOE}	PO_2 at loss of equilibrium
PO_2	Partial pressure of O_2
RI	Regulation index
t_{LOE}	Time to loss of equilibrium

6.3 Introduction

Hypoxia is a pervasive stressor in aquatic environments that can originate from natural and anthropogenic events (Breitburg et al., 2009; Diaz and Breitburg, 2009; Diaz and Rosenberg, 2008). Severe bouts of low oxygen availability are often implicated in fish kills, and hypoxic episodes can have broad ecological implications by reducing available habitat, altering species distributions, and changing trophic relationships (Breitburg et al., 2009; Mallin et al., 2006). Even brief exposure to less severe hypoxia can also affect fish physiology. Hypoxia-prone zones like tidal pools and estuaries are typically occupied by relatively hypoxia-tolerant organisms (Bickler and Buck, 2007; Chapman et al., 2002; Chapman et al., 1995; Mandic et al., 2009; Pollock et al., 2007; Richards, 2011; Wu, 2002).

Several metrics are used as indices of hypoxia tolerance in aquatic organisms. Many species maintain resting rates of oxygen consumption (MO_2) across a range of high O_2 tensions (PO_2) but will exhibit progressive declines in MO_2 at low PO_2. The transition from oxyregulation to oxyconformation of MO_2, which occurs at a PO_2 that is termed the critical O_2 tension (P_{crit}), is one common measure of hypoxia tolerance (Regan et al., 2019; Richards, 2009; Rogers et al., 2016). P_{crit} is often calculated as the breakpoint in a two-segmented linear regression (Yeager and Ultsch, 1989). However, studies have found that some species do not exhibit true oxyregulation; some species appear to oxyconform, in which MO_2 declines progressively as PO_2 falls from normoxic levels (Urbina et al.,

2012; Wood, 2018), and the patterns of MO_2 variation cannot be adequately described by two-segmented regression. For this and other reasons, the value of P_{crit} as an index of hypoxia tolerance has been debated recently (Regan et al., 2019; Wood, 2018). Alternative metrics for summarizing the PO_2 dependence of MO_2, such as the regulation index (a measure of relative degree of oxyregulation), have been proposed to overcome these criticisms (Mueller and Seymour, 2011; Wood, 2018), but these metrics may represent different physiological information (Regan et al., 2019). Hypoxia tolerance is also reflected by the ability to resist the loss of equilibrium in severe hypoxia, as either the time to loss of equilibrium (LOE) at a constant level of severe hypoxia (t_{LOE}) or the PO_2 at LOE (P_{LOE}) during progressive hypoxia (Borowiec et al., 2016; Crans et al., 2015; Dhillon et al., 2013; Mandic et al., 2013; McBryan et al., 2016). These various indices of hypoxia tolerance have sometimes (Dan et al., 2014; Mandic et al., 2013; Yang et al., 2013), but often not (Crans et al., 2015; Dhillon et al., 2013; Fu et al., 2014; Mathers et al., 2014; Speers-Roesch et al., 2013), been found to co-vary across species, likely because different indices of tolerance are underlaid in part by distinct mechanisms (Borowiec et al., 2016; Nilsson and Renshaw, 2004).

Variation in hypoxia tolerance results from developmental plasticity, adult phenotypic plasticity, and/or evolutionary innovation, in association with variation in many underlying physiological traits. Adult fish can show substantial plasticity that improves hypoxia tolerance in response to chronic hypoxia exposure, in association with morphological and physiological changes in a number of underlying traits involved in

oxygen uptake, transport, and utilization (Borowiec et al., 2015, see Chapter 2 of this volume; Burnett et al., 2007; Du et al., 2016; Fu et al., 2011; Greaney et al., 1980; Martinez et al., 2006; Søllid et al., 2003; Sollid and Nilsson, 2006). Exposure to hypoxia during early development can also can elicit changes in hypoxia tolerance and in other phenotypic traits that persist into adulthood (Blank and Burggren, 2014; Heinrich et al., 2011; Robertson et al., 2014). Enhanced hypoxia tolerance can also evolve over generations, such as when species evolve to become more specialized for life in hypoxia-prone environments, and be associated with evolved changes in the underlying determinants of O_2 transport and utilization (Hopkins and Powell, 2001; Mandic et al., 2009; Regan et al., 2017b; Richards, 2011). However, we know much less about the interactions between these processes – namely, the extent to which the plastic responses to chronic hypoxia might differ between species. Some evidence from African cichlids suggests that adaptation to hypoxia-prone swamps can attenuate the changes in brain size in response to developmental hypoxia (Chapman et al., 2008; Crispo and Chapman, 2010), but we know relatively little about the magnitude of interspecific variation in the plasticity of hypoxia tolerance across closely related species.

Killifish from the family Fundulidae (Fig. 6.1) are well suited for evaluating the roles of phenotypic plasticity and evolutionary innovation in tolerance of challenging environments. Species of this family are widely distributed and occupy habitats spanning a range of dissolved oxygen, salinity, pH, temperature, and other environmental factors (Burnett et al., 2007; Whitehead, 2010; Whitehead et al., 2013a). Considerable variation

in physiological tolerance of environmental stressors occurs across this family, and even closely related sister taxa can have very different tolerances (Griffith, 1974; Nordlie, 2006; Whitehead, 2010; Whitehead et al., 2013a), making Fundulidae a particularly useful model for evolutionary physiology (Burnett et al., 2007). *Fundulus heteroclitus* exhibits significant plasticity to chronic hypoxia (Borowiec et al., 2015, see Chapter 2 of this volume; Borowiec et al., 2018, see Chapter 3 of this volume, Du et al., 2016; Martinez et al., 2006) as well as intraspecific variation in hypoxia tolerance (McBryan et al., 2016), but it is unclear how hypoxia tolerance and its plasticity varies across the family. Therefore, our first two objectives were to better understand (i) the interspecific variation in hypoxia tolerance across Fundulidae and (ii) whether there is interspecific variation in the plasticity of hypoxia tolerance in response to chronic hypoxia. Finally, given recent debate surrounding P_{crit} and the most appropriate indices of hypoxia tolerance in fishes (Regan et al., 2019; Wood, 2018), our third objective was (iii) to examine the relationships between different indices of hypoxia tolerance across a variety of taxa and patterns of hypoxia exposure.

6.4 Materials & methods

6.4.1 Study animals and husbandry

Wild populations of killifish were collected with minnow traps, dip nets, or seine nets. *Fundulus confluentus* and *F. heteroclitus* were collected from Jekyll Island, GA, USA

(31.1039°N; 81.4061°W) with minnow traps. *Lucania parva* and *L. goodei* were collected from the Wakulla River, FL, USA (30.1761°N, 84.245°W) using dip nets. *F. diaphanus* and *F. rathbuni* were collected from Lake Opinicon, ON, Canada (44.559°N, −76.328°W) and Chapel Hill, North Carolina, USA (35.9266474N, -79.0318428W), respectively, with a beach seine. Fish were treated with a 24 h cupramine soak followed by 2 weeks of praziquantel treatment upon arrival to Louisiana State University. Fish were slowly acclimated to common 0.1 ppt conditions over the course of 1-2 weeks after arrival. Experiments were carried out at Louisiana State University for all taxa except *F. diaphanus*, for which experiments were carried out at McMaster University, and care was taken to provide consistent housing conditions and equivalent experimental treatments at both sites.

At Louisiana State University, fish were initially housed in a filtered recirculating rack system in aerated (normoxic) water (~20 kPa, 8 mg O_2/l) with a salinity of 0.1 ppt, at room temperature (22-26 °C), a 12 h light:12 h dark photoperiod, and were then transferred to 35 l aquaria for experimental acclimations. At McMaster University, fish were initially housed in 35 l glass aquaria under the conditions described above and were kept in these aquaria for experimental acclimations. Fish at both sites were fed daily with commercial fish pellets (Cargill, Minneapolis, MN, USA). Water chemistry (ammonia, nitrates, nitrites, and pH) was monitored at least once per week, and water changes were performed as necessary to maintain good water quality. Acclimations to normoxia and hypoxia (see below), respirometry, and hypoxia tolerance measurements were conducted

in aquaria at least one month after arrival to McMaster University or Louisiana State University. All procedures for collecting wild fish and for subsequent experimental treatments were approved by the institutional animal ethics boards of each institution.

6.4.2 Chronic hypoxia exposures

A subset of species (*F. rathbuni*, *L. parva*, *L. goodei*) were also exposed for 28 d to constant hypoxia or to nocturnal intermittent hypoxia (12 h hypoxia: 12 h normoxia, matched to the photoperiod), as previously described (Borowiec et al., 2015, see Chapter 2 of this volume; Borowiec et al., 2018, see Chapter 3 of this volume). Constant hypoxia (2 kPa O_2, 0.8 mg O_2/l) was maintained by bubbling the water with nitrogen gas, mediated by a feedback loop using a fibreoptic oxygen sensor (Loligo Systems, Tjele, Denmark) to regulate the action of a solenoid valve controlling the flow of nitrogen. Intermittent hypoxia was maintained using the same feedback loop as was used for constant hypoxia, except that hypoxia (2 kPa) was only maintained at night (7 pm to 7 am local time), and the water was bubbled with air to reoxygenate and maintain normoxia during the day. Fish were prevented from accessing the water surface using a plastic grid barrier over which we laid bubble wrap to minimize the diffusion of O_2 from the atmosphere into the water in the aquarium. Respirometry and hypoxia tolerance measurements (see below) were conducted after the completion of chronic exposures.

6.4.3 Respirometry and hypoxia tolerance measurements

Stop-flow respirometry was used to measure resting O_2 consumption rate (MO_2), critical O_2 tension (P_{crit}), regulation index (RI), and PO_2 at loss of equilibrium (P_{LOE}) (Fig. 6.2A), using methods consistent to those we have described previously for *F. heteroclitus* (Borowiec et al., 2015, see Chapter 2 of this volume). Fish were habituated overnight in normoxia to a 70 ml cylindrical respirometry chamber that was situated in a large darkened buffer tank. The chamber was connected to a flush pump that circulated water from the buffer tank through the chamber in a 'flushing circuit'. A second pump circulated water from the chamber in a closed loop (a 'recirculating circuit') across a flow-through fibre-optic O_2 sensor (PreSens, Regensburg, Germany). Water flow through both circuits were driven by pumps controlled by AutoResp software (Loligo Systems). Pumps in both the flushing and recirculating circuit were active during the overnight habituation to the respirometry chamber.

MO_2 measurements began the following morning, and sequential activation and deactivation of the pumps allowed measurement of the change in O_2 concentration due to fish respiration. During flush periods, both pumps were active, the chamber received a steady flow of water from the buffer tank, and no measurements of MO_2 were conducted. During measurement periods, the flushing pump was deactivated, but continued pumping through the recirculating circuit allowed for measurement of the decline in water oxygen content due to fish respiration. First, resting MO_2 (indicated by "1" in Fig. 6.2A) was

measured in normoxia. We then measured MO_2 throughout a progressive stepwise hypoxia protocol, in which the PO_2 of the buffer tank was reduced in ~2 kPa steps from ~20 kPa to 2 kPa (~15 min per step) using the O_2 control system described above. After MO_2 was measured at 2 kPa, the chamber was isolated from the buffer tank (by deactivating the flushing circuit) and the fish consumed the remaining O_2 until loss of equilibrium, and the P_{LOE} was recorded (indicated by "4" in Fig. 6.2A). During this period, we recorded MO_2 at roughly 1.5, 1.0, and 0.5 kPa, but measurements were not possible at each of these nominal PO_2 in every individual (e.g., if the fish had earlier lost equilibrium). A modest number of small fish could not consume O_2 below 0.5 kPa, so we had to open the chamber to the buffer tank and bubble the tank with nitrogen until the fish reached LOE.

MO_2 was calculated from the change in chamber O_2 concentration over time, as previously recommended (Clark et al., 2013), and are expressed relative to body mass. P_{crit} was calculated from the MO_2 and PO_2 data using the R package "segmented" (Muggeo, 2008), which allows for the identification and calculation of multiple breakpoints within a single MO_2-PO_2 curve. To calculate the P_{crit} of an individual fish, we used the average MO_2 measurement from 2-3 replicates for the setpoint PO_2 at each step. The segmented regression model was fitted to a general linear model of the data (Muggeo, 2008). Most individuals exhibited the expected two-segment association between MO_2 and PO_2, such that P_{crit} could be calculated as the single breakpoint using the MO_2 data across all PO_2 (i.e., in the manner described by Yeager and Ultsch 1989).

However, for some individuals from a subset of species, there were two PO_2 breakpoints in the MO_2-PO_2 relationship (indicated by "2" and "3" in Fig. 6.2A, respectively) rather than a single breakpoint. This pattern resulted from a decline in MO_2 across a narrow range of PO_2 just below normoxia, followed by a stabilization of MO_2 across a broader range of intermediate PO_2 (i.e., an absence or appreciable reduction in the slope of decline), and then another phase of more steeply declining MO_2 (Fig. 6.2A). There has recently been criticism of the lack of standardized approaches and a tendency for data pruning in calculations of P_{crit}, possibly in an effort to force a two-segment association to MO_2-PO_2 data that do not exhibit this pattern of variation (Wood, 2018). With this criticism in mind, we calculated the PO_2 at which each of the two apparent breakpoints occurred, and designated the breakpoint that occurred at the lower PO_2 as the P_{crit}. For only a very small number of individuals across all treatment groups (N=1 each of *F. rathbuni* and *L. parva*), the model could not converge upon a P_{crit} value as either the single breakpoint or the lower of two breakpoints in the MO_2-PO_2 relationship, so we do not report P_{crit} values for these individuals.

We also used the MO_2 and PO_2 data to calculate RI for each individual, which provides a relative measure of the degree of oxyregulation by comparing the MO_2 measured across PO_2 to the MO_2 expected from perfect oxyconformation (Mueller and Seymour, 2011). The lines of perfect oxyconformation and perfect oxyregulation were the lines from the MO_2 recorded at a PO_2 of ~20 kPa and the origin (0, 0) or a horizontal line at that MO_2 to a PO_2 of zero, respectively. RI was calculated as the area bound by the individual's

measured MO_2-PO_2 relationship and the oxyconformity line (indicated by the shaded region in Fig. 5.2A), divided by the triangular area bound by the oxyregulation and oxyconformity lines. Therefore, RI of 1 describes perfect maintenance of the MO_2 recorded at 20 kPa during hypoxia exposure, whereas RI of 0 describes perfect oxyconformation.

