

**THE HYPOXIC METABOLIC RESPONSE: HOW TIME AND PO_2 SHAPE THE
WAY FISHES COMBINE AEROBIC, ANAEROBIC AND DEPRESSED
METABOLISM IN HYPOXIC ENVIRONMENTS**

by

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ABSTRACT

Animals rely on O₂ to balance cellular ATP supply and demand. In O₂-limited hypoxic environments, survival depends on the maintenance of this balance and is accomplished through some combination of aerobic metabolism, anaerobic metabolism and metabolic rate depression (MRD). My thesis studied how fishes combine these three metabolic strategies as a total hypoxic metabolic response (HMR) to survive hypoxic environments that vary in O₂ level (P_wO_2) and duration.

Calorimetry is required to accurately measure the metabolic rates (MR) of hypoxia (or anoxia)-exposed fishes that are partially reliant on anaerobic glycolysis and/or MRD. Thus, I started by building a novel calorimeter that simultaneously measures indices of aerobic metabolism, anaerobic metabolism and MRD, and used it for the remainder of my thesis projects.

Using goldfish, I found that time influences how P_wO_2 affects HMR. Under acute and continually decreasing P_wO_2 conditions, goldfish maintained routine O₂ uptake rates ($\dot{M}O_2$) to ~3.0 kPa P_wO_2 (*i.e.*, P_{crit}), but sustained routine MR to 0.5 kPa by up-regulating anaerobic glycolysis. Under constant hypoxia (1 or 4 h) at a variety of P_wO_2 s, however, goldfish maintained routine $\dot{M}O_2$ to ~0.7 kPa and consequently reduced their reliance on anaerobic glycolysis. I confirmed this rapidly enhanced O₂ uptake ability in subsequent experiments by using different rates of hypoxia induction (RHI) to vary the amount of time goldfish spent at hypoxic P_wO_2 s. Gradual RHIs yielded greater lamellar surface areas, haemoglobin-O₂ binding affinities, and subsequently, lower P_{crit} s than rapid RHIs. However, goldfish only induced MRD below 0.7 kPa.

To test the idea that MRD is reserved for extreme hypoxia, I compared two threespine stickleback populations from two isolated lakes: one that experiences deep, long-term hypoxia due to winterfreeze (Alta Lake), and the other that does not (Trout Lake). The two populations did not differ in P_{crit} or capacities for anaerobic metabolism, but Alta Lake

sticklebacks, which were 2-fold more hypoxia-tolerant than Trout Lake sticklebacks, employed hypoxia-induced MRD while Trout Lake sticklebacks did not.

My results reveal that the HMR varies with an animal's biology and the abiotic aspects of its natural hypoxic environment in a way that may optimize hypoxic survival.

LAY SUMMARY

Animals rely on O₂ to maintain the balance of energy supply and demand. O₂-limited hypoxic environments threaten this balance, and so a hypoxia-exposed animal's survival depends on its ability to use aerobic metabolism, anaerobic metabolism and/or metabolic depression to maintain this balance. My thesis investigated how fishes combine these three metabolic strategies in a total hypoxic metabolic response (HMR), a crucial yet unknown dimension of hypoxic survival. Using goldfish and threespine stickleback as study species, I found that the HMR is plastic, influenced by the biology of the animal and the abiotic aspects of its hypoxic environment. Furthermore, the HMRs of goldfish and different stickleback populations appear finely tuned to the animals' respective natural hypoxic environments, which vary from one another considerably. A species' HMR may therefore be predictable based on its particular natural hypoxic environment. This knowledge may benefit conservation efforts as the world's aquatic environments become increasingly hypoxic.

PREFACE

A version of Chapter 2 has been published as: Regan, M.D., Gosline, J.M. & Richards, J.G. (2013). A simple and affordable calorimeter for measuring the metabolic rates of fishes. *Journal of Experimental Biology* 216: 4507-4513. I co-designed the calorimeter with the help of Drs. John M. Gosline and Jeffrey G. Richards. I designed and carried out all experiments and analyzed their data. I wrote the manuscript with editorial input from Drs. Gosline and Richards.

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All experiments in this thesis were approved by UBC's Animal Care Committee (Protocol A13-0309).

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*For Emmanuelle,
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Chapter 1

General introduction

The maintenance of stable cellular conditions and proper cellular function requires a high turnover of energy in the form of ATP. This ATP can be supplied aerobically *via* oxidative phosphorylation or anaerobically *via* substrate-level phosphorylation (*e.g.*, anaerobic glycolysis). Aerobic pathways yield up to 18-fold more ATP per unit of substrate than anaerobic pathways, and perhaps because of this, almost all animals on earth have evolved an ultimate reliance on O₂ to supply their cells with sufficient quantities of ATP to match cellular ATP demand. But many environments on earth are low in O₂ (*i.e.*, hypoxic), and despite the critical importance of O₂ in supplying cellular ATP, a range of anatomically and physiologically specialized animals can be found living in many of them (Bickler and Buck, 2007; Ramirez et al., 2007). These environments include the subterranean, the high-altitude and the aquatic, and the animals living here risk their abilities to supply ATP aerobically, and consequently, risk upsetting the balance of energy supply and demand.

The fact that these animals are living in these environments suggests they routinely maintain their cellular ATP balance (Boutilier, 2001). There are three general mechanisms used to do this at low levels of environmental O₂: sustained aerobic metabolism, activation of anaerobic metabolism and metabolic rate depression (MRD). Some combination of these mechanisms allows an animal to maintain energy balance and survive a hypoxic exposure. Much work has been put towards understanding each of these mechanisms in isolation, but very little work has focused on how they are used simultaneously and in combination in what I will refer to as a hypoxic metabolic response (HMR). My thesis will investigate the HMR of fishes, and how it varies with exposure to different hypoxic environments. But before delving into the HMR and the mechanisms it comprises, it would be prudent to first clarify what is meant by ‘hypoxia’.

1.1 Environmental hypoxia

Environmental hypoxia is most simply defined as a partial pressure of O₂ (PO_2 , P_wO_2 for water) lower than full air saturation (*i.e.*, less than 157 mmHg or 21 kPa at sea level and 20°C, less any vapour pressure for terrestrial animals). Such environments are naturally prevalent in aquatic systems owing to the density stratification of the water column, O₂ consumption on the part of decomposing organic matter, ice cover, algal blooms, tidal cycles, and water body isolation (Diaz and Breitburg, 2009). Furthermore, anthropogenic practices resulting in rising global temperatures and increased eutrophication events have increased the prevalence, severity and duration of these hypoxic events (Boesch, 2002; Diaz and Breitburg, 2009; Diaz and Rosenberg, 1995; Smith et al., 2006). From a general perspective, defining hypoxia in this absolute sense is useful when comparing the responses and/or tolerances of different species to a particular PO_2 . From an individual's perspective, however, defining hypoxia involves determining the PO_2 at which physiological function is first compromised (Farrell and Richards, 2009), and this requires accounting for the various hypoxic responses that contribute to the individual's HMR.

Hypoxic environments vary in their hypoxic severities (PO_2), hypoxic durations, and rates of hypoxic induction (RHIs). Among aquatic systems, there is high variation in hypoxic environments. For example, oceanic O₂ minimum zones are stable hypoxic environments that are characterized by chronic and severe P_wO_2 s (≤ 4.2 kPa; Seibel, 2011). At the other end of the spectrum, tidepools located high in the intertidal zone are in a constant state of flux, oscillating daily between ~ 80 kPa and anoxia, and rarely holding stable at any P_wO_2 (Richards, 2011). And between these on the spectrum of hypoxic environments are winterfreeze lakes, which gradually descend towards anoxia over ~ 2 months and then hold there until spring thaw because O₂ cannot move into the water until the ice melts (Vornanen, 2004). With such variation in PO_2 , RHI and duration, there is probably no such thing as a 'grand unified' strategy of hypoxia tolerance. Rather, the hypoxia defense responses (including HMR) of a species are probably shaped by the particular hypoxic environment to which the species is adapted; because these environments differ, so too will the hypoxic survival strategies of the species that inhabit them. A broad survey of the literature reveals that the hypoxia defense strategies employed by fishes do indeed vary, and this may be the

result of adaptation to different types of hypoxic environments. But the inconsistency of the hypoxic exposure protocols across studies, and the fact that the majority of hypoxia studies focus on hypoxic severity (fewer on duration, and almost non on RHI) make such broad comparisons difficult.