Time to loss of equilibrium (t_{LOE}) at a sustained level of severe hypoxia was measured in all species (Fig. 6.2B). Fish were held individually in small chambers in an aquarium of aerated (normoxic) water for an overnight period. During this period, water was continuously circulated through the chambers with aquarium pumps. The following morning, buffer tank PO_2 was rapidly decreased to 0.6 kPa (0.3 mg O_2/l), which typically took less than 5-15 min, by the rapid bubbling of nitrogen gas. This PO_2 was held steady until the fish lost equilibrium, and t_{LOE} was calculated beginning from the time when the PO_2 in the aquaria first reached 0.6 kPa ("5" in Fig. 6.2B). We chose 0.6 kPa because it likely represents a considerable acute hypoxia stressor for even hypoxia acclimated fish, being well below the P_{crit} of *F. heteroclitus* acclimated for 7 d to 2 kPa hypoxia (Borowiec et al., 2015, see Chapter 2 of this volume). Accordingly, animals that lost equilibrium before the PO_2 reached 0.6 kPa were assigned a negative t_{LOE}, which was reflective of the difference in time between when loss of equilibrium occurred and when the aquarium reached 0.6 kPa.

6.4.4 Statistics and phylogenetic analyses

Data were first checked for normality using a Shapiro-Wilk test (data not shown). For data across normoxia-acclimated fish, we used one-way ANOVA (on ranks in cases when normality was not confirmed) followed by Dunn's or Dunnett post-hoc tests (as appropriate to the type of ANOVA used), to examine the change in MO_2 as a function of PO_2 within a species or to compare hypoxia tolerance metrics between taxa. We similarly used two-way ANOVA to test for effects of hypoxia acclimation, species, and their interaction. We used least squares linear regressions to test for relationships between body mass and MO_2 or indices of hypoxia tolerance, and for relationships between MO_2 and indices of hypoxia tolerance. These statistical analyses were performed using R Studio or GraphPad Prism software (La Jolla, CA, USA).

Phylogenetically independent contrasts were calculated using Mesquite (Maddison and Maddison, 2009) using the PDAP module (Midford et al., 2008). We used a previously published, robust maximum likelihood phylogeny of the Fundulidae based on the consensus sequence of the cytochrome b gene for each species (Whitehead, 2010) (Fig. 6.1). We pruned the tree to only include species for which we measured all character data (species names in black text in Fig. 6.1), and we used this tree for phylogenetically independent contrast analysis. Positivized unstandardized contrasts were calculated from absolute data and then standardized by dividing them by the standard deviation of that

contrast (e.g. square root of the sum of corrected branch lengths), as previously recommended (Garland et al., 1992; Maddison and Maddison, 2009). We used least square linear regressions on standardized contrasts to test in a phylogenetically independent manner for the same relationships between body mass, MO_2, and indices of hypoxia tolerance that are described above.

6.5 Results

6.5.1 Variation in hypoxia tolerance across Fundulidae

We quantified the changes in resting MO_2 during progressive hypoxia (Fig. 6.3) in 6 species from multiple lineages of fundulid killifish (Fig. 6.1), in fish that were well acclimated to normoxia in freshwater (0.1 ppt). The response of MO_2 to declining PO_2 varied between species (Fig. 6.3), but all species had a significant main effect of PO_2 on MO_2 (Fig. 6.3A, *F. rathbuni*, $F_{[12,171]} = 4.30$, $p < 0.0001$; Fig. 6.3B, *F. diaphanus*, $H_{[13]} = 37.29$, $p = 0.0002$; Fig. 6.3C, *L. parva*, $H_{[13]} = 46.26$, $p < 0.0001$; Fig. 6.3D, *L. goodei*, $H_{[13]} = 33.74$, $p = 0.0007$; Fig. 6.3E, *F. heteroclitus*, $H_{[13]} = 21.61$, $p = 0.0421$; Fig. 6.3F, *F. confluentus*, $H_{[13]} = 34.77$, $p = 0.0005$). There was variation in the PO_2 at which MO_2 first exhibited statistically significant declines relative to MO_2 in normoxia, with the extremes represented by *L. parva*, the species that reduced MO_2 at the highest PO_2 (occurring at ~10 kPa), and *F. heteroclitus* and *F. confluentus*, species that maintained MO_2 statistically similar to resting MO_2 until very low (~0.5 kPa) PO_2.

Along with the variation in the PO_2 at which MO_2 first decreased from normoxic MO_2, the general pattern of the MO_2-PO_2 curve also differed between taxa. For example, in *F. diaphanus* and *L. goodei*, MO_2 changed very little until it declined at low PO_2 (Fig. 6.3B,D). Other species, like *F. confluentus* and *F. heteroclitus*, seemed to show a weaker pattern of oxyregulation with slight declines in MO_2 at high levels of declining PO_2 (Fig. 6.3E, F), but nevertheless showed the typical two-segment MO_2-PO_2 relationship with a single breakpoint PO_2 that we considered to be P_{crit}. However, some species (e.g., *F. rathbuni, L. parva*) exhibited an early decline in MO_2 during mild hypoxia (PO_2 just below normoxia) followed by stabilization of MO_2 in moderate hypoxia (the delineation between the two occurring at an upper breakpoint PO_2), and then a second sharper decline in MO_2 at low PO_2 below P_{crit} (Fig. 6.3A, C, F). In such cases, in which there were three clear segments in the MO_2-PO_2 curve, we considered the lower breakpoint to represent P_{crit}.

There was a significant main effect of species on resting MO_2 in normoxia ($H_{[6]} = 49.64$, $p < 0.0001$) and this appeared to be partially driven by the relatively high MO_2 of *L. parva* and *L. goodei,* as well as the low MO_2 of *F. diaphanus* compared to other taxa (Fig. 6.3). This variation appeared to result from the relationship between MO_2 and body mass. As expected, resting MO_2 in normoxia was negatively correlated with body mass, such that larger animals had lower mass-specific O_2 demands (Fig. 6.4A), and this relationship remained significant after accounting for evolutionary relatedness between taxa using

phylogenetically independent contrasts (Fig. 6.4B). These relationships remained true even after removing *Fundulus diaphanus* from the regression in Fig. 6.4A, or after removing the strong *Fundulus diaphanus-Funduus rathbuni* contrast from the regression in Fig. 6.4B (data not shown).

Indices of hypoxia tolerance varied appreciably between species (Fig. 6.5). There were highly significant main effects of species on t_{LOE} ($H_{[6]} = 71.23$, $p < 0.0001$) (Fig. 6.5A), P_{LOE} ($H_{[6]} = 31.34$, $p < 0.0001$) (Fig. 6.5B), and P_{crit} ($H_{[6]} = 26.56$, $p < 0.0001$) (Fig. 6.5C), all of which suggested that *F. confluentus* was the most hypoxia tolerant species (longest t_{LOE}, lowest P_{LOE}, and tied for lowest P_{crit}), *F. rathbuni* and *F. diaphanus* were the least hypoxia tolerant (shortest t_{LOE}, highest P_{LOE}, highest P_{crit}), and the other species had intermediate tolerance. RI also varied across species (Fig. 6.5D), as reflected by a significant main effects of species ($H_{[6]} = 16.98$, $p = 0.0045$), but did not follow a similar pattern to P_{crit} or to the other indices of hypoxia tolerance. RI was generally greater than zero, suggesting that all species exhibit some degree of oxyregulation in hypoxia. Although our different hypoxia tolerance metrics generally agreed with each other (except RI) in species representing the extremes of hypoxia tolerance (i.e., *F. confluentus* and *F. rathbuni*), the rank order of species with intermediate levels of tolerance was inconsistent.

Differences in body mass did not appear to make a major contribution to the interspecific variation in hypoxia tolerance. The correlations of t_{LOE}, P_{LOE}, or P_{crit} to body mass were

not significant for the allometric regressions using either absolute values or phylogenetically independent contrasts (Table 5.1). The allometric regressions of RI to body mass were significant before (Fig. 6.6A) and after (Fig. 6.6C) phylogenetic correction, suggesting that larger species have a higher RI (Table 6.1). Fish with a lower mass-specific MO_2 also had a higher RI (Fig. 6.6B), but this result was only significant after phylogenetic correction (Fig. 6.6D, Table 6.1). However, these significant correlations were entirely driven by the single contrast between the sister taxa pair of *F. rathbuni* and *F. diaphanus*, which differ appreciably in body mass (Fig. 6.4A), MO_2 (Fig. 6.3), and RI (Fig. 6.5).

6.5.2 Relationships between indices of hypoxia tolerance

Given the substantial variation in metrics associated with hypoxia tolerance across Fundulidae (Fig. 6.5), we examined how different indices of hypoxia tolerance correlated with each other (Table 6.1). P_{LOE} and t_{LOE} exhibited very similar patterns of variation across species (Fig. 6.5A, B), and the negative correlation between the absolute values of these traits neared significance ($p = 0.076$). There was also a significant positive correlation between the absolute values of P_{LOE} and P_{crit} (Fig. 6.7A), but this correlation was not significant after phylogenetic correction (Fig. 6.7C, Table 6.1). Regulation index showed a significant negative correlation with P_{crit} after accounting for evolutionary relatedness between taxa (Fig. 6.7D) using phylogenetically independent contrasts, but the correlation of absolute data was not significant (Fig. 6.7B).

6.5.3 Effects of hypoxia acclimation on hypoxia tolerance

We examined the plasticity of hypoxia tolerance in response to chronic hypoxia exposure in a subset of species – *F. rathbuni*, *L. goodei*, and *L. parva* – that appeared to differ in hypoxia tolerance in normoxia (Fig. 6.5). This was done using two distinct patterns of chronic hypoxia, constant sustained hypoxia and diel cycles of nocturnal hypoxia ('intermittent hypoxia,' 12 h hypoxia: 12 h normoxia, matched closely to the photoperiod). Hypoxia acclimation appeared to affect the qualitative pattern of the response of MO_2 to declining PO_2, particularly in *F. rathbuni* and *L. parva*, such that animals generally reduced MO_2 less at higher PO_2 (i.e. a less pronounced drop in MO_2 above the upper PO_2 breakpoint) after acclimation to constant and/or intermittent hypoxia (Fig. 6.8). The change in the MO_2-PO_2 curve was most dramatic in *F. rathbuni*, where the PO_2 at which MO_2 was first statistically different from MO_2 at 20 kPa decreased from 8 kPa in normoxia acclimated fish to ~0.5 kPa in hypoxia acclimated fish (Fig. 6.8A, D, G).

Hypoxia acclimation led to significant improvements in some (though not all) indices of hypoxia tolerance, as reflected by significant main effects of hypoxia acclimation on t_{LOE} ($F_{[2, 117]} = 22.32$, $p < 0.0001$), P_{LOE} ($F_{[2, 80]} = 17.73$, $p < 0.0001$) and RI ($F_{[2, 91]} = 10.10$, $p = 0.0001$), but not on P_{crit} ($F_{[2, 89]} = 1.21$, $p = 0.30$) (Fig. 6.9). Although there was some qualitative variation between the responses to constant hypoxia and intermittent hypoxia, both patterns of hypoxia exposure generally appeared to improve most metrics of hypoxia

tolerance. These changes were not associated with any significant changes in resting MO_2, which was unaffected by hypoxia acclimation ($F_{[2, 91]} = 1.52$, $p = 0.23$), and there was no species × environment interaction for this trait ($F_{[4, 91]} = 0.47$, $p = 0.76$) (Fig. 6.9E). As a result, the species differences in resting MO_2 that were observed in normoxia (Fig. 6.3) were found to persist across acclimation environments (significant main effect of species, $F_{[2, 91]} = 27.52$, $p < 0.0001$).

There were species differences in the effects of chronic hypoxia on some indices of tolerance (Fig. 6.9). The strongest example of these differences was the highly significant species × environment interaction for t_{LOE} ($F_{[4, 117]} = 8.57$, $p < 0.0001$), for which there was also a significant main effect of species ($F_{[2, 117]} = 95.81$, $p < 0.0001$). This significant interaction was driven by the large increase in t_{LOE} in *L. goodei* in response to both patterns of chronic hypoxia and the increase in *L. parva* in response to intermittent hypoxia, with no effects of hypoxia acclimation in *F. rathbuni* (Fig. 6.9A). P_{crit} did not show a significant main effect of species ($F_{[2, 89]} = 0.48$, $p = 0.62$), but there was a significant species × environment interaction ($F_{[4, 89]} = 2.90$, $p = 0.026$) driven by the strong reduction in P_{crit} in *F. rathbuni* after hypoxia acclimation (Fig. 6.9C). Neither P_{LOE} ($F_{[4, 80]} = 1.90$, $p = 0.12$) nor RI ($F_{[4, 91]} = 1.84$, $p = 0.13$) had a significant species×hypoxia acclimation interaction, but in both cases, the significant main effects of hypoxia acclimation on hypoxia tolerance appeared to be driven by responses in only one or two of the three species. The effects of hypoxia acclimation on P_{LOE} were driven by the much stronger reductions in *F. rathbuni* than in either *Lucania* species (Fig. 6.9B). The effects

of hypoxia acclimation on regulation index were driven by increases in both *F. rathbuni* and *L. parva* after constant hypoxia, and in only *F. rathbuni* after intermittent hypoxia (Fig. 6.9D). These patterns of variation suggest that the responses of each index of hypoxia tolerance to hypoxia acclimation are uncoupled, and there is interspecific variation in the magnitude of these responses.

6.6 Discussion

We compared across closely related taxa and in response to hypoxia acclimation to investigate the interspecific variation and plasticity of hypoxia tolerance in fundulid killifishes. MO_2 and various metrics of hypoxia tolerance (t_{LOE}, P_{LOE}, P_{crit}, and RI) varied substantially across species (Figs. 6.3, 6.5). No single metric could fully describe the variation across species (Fig. 6.5), and the interspecific variation in different indices of hypoxia tolerance often did not correlate (Fig. 6.7, Table 6.1). Hypoxia acclimation generally improved hypoxia tolerance based on some indices (t_{LOE}, P_{LOE}, and RI), but there was interspecific variation in the magnitude of this plasticity and in the indices of tolerance that were altered by chronic hypoxia (Fig. 6.8, 6.9). Our results suggest that hypoxia tolerance is a complex trait that is best appreciated by fully characterizing the MO_2-PO_2 relationship and by considering multiple indices of hypoxia tolerance.

6.6.1 Evolution of hypoxia tolerance across Fundulidae killifishes

There was appreciable interspecific variation in hypoxia tolerance (Fig. 6.5). Relative to other teleost fish, the P_{crit} of fundulid species ranged from comparable (*F. heteroclitus, F. rathbuni, F. diaphanus, L. parva*) to far below (*F. confluentus, L. goodei*) the typical P_{crit} across various species of fish held at a similar temperature, based on a recent metanalysis (Rogers et al., 2016). The P_{LOE} and t_{LOE} reported here in *Fundulus* and *Lucania* are lower than previous measurements in centrarchids (Borowiec et al., 2016; Crans et al., 2015), and are comparable to some previous measurements across common carp and other cyprinids (Dhillon et al., 2013; Fu et al., 2014), suggesting that Fundulidae killifish are relatively tolerant of extreme hypoxia. Among the species examined in this study, *F. confluentus* was the most hypoxia tolerant overall and the closely related pair of *F. rathbuni* and *F. diaphanus* had the weakest tolerance (according to the pattern of variation in t_{LOE}, P_{LOE}, and P_{crit}). This may reflect differences in the native habitat between these species: *F. rathbuni* and *F. diaphanus* are typically constrained to freshwater streams or lakes (which are often well oxygenated by can become hypoxic, such as during seasonal ice-cover for *F. diaphanus*), whereas *F. confluentus* are widely distributed across the highly variable, and frequently hypoxic (Diaz, 2001) estuaries of the southern Atlantic and the Gulf of Mexico (Griffith, 1974; Nordlie, 2006; Whitehead, 2010). Better characterization of the habitat occupied by different fundulid species is needed to better understand the relationship between species distribution and hypoxia

tolerance in this family, but similar associations have been observed in other taxa. For example, variation in traits associated with hypoxia tolerance are related with differences in distribution between North American *Lepomis* sunfish, based on the exclusion of the less tolerant bluegill but not the more tolerant pumpkinseed from northern lakes that experience winterkill events (Farwell et al., 2007).