1.2 The metabolic responses of fishes to hypoxia

1.2.1 Aerobic metabolism

Aerobic metabolism centers on oxidative phosphorylation, the O₂-dependent process by which ATP is produced in the mitochondria. Because fishes rely predominantly on aerobic metabolism under normoxic, steady state conditions, O₂ uptake rate from the environment ($\dot{M}O_2$) is widely used as a proxy for metabolic rate (Nelson, 2016). There are different levels of metabolic rate that can be supported aerobically (see Fig. 1-1). Standard metabolic rate (SMR) is the $\dot{M}O_2$ of an awake, post-absorptive, and entirely inactive ectothermic animal in its thermoneutral zone (Chabot et al., 2016). It is equivalent to the basal metabolic rates of endothermic animals, and essentially represents the minimal cost of living at a particular temperature. At the other end of the spectrum, maximum metabolic rate (MMR) is the highest $\dot{M}O_2$ an animal can attain in a given environment (Norin and Clark, 2016). The difference between SMR and MMR represents an animal's aerobic scope, an $\dot{M}O_2$ range that supports higher level functions such as growth, digestion, locomotion and reproduction (Claireaux and Chabot, 2016). Within the aerobic scope lies routine metabolic rate (RMR). Similar to SMR, RMR is the $\dot{M}O_2$ of an awake, post-absorptive, and entirely inactive ectothermic animal in its thermoneutral zone, but RMR also accounts for small movements that are typical of fish under experimental conditions (Chabot et al., 2016). The costs of these movements are typically low, so RMR tends to be far closer to SMR than to MMR (Chabot et al., 2016; Fig. 1-1).

Maintaining aerobic metabolism at any rate requires transporting O₂ from its origin in the environment to its terminus at the mitochondria. In water-breathing fishes, this process occurs *via* a five-step cascade: 1, breathing, which brings O₂ into contact with the respiratory (gill) surface; 2, branchial diffusion across the water-blood barrier; 3, circulation throughout the body in the bloodstream; 4, tissue diffusion across the blood-mitochondria interface; and 5, use in the mitochondria as an electron acceptor, ultimately producing ATP (Weibel, 1984).

Optimizing this cascade to more efficiently move O₂ from the environment to the mitochondria can aid hypoxic survival, and hypoxia-adapted animals have evolved traits at each step to do so.

Most animals possess mechanisms that enhance O₂ extraction and delivery to tissues as environmental P_{O_2} is reduced. For fishes, these mechanisms include increases to gill surface area (Sollid et al., 2003), haemoglobin (Hb) synthesis (Gracey et al., 2001) and concentration in the blood (Affonso et al., 2002), hematocrit (Lai et al., 2006; Turko et al., 2014), Hb-O₂ binding affinity (Turko et al., 2014), and ventilation frequency and amplitude (Holeton and Randall, 1967; Itazawa and Takeda, 1978; Tzaneva et al., 2011; Vulesevic and Perry, 2006), as well as a redistribution of blood supply to critical tissues (Sundin et al., 1995). Together, these mechanisms allow fishes to maintain stable $\dot{M}O_2$ s across a range of P_wO_2 s (called oxyregulation). Should environmental O₂ levels continue to decrease, however, a P_wO_2 will be reached at which the fish's compensatory mechanisms of O₂ uptake can no longer maintain stable $\dot{M}O_2$. This P_wO_2 is called the critical O₂ level (P_{crit}), and at P_wO_2 s below this, the fish's $\dot{M}O_2$ becomes dependent on the environmental O₂ level (called oxyconformation; Claireaux and Chabot, 2016). In the context of the aerobic hierarchy shown in Fig. 1-1, a fish has three corresponding P_{crit} values: $P_{crit-Std}$, $P_{crit-Max}$ and $P_{crit-Rtn}$, the lowest P_wO_2 s at which $\dot{M}O_2$ can support SMR, MMR and RMR, respectively (Fig. 1-1). Investigators typically use $P_{crit-Std}$ (Claireaux and Chabot, 2016) or $P_{crit-Rtn}$ (McBryan et al., 2013) when discussing P_{crit} in the context of hypoxia tolerance. Unless otherwise indicated, I will use $P_{crit-Rtn}$ (referred to simply as ' P_{crit} ') throughout this thesis owing to the difficulty in achieving true SMR under experimental conditions (but see Chabot et al. 2016).

At P_{crit} , the fish's aerobic scope is either at or near zero (for SMR- and RMR-based estimates, respectively), and at P_wO_2 s below P_{crit} , the fish's ability to supply ATP aerobically is limited (Farrell and Richards, 2009). P_{crit} therefore reflects a fish's ability to acquire and use environmental O₂ at stable rates as a function of P_wO_2 , with a lower P_{crit} value indicating a greater ability to do so in hypoxic environments. Among species, P_{crit} values tend to be lower in hypoxia-tolerant species than in non-tolerant species (Mandic et al., 2009b; Nilsson and Östlund-Nilsson, 2008; Speers-Roesch et al., 2012a), and this is often the result of variation in traits related to O₂ extraction (Mandic et al., 2009b; Nikinmaa, 2001; Perry and Reid, 1992; Petersen and Gamperl, 2011; Sollid et al., 2003; Takeda, 1990). Even within an

individual, P_{crit} can be reduced as a result of hypoxic acclimation (Borowiec et al., 2015; Sollid et al., 2003). This relationship of P_{crit} and hypoxia tolerance has led some to use P_{crit} as a metric of hypoxia tolerance *per se*, but the completeness of this connection is (rightly) debated because P_{crit} does not incorporate the contributions of anaerobic metabolism and MRD to hypoxia tolerance (Claireaux and Chabot, 2016; Rogers et al., 2016; Speers-Roesch et al., 2013; Urbina and Glover, 2013).

In any case, a low P_{crit} is beneficial because it allows the animal to maintain RMR (or SMR) in more hypoxic environments while avoiding a reliance on anaerobic glycolysis and/or MRD. But for any fish, should environmental P_wO_2 decrease below its P_{crit} , $\dot{M}O_2$ will decrease, and with it, the fish's ability to produce ATP aerobically. The balance of cellular ATP supply and demand therefore becomes more reliant on anaerobic glycolysis and/or MRD, and solely reliant on these should P_wO_2 fall to 0 kPa.

1.2.2 Anaerobic metabolism

Fishes have two primary methods of generating ATP anaerobically: substrate-level phosphorylation *via* creatine phosphate (CrP) and anaerobic glycolysis (hereafter referred to as glycolysis). Substrate-level phosphorylation *via* CrP occurs in the cytosol of certain tissues (*e.g.*, muscle) and involves the rapid, direct transfer of phosphate from CrP to ADP. Because the cell's CrP reserves are small, CrP can be quickly depleted, forcing it to rely on glycolysis for the anaerobic supply of ATP should aerobic process remain constrained (Wang and Richards, 2011).

Glycolysis, defined as the splitting of sugars, occurs in the cytosol. The carbohydrate source for anaerobic glycolysis is glycogen, an endogenously stored branched polysaccharide from which glucose monomers are cleaved by glycogen phosphorylase for entry into the glycolysis pathway. Because hypoxia exposure suppresses the appetite and digestive functions of fishes (Wang et al., 2009), species with larger glycogen stores (*e.g.*, goldfish, carp, tilapia) can fuel glycolysis for longer time periods than species with smaller glycogen stores (*e.g.*, rainbow trout), and this prolongs their hypoxic/anoxic survival time (Richards, 2009).

When O_2 is present in the mitochondria, the two products of glycolysis are transported into the mitochondria, where pyruvate is used as the starting compound for the

TCA cycle and a reducing equivalent (from NADH) is used for the electron transport chain. The resulting NAD^+ remains in the cytosol where it is again available for further glycolytic activity. When O_2 levels in the mitochondria are low, however, the electron transport system becomes unable (or less able) to accept reducing equivalents and NADH accumulates in the cytosol, reducing NAD^+ and potentially halting glycolysis altogether. To avoid this, NADH is recycled back into NAD^+ in a lactate dehydrogenase-catalyzed reaction that simultaneously converts pyruvate into lactate. When lactate formation rate exceeds its removal rate (as during hypoxia), it accumulates in the tissue. The level of lactate in a tissue is therefore indicative of its O_2 -independent glycolytic activity and may be used to estimate its reliance on anaerobic ATP production.

The formation of lactate results in a parallel and equimolar production of protons from ATP hydrolysis (Hochachka and Somero, 2002). These protons may negatively impact the health and, consequently, hypoxia tolerance of the fish (Driedzic and Gesser, 1994; Nilsson and Östlund-Nilsson, 2008). Most species rely on traditional modes of acid-base regulation (*e.g.*, intrinsic buffering) to cope with this acidosis, but a few especially hypoxia/anoxia tolerant fish species (crucian carp, goldfish, bitterling, and possibly lanternfishes and desert pupfishes) have evolved the ability to produce ethanol, not lactate and protons, as the ultimate end-product of glycolysis (Heuton et al., 2015; Nilsson, 1988; Shoubridge and Hochachka, 1980; Torres et al., 2012; Wissing and Zebe, 1988). This ability reduces the risk of a metabolic acidosis when these species are exposed to long bouts of hypoxia, and goes a long way to explaining their exceptional hypoxia tolerances (Vornanen et al., 2009).

Glycolysis is beneficial in O_2 -limited environments because it allows for an O_2 -independent supply of ATP. But the relative inefficiency of glycolysis and fact that glycogen, the required fuel for glycolysis, is of finite supply will invariably lead to a problem: should the hypoxic exposure last too long, even the large glycogen stores of a species like goldfish risk being exhausted. To compensate, energetic demands need to decrease.

1.2.3 Metabolic rate depression

An effective way to extend the lifetime of a finite fuel supply is to reduce the rate at which it is used. For an animal to survive a particularly long and/or severe bout of hypoxia,

this means reducing its ATP consumption rate so as to extend the time period over which its finite glycogen stores can be used. This is accomplished through MRD, which is defined as a reduction in metabolic rate below standard metabolic rate (SMR; Hochachka and Somero, 2002; Richards, 2009). MRD is widely used among species spanning the phyla (Guppy and Withers, 1999), but this discussion will focus on how ectothermic vertebrates use hypoxia-induced MRD.

MRD is made manifest through adjustments at the behavioural, physiological, and biochemical levels. Behaviourally, metabolic rate can be reduced through reductions in locomotor activity, feeding, mating and courtship behaviour, and reductions in these behaviours are typical of hypoxia-exposed fishes (Brett and Groves, 1979; McKenzie et al., 1995; Nilsson et al., 1993; Pedersen, 1987; Schurmann and Steffensen, 1994; Wang et al., 2009; Wu, 2009). Physiologically, metabolic rate can be reduced through reductions in growth, digestion, specific dynamic action, gonad development and gametogenesis, and ventilatory effort once P_wO_2 has dropped below P_{crit} (Fitzgibbon et al., 2007; Wang et al., 2009). And biochemically, metabolic rate can be further depressed below SMR through reductions in the major cellular ATP-consuming pathways, including gluconeogenesis, protein turnover and the maintenance of membrane ion gradients. These processes respectively account for 17%, 29% and 53% of the total cellular energy demand of isolated turtle hepatocytes under normoxic conditions (Hochachka et al., 1996). However, when these hepatocytes are exposed to anoxia, these demands are reduced by 100%, 75% and 93%, respectively, resulting in a 90% reduction in total cellular energy demand (Hochachka et al., 1996). The contributions these processes make to total cellular energy demand, and the degree to which they are down-regulated, likely vary across tissues and species (see Bickler and Buck, 2007; Hylland et al., 1997; Vornanen et al., 2009). But so long as they are down-regulated, the result will be a depressed metabolic rate and a prolonged survival time in hypoxia/anoxia.

Despite MRD's effectiveness of maintaining ATP balance in hypoxic conditions, not all fish species are capable of employing it. For example, zebrafish (*Danio rerio*), a species native to stagnant, shallow water bodies that regularly become hypoxic (Spence et al., 2008), do not depress metabolic rate when exposed to at least two different sub- P_{crit} P_wO_2 s (Stangl and Wegener, 1996; Regan and Richards, unpublished). The reason they have not evolved (or

alternatively, secondarily lost) an ability to induce MRD is not known, but it may relate to MRD's inherent physiological and ecological costs. For torpid mammals, these include oxidative damage (Carey et al., 2000), reduced growth, repair and immunocompetence (Burton and Reichman, 1999), cognitive impairments stemming from neuronal damage (Popov et al., 1992), ceased reproduction (Humphries et al., 2003), and increased susceptibility to predation by aquatic and aerial predators stemming from significantly reduced motor activity (Humphries et al., 2003). These costs may be more or less relevant to a given species depending on its ecological environment and its ability to mitigate them. For a species like zebrafish that is unable to employ MRD, it may be that the costs of MRD outweigh its benefits, especially if there are other ways to maintain ATP balance in their particular hypoxic environment. But almost no work has been done to quantify the costs and benefits that come with hypoxia-induced MRD in fishes.

1.3 The hypoxic metabolic response: the concurrent use of aerobic metabolism, anaerobic metabolism and metabolic rate depression in hypoxic environments

Surprisingly few studies have measured how hypoxia-exposed fishes concurrently use aerobic metabolism, anaerobic metabolism and MRD as a total HMR. The few studies that have suggest that different species have different HMRs. Common sole (*Solea solea*) appear to induce anaerobic glycolysis at the same P_wO_2 as their P_{crit} for $\dot{M}O_2$ (2.5 kPa), entering a progressively more depressed metabolic state as P_wO_2 is further reduced (Dalla Via et al., 1994; van den Thillart et al., 1994). On the other hand, Amazonian oscars (*Astronotus ocellatus*) only become reliant on anaerobic glycolysis at 1.3 kPa P_wO_2 , despite their P_{crit} of $\dot{M}O_2$ being at 4.2 kPa (Muusze et al., 1998).

Together, these results suggest that the two species have different glycolytic control mechanisms (*i.e.*, a typical Pasteur effect for sole; a reverse Pasteur effect for oscar), but the different hypoxia exposure protocols employed by each study make this difficult to interpret. Specifically, the common sole studies exposed single fish to 12 h at a single hypoxic P_wO_2 , while the oscar study exposed single fish to progressively deepening hypoxia over ~24 h, holding them at each of seven hypoxic P_wO_2 s for ~3 h each. With only a single end-point for the lactate sample, the dynamics of lactate accumulation (*i.e.*, anaerobic reliance) throughout the exposures is unclear. Also, in the case of oscar, the representative trace of $\dot{M}O_2$ makes it

clear that aerobic reliance is affected by time at each hypoxic P_wO_2 . Finally, MRD was not directly measured in these studies, but rather deduced from estimated ATP turnover rates that were calculated from the $\dot{M}O_2$ and lactate data. Real-time measurements of hypoxic metabolic rates, including depressed metabolic rates, require the use of direct calorimetry (discussed in section 1.4).