In general, the different indices of hypoxia tolerance often did not correlate with each other (Table 6.1), as previously observed in other taxa (Crans et al., 2015; Dhillon et al., 2013; Speers-Roesch et al., 2013), likely because these different metrics are partially underlaid by distinct physiological mechanisms (Borowiec et al., 2016; Nilsson and Renshaw, 2004). Fish that maintain resting MO_2 into deeper levels of hypoxia (perhaps from having higher O_2 transport capacity) would be expected to have lower P_{crit} and higher RI (Perry et al., 2009; Richards, 2009). If the mechanisms supporting this ability are associated with increases in cellular O_2 levels, they may also contribute to helping fish resist losing equilibrium in hypoxia, which could help explain the correlation between P_{crit} and P_{LOE} observed here (Fig. 6.7A). However, the correlation between the phylogenetically independent contrasts of these traits was not significant, nor were many other correlations between P_{LOE} or t_{LOE} and traits expected to have a respiratory component (P_{crit} and RI) (Table 6.1). Therefore, the interspecific variation in P_{LOE} and t_{LOE} reported here (Fig. 6.5) is likely explained at least partly by physiological factors independent of the ability to maintain aerobic metabolism in hypoxia.

Variation in the ability to maintain equilibrium in hypoxia could have instead resulted from variation in the relative use of metabolic depression or anaerobic metabolism to help match cellular ATP supply and demand, or in the relative development and sensitivity to metabolic acidosis, rather than variation in the ability to maintain cellular O_2 supply. Metabolic depression can reduce O_2/ATP demands and stretch limited fuel stores through a general reduction in energetically expensive processes in the cell (Guppy and Withers, 1999; Hochachka et al., 1996; Nilsson and Renshaw, 2004). Interspecific variation in the use of metabolic depression could have contributed to some of the observed variation in hypoxia tolerance, although resting MO_2 in normoxia was not generally observed to be associated with variation in hypoxia tolerance (e.g. the hypoxia intolerant *F. diaphanus* had the lowest resting MO_2 of all species, and *L. goodei* and *L. parva*, which had by far the highest resting MO_2, were intermediate in their hypoxia tolerance). Variation in the capacity and availability of fuel reserves to support anaerobic metabolism could also have been important; in sculpins, for example, interspecific variation in t_{LOE} is associated with variation in glycogen reserves and LDH activity in the brain (Mandic et al., 2013; Speers-Roesch et al., 2013). The detrimental effects of metabolic acidosis may have differed between species as well; among triplefin fish, for example, hypoxia-tolerant species are less susceptible to acidosis-induced mitochondrial dysfunction (Devaux et al., 2019).

There was not always a consistent association between P_{LOE} and t_{LOE} (Table 6.1), largely because there appeared to be discordances between these traits in some species (e.g., *F. diaphanus* had high P_{LOE} but intermediate t_{LOE}). This may be reflective of differences in

the underlying physiological mechanism of what causes LOE in each situation, at least in some species. For example, t_{LOE} has been associated with resistance of brain ATP depletion and anaerobic capacity, whereas the mechanisms underlying P_{LOE} are less well-understood (Mandic et al., 2013; Speers-Roesch et al., 2013). A potential cause of such differences may lie in disparities in the rate of hypoxia induction between protocols, which was ~2 h during the stepwise reductions in PO_2 that led up to our measurements of P_{LOE} but was <15 min for measurements of t_{LOE}. Variation in the rate of hypoxia induction over the same order of magnitude has been shown to affect P_{crit} in goldfish, such that slower rates of induction (8 h) were beneficial because they provided more time to reduce P_{crit}, *via* increases in gill surface area and haemoglobin-O_2 binding affinity, whereas faster rates of induction (~0.5 to 1.5 h) did not (Regan and Richards, 2017). Alternatively, some species may have suffered detrimental effects of the prolonged exposure to moderate hypoxia that occurred in the stepwise hypoxia protocol that was used to determine P_{LOE}, such that the slower rate of hypoxia induction was a disadvantage for some species compared to others. This latter possibility may explain a puzzling finding reported here, that *F. diaphanus* were able to maintain equilibrium for nearly 20 min on average in the t_{LOE} experiment, when PO_2 was rapidly reduced to 0.6 kPa (Fig. 6.5A), but they exhibited a P_{LOE} of ~0.8 kPa in the stepwise-hypoxia experiment when hypoxia was induced more slowly.

There was a negative correlation between P_{crit} and regulation index, but only after phylogenetic correction (Fig. 6.7D). We had expected a much stronger association

between these traits, given that they both describe how well an animal is able to maintain resting MO_2 in acute hypoxia. The regulation index has been proposed by some to be a preferable alternative to P_{crit} (Mueller and Seymour, 2011; Wood, 2018). However, RI was the only metric that did not distinguish the most tolerant killifish species (*F. confluentus*) from the least tolerant (*F. rathbuni*) (Fig. 6.5). Our data also suggest that the RI does not adequately describe the MO_2 responses of some species to hypoxia. For example, the very low RI (~0.25) of *L. parva* could be mistakenly interpreted as evidence for oxyconformity of this species, but the MO_2-PO_2 relationship clearly shows that this species maintains a stable MO_2 across a broad range of PO_2 from 10 kPa down to its P_{crit} ~3 kPa (Fig. 6.3). *F. rathbuni* and *F. diaphanus*, two closely related species that have similar P_{crit} and similarly poor hypoxia tolerance (as also reflected by P_{LOE} and t_{LOE}), have very different RI values due to the "upper breakpoint" pattern seen in *F. rathbuni* but not in *F. diaphanus*. Our data suggest that neither P_{crit} nor RI can adequately represent the nuances of the MO_2-PO_2 relationship that we observed in some species of killifish, and that the pattern of interspecific variation in RI is largely inconsistent with multiple other indices of hypoxia tolerance. As such, RI may have limited value as a metric of hypoxia tolerance for *Fundulus* species.

Although some of the distinctions between P_{crit} and RI likely arise from the upper breakpoint pattern seen in the MO_2-PO_2 relationship for some species, the cause of this pattern is not entirely clear. One possibility is that some species are especially sensitive to the very subtle effects of disturbance at the beginning of an experiment (e.g., inactivation

of the flush pump, etc.), such that resting normoxic MO_2 was elevated and fish only returned to a stable standard metabolic rate after they became accustomed to these minor disturbances after a few flush-measurement cycles. The species that exhibited this upper breakpoint pattern (*L. parva*, *L. goodei*, and *F. rathbuni*) also appeared to be relatively skittish in laboratory conditions and may be more sensitive to minor disturbance, providing some anecdotal support for this possibility. Another possibility is that animals were sensing and responding to minor changes in PO_2 above P_{crit}. Regardless of the underlying cause of the upper breakpoint pattern, it tends to reduce RI and thus exaggerates the apparent level of oxyconformity, likely without affecting P_{crit} (if calculated using three-segment as done here), P_{LOE}, or t_{LOE}. Regardless, the R-script used in this study was suitable for modeling both patterns of respirometry data even though the cause of each pattern is unclear.

Overall, our findings suggest that multiple indices of hypoxia tolerance are needed to appreciate interspecific variation in how fish cope with hypoxia, even when comparing very closely-related species as was done in this study. Hypoxia tolerance is underlaid by several distinct mechanisms, and no single metric can fully represent the various ways in which fish respond to and cope with hypoxia. We agree with recent suggestions (Regan et al., 2019; Wood, 2018) that full characterization of the MO_2-PO_2 relationship can provide valuable insight that is not represented by the metrics that are calculated from this relationship (i.e., P_{crit} and RI). P_{LOE} and t_{LOE}, indices that are measured directly and reflect the ability to survive hypoxia, are also critical indices of hypoxia tolerance that are often

not correlated with P_{crit} or RI (Table 6.1) (Speers-Roesch et al., 2013). These metrics for survival in hypoxia should be included in future studies aiming to characterize hypoxia tolerance.

6.6.2 Hypoxia acclimation improves hypoxia tolerance

Hypoxia acclimation generally improved hypoxia tolerance, as reflected by the various indices of tolerance measured here, including reduced P_{LOE}, increased t_{LOE}, and increased RI. Broadening of the PO_2 range for sustaining resting metabolism and/or body posture are common responses to hypoxia acclimation in fishes (Borowiec et al., 2015, see Chapter 2 of this volume; Borowiec et al., 2018, see Chapter 3 of this volume; Fu et al., 2011; Regan et al., 2017b; Richards, 2009), and could reflect the combined impact of physiological changes that increase branchial O_2 uptake or circulatory O_2 transport (Matey et al., 2008; Perry et al., 2009; Wells, 2009), adjust the use of anaerobic metabolism (Richards, 2009; Richards et al., 2008; Vornanen et al., 2009), and actively reduce cellular ATP demands (Boutilier, 2001; Hochachka et al., 1996; Richards, 2010). In *F. heteroclitus*, the mechanisms used to improve hypoxia tolerance after acclimation appear to differ between patterns of hypoxia exposure. Acclimation to constant hypoxia induces a pronounced ~50% reduction in whole-animal MO_2, well beyond the reduction observed in naïve fish exposed to acute hypoxia, in association with reductions in gill-filament length and in the oxidative capacity of the muscle (Borowiec et al., 2015, see Chapter 2 of this volume; Borowiec et al., 2018, see Chapter 3 of this volume). Whether

similar mechanisms underlaid the improvements in hypoxia tolerance observed here is unclear, insofar as resting MO_2 was unaffected by acclimation across species (Fig. 6.9). If these species are capable of metabolic depression, its use may be reserved for more severe levels of hypoxia, as observed in goldfish (Regan et al., 2017a). Acclimation of *F. heteroclitus* to intermittent hypoxia, by contrast, leads to increases in MO_2 in hypoxia, and to increases in the oxidative and gluconeogenic capacities of the liver that could hasten the speed of recovery from anaerobic metabolism (Borowiec et al., 2015, see Chapter 2 of this volume; Borowiec et al., 2018, see Chapter 3 of this volume). These distinct coping mechanisms enacted by different patterns of hypoxia exposure are both effective at maintaining cellular ATP content and avoiding metabolic acidosis during hypoxia (Borowiec et al., 2018, see Chapter 3 of this volume), and at improving hypoxia tolerance (Borowiec et al., 2015, see Chapter 2 of this volume).

There were interspecific differences in the magnitude of the response to chronic hypoxia, suggesting that there is evolutionary variation in the phenotypic plasticity associated with hypoxia acclimation across fundulid killifishes (Figs. 6.8, 6.9). *F. rathbuni* exhibited greater plasticity than *L. parva* and *L. goodei* for several traits (P_{LOE}, P_{crit}, RI). One explanation for this may be differences in the signal for plasticity across taxa (e.g., *F. rathbuni* may experience a greater decrease in tissue PO_2 during hypoxia acclimation). Another possible explanation is that each species differs in its inherent capacity for plasticity (e.g., *F. rathbuni* may be more responsive to a given reduction in tissue PO_2), potentially due to interspecific variation in the activation and capacity of different coping

mechanisms during exposure to chronic hypoxia. Differences in the capacity for plasticity in response to salinity change are known to exist between populations and species of fundulid killifish (Whitehead et al., 2011; Whitehead et al., 2013b), and our results here suggest that this is also the case for the plastic response to hypoxia.

Phenotypic plasticity can facilitate (or sometimes impede) colonization of novel environments, and the magnitude of plasticity can evolve (Fordyce, 2006; Ghalambor et al., 2007; Storz et al., 2010). The observed improvements in hypoxia tolerance associated with hypoxia acclimation likely help fundulid killifish colonize hypoxic environments in the wild. Most of the killifish species studied here were relatively tolerant of hypoxia by standard measures, and many inhabit environments that can become quite hypoxic, including estuaries (*F. heteroclitus*, *F. confluentus, L. parva*) and freshwater lakes that experience seasonal ice-cover and hypoxia (*F. diaphanus*) (Hasler et al., 2009; Nordlie, 2006; Whitehead, 2010). Our findings suggest that hypoxia tolerance is plastic across fundulids, but the manifestation of plasticity can differ between species, which could contribute to the broad distribution of this family across North America (Nordlie, 2006; Whitehead, 2010).

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6.8 Competing interests

We declare no competing interests.

6.9 Author contributions

Conceptualization: B.G.B., G.R.S.; Methodology: B.G.B., G.R.S.; Validation: B.G.B.; Formal analysis: B.G.B.; Investigation: B.G.B., R.D.H.; Resources: G.R.S., F.G.; Writing - original draft: B.G.B.; Writing - review & editing: B.G.B., R.D.H., C.D.H., F.G., and G.R.S.; Visualization: B.G.B., G.R.S.; Supervision: G.R.S., F.G., and C.D.H.; Funding acquisition: G.R.S., B.G.B., R.D.H., and F.G.