Few studies have employed calorimetry to measure the metabolic rates of fishes, and of those that have, only two have made some attempt to measure HMR as a function of P_wO_2 (non have done so as a function of RHI or hypoxic duration). Tilapia (*Oreochromis mossambicus*) appear to have a P_{crit} of $\dot{M}O_2$ between 3.2 and 1 kPa and induce MRD (as indicated by metabolic heat) in the same P_wO_2 range (van Ginneken et al., 1997). No indices of anaerobic glycolysis were measured in this study, but estimates based on oxycaloric equivalents suggest the fish become more reliant on glycolysis in this same P_wO_2 range. On the other hand, goldfish (*Carassius auratus*) appear to have a P_{crit} of $\dot{M}O_2$ of ~4.2 kPa while MRD is only induced at ~2.1 kPa (van Ginneken et al., 2004). The authors do not speculate on anaerobic reliance, but it is probably the case that the maintained metabolic heat between 4.2 and 2.1 kPa is owing to an increased reliance on anaerobic glycolysis.

These results suggest that while goldfish and tilapia maintain SMR to similar P_wO_2 s, potentially different HMRs are used; goldfish employ anaerobic glycolysis to maintain SMR once O_2 becomes limiting, while tilapia arrest metabolic rate once O_2 becomes limiting and enter MRD. However, the lack of reliable anaerobic metabolite measurements makes this conclusion speculative, especially with regard to the effect of duration. In fairness, these studies' objectives were to determine the effects of light and P_wO_2 on activity and total metabolic rate (tilapia) and to characterize the relationship of heart rate, metabolic rate and P_wO_2 (goldfish). They are nevertheless informative with respect to HMR, but a better experimental design for the study of HMR would be to simultaneously measure $\dot{M}O_2$, metabolic heat and glycolytic end-products in the same species as a function of P_wO_2 , RHI and hypoxic duration.

1.4 Thesis objectives and hypotheses

The objective of my thesis was to determine how fishes combine their use of aerobic metabolism, anaerobic metabolism and MRD in hypoxic environments, and how this use is

affected by variation in hypoxic severity, RHI and duration. I used goldfish as my model fish species because they are exceptionally tolerant of hypoxia, are capable of MRD (Addink et al., 1991; van Ginneken 2004), and are well studied with many literature values for comparison (Chapters 3, 4). To further explore the effect of hypoxic duration on HMR (MRD in particular), I used two isolated populations of threespine sticklebacks (*Gasterosteus aculeatus*) that are native to lakes with different O_2 regimes (Chapter 5). But because most of this work required the use of calorimetry, my thesis began with the construction of a novel calorespirometer (Chapter 2).

Chapter 2 describes the design and construction of a novel calorespirometer I built in collaboration with Dr. John Gosline. This apparatus was necessary for the accurate measurement of hypoxic metabolic rate (including MRD) because, under severe hypoxia when anaerobic pathways are contributing to ATP production, respirometric measurements using $\dot{M}O_2$ alone will inevitably underestimate metabolic rate. Separating ATP turnover from $\dot{M}O_2$ is therefore necessary when measuring the metabolic rates of animals in hypoxic environments, and the most effective way to do so is by measuring metabolic heat. The catabolic processes— aerobic and anaerobic—that produce cellular ATP are inefficient and generate heat as a byproduct. The same is true for the many energy-consuming processes of the cell. The amount of heat lost by an organism is therefore directly related to its total ATP turnover rate (Kaiyala and Ramsay, 2011; McLean and Tobin, 1987; Mendelsohn et al., 1964), and measuring this heat *via* calorimetry provides the most accurate measurement of the organism's metabolic rate.

In Chapter 3, I explored the effect of hypoxic severity on HMR, hypothesizing that MRD is employed by goldfish at hypoxic P_wO_2 s (not just anoxia) and initiated at P_wO_2 just below P_{crit} for $\dot{M}O_2$, where the negative impacts of reduced aerobic capacity and increased anaerobic reliance begin to accrue. I tested this hypothesis using a combination of closed-chamber and flow-through calorespirometry to measure $\dot{M}O_2$ (*i.e.*, aerobic metabolism) and metabolic heat (*i.e.*, MRD) at different P_wO_2 s, as well as terminal sampling experiments to measure concentrations of accumulated and excreted glycolytic end-products (*i.e.*, anaerobic metabolism) at these same P_wO_2 s.

In Chapter 4, I explored the effect of RHI on HMR, hypothesizing that, compared with rapid RHIs, gradual RHIs will afford an organism more time to alter plastic phenotypes

associated with O_2 uptake and subsequently reduce their P_{crit} of $\dot{M}O_2$. I tested this hypothesis by determining P_{crit} values for goldfish exposed to short, typical and long duration P_{crit} trials to represent different RHIs, and then ran parallel hypoxic exposures of different RHIs to investigate morphological and physiological traits of goldfish that might play causal roles in a RHI-related shift in P_{crit} .

In Chapters 3 and 5, I explored the effect of hypoxic duration using two different approaches. In Chapter 3, I used the methods described above to characterize the HMR of goldfish at a variety of P_wO_2 s over acute, 1 h and 4 h timescales. In Chapter 5, I used the threespine stickleback model system to investigate how HMR varies in two isolated stickleback populations that are native to different lakes with different hypoxic regimes, one that experiences winterfreeze-induced long-term hypoxia (Alta Lake), and another that does not experience long-term hypoxia (Trout Lake). Specifically, I hypothesized that the Alta Lake sticklebacks would employ MRD to achieve a greater hypoxia tolerance than the Trout Lake sticklebacks, and I tested this hypothesis by measuring indices of aerobic metabolism, anaerobic metabolism and MRD as described above, and running time-to-loss of equilibrium experiments to quantify the two populations' hypoxia tolerances.

The general outcome of my thesis is a comprehensive understanding of how goldfish simultaneously use aerobic metabolism, anaerobic metabolism and MRD to survive hypoxic environments that vary in severity, RHI and duration. In particular, it advances our understanding of how time influences the use of aerobic metabolism in hypoxia (Chapter 4), and what type of hypoxic environments might favour the least-studied of the three metabolic mechanisms of hypoxia defense, MRD (Chapters 3 and 5). My thesis also makes methodological contributions to the field in the form of a novel calorimeter, the demonstration of RHI as a significant contributor to the outcome of P_{crit} experiments, and the identification of the threespine stickleback model as a powerful system with which to investigate the mechanisms and evolution of hypoxia tolerance.

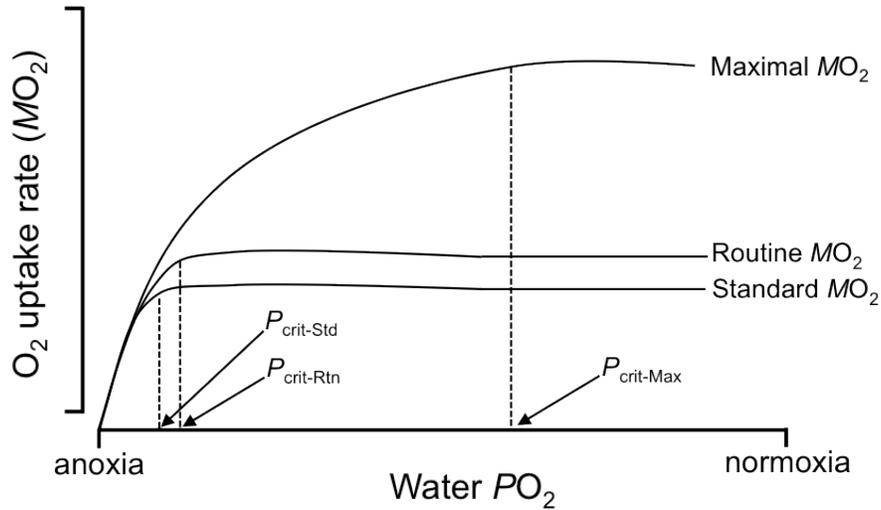


Figure 1-1. The aerobic metabolic responses of a typical fish to decreasing water PO_2 at three different levels of aerobic metabolism. Solid curves represent the required $\dot{M}O_2$ s to support maximal, routine and standard metabolic rates. Each level of metabolism has its own critical O₂ tension (P_{crit}), the water PO_2 at which the fish's compensatory mechanisms of O₂ extraction and use become insufficient to support maximal, routine or standard $\dot{M}O_2$, respectively. Supporting each respective level of metabolism at water PO_2 s below P_{crit} requires increased reliance on anaerobic ATP supply pathways.

Chapter 2

A simple and affordable calorespirometer for measuring the metabolic rates of fishes

2.1 Summary

Calorimetry is the measurement of the heat liberated during energy transformations in chemical reactions. When applied to living organisms, it measures the heat released due to the energy transformations associated with metabolism under both aerobic and anaerobic conditions. This is in contrast to the often-used respirometric techniques for assessing energy turnover, which can only make precise measurements under fully aerobic conditions. Accordingly, calorimetry is considered the “gold standard” for quantifying metabolic rate, yet despite this, it remains a seldom-used technique among comparative physiologists. The reasons for this are related to the expense and perceived difficulty of the technique. I have designed and constructed an inexpensive flow-through calorespirometer capable of detecting rates of metabolic heat loss and oxygen uptake ($\dot{M}O_2$) in fishes under a variety of environmental conditions over long-term experiments. The metabolic heat of the fish is detected as a (micro)voltage by a collection of Peltier units wired in series, while oxygen optodes placed on the inflowing and outflowing water lines are used for the calculation of $\dot{M}O_2$. The apparatus is constructed in a differential fashion to account for ambient temperature fluctuations. This paper describes the design and construction of the calorespirometer for ~\$1,300 CAD. Using the goldfish (*Carassius auratus auratus*), I show that the calorespirometer is sensitive to changes in metabolic rate brought about by pharmacological manipulation and severe hypoxia exposures.

2.2 Introduction

The accurate measurement of metabolic rate has tremendous value across many disciplines in the life sciences. The rate at which an organism consumes and utilizes energy provides insight into its biology from the level of its cells to its ecology (Hochachka and Somero, 2002; Brown et al., 2004). The most widely used method for assessing metabolic rate is through the measurement of oxygen (O_2) uptake rate ($\dot{M}O_2$), which, under aerobic conditions, provides a reasonably good estimate of metabolic rate. However, under circumstances like hypoxia, where the metabolic rate of an organism cannot be solely supported by aerobic metabolism and anaerobic processes are utilized to buffer ATP turnover, measurements of $\dot{M}O_2$ could drastically underestimate metabolic rate. This is most evident in cases of anoxia-tolerant organisms like the painted turtle (*Chrysemys picta*), crucian carp (*Carassius carassius*), and goldfish (*Carassius auratus auratus*), where attempts to quantify metabolic rate *via* respirometry in anoxia are futile due to the organism's complete reliance on anaerobic processes to support energy turnover. Like aerobic pathways, however, these pathways yield heat as a byproduct, and the total amount of heat lost by an animal to its environment is proportional to its total energy turnover (minus that conserved in carbon bonds) (Mendelsohn, 1964; Mclean and Tobin, 1987; Kaiyala and Ramsay, 2011). Measuring this heat *via* calorimetry is therefore an effective way of estimating an animal's metabolic rate in situations where aerobic metabolism may be compromised.

Despite direct animal calorimetry being the “gold standard for quantifying the fire of life” (Kaiyala and Ramsay, 2011), it is a seldom-used technique owing to its reputed difficulty and expense when compared with respirometry. These difficulties are especially true when working with ectothermic animals like fishes, whose lower metabolic rates produce less heat compared with similarly sized endotherms. Measuring these low levels of heat requires an especially sensitive calorimeter, and to date, these have been extremely expensive to purchase. Efforts have been made over the years to produce inexpensive systems to measure heat in fishes (Davies, 1966; Stevens and Fry, 1970), but they have not been described in sufficient detail to facilitate their reconstruction. With high-density thermocouple Peltier units being widely available, it should be possible to construct a relatively simple and inexpensive calorimeter of high sensitivity. This chapter describes the construction and testing of such an apparatus, capable of converting a fish's metabolic heat to

a voltage through use of Peltier units and the thermoelectric effect (more specifically, the Seebeck effect). Furthermore, the fish chamber is designed to operate under flow-through conditions to enable environmental manipulations and the simultaneous measurement of inflowing and outflowing partial pressure of O₂ (PO_2), which can be used to calculate $\dot{M}O_2$ (hence, calorespirometer). I tested the calorespirometer's function using goldfish, a species that is well known to undergo metabolic rate depression in response to hypoxia/anoxia exposure (van Waversveld et al., 1988; Addink et al., 1991; Stangl and Wegener, 1996; Richards, 2009).

2.3 Materials and methods

2.3.1 Theory and overview

The Seebeck effect allows a heat flux to be converted to a voltage as it passes through a thermally conductive element such as a thermocouple. Peltier units are composed of a number of antimony telluride thermocouples connected in series that, with a Seebeck coefficient of 213 $\mu\text{V Kelvin}^{-1}$ (K), are highly sensitive to temperature change. In building my calorespirometer, I placed a collection of Peltier units between a small fish/reference chamber and a large mass of aluminum so that the metabolic heat produced by the fish would flow through the Peltier units and into the mass of aluminum. The calorespirometer was assembled in a differential fashion with identical fish and reference chambers attached on either side of the aluminum mass. To measure metabolic heat loss from the fish, both chambers were treated identically apart from the presence of a fish (or resistor; see heat calibration and measurements section below) in one side, and I monitored the net voltage between the two chambers. This differential configuration accounted for any fluctuation in ambient temperature. Below, I detail the construction of the apparatus and its major components, and explain how it was assembled to optimize performance. A complete list of its essential and accessorizing components and their costs is shown in Table 2-1.

2.3.2 Calorimeter

For this section, “calorimeter” will refer exclusively to the component of the calorespirometer responsible for the detection of heat and its conversion to a voltage. This component, shown in Fig. 2-1A, B, was assembled symmetrically with two identical sides centered on a block of aluminum (98 x 48 x 48 mm). Two Peltier units (approx. 40 x 40 x 4.7 mm; 127 couples; Custom Thermoelectric Peltier module 12711-5L31-03CQ) were affixed to each side of this block using an ultra thin layer of silver conductive epoxy (MG Chemical #8331) and connected in series so as to maximize the voltage reading (Fig. 2-1A). A brass block (approx. 78 x 26 x 26 mm) was affixed to the opposite side of each group of Peltier units using the silver conductive epoxy. Brass was ideal for this component as its hardness and machinability allowed for especially thin walls and its high thermal conductivity optimized heat flow. The brass blocks had a 25 mm diameter bore into which a fish or reference chamber could be inserted. Together with the Peltier units and the brass blocks, the central block of aluminum was bolted to another aluminum block (approx. 98 x 152 x 48 mm) into which two cylinders were bored (approx. 25 mm) and through which the fish chamber and reference chamber could be inserted into and removed from the calorimeter’s brass blocks (Fig. 2-1B).