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6.11 Figures & figure legends

Figure 6.1

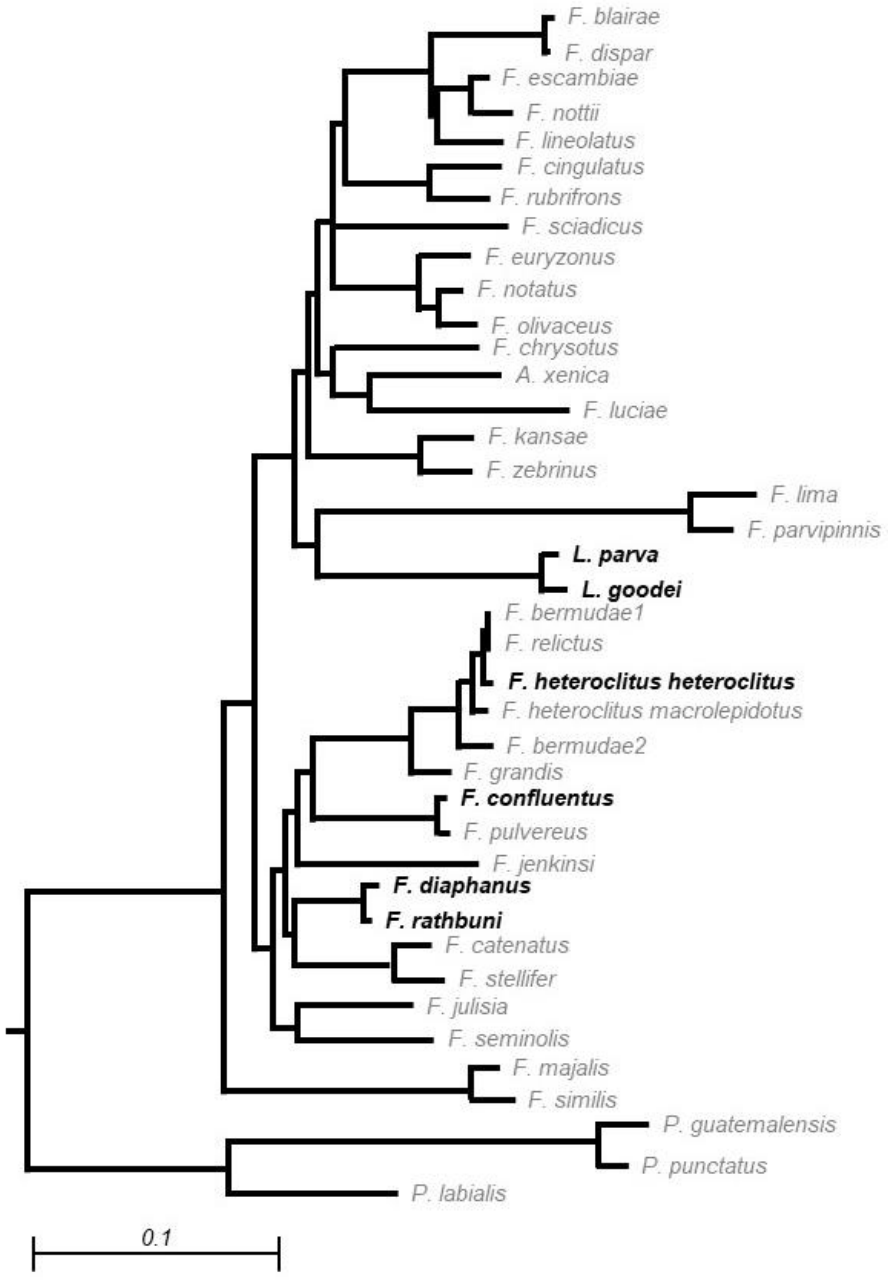

Fig. 6.1 Phylogeny of *Fundulidae* killifishes. Determined by maximum likelihood using the consensus sequence of the cytochrome b gene for each species, generated as described previously (Whitehead, 2010). The species used in this study are indicated by **black bolded text**. Branch lengths are representative of the relative evolutionary distance between taxa.

Figure 6.2

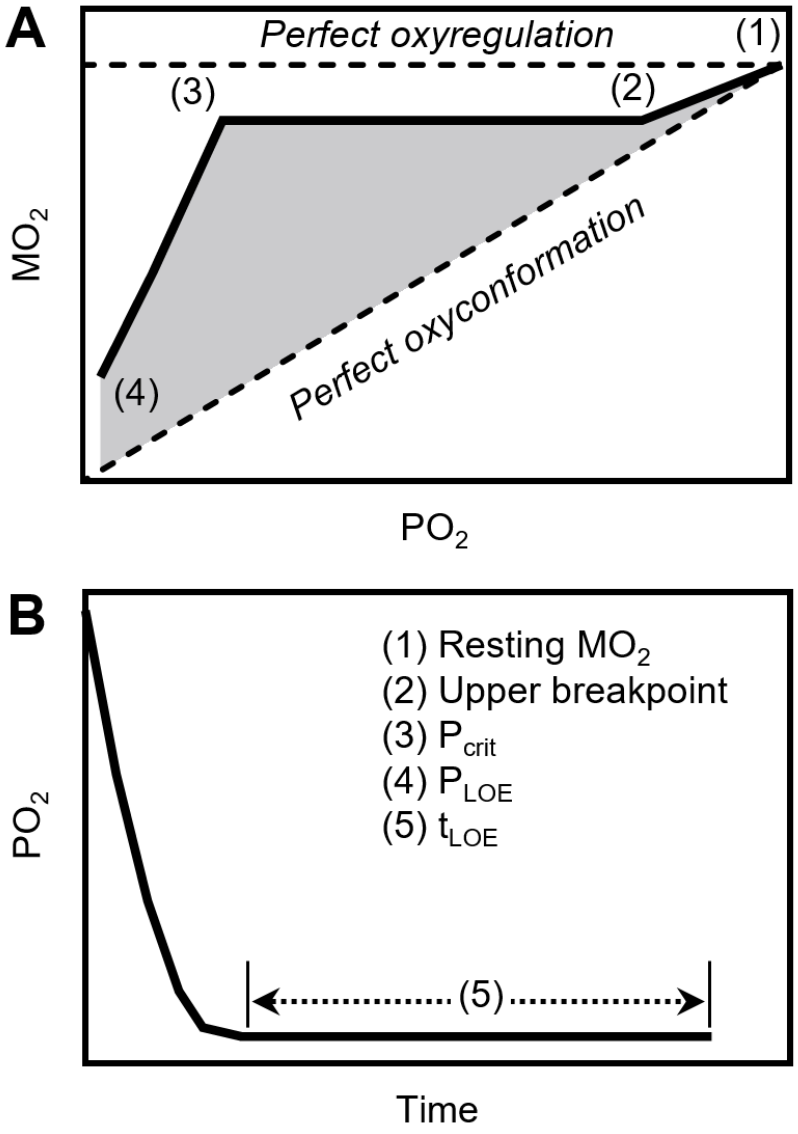
A
Perfect oxyregulation
(1)
(2)
(3)
(4)
Perfect oxyconformation
MO2
PO2
B
(1) Resting MO2
(2) Upper breakpoint
(3) Pcrit
(4) PLOE
(5) tLOE
(5)
PO2
Time

Fig. 6.2 Hypothetical illustration of the relationship between O_2 consumption rate (MO_2) and O_2 pressure (PO_2), as well as the hypoxia tolerance metrics measured in this study. (A) Resting MO_2 was determined for each fish in normoxia (1), and a progressive hypoxia protocol was used to determine the critical O_2 tension (P_{crit}, the lower breakpoint in the MO_2-PO_2 relationship) (3), and the PO_2 at which the fish displays loss of equilibrium (P_{LOE}) (4). A minority of individuals also showed an upper breakpoint (2) in the MO_2-PO_2 relationship (see text for further details). The overall response of MO_2 to progressive hypoxia was used to calculate the regulation index, the ratio of the area bound between the measured MO_2 and the line of perfect oxyconformation (indicated by the shaded region) and the area bound between the lines of perfect oxyregulation and perfect oxyconformation. (B) In a separate experiment, time to LOE (t_{LOE}, 5) was calculated as the time elapsed between when the water PO_2 first reached 0.6 kPa and when the fish lost equilibrium.

Figure 6.3

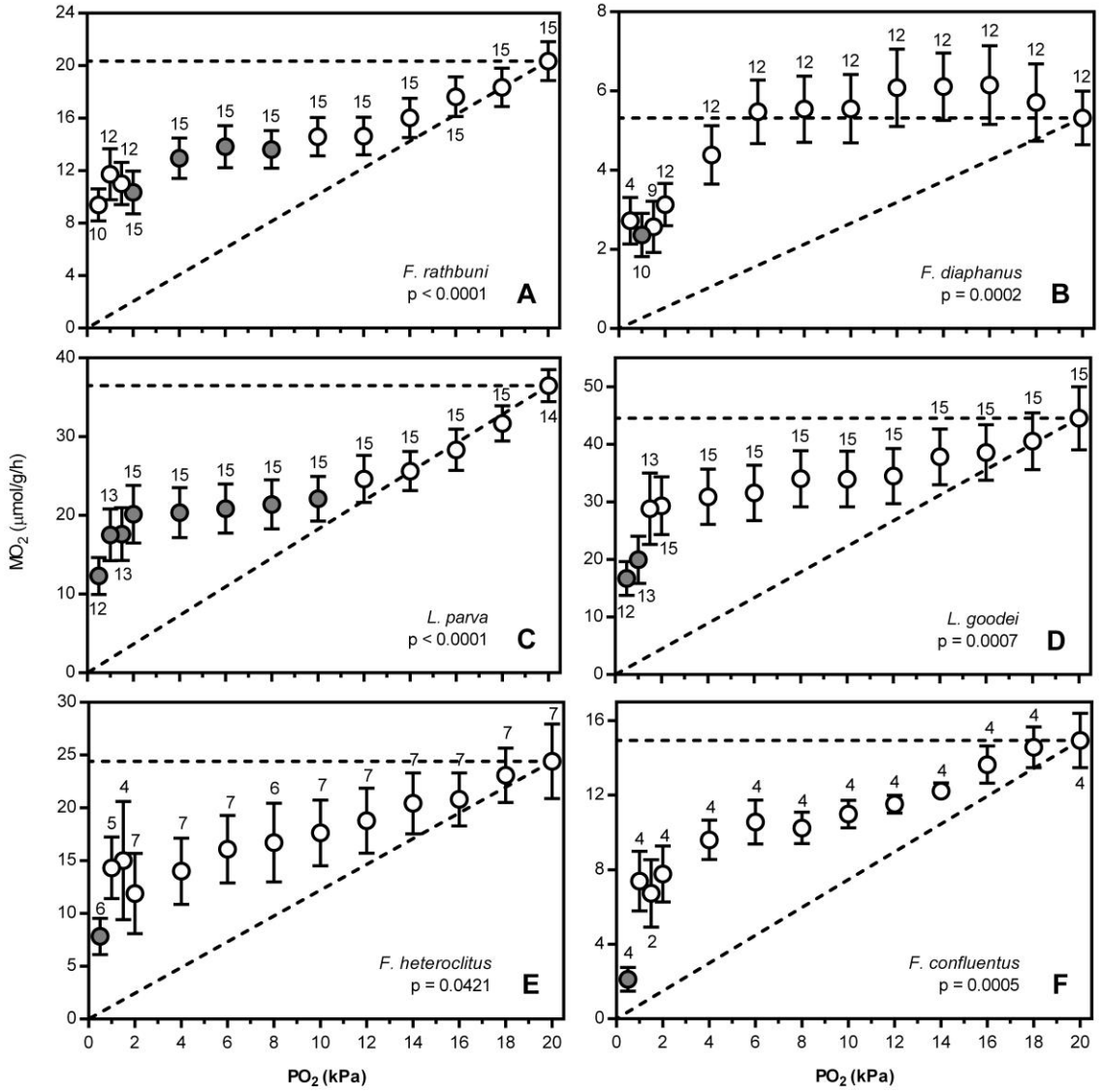
F. rathbuni
p < 0.0001
A
F. diaphanus
p = 0.0002
B
L. parva
p < 0.0001
C
L. goodei
p = 0.0007
D
F. heteroclitus
p = 0.0421
E
F. confluentus
p = 0.0005
F
MO2 (µmol/g/h)
PO2 (kPa)
PO2 (kPa)

Fig. 6.3 Response of O_2 consumption rate (MO_2) to declines in O_2 pressure (PO_2) in 6 killifish species acclimated to normoxia. Dashed lines represent the MO_2 expected with perfect oxyregulation (maintenance of resting MO_2) and perfect oxyconformation (linearly decline of MO_2 in hypoxia, to $MO_2 = 0$ at $PO_2 = 0$). Data are presented as means ± s.e.m. with samples sizes reported above each data point (see Materials and Methods). The p-values for the main effects of PO_2 from one-way ANOVA are indicated on the panels. MO_2 measurements that are statistically different from resting MO_2 in normoxia (20 kPa) via post-hoc tests are indicated by gray symbols, and measurements that are not statistically different from resting MO_2 are indicated by white symbols.

Figure 6.4

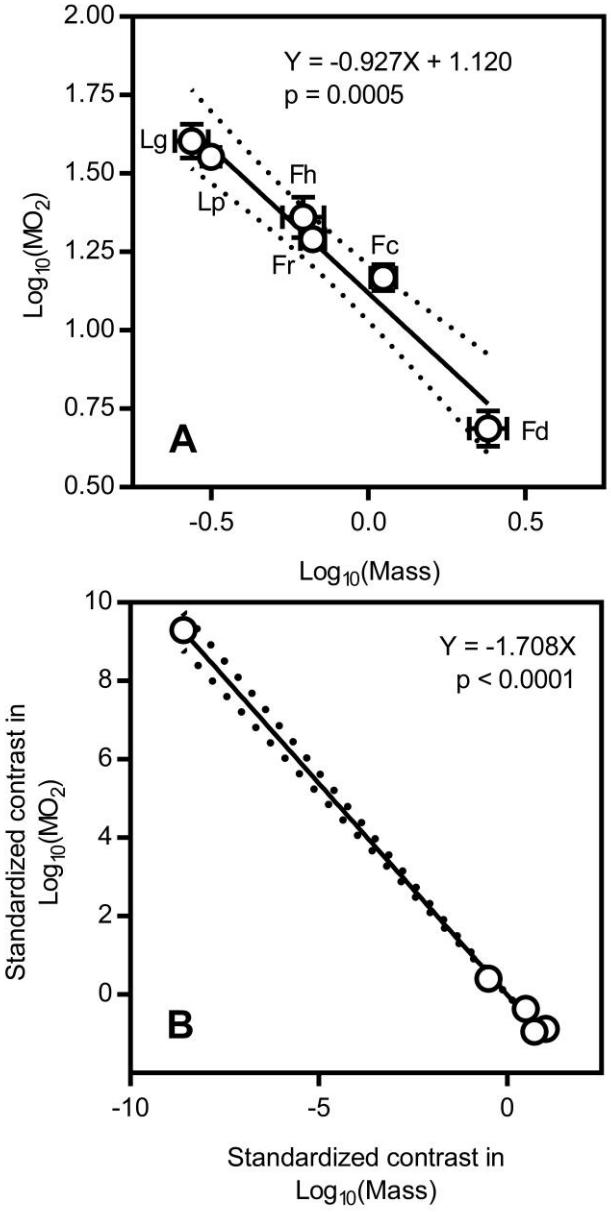
Y = -0.927X + 1.120
p = 0.0005
Lg
Lp
Fh
Fr
Fc
Fd
A
Log10(MO2)
Log10(Mass)
Y = -1.708X
p < 0.0001
B
Standardized contrast in Log10(MO2)
Standardized contrast in Log10(Mass)

Fig. 6.4 Relationship between O_2 consumption rate (MO_2) and body mass using (A) absolute values (means ± s.e.m.) and (B) phylogenetically-independent contrasts. Least squares linear regressions (solid line) and 95% confidence bands (indicated by dotted lines) are shown, with equations and p-values reported on each panel. Species abbreviations and sample sizes for absolute values were as follows: Fc, *F. confluentus* (4); Fd, *F. diaphanus* (12); Fh, *F. heteroclitus* (7); Fr, *F. rathbuni* (15); Lg, *L. goodei* (15); Lp, *L. parva* (15). Other statistical information is reported in Table 5.1.