2.3.4 Fish and reference chambers

Identical 32 mL flow-through chambers were constructed to serve as the fish chamber and the reference chamber (Fig. 2-1B). These chambers were constructed of stainless steel tubing (approx. 77 mm length, 25 mm OD, 24 mm ID, 0.5 mm wall thickness; McMaster-Carr #6622K152), with a stainless steel cap of 0.5 mm thickness permanently welded to the upstream end of the chamber. Inserted through this cap were stainless steel inflow and outflow water lines (1 mm OD, 0.8 mm ID, 0.1 mm wall thickness), the inflow water running along the chamber’s bottom all the way to the downstream end, and the outflow water line situated at the chamber’s top and mounted flush with the stainless steel cap at the upstream end of the chamber. The water lines were oriented this way to optimize mixing within the chamber and to allow for an easy path of exit for any gas bubbles that may enter the chamber. Finally, a removable Plexiglas cap equipped with a rubber gasket was placed at the downstream end of the chamber through which the fish could be inserted and removed. This

cap could also accommodate a PO_2 optode (Ocean Optics OR125) that was used to measure the water PO_2 (P_wO_2) within the chamber. Apart from this optode, the fish chamber and reference chamber were identical.

2.3.5 *Respirometer*

For this section, “respirometer” will refer exclusively to the component of the calorespirometer responsible for the measurement of PO_2 and determination of $\dot{M}O_2$. This component was built in a flow-through fashion and incorporated exclusively on the fish chamber side of the calorimeter. Small stainless steel chambers of 1 mL (Fig. 2-1C) were built to accommodate PO_2 optodes (Ocean Optics OR125) on both the inflow and outflow water lines immediately adjacent the bored-out aluminum block (*i.e.*, as close to the fish chamber as possible), and the difference between the P_wO_2 values measured by these optodes allowed for the calculation of the fish’s $\dot{M}O_2$.

No heat or electrical signals from the activated PO_2 optodes could be detected by the calorimeter, thus their use did not affect measurements of metabolic heat loss.

2.3.6 *Setup and optimization*

To provide a thermally stable environment for the calorespirometer, it was placed within an insulated ice chest (Coleman 6-Day Xtreme™) inside an additional enclosure (foam insulation, 5.08 cm in thickness), located within a temperature controlled ($20 \pm 0.1^\circ\text{C}$) environmental chamber measuring 3 x 3 x 2.5 m. The insulated ice chest was lined with aluminum blocks totaling approximately 40 kg, and the calorespirometer was placed in the centre of the chest. The aluminum was used as a heat sink, drawing heat from the fish and reference chambers through the Peltier units. The aluminum’s high thermal inertia, a function of its mass, thermal conductivity ($237 \text{ W m}^{-1} \text{ K}^{-1}$) and (molar) heat capacity ($24.2 \text{ J mol}^{-1} \text{ K}^{-1}$), made it an especially effective heat sink and ensured the Peltier units accounted for as much of the fish’s metabolic heat as possible.

As heat from the chambers flowed through the Peltier units, the net voltage was measured using a Keithley Model 147 nanovoltmeter. The leads from the Peltier units were soldered to the pure copper lead from the voltmeter and this junction was affixed to the aluminum mass using electrical tape to minimize its possible (albeit small) effect on the

measured voltage. The amplified signal was then digitally converted using a DATAQ DI-148 data acquisition system and recorded on a Dell Precision M4300 laptop computer using DATAQ WinDaq software.

2.3.7 Water supply and gas mixing

The water supplying the fish and reference chambers was sourced from a common 2 L recirculating volume. This volume was held in an insulated ice chest identical to the one housing the calorimeter (minus the aluminum) and placed adjacently. Water was drawn out of the beaker by a peristaltic pump (Gilson Minipuls 3), pushed into a gas equilibration chamber (see below), and then into the stainless steel tubing supplying the fish and reference chambers. Water flowed into and out of the chambers as described previously, and was returned to the original 2 L beaker for recirculation.

To manipulate the gas tension in the fish and reference chambers, gas mixing was done using a precision gas mixer (Corning 192) and the mixed gas was equilibrated with the water supply in two ways. First, the mixed gas was bubbled into the 2 L recirculating supply volume, and second, the mixed gas flowed into a 1.5 L glass gas equilibration chamber within which the supply water flowed through Silastic tubing before flowing into the stainless steel tubing supplying the fish and reference chambers (Fig. 2-2).

2.3.8 Heat calibration and measurements

To calibrate the calorimeter, I used three different resistors (9890 Ω , 19 980 Ω and 39 560 Ω ; 5% tolerance; resistances measured using a Fluke 73 multimeter whose accuracy was calibrated using 16 different resistors of 1% tolerance) and the following equation:

$$Q = V^2 / R$$

where Q is heat flow (in watts), V is voltage applied to a resistor (in volts), and R is the resistance of the resistor (in ohms). The resistors were each embedded in an epoxy-filled glass test tube to protect them from the water. During calibration, one of the glass/epoxy-embedded resistors was placed into the fish/reference chamber, the whole unit inserted into the calorimeter and held under experimental conditions (water flow rate of 22 mL h⁻¹;

normoxia; inflow, chamber, and outflow PO_2 optodes running) in order to account for any lost metabolic heat owing to the flow-through design. A voltage (measured at 4.98 V and produced by a C-TON Industries PW2-5 model power supply *via* a two wire system of negligible resistance [$< 0.35 \Omega$] when compared with the resistances of the resistors) was applied to the resistor yielding heat flows of 2.528 mW, 1.251 mW and 0.632 mW for the 9890 Ω , 19 980 Ω and 39 560 Ω resistors, respectively. Once the measured voltage from the calorimeter stabilized, it was recorded. Calibration with each resistor was performed three times and a calibration curve relating applied heat (in watts) to measured voltage was constructed and used to convert the metabolic heat of fishes, measured in millivolts, to milliwatts.

2.3.9 Oxygen uptake measurements

Oxygen uptake rates (moles of O_2 consumed per hour per gram of tissue) were calculated from measurements of inflow and outflow P_wO_2 , water flow rate, and animal weight according to the Fick principle:

$$\dot{M}O_2 = [\Delta P_wO_2 \cdot \alpha O_2 \cdot \text{flow}] / [\text{animal weight}]$$

where ΔP_wO_2 is the difference in P_wO_2 between inflowing and outflowing water (in mmHg), αO_2 is the solubility coefficient of O_2 in water at the experimental temperature (1.8230 $\mu\text{mol mmHg}^{-1} \text{ l}^{-1}$ at 20°C), flow is measured in L h^{-1} , and animal weight is measured in grams. Partial pressure measurements of mmHg were later converted to kPa.

2.3.10 Experimental animals

Goldfish (*Carassius auratus auratus*) of 0.756 ± 0.087 g (mean \pm SEM, $n = 5$) wet body weight were acquired from a commercial fish dealer and held at the Department of Zoology's Aquatic Facility at The University of British Columbia, Vancouver, BC, Canada. Fish were held at a stocking density of $< 0.4 \text{ g L}^{-1}$ in a 76 L recirculating system and maintained in well-aerated, dechlorinated City of Vancouver tap water at 20°C under a 12:12 h light:dark cycle. Water in the recirculating system was replaced every 7 to 10 days. Fish were fed to satiation daily (Nutrifin Max Goldfish Flakes) except 24 h before being

transferred to the calorespirometer, during which period feeding was suspended. The University of British Columbia Animal Care Committee approved all procedures involving fish (protocol A13-0309).

2.3.11 *Experimental protocols*

Before each experiment, the PO_2 optodes were calibrated in air and 100% nitrogen. A single goldfish was then inserted into the fish chamber *via* the removable cap, and the chamber was sealed and slid into place within the calorespirometer's brass block. The peristaltic pump was turned on, supplying both fish and reference chambers with oxygenated water at a rate of 22 mL h^{-1} . Water temperature was maintained at 20°C throughout all experimental trials. The fish was allowed to habituate to the chamber for 16 to 18 hours, which was sufficient time to allow for both the thermal equilibration of the calorespirometer and the recovery of the fish from handling stress. After the habituation period, I conducted several experiments designed to test the calorimeter's function. To ensure it was capable of detecting variation in metabolic heat produced by fishes, I used carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; Sigma-Aldrich C2920) in an attempt to increase metabolic rate *via* mitochondrial uncoupling, and benzocaine (Sigma-Aldrich E1501), an anaesthetic, to decrease metabolic rate. These experiments were repeated three times. The next set of experiments were carried out to ensure the apparatus was capable of detecting the previously observed O_2 dependent changes in metabolic heat produced by goldfish (van Waversveld et al., 1988; van Waversveld et al., 1989; Addink et al., 1991). Water PO_2 was decreased from approximately 40 kPa to 0-0.25 kPa, where it was held for 1.5 h before being returned to normoxia. This experiment was repeated five times.

Although the baseline heat signal remained stable over the duration of each run, it fluctuated between runs by $\pm 0.03 \text{ mV}$. In order to accurately determine the baseline heat signal for each experiment, I introduced an overdose of anesthetic in the fish chamber (final concentration $\sim 300 \mu\text{mol l}^{-1}$ benzocaine) to euthanize the fish in the chamber at the end of the experiment. The fish's metabolic heat quickly subsided after the addition of the anesthetic, stabilizing at a baseline value within $\sim 25 \text{ min}$ (preliminary experiments showed no further decrease in heat loss rate over 3 hours). After $\sim 1 \text{ h}$ of stable baseline reading, the fish was removed from the calorespirometer and the experiment concluded.

2.3.12 Data and Statistical Analysis

Statistical analyses consisted of one-way analysis of variance that was performed using SigmaStat version 4.0.

2.4 Results and discussion

2.4.1 Metabolic heat

The calorimeter was both stable and sensitive. Under flow-through conditions of 22 mL h⁻¹ and 20°C but without a fish present, heat flow measurements showed very small oscillations (± 0.35 mW) around the baseline and there was no net drift in baseline heat detected over 72 hours (data not shown). Changes in PO_2 of inflowing water (between 0 and 40 kPa) and turning the PO_2 probes on and off had no effect on heat flow (data not shown).

The heat calibration generated a linear relationship between applied wattage and measured voltage (Fig. 2-3; equation of the line $mV = 0.1371 \cdot mW$) that could be used to convert the metabolic heat of a fish, measured in millivolts, to milliwatts. The heat pulses also revealed the calorimeter's sensitivity to be 141.15 $\mu V mW^{-1}$ at a water flow rate of 22 mL h⁻¹, a sensitivity in close agreement with that of the only known commercially available calorimeter that can accommodate a fish (as used in Addink et al., 1991).

The next step involved inserting a fish into the fish chamber to determine if the apparatus was capable of measuring its metabolic heat under fully oxygenated conditions. The representative trace in Fig. 2-4 shows that approximately 15 h were required for the fish to habituate to its new environment (Fig. 2-4) and for the calorimeter to thermally equilibrate after insertion of the fish (time zero on Fig. 2-4). During these preliminary trials, water PO_2 was maintained at ~40 kPa to ensure adequate oxygen delivery and compensate for the low water flow rate (22 mL h⁻¹). The fish's rate of metabolic heat loss stabilized by 15 hours and remained relatively constant at ~1.5 mW g⁻¹ (Fig. 2-4), with sporadic increases in heat likely due to episodes of activity.

To ensure I could detect variation in metabolic heat loss, pharmacological agents with known effects on metabolism were introduced to the fish and reference chambers by briefly transferring the inflow lines from the 2 L water supply beaker to a vessel holding the pharmacological agent. FCCP is an uncoupling agent that increases the permeability of the

mitochondrial inner membrane, dissipating the proton gradient used to drive ATP production *via* oxidative phosphorylation. I predicted this would increase the fish's metabolic heat, and in fact, sequential additions of 4 $\mu\text{mol L}^{-1}$ FCCP (up to 12 $\mu\text{mol L}^{-1}$ FCCP) resulted in incremental increases in metabolic heat, up to a $60.5 \pm 10.3\%$ increase compared with controls ($P < 0.001$; Table 2-2). Similarly, benzocaine, a widely used anaesthetic, was administered in the same way with the prediction that it would decrease metabolic heat. A single dose of $\sim 100 \mu\text{mol L}^{-1}$ benzocaine resulted in a $68.6 \pm 5.1\%$ decrease in heat loss compared with controls ($P < 0.001$).

Goldfish have been shown to reversibly depress their metabolic rate by 70 to 80% when exposed to anoxia (van Waversveld et al., 1988; van Waversveld et al., 1989; Addink et al., 1991; Stangl and Wegener, 1996), and my results are consistent with these previous findings (Figs. 2-4, 2-5A). At the end of the 15 hour habituation period, water PO_2 was decreased over 90 min to between 0 and 0.25 kPa. The fish was then held at this PO_2 for 1.5 hours during which metabolic heat loss decreased and stabilized at an average value that was $\sim 30\%$ that of the average resting level (Fig. 2-5A). When PO_2 was returned to ~ 40 kPa, metabolic heat returned to levels that were equal to pre-hypoxia levels. Following a 2 h recovery period, the fish was euthanized with an overdose of anaesthetic to determine the baseline heat signal as described previously.

2.4.2 Oxygen uptake rate

The trends for $\dot{M}O_2$ measurements paralleled those for the metabolic heat measurements discussed above. Specifically, high $\dot{M}O_2$ values were measured over the initial five hours after the fish was introduced to the calorimeter, gradually decreasing to stable levels after 12 to 15 hours in the calorimeter (Fig. 2-4). My mass-specific routine $\dot{M}O_2$ values are higher than those reported elsewhere for goldfish (van Waversveld et al., 1988), but this variance is likely accounted for by differences in size (our fish are 12 times smaller than those used by van Waversveld et al., 1988), fasting regime, and habituation time and conditions between the studies. Upon exposure to anoxia/hypoxia, $\dot{M}O_2$ fell to near-zero levels, returning to routine levels upon the reintroduction of O_2 (Fig. 2-5B).

In order to maximize the sensitivity of my calorimeter for heat detection, I used a low rate of water flow through the fish and reference chambers, which affected the time

domain over which $\dot{M}O_2$ could be measured. After a change in inflowing PO_2 , about 60 minutes were required for the PO_2 in the outflowing water to stabilize, and thus, during this equilibration period, calculations of $\dot{M}O_2$ were inaccurate. Apart from this period, the fish's O_2 consumption could be accurately and constantly measured in real time in parallel with its rate of metabolic heat loss. It is important to note that this ~60 min equilibration period was not needed for the measurement of metabolic heat; the calorimeter responded instantly to changes in heat and stabilized within ~25 min (Fig. 2-3 inset).

2.4.3 *Tips on effective calorespirometry*

Despite the calorespirometer's straightforward design and assembly, much attention was needed when preparing the apparatus for experimental use. Central to most of this was the extreme thermal sensitivity of the Peltier units. The differential design of my calorespirometer should theoretically account for fluctuations in ambient temperature, but effort was still required to ensure all heat produced by electrical equipment in the environmental chamber (*e.g.*, computer, voltmeter, peristaltic pump, etc.) was evenly distributed across the enclosed, insulated ice chest. Fans and heat funnels were used for this, and any vulnerable parts on the insulated ice chest (especially drilled holes for the passage of water lines and electrical cables) were patched with form-fitting foam insulation. This was particularly important for holes in close proximity to the Peltier units. Although it was not a problem with my setup, care should also be taken to ensure the voltage reading is not being affected by electrical activity on the circuit into which the voltmeter is plugged.