Figure 6.5

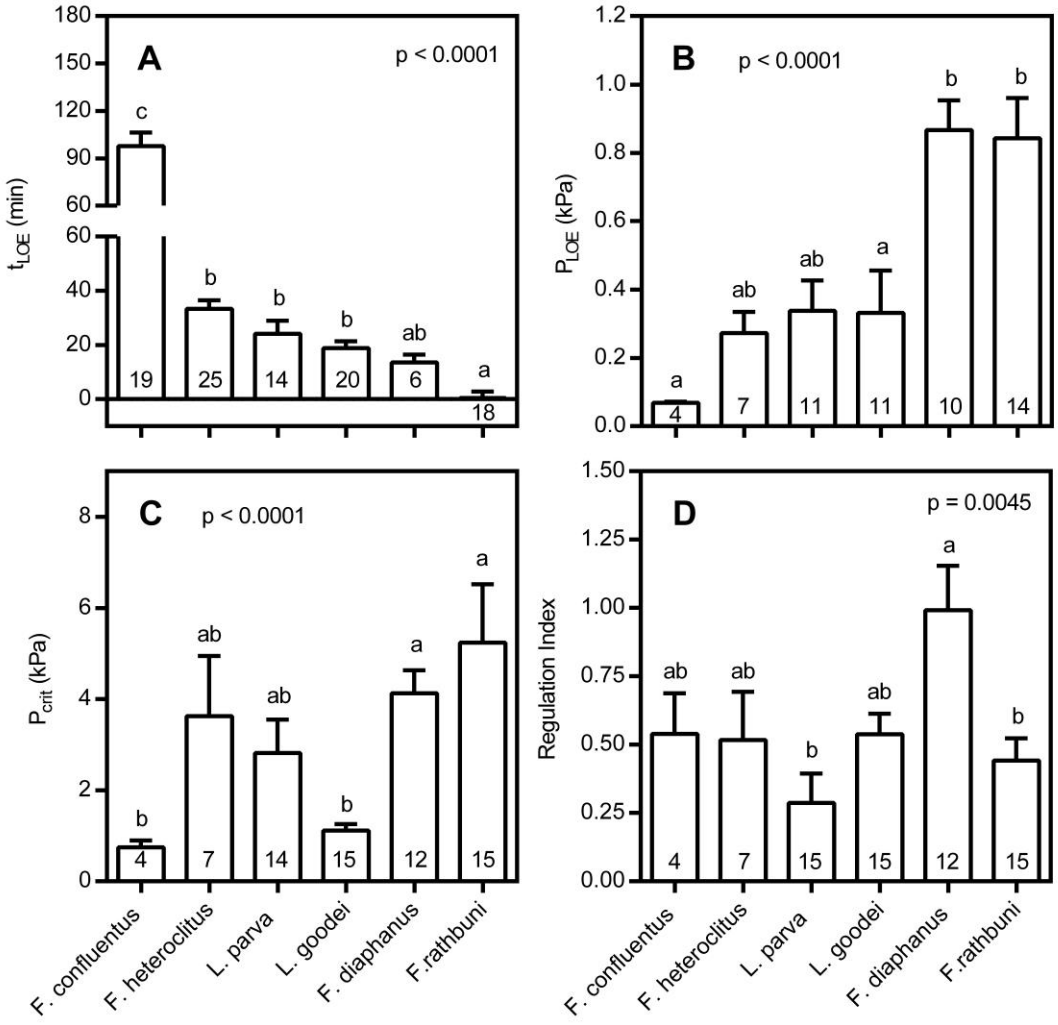
A
p < 0.0001
t_{LOE} (min)
c
b
b
b
ab
a
19
25
14
20
6
18
B
p < 0.0001
P_{LOE} (kPa)
a
ab
ab
a
b
b
4
7
11
11
10
14
C
p < 0.0001
P_{crit} (kPa)
b
ab
ab
b
a
a
4
7
14
15
12
15
D
p = 0.0045
Regulation Index
ab
ab
b
ab
a
b
4
7
15
15
12
15
F. confluentus
F. heteroclitus
L. parva
L. goodei
F. diaphanus
F.rathbuni

Fig. 6.5 Variation in (A) time to loss of equilibrium (t_{LOE}), (B) PO_2 at loss of equilibrium (P_{LOE}), (C) critical O_2 tension (P_{crit}), and (D) regulation index across 6 killifish species. Data are presented as means ± s.e.m., and samples sizes are reported within each bar. The p-values for the main effects of species in one-way ANOVA are reported on each panel (see Materials and Methods), and dissimilar letters indicate a significant pairwise difference between species according to post-hoc tests.

Figure 6.6

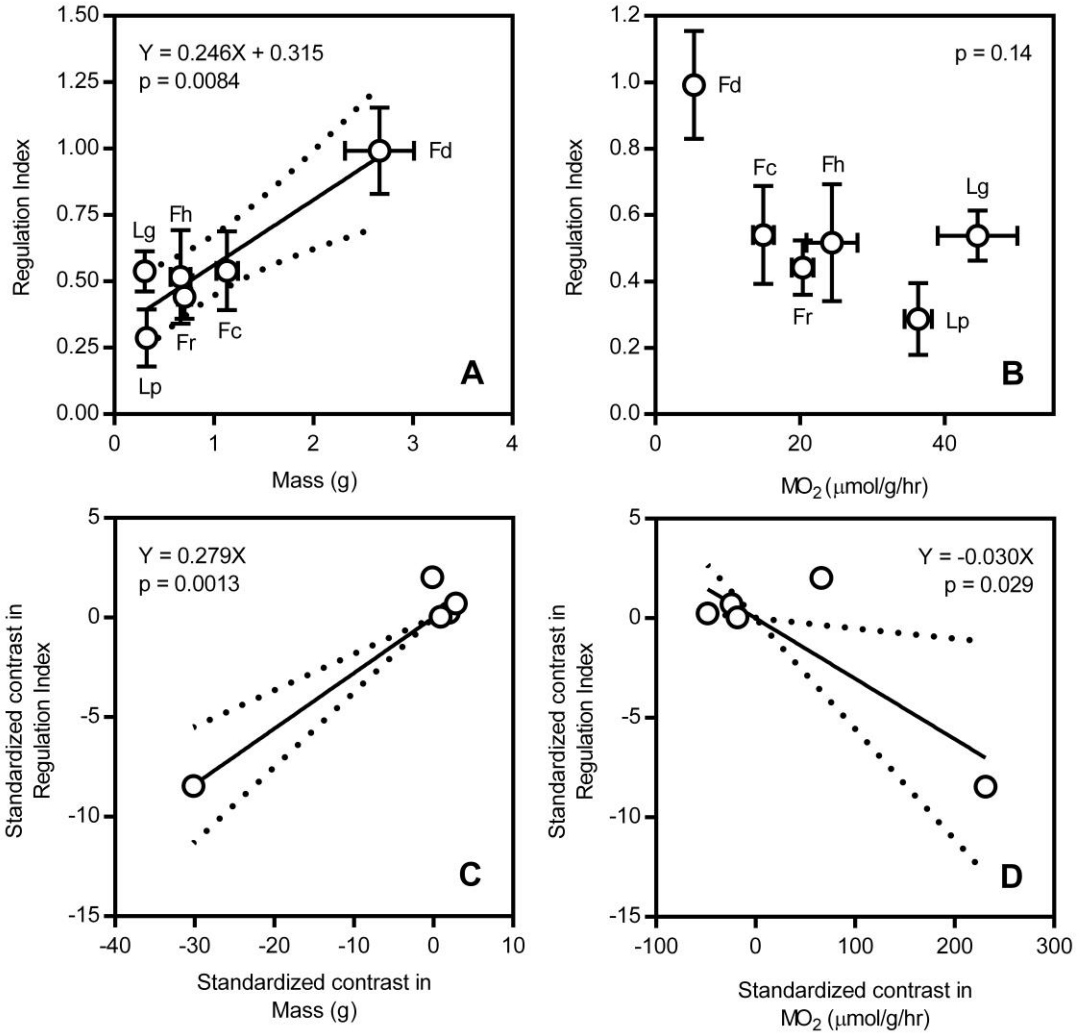
Y = 0.246X + 0.315
p = 0.0084
Regulation Index
Mass (g)
Fd
Fh
Lg
Fr
Fc
Lp
A
p = 0.14
MO2 (μmol/g/hr)
B
Y = 0.279X
p = 0.0013
Standardized contrast in Regulation Index
Standardized contrast in Mass (g)
C
Y = -0.030X
p = 0.029
Standardized contrast in MO2 (μmol/g/hr)
D

Fig. 6.6 Correlations between RI and body mass and between RI and MO_2 using (A, B) absolute values (means ± s.e.m.) and (C, D) phylogenetically-independent contrasts. Least squares linear regressions (solid line) and 95% confidence bands (indicated by dotted lines) are shown, with equations and p-values reported on each panel. Species abbreviations and sample sizes for absolute values were as follows: Fc, *F. confluentus* (4); Fd, *F. diaphanus* (12); Fh, *F. heteroclitus* (7); Fr, *F. rathbuni* (15); Lg, *L. goodei* (15); Lp, *L. parva* (15). Other statistical information is reported in Table 5.1.

Figure 6.7

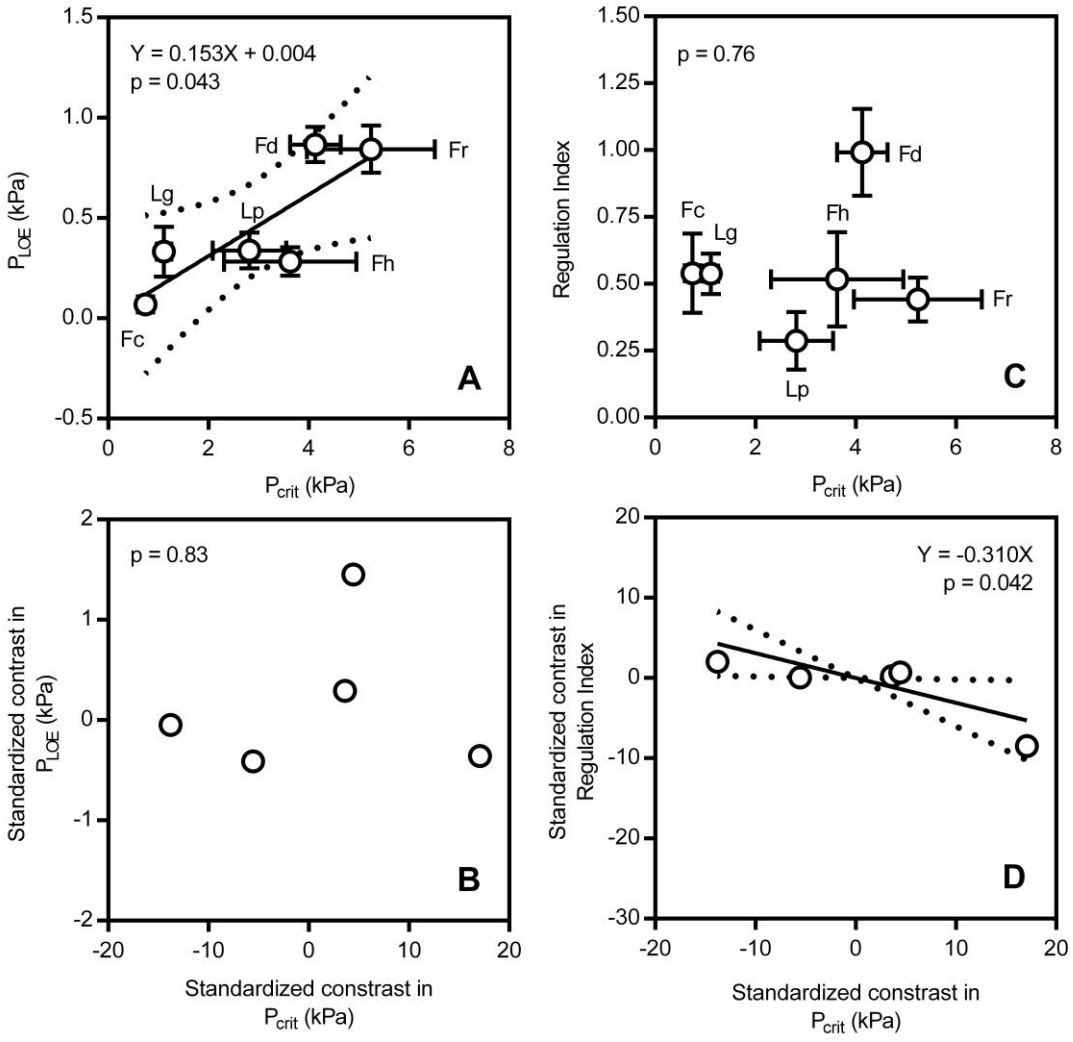
Y = 0.153X + 0.004
p = 0.043
Fd
Fr
Lg
Lp
Fh
Fc
A
P_{LOE} (kPa)
P_{crit} (kPa)
p = 0.76
Regulation Index
Fd
Fc
Lg
Fh
Fr
Lp
C
P_{crit} (kPa)
p = 0.83
Standardized contrast in P_{LOE} (kPa)
B
Standardized constrast in P_{crit} (kPa)
Y = -0.310X
p = 0.042
Standardized contrast in Regulation Index
D
Standardized constrast in P_{crit} (kPa)

Fig. 6.7 Correlations between P_{crit} and P_{LOE} or P_{crit} and RI using (A, C) absolute values (means ± s.e.m.) and (B, D) phylogenetically-independent contrasts. Least squares linear regressions (solid line) and 95% confidence band (indicated by dotted lines) are shown, with equations and p-values reported on each panel. Species abbreviations and sample sizes for absolute values (A, B) were as follows: Fc, *F. confluentus* (4, 4); Fd, *F. diaphanus* (10, 12); Fh, *F. heteroclitus* (7, 7); Fr, *F. rathbuni* (14, 15); Lg, *L. goodei* (11, 15); Lp, *L. parva* (11, 15). Other statistical information is reported in Table 5.1.

Figure 6.8

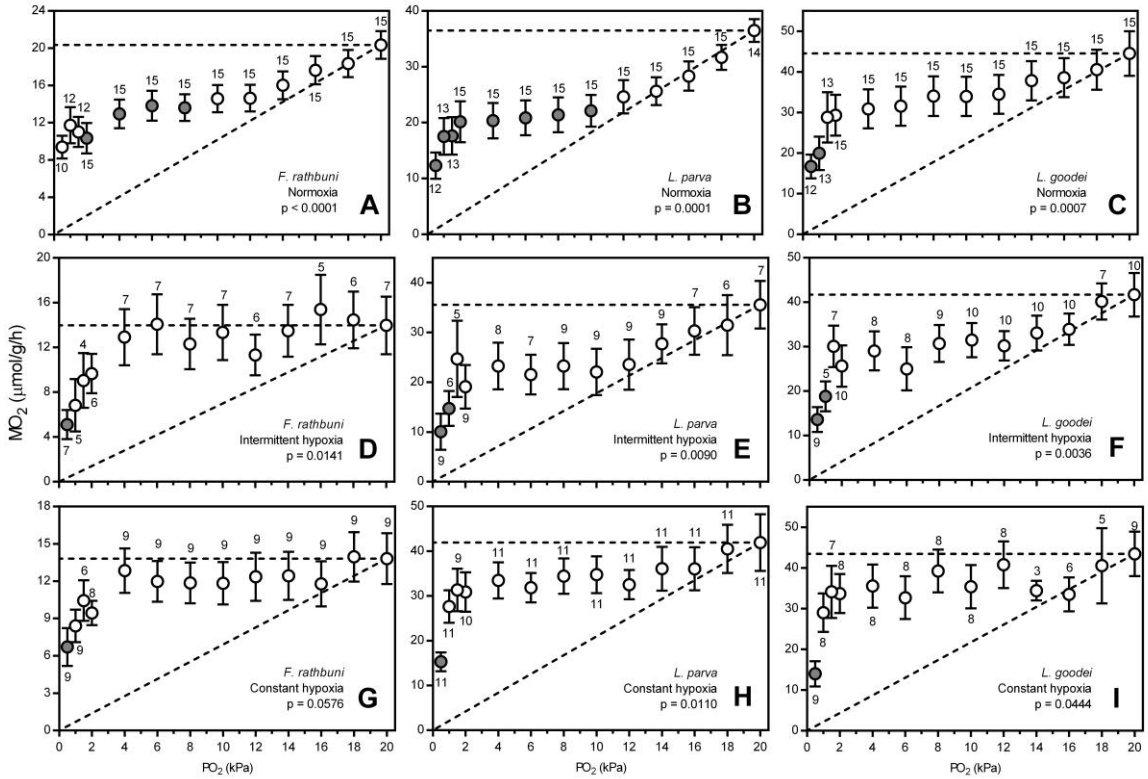
MO2 (µmol/g/h)
PO2 (kPa)
F. rathbuni
Normoxia
p < 0.0001
A
L. parva
Normoxia
p = 0.0001
B
L. goodei
Normoxia
p = 0.0007
C
F. rathbuni
Intermittent hypoxia
p = 0.0141
D
L. parva
Intermittent hypoxia
p = 0.0090
E
L. goodei
Intermittent hypoxia
p = 0.0036
F
F. rathbuni
Constant hypoxia
p = 0.0576
G
L. parva
Constant hypoxia
p = 0.0110
H
L. goodei
Constant hypoxia
p = 0.0444
I

Fig. 6.8 Response of O_2 consumption rate (MO_2) to progressive hypoxia in 3 killifish species. (A-C) MO_2-PO_2 relationships in fish acclimated to normoxia (statistical results in text). MO_2-PO_2 relationships after acclimation to nocturnal intermittent hypoxia (12 h hypoxia: 12 h normoxia) in (D) *F. rathbuni* ($H_{[13]} = 25.15$, $p = 0.0141$), (E) *L. parva* ($H_{[13]} = 26.55$, $p = 0.0090$), and (F) *L. goodei* ($H_{[13]} = 29.24$, $p = 0.0036$). MO_2-PO_2 relationships after acclimation to constant hypoxia in (G) *F. rathbuni* ($H_{[13]} = 20.54$, $p = 0.0576$), (H) *L. parva* ($F_{[12,127]} = 2.30$, $p = 0.011$), and (I) *L. goodei* ($H_{[13]} = 21.43$, $p = 0.0444$). Dashed lines represent the MO_2 expected with perfect oxyregulation (maintenance of resting MO_2) and perfect oxyconformation (linearly decline of MO_2 in hypoxia, to $MO_2 = 0$ at $PO_2 = 0$). Data are presented as means ± s.e.m. with samples sizes reported above each data point (see Materials and Methods). The p-values for the main effects of PO_2 in one-way ANOVA are indicated on the panels. MO_2 measurements that are statistically different from resting MO_2 in normoxia (20 kPa) via post-hoc tests are indicated by gray symbols, and measurements that are not statistically different from resting MO_2 are indicated by white symbols.

Figure 6.9

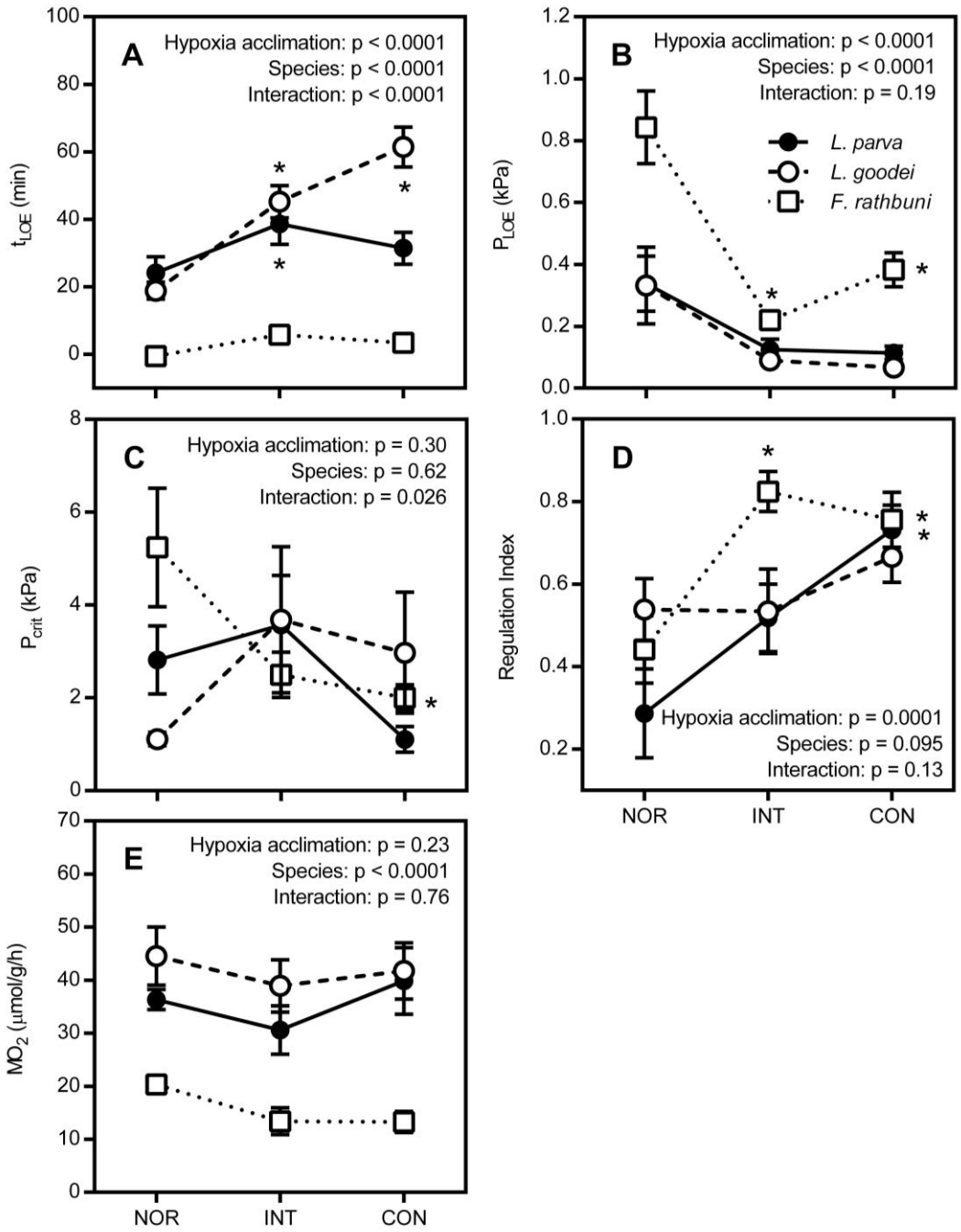
A
Hypoxia acclimation: p < 0.0001
Species: p < 0.0001
Interaction: p < 0.0001
t_{LOE} (min)
B
Hypoxia acclimation: p < 0.0001
Species: p < 0.0001
Interaction: p = 0.19
L. parva
L. goodei
F. rathbuni
P_{LOE} (kPa)
C
Hypoxia acclimation: p = 0.30
Species: p = 0.62
Interaction: p = 0.026
P_{crit} (kPa)
D
Hypoxia acclimation: p = 0.0001
Species: p = 0.095
Interaction: p = 0.13
Regulation Index
E
Hypoxia acclimation: p = 0.23
Species: p < 0.0001
Interaction: p = 0.76
$\dot{M}O_2$ (μmol/g/h)
NOR
INT
CON

Fig. 6.9 Effects of acclimation for 28 days to nocturnal intermittent hypoxia (12 h hypoxia at 2 kPa O_2: 12 h normoxia; 'INT') or constant hypoxia ('CON') at 2 kPa O_2 on (A) time to loss of equilibrium (t_{LOE}), (B) PO_2 at loss of equilibrium (P_{LOE}), (C) critical O_2 tension (P_{crit}), (D) regulation index, or (E) resting O_2 consumption rate in 3 killifish species. The p-values for the main effects of species, hypoxia acclimation, and their interaction in two-way ANOVA are reported on each panel. * denotes a significant ($p<0.05$) within-species pairwise differences from the normoxia-acclimated animals via post-hoc tests. Samples sizes for normoxia, intermittent hypoxia, and constant hypoxia, respectively, are as follows: *F. rathbuni*, (A) 19, 15, 14, (B) 14, 7, 9, (C) 15, 7, 8, (D) 15, 7, 9; (E) 15, 7, 9; *L. goodei*, (A) 20, 15, 10, (B) 11, 10, 8, (C) 15, 10, 9, (D) 15, 10, 9, (E) 15, 10, 9; *L. parva*, (A) 14, 11, 8, (B) 11, 8, 11, (C) 14, 9, 11, (D) 15, 9, 11, (E) 15, 9, 11

6.12 Tables

Table 6.1: Relationships between body mass, O_2 consumption rate, and hypoxia tolerance metrics in normoxia-acclimated fish

			Absolute values			Phylogenetically independ.		
X	Y	Slope	b	r^2	p	Slope	r^2	p
$Lg(M_b)$	$Lg(MO_2)$	-0.927	1.120	0.964	0.0005	-1.708	0.999	<0.0001
M_b	P_{crit}	0.316	3.597	0.018	0.80	-0.287	0.022	0.60
M_b	RI	0.246	0.315	0.854	0.0084	0.279	0.937	0.0013
M_b	P_{LOE}	0.189	0.273	0.265	0.30	0.016	0.034	0.54
M_b	t_{LOE}	1.101	28.86	0.003	0.92	8.884	0.288	0.20
MO_2	P_{crit}	-0.019	4.377	0.018	0.80	0.002	-0.059	0.98
MO_2	RI	-0.011	0.822	0.451	0.14	-0.030	0.714	0.029
MO_2	P_{LOE}	-0.009	0.686	0.173	0.41	-0.002	0.042	0.53
MO_2	t_{LOE}	-0.492	43.07	0.042	0.70	-0.831	0.535	0.076
P_{crit}	RI	0.022	0.489	0.003	0.76	-0.310	0.659	0.042
P_{crit}	P_{LOE}	0.153	0.004	0.683	0.043	0.008	-0.059	0.83
P_{crit}	t_{LOE}	-13.01	81.91	0.614	0.065	-2.067	-0.059	0.64
RI	P_{LOE}	0.655	0.094	0.226	0.34	0.053	0.018	0.57
RI	t_{LOE}	-11.39	37.40	0.006	0.89	20.48	0.376	0.15
P_{LOE}	t_{LOE}	-81.06	68.00	0.587	0.076	-51.52	-0.022	0.56

b, intercept on the y-axis; independ., independent; Lg, Log_{10}; Mb, body mass (g); MO_2, O_2 consumption rate (μmol/g/hr); P_{crit}, critical O_2 tension (kPa); P_{LOE}, O_2 tension at loss of equilibrium (kPa); RI, regulation index; t_{LOE}, time to loss of equilibrium at 0.6 kPa (min).

6.13 References

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CHAPTER 7

General discussion

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Fish encounter several different patterns of hypoxia in the wild, including constant hypoxia and diel cycles of intermittent hypoxia. Using an integrative approach that included work from many levels of biological organization, I investigated how acclimation to different patterns of hypoxia modified hypoxia tolerance, and the candidate underlying traits involved in the maintenance of energetic balance and protection from oxidative stress in *Fundulus* killifish.

Fundulus heteroclitus has an effective physiological strategy to cope with bouts of intermittent hypoxia that is unique from the coping strategy in response to constant hypoxia. This is the most extensive investigation to date on how fish cope with the energetic and oxidative challenges of intermittent hypoxia, and how these responses differ from constant hypoxia. This thesis contributes substantial insight into the general mechanisms by which animals can respond to an ecologically important but understudied component of natural encounters with aquatic hypoxia.

7.1 Hypoxia patterns matter

7.1.1 Natural variability in the aquatic environment

For several technical and theoretical reasons, physiologists have historically focused on relatively simple manipulations of environmental conditions in the laboratory (Morash et al., 2018), such as acute hypoxia and stable patterns of constant hypoxia. While this work

has added a great deal to our understanding of how animals work, it limits our ability to understand how animals live in realistically complex environments. Growing recognition of the importance of environmental variability on physiological responses has begun to address this gap (Denny, 2017; Koussoroplis et al., 2017; Morash et al., 2018), but there is still much to be learned about how the physiological effects of variable conditions differ (or do not differ) from stable conditions. The rapid pace of climate change, and subsequent unprecedented alterations in aquatic habitats worldwide (Breitburg et al., 2018; Cloern et al., 2016; Doney et al., 2012; Ficke et al., 2007) underscores the importance of understanding variable environments and environmental variability.

Like most environmental stressors, environmental hypoxia is typically studied under stable, 'average' conditions such as constant hypoxia (Morash et al., 2018). This thesis demonstrates that even a relatively simple pattern of intermittent hypoxia leads to unique physiological changes compared to both a single bout of hypoxia-reoxygenation and acclimation to a constant pattern of hypoxia. Future work investigating how the pattern of environmental stress influences the physiological coping strategy used by animals has clear scientific and ecological implications.

7.2.1 The economic implications of aquatic hypoxia

The natural aquatic environment is extremely variable. Hypoxic events are becoming more severe and more frequent in ecosystems around the world due global climate change

and other anthropogenic influences (Breitburg et al., 2018; Diaz, 2001; Diaz and Breitburg, 2009). In addition to their ecological effects, hypoxic dead zones and other hypoxic events have major economic implications, and these occur through mechanisms such as fish kills and reduced catch rates from fisheries (Diaz and Breitburg, 2009; Diaz and Rosenberg, 2008; Díaz and Rosenberg, 2011).

Most hypoxic fish kills along the Atlantic coast of North America are of species of low economic value (Díaz and Rosenberg, 2011; Thronson and Quigg, 2008), but this does not account for the costs associated with the declining food stocks and poor recruitment of more valuable species (Díaz and Rosenberg, 2011; Thronson and Quigg, 2008). There is also evidence that hypoxic fishing grounds have reduced catch rates, as fish avoid hypoxic habitats (Kraus et al., 2015), and that hypoxia can reduce overall fish biomass through reductions in fish numbers and/or individual size. Both of these effects can place further pressure on ecosystems vulnerable to overfishing and other stressors (Diaz, 2001; Díaz and Rosenberg, 2011; Huang et al., 2010), leading to loss of catch, recruitment failure, and even complete loss of key fishery species (Díaz and Rosenberg, 2011; Karlson, 2002; Mee, 1992). Much of the work describing the ecological effects and economic implications of hypoxia focuses on dead zones (e.g. the Gulf of Mexico, the Baltic Sea) or other dramatic, extreme cases of aquatic hypoxia that can occur seasonally (e.g. hypoxia induced by algal blooms in Lake Erie). Better understanding of an ecologically common but poorly studied pattern of hypoxia such as repeated normoxia-hypoxia cycles may inform conservation efforts and future fisheries practices.

7.2 How does *F. heteroclitus* cope with diurnal hypoxia?

Intermittent hypoxia is a unique challenge from constant hypoxia that induces a distinct physiological coping strategy to balance cellular energy supplies with metabolic demand. In *Fundulus heteroclitus*, this involves extensive alterations to the O_2 transport system and metabolism (Borowiec et al., 2015, see Chapter 2 of this volume; Borowiec et al., 2018, see Chapter 3 of this volume; Borowiec and Scott, in prep., see Chapter 4 of this volume). Acclimation to either intermittent hypoxia or constant hypoxia also alters the homeostasis of reactive oxygen species and oxidative status in a tissue-specific way (Borowiec and Scott, in prep., see Chapter 5 of this volume). Future work in this area promises to provide a more integrative and ecologically relevant understanding of how fish physiology is altered by different patterns of hypoxia exposure, and the physiological trade-offs necessary for successful acclimation to intermittent hypoxia.

7.2.1 Maintenance of resting metabolic rate during hypoxia

Killifish acclimated for 28 d to severe intermittent hypoxia show little to no reliance on metabolic depression, and maintain resting (normoxic) O_2 consumption rates through most of the nighttime hypoxic period (Borowiec et al., 2018, see Chapter 3 of this volume). This maintenance of resting MO_2 comes at the cost of a significant reduction in factorial aerobic scope in hypoxia, resulting in fish acclimated to intermittent hypoxia

operating at over 50% of MO_{2max} in hypoxia for much of the hypoxic period (Borowiec and Scott, in prep., see Chapter 4 of this volume). These high O_2 consumption rates are supported at least in part by increased whole blood haemoglobin content in hypoxia (Borowiec and Scott, in prep., see Chapter 4 of this volume). Fish acclimated to normoxia and to intermittent hypoxia show similar increases in haematocrit during 2 kPa hypoxia bouts (Borowiec and Scott, in prep., see Chapter 4 of this volume), but only the latter maintain resting MO_2 during hypoxic conditions, suggesting that fish acclimated to intermittent hypoxia may alter other components of the oxygen transport cascade in support of higher routine MO_2 in hypoxia such as branchial O_2 uptake, circulatory O_2 transport, and/or mitochondrial O_2 utilization (Hughes, 1973; Perry et al., 2009; Wu, 2002) (see section 7.2.2., "Altered O_2 transport capacity in fish acclimated to intermittent hypoxia" for a further discussion of this area).

Aerobic scope, the factorial or absolute difference between resting MO_2 and $MO_{2,max}$, is suggested to be a key fitness-related trait (Claireaux and Lefrançois, 2007; Clark et al., 2013; Farrell and Richards, 2009). Under routine (normoxic) circumstances, aerobic scope is thought to reflect the capacity for an animal to direct energy towards non-maintenance costs like growth, reproduction, and predator avoidance (Bishop, 1999; Clark et al., 2013). Interestingly, fish acclimated to intermittent hypoxia have a reduced factorial aerobic scope relative to normoxia-acclimated fish under hypoxic conditions, and this is driven by maintenance of high resting MO_2 without a concurrent increase in maximal O_2 consumption rate (Borowiec and Scott, in prep., see Chapter 4 of this

volume). This loss of factorial aerobic scope may reflect a relaxation of metabolic depression that is necessary for acclimation to intermittent bouts of hypoxia (i.e. allowing killifish to achieve high metabolic rates during recovery in normoxia) or be a function of the greater hypoxia resistance of resting MO_2 compared to $MO_{2,max}$ (i.e. recovery of $MO_{2,max}$ lags behind recovery of resting MO_2 in fish acclimated to intermittent hypoxia). If the latter is true, then there may be a trade-off between maintenance of aerobic scope and maintenance of normoxic rates of resting MO_2 in fish acclimated to intermittent hypoxia, and this may have important ecological implications (e.g. maintenance of reproductive output, increased susceptibility to nocturnal predators), especially if it also occurs during the normoxic phase of the cycle. Investigation of this possibility would be an interesting area for future work.

Fish acclimated to intermittent hypoxia also experience a substantial and extended period of increased MO_2 during reoxygenation (Borowiec et al., 2018, see Chapter 3 of this volume). While this has superficial similarities to a classic post-hypoxic oxygen debt response (Genz et al., 2013; Lewis et al., 2007; Scarabello et al., 1991; van den Thillart and Verbeek, 1991), the lack of concordance between the magnitude of increase in post-hypoxic MO_2 and tissue lactate accumulation during hypoxia suggests that recovery from anaerobic metabolism is not the main driver of this response, as has been suggested by some previous investigations (Genz et al., 2013; Lewis et al., 2007). Instead, this may reflect the metabolic costs of other processes involved in recovery from hypoxia, including correction of [GSH]:[GSSG], oxidative damage (Borowiec et al., in prep., see

Chapter 5 of this volume), or perhaps correction of other disturbances associated with responses that support large changes in metabolism (e.g. ion or osmotic disturbance associated with increases gill perfusion) that were not formally investigated in this thesis (Iftikar et al., 2010; Matey et al., 2011; Matey et al., 2008; Robertson et al., 2015; Wood et al., 2009), and alterations in transcription and translation associated with reoxygenation (Dowd et al., 2010; Rytkönen et al., 2012). Understanding the underlying components of this characteristic response in fish acclimated to intermittent hypoxia would help elucidate the mechanisms of acclimation to repeated hypoxia-reoxygenation cycles in fish and may reveal some of the physiological trade-offs and potential ecological implications involved with routine encounters with variation in O_2 availability.

How aerobic scope varies during the reoxygenation phase of intermittent hypoxia, where fish can reach O_2 consumption rates nearly double those observed under resting conditions (Borowiec et al., 2018, see Chapter 3 of this volume), and approximately 75% of maximum MO_2 of normoxia-acclimated *F. heteroclitus* held in similar conditions (Healy and Schulte, 2012) is less clear. For example, fish acclimated to intermittent hypoxia may allocate a substantial proportion of their aerobic capacity to recover from bouts of hypoxia, especially during the early phase of reoxygenation (Borowiec et al., 2018, see Chapter 3 of this volume), and thus see a loss of aerobic scope early in the recovery phase. Loss of aerobic scope has been suggested to limit the capacity of some species to cope with environmental challenges such as warming temperatures (Pörtner, 2010; Pörtner and Lannig, 2009; Rummer et al., 2014). Aerobic scope may limit how

quickly fish acclimated to intermittent hypoxia may recover from hypoxia bouts, and/or impair or shift the timing of other energetically expensive processes such as reproduction, feeding, and digestion (López-Olmeda et al., 2012; Montoya et al., 2010; Vera et al., 2007). An interesting area of future work would be to investigate how circadian-linked processes are shifted in fish acclimated to intermittent hypoxia, how interactions between variation in environmental O_2 availability and aerobic scope guide the timing of these processes. Another potential direction for future work already in progress is to identify the physiological traits (e.g. heart morphology, the extent of the gill interlamellar cell mass) that allow fish acclimated to intermittent hypoxia to sustainably operate at such a high proportion of $MO_{2,max}$ throughout the majority of the hypoxia-reoxygenation cycle.

7.2.2 Altered O_2 transport capacity in fish acclimated to intermittent hypoxia

Fish exposed to hypoxia often attempt to counteract the effects of hypoxia on aerobic metabolism by increasing branchial O_2 uptake (e.g. increasing gill ventilation or lamellar perfusion) and/or by increasing O_2 transport by the circulatory system to the tissues (e.g. blood haemoglobin content, or hameglobin-O_2 binding affinity) (Perry et al., 2009) (see Chapter 1 of this volume for a review in this area). During hypoxia, fish acclimated to intermittent hypoxia show a substantial increase in blood O_2 carrying capacity (haematocrit, whole-blood haemoglobin), and this seems to be largely mediated through the release of red blood cells into the circulation from the spleen (Borowiec and Scott, in prep., see Chapter 4 of this volume). Moreover, this nightly modulation of blood

haemoglobin content appears to be supported by filtering and re-storing red cells from the circulation in normoxia, allowing for rapid modulation of O_2 carrying capacity depending on water O_2 content. Elevated haematocrit during nighttime hypoxia but not daytime normoxic periods has also been recorded in southern flounder (*Paralichthys lethostigma*) (Taylor and Miller, 2001) and the common carp (*Cyprinus carpio*) (Lykkeboe and Weber, 1978) as well as in rodents (Kuwahira et al., 1999). Restricting periods of high haematocrit to hypoxic phases only may therefore be a common strategy to maintain O_2 transport capacity during bouts of intermittent hypoxia while limiting the cardiovascular burden of prolonged polycythemia and elevated blood viscosity during intervening normoxic periods when having a high haemoglobin content is less useful.

Blood O_2 carrying capacity can also be modulated irrespectively the number of O_2 binding sites through alterations in haemoglobin-O_2 binding affinity. For example, *C. carpio* exposed to nocturnal bouts of hypoxia also reduce the concentration of GTP, a negative allosteric modifier of haemoglobin-O_2 binding, in red blood cells (Lykkeboe and Weber, 1978), which could also allow for increased blood O_2 content while avoiding extended periods of polycythemia. This does not occur in *F. heteroclitus*, as ATP, GTP, and total NTP levels are similar across acclimation groups (Borowiec and Scott, in prep., see Chapter 4 of this volume) during both the day and night phases of a nocturnal hypoxia-reoxygenation cycle. Haemoglobin O_2-binding affinity is highly sensitive to a number of conditions, including pH and chloride (Jensen, 2004; Nikinmaa, 2001), and in some species can be altered by isoform shifts induced by hypoxia exposure (Pan et al.,

2017; Rutjes et al., 2007; van den Thillart et al., 2018). The absence of substantial intergroup variation between ATP and GTP within red blood cells does not preclude other mechanisms of modulating haemoglobin-O_2 binding affinity in this system.

Fish acclimated to intermittent hypoxia have the same $MO_{2,max}$ and increase in haematocrit during hypoxia as fish exposed to acute hypoxia (Borowiec and Scott, in prep., see Chapter 4 in this volume). While it is possible that maximal O_2 transport capacity is similar between fish exposed to acute hypoxia and acclimated to intermittent hypoxia (i.e. there is no further improvement in O_2 transport capacity by acclimation to intermittent hypoxia), the MO_2 profiles during a hypoxia-reoxygenation cycle suggest this may not be the case (Borowiec et al., 2018, see Chapter 3 of this volume), as fish acclimated to acute hypoxia are unable to maintain resting MO_2 during the hypoxia bout, and do not show the same magnitude of increase in MO_2 during recovery. Further characterization of traits related to O_2 transport capacity in the heart, spleen, and gills will investigate this possibility (Borowiec and Scott, in prep., see Chapter 4 in this volume).

Alternatively, the reduction in MO_2 during acute hypoxia may be facultative, and not limited by O_2 transport capacity, for at least part of the 12 h hypoxia bout. The P_{crit} of normoxia-acclimated *F. heteroclitus* is well above the 2 kPa hypoxia used during the exposure (Borowiec et al., 2015, see Chapter 2 of this volume), and this may explain the initial reduction in MO_2 of these animals (Borowiec et al., 2018, see Chapter 3 of this volume). However, increases in haematocrit and other respiratory responses to acute

hypoxia that occur over the 6 h of hypoxia prior to the measurement of $MO_{2,max}$ may allow for partial acclimation to hypoxia and recovery of aerobic scope. The rate of O_2 induction during a standard P_{crit} trial can influence the measured P_{crit} in goldfish, such that slower rates of O_2 induction (e.g. more time in hypoxia) lead to lower P_{crit} due to alterations in the O_2 transport cascade such as increased gill surface area and haemoglobin–O_2 binding affinity that occur during the trial (Regan and Richards, 2017). A similar response in killifish exposed to acute hypoxia would explain how these animals maintain a substantial aerobic scope following exhaustive chase despite being exposed to sub-P_{crit} PO_2 for 6 h. Facultative metabolic suppression during hypoxia may help extend fuel stores and slow the development of acidosis, both of which could result from the extensive use of anaerobic metabolism observed in the acute hypoxia group (Borowiec et al., 2018, see Chapter 3 of this volume). How facultative metabolic suppression could be activated and maintained in fish under acute hypoxia conditions is unclear, and would be an interesting area for future work.

Finally, $MO_{2,max}$ induced by exhaustive exercise may not have elicited the full capacity of the O_2 transport system, leading to underestimation of $MO_{2,max}$ and obscuring potential differences between the acclimation groups. Killifish trunk muscle is predominantly composed of glycolytic fibre types (Borowiec et al., 2015, see Chapter 2 of this volume), as in other fish (Greek-Walker and Pull, 1975), and the low mitochondrial abundance of these fibre types may limit the rate at which O_2 can be consumed and ATP supplied to fuel recovery in this tissue. It is therefore possible that an alternative protocol for

determining $MO_{2,max}$ that causes more global alterations in tissue metabolism (e.g. a critical swimming speed trial, or an acute temperature increase) may provide a better index of maximum O_2 transport capacity, and better demonstrate any variation in O_2 transport capacity between groups. Critical swimming speed protocol produces higher $MO_{2,max}$ than a chase protocol in a relatively aerobic coral reef fish (Roche et al., 2013), whereas chase protocols produce higher or similar $MO_{2,max}$ compared to a critical swimming speed test in relatively sluggish Atlantic cod (Reidy et al., 1995; Sylvestre et al., 2007). I suspect that our chase and air exposure protocol was sufficient to induce $MO_{2,max}$ in killifish for two reasons. Firstly, our $MO_{2,max}$ measurements in normoxia-acclimated killifish are comparable to previously published values in the same subspecies (albeit at a different salinity) (Healy and Schulte, 2012). Secondly, like Atlantic cod, most killifish muscle is anaerobic (Borowiec et al., 2015, see Chapter 2 of this volume), and MO2,max from a critical swimming speed test would likely be similar or lower than a chase protocol, especially since killifish are difficult to motivate to swim long distances (Borowiec, unpublished observations).

Beyond haemoglobin concentration and affinity, there are several other mechanisms by which *F. heteroclitus* acclimated to intermittent hypoxia can improve O_2 transport capacity such as expression of alternative haemoglobin isoforms (Pan et al., 2017; Rutjes et al., 2007; van den Thillart et al., 2018), increases in lamellar surface area (e.g. loss of interlamellar cell mass) (Dhillon et al., 2013; Fu et al., 2011; Matey et al., 2008; Nilsson et al., 2012; Søllid et al., 2003), alterations in gill perfusion or ventilation, or other

modifications to processes dictating O_2 transport and utilization, and characterizing some of these traits is a current line of study (Borowiec and Scott., in prep., see Chapter 4 in this volume).

7.2.3 No heavy recruitment of anaerobic metabolism

The ability of fish acclimated to 28 d of intermittent 2 kPa hypoxia to maintain resting MO_2 throughout the hypoxia-reoxygenation cycle may have mitigated the need to recruit anaerobic metabolism to supplement tissue ATP supplies during hypoxia (Richards, 2009), and thus allowed killifish to avoid the development of acidosis and maintain low levels of lactate in their tissues during hypoxia bouts (Borowiec et al., 2018, see Chapter 3 of this volume). Despite the presumably low need to use anaerobic metabolism compared to fish exposed to acute hypoxia, fish acclimated to intermittent hypoxia also had features that could be associated with enhanced recovery from anaerobic metabolism, including increased capillarity of the glycolytic (but not oxidative) muscle, which could facilitate both better tissue perfusion during hypoxia and better lactate clearance during the daily normoxic periods between hypoxia bouts (Borowiec et al., 2015, see Chapter 2 of this volume), and increased the activities of several enzymes in the liver involved in gluconeogenesis and lactate oxidation (Borowiec et al., 2015, see Chapter 2 of this volume), which could foreseeably support the capacity to use and recover from anaerobic metabolism. Whether fish acclimated for 28 d to 2 kPa intermittent hypoxia show similar changes in liver enzyme activity (which was only investigated followed 7 d of

intermittent 2 kPa hypoxia) and muscle histology (observed following 28 d of intermittent 5 kPa hypoxia), or if these alterations represent an intermediate strategy for coping with short-term cycles of intermittent hypoxia, is unclear and future work on how the responses to constant hypoxia and intermittent hypoxia vary and develop over the course of the 28 d acclimation period would be valuable in better characterizing the acclimation response to intermittent hypoxia.

While we did not investigate how the activity of metabolic enzymes or muscle histology was altered by 28 d acclimation to 2 kPa intermittent hypoxia, maintaining an enhanced capacity to recover from anaerobic metabolism may still be advantageous in these animals, even though if it is underutilized during routine bouts of nocturnal hypoxia (see below for discussion of the caveats in how we estimated the use of anaerobic metabolism during hypoxia). For example, maintenance of a high capacity to use and recover from anaerobic metabolism despite minimal lactate accumulation in several tissues (which is suggestive of relatively low use of anaerobic glycolysis) during routine hypoxia bouts may act as a safeguard against further or more severe bouts of hypoxia in the naturally variable estuarine environment (Tyler et al., 2009) and/or maintain the capacity for bursts of high activity fueled by anaerobic metabolism such as fast-start burst swimming (Domenici and Blake, 1997; Johnston et al., 1977; Wakeling, 2001), and thus allow killifish to rapidly respond to further environmental and/or physiological challenges. This flexibility may be especially important during periods when aerobic scope is limited in fish acclimated to intermittent hypoxia, such as during nightly hypoxia (Borowiec and

Scott, in prep., see Chapter 4 in this volume) and potentially during the early recovery phase of reoxygenation (Borowiec et al., 2018, see Chapter 3 of this volume).

An important caveat is that we did not measure anaerobic metabolic rate directly (i.e. through calorimetry) and instead used to appearance of classic markers of anaerobic metabolism (phosphocreatine, lactate, reduced pH_i) as an indication of the use of anaerobic metabolism. It is therefore possible that fish acclimated to intermittent hypoxia did indeed have a high rate of anaerobic metabolism during hypoxia bouts, but that this increased rate was match by an enhanced capacity to recover from anaerobic metabolism (e.g. elevated capacities to defend acid-base status, or for lactate oxidation and/or gluconeogenesis as we observed in Borowiec et al., 2015), resulting in no net change in markers of anaerobic metabolism.

7.2.4 Decoupling of ROS signalling from oxidative damage

Acclimation to intermittent hypoxia or constant hypoxia substantially alters ROS homeostasis and the cellular redox environment without significantly increasing oxidative damage, and this appears to depend largely upon maintenance or improvement in antioxidant capacity (Borowiec and Scott., in prep., see Chapter 5 of this volume). Our previous work suggested that this elevated ROS signal is not due to increased ROS emission from the mitochondria, as killifish acclimated to either pattern of 5 kPa hypoxia have lower mitochondria ROS emission rates than normoxia-acclimated animals (Du et

al., 2016), though whether this also holds true for fish acclimated to 2 kPa hypoxia is unclear. Potential sites of extramitochondrial ROS production vary across tissues, but include various oxidases, oxygenases, free metal ions, and several other sources (Barbieri and Sestili, 2012; Nathan and Cunningham-Bussel, 2013). Better characterization of the source and type of reactive species released is crucial to understanding the potential signalling implications of acclimation to hypoxia. For example, our ROS assay was a relatively non-specific measure of total oxyradical signal within crude tissue homogenates, and is known to cross react with reactive nitrogen species or reactive sulfur species (Chen et al., 2010; Jakubowski and Bartosz, 2000). Reactive nitrogen species have a variety of signalling targets (Adams et al., 2015), and some species show increases in blood nitrite (which can be converted to reactive nitrogen species *via* nitric oxide) during O_2 limited conditions (Gladwin et al., 2006; Hansen and Jensen, 2010; Sandvik et al., 2012; van Faassen et al., 2009). Nitric oxide, and perhaps reactive nitrogen species, may facilitate some of the adaptive cardiovascular (Jacobsen et al., 2012) or mitochondrial (Misfeldt et al., 2009; Moncada and Erusalimsky, 2002; Umbrello, et al. 2013) responses associated with hypoxia or anoxia tolerance (Jacobsen et al., 2012; Umbrello et al., 2013), and could foreseeably be differentially regulated by acclimation to constant hypoxia or intermittent hypoxia. For example, nitric oxide has been implicated in energy supply-demand matching through reductions in MO_2, increased expression of enzymes involved in glycolysis, and increased glycolytic ATP production (through inhibition of PDH) (Umbrello et al., 2013). NO signalling during hypoxia could therefore

be an important component of the acclimation response to both intermittent hypoxia and constant hypoxia in killifish.

Rather than representing a sign of oxidative stress, the adjustments in reactive species homeostasis and oxidative status after hypoxia acclimation may represent beneficial adjustments that killifish use to cope with chronic hypoxia. ROS are well-known signalling molecules that interact with several oxygen and redox-sensitive intracellular pathways and transcription factors, including HIF-1α and NF-κB (Costantini, 2019; D'Autréaux and Toledano, 2007; Prabhakar et al., 2007; Schieber and Chandel, 2014; Wolin et al., 1999) and may be directly regulated by the mitochondria (Munro and Treberg, 2017; Treberg et al., 2019). The implications of these interactions in modulating the hypoxia coping response in fish is not-well understood, though HIF-1α is known to modulate cellular and systems-level responses to hypoxia such as angiogenesis, energy metabolism, and the hypoxic chemoreflex (Iyer et al., 1998; Nikinmaa et al., 2004; Nikinmaa and Rees, 2005; Robertson et al., 2014; Semenza, 2000; Semenza, 2006; Semenza and Prabhakar, 2007; Wang et al., 1995), and altered HIF-1α signalling is implicated in some of the unique effects of intermittent hypoxia on mammals (Peng et al., 2006; Semenza and Prabhakar, 2007). However, the similar levels of ROS and HIF-1α protein abundance we observed in fish acclimated to intermittent hypoxia and constant hypoxia suggest that while this pathway may be important for responding to hypoxia in general in fish, it does not seem to contribute to the unique aspects of intermittent hypoxia in fishes (Borowiec et al., 2018, see Chapter 3 of this volume; Du et al., 2016; Borowiec

and Scott, in prep., see Chapter 5 of this volume). An interesting future direction in this area would be to investigate how the abundance or sensitivity of other known ROS and/or redox sensors (e.g. thiol-proteins, proteins reliant on NAD^+/NADH) are altered by acclimation to different patterns of hypoxia, which could allow differential responses despite similar oxyradical signalling within a cell.

7.3 Other components of the intermittent hypoxia coping strategy

This investigation focused on how killifish coped with two distinct physiological challenges associated with hypoxia and/or hypoxia reoxygenation cycles – the development of an ATP supply-demand imbalance (Borowiec et al., 2015, see Chapter 2 of this volume; Borowiec et al., 2018, see Chapter 3 of this volume; Borowiec and Scott, in prep., see Chapter 4 in this volume) and oxidative stress (Borowiec and Scott, in prep., see Chapter 5 of this volume). There are likely other physiological challenges, such as maintenance of acid-base balance, associated with either the direct effects of hypoxia and hypoxia-reoxygenation cycles and/or the consequences of the adjustments made to survive these stressors. For example, we did not investigate if acid-base regulation is altered by acclimation to different patterns of hypoxia, and whether fish acclimated to intermittent hypoxia maintain a high capacity to recover from acidosis that is comparable to their capacity to recover from the use of anaerobic metabolism. While we did observe that fish acclimated to intermittent hypoxia avoid the development of acidosis in their tissues during hypoxia exposure (Borowiec at al., 2018, see Chapter 3 in this volume),

whether this is due to enhanced acid-base buffering capacity or simply a reduced recruitment and use of anaerobic metabolism is unclear. Understanding these "secondary" coping responses to intermittent hypoxia promises to be an interesting future direction for work in this area and would provide a better understanding of the complexities of the acclimation response to intermittent hypoxia in fishes.

7.4 Do other fishes have multiple hypoxia coping strategies?

7.4.1 Evolution and plasticity of hypoxia tolerance

The magnitude of phenotypic flexibility can vary between individuals due to genetic, developmental, or acclimation effects. Phenotypic flexibility can also vary between species and even evolve (Fordyce, 2006; Ghalambor et al., 2007; Storz et al., 2010), and this may include the capacity for different coping strategies for different patterns of hypoxia. The extent to which acclimation to intermittent hypoxia or constant hypoxia modified hypoxia tolerance varied across Fundulidae killifishes (Borowiec et al., in review, see Chapter 6 in this volume). One explanation for these differences in the magnitude of the acclimation response may be differences in the signal for plasticity across taxa. For example, the relatively intolerant *F. rathbuni* (Borowiec et al., in review, see Chapter 6 in this volume) may experience a greater decrease in tissue PO_2 during acclimation to a common PO_2 (e.g. due to an inability for O_2 transport capacity to meet O_2 demands), and this may have induced a more dramatic hypoxia acclimation response

for traits underlying hypoxia tolerance. Another potential explanation is that species differ in their inherent capacity for plasticity due to interspecific variation in the activation and/or capacity of different coping mechanisms during exposure to chronic hypoxia.

7.4.2 Interspecific variation in the response to intermittent hypoxia

An intriguing possibility is that these differences in the plasticity of hypoxia tolerance may also reflect variation in the underlying physiological traits involved in hypoxia acclimation response. For example, the widely-distributed estuarine *F. heteroclitus* (sourced from NH, USA) responds to acclimation to constant hypoxia with strong metabolic depression (Borowiec et al., 2018, see Chapter 3 of this volume), whereas *Lucania parva* (sourced from FL, USA), which is distributed only in southern estuaries, does not (Borowiec et al., in review, see Chapter 6 in this volume). MO_2 depression is an effective hypoxia coping strategy (Bickler and Buck, 2007; Boutilier, 2001; Hochachka et al., 1996; Hochachka and Dunn, 1983), but often comes at the cost of impairment of other processes like reproduction, locomotion, and cognition (Johansson et al., 1997; Nilsson et al., 1993). Variation in the capacity for MO_2 depression between *F. heteroclitus* relative to *L. parva* may reflect differences in the context of hypoxia experienced in these species (e.g. ice-cover, seasonal variation in temperature, etc.) and the subsequent fitness trade-off between metabolic depression and maintenance of these processes (i.e. metabolic depression may be a less viable strategy in the warm waters expected in southern estuaries). Understanding how different patterns of hypoxia interact with other stressors

within an environment, and how those interactions may modulate the acclimation responses to those stressors would be a useful area of future study and would provide a broader ecological understanding of the selective pressures that shape the two hypoxia coping strategies.

Along the same lines, another interesting area of future study would be to investigate other animals from highly variable environments that may be adapted specifically to intermittent patterns of hypoxia (compared to hypoxia in general), such as intertidal sculpins and plainfin midshipman (Bose et al., 2019; Craig et al., 2014; Richards, 2011). Moreover, it would be interesting to investigate if these putative "intermittent hypoxia specialists" also alter their hypoxia coping strategy depending on the pattern of hypoxia exposure, and if these strategies are consistent with the strategy observed in *F. heteroclitus*. Preliminary work on plainfin midshipman suggests that the mechanisms of acclimation to intermittent hypoxia may vary between species, as these animals are commonly described as sluggish during low tide and aquatic hypoxia (Arora, 1948; Le Moine et al., 2014), perhaps as a result of metabolic depression (Bose et al., 2019; Houpt et al., in review).

7.5 Intermittent hypoxia vs. constant hypoxia in fishes as an experimental approach

The main objective of this investigation was to understand the mechanisms of acclimation to intermittent hypoxia in an estuarine fish, and how the acclimation response to intermittent hypoxia differed from the response to constant hypoxia. While accomplishing these objectives, we also used our model system to investigate broader questions about how oxidative stress interacts with hypoxia and/or hypoxia-reoxygenation in two representative tissues (Borowiec and Scott, in prep., see Chapter 5 of this volume) and the repeatability and reliability of different indices of hypoxia tolerance in fishes (Borowiec et al., in review, see Chapter 6 of this volume). Therefore, in addition to contributing substantial insight into an ecologically important but understudied component of natural encounters with hypoxia (pattern), we also made useful and timely contributions (Costantini, 2019; Regan et al., 2019; Wood, 2018) to hotly debated areas of comparative physiology, including the interactions between hypoxia and oxidative stress, and the value of and relationships between different indices of hypoxia tolerance.

Fundulus killifish acclimated intermittent and constant patterns of environmental stress are a tractable model organism for understanding the physiological effects of a variety of ecologically relevant questions in the Anthropocene (Burnett et al., 2007), such as the impacts of pollution exposure, and how animals manage to respond to multiple simultaneous environmental stressors.

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The End.