The accurate and precise determination of a fish's metabolic rate demands a baseline heat signal that is known and stable. It is possible that with a highly controlled environment and a faithful duplication of experimental setup procedures and the orientation of all components, an identical inter-experiment baseline heat signal can be generated. However, despite my efforts, I noticed an inter-experiment fluctuation in baseline heat signal by ± 0.03 mV (although mean intra-experimental baseline drift was negligible). This required the fish to be euthanized *via* an overdose of anaesthetic at the end of each experiment as described previously. Although this is not ideal, the accurate and precise determination of the fish's metabolic rate required it. It is possible that this approach may be needed in other calorespirometers built from this design.

Finally, a calorespirometer like the one described here will inevitably come with a few limitations that need addressing. First, the flow-through design that allows for environmental manipulation and long experimental durations means some of the metabolic heat produced by the fish will be washed downstream, resulting in a possible underestimation of its metabolic rate. This effect will be minimized through the use of a relatively low flow rate, and all but eliminated by performing the heat calibration process at the experimental flow rate (See Materials and Methods). Second, the use of a low water flow rate could result in the accumulation of metabolic end products (*e.g.* CO₂) in the fish chamber that could have their own effects on the organism. In my hands, measured *PCO*₂ values never exceeded 1.1 kPa in a typical 24 hour experiment and thus did not likely have a negative effect on the fish's metabolic rate (Fry et al., 1947). Should the outflowing water contain high *PCO*₂ or metabolic waste, a higher flow rate is recommended, though this will decrease the calorimeter's sensitivity. Third, as discussed above, there are different time delays for the measurements of $\dot{M}O_2$ and heat loss that must be taken into account when assessing metabolic rate. In general, if both measurements are required, the time resolution for measurements will be approximately 1 to 2 h. And finally, due to the long habituation time required for accurate measurements of $\dot{M}O_2$ and metabolic heat, the animals are in a fasted state. Duration of fasting has been shown to influence metabolic rate (Davies, 1966), so care must be taken to ensure that all animals are treated similarly.

2.4.4 Concluding remarks

I have constructed a calorespirometer that is capable of simultaneously measuring the $\dot{M}O_2$ and metabolic heat of fishes, making it possible to measure metabolic rate in environments that compromise aerobic ATP supply pathways. Combined with its low cost of construction and simple, modifiable design, this apparatus is obtainable to most researchers and has the potential to shed light on the metabolic responses of a broad range of species in any number of environments.

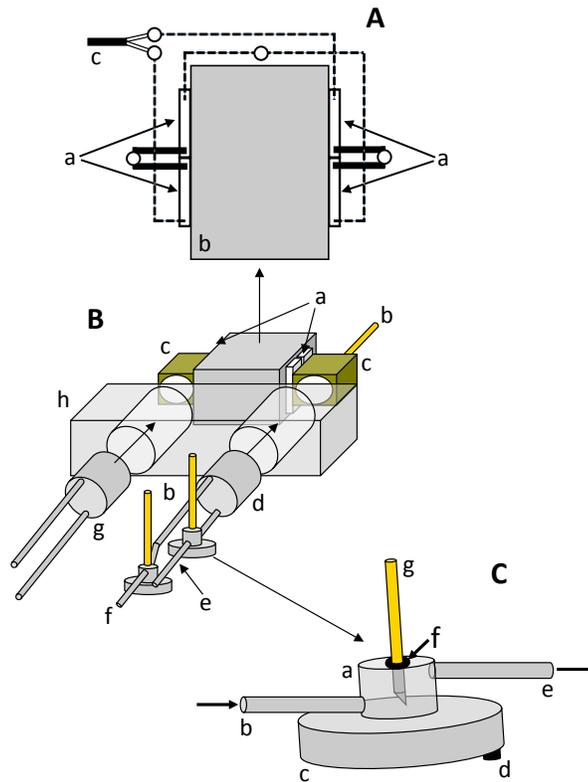


Figure 2-1. A wiring diagram (A) and schematics for the functional component of the calorimeter (B) and the PO_2 optode chambers (C). Wiring diagram of the four Peltier units (fish chamber side on right, reference chamber side on left). Dashed lines represent positive wires, thick black lines (towards the center) represent negative wires, and white circles represent the soldered junctions between wires (each affixed to the aluminum mass; see Materials and Methods for details). a, Peltier units (Custom Thermoelectric Peltier module 12711-5L31-03CQ); b, central aluminum block; c, pure copper lead to voltmeter. (B) Schematic of the functional component of the calorimeter. a, Peltier units; b, PO_2 optodes (simplified; see (C) below); c, bored out brass blocks; d, 32 mL fish chamber; e, inflowing stainless steel water line; f, outflowing stainless steel water line; g, 32 mL reference chamber; h, aluminum block. This portion of the calorimeter is embedded within a 40 kg mass of aluminum located within a highly insulated ice chest. (C) Detailed schematic of the PO_2 optode chamber. a, 1 mL stainless steel water chamber; b, inflow water line; c, Plexiglas base; d, affixed rubber stopper to tilt chamber and promote the exit of any gas bubbles; e, outflow water line; f, rubber gasket; g, PO_2 optode tip.

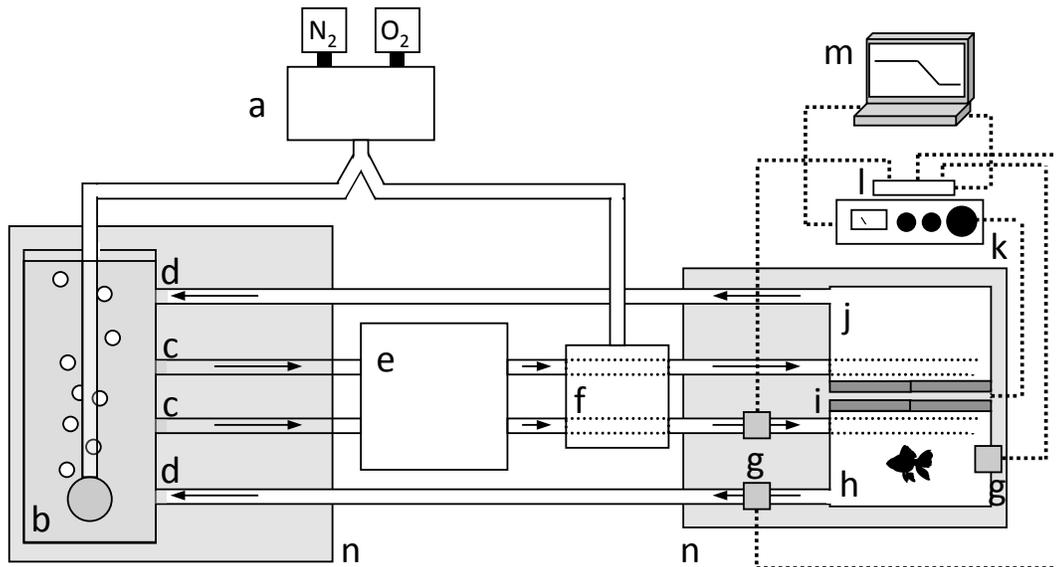


Figure 2-2. A two-dimensional schematic detailing the calorespirometric setup. a, precision gas mixer; b, 2 L water volume supplying the fish and reference chamber; c, inflowing water lines; d, outflowing water lines; e, peristaltic pump; f, PO_2 equilibration chamber; g, PO_2 optodes; h, fish chamber; i, Peltier units; j, reference chamber; k, nanovoltmeter; l, data acquisition system; m, data acquisition computer; n, insulated ice chests. The calorespirometer (including PO_2 optodes) and water supply are housed within foam insulation-enshrouded ice chests, themselves within a thermally regulated environmental chamber with ambient fluctuations of not more than 0.1°C . All heat-producing electrical equipment is housed outside of the insulated ice chests.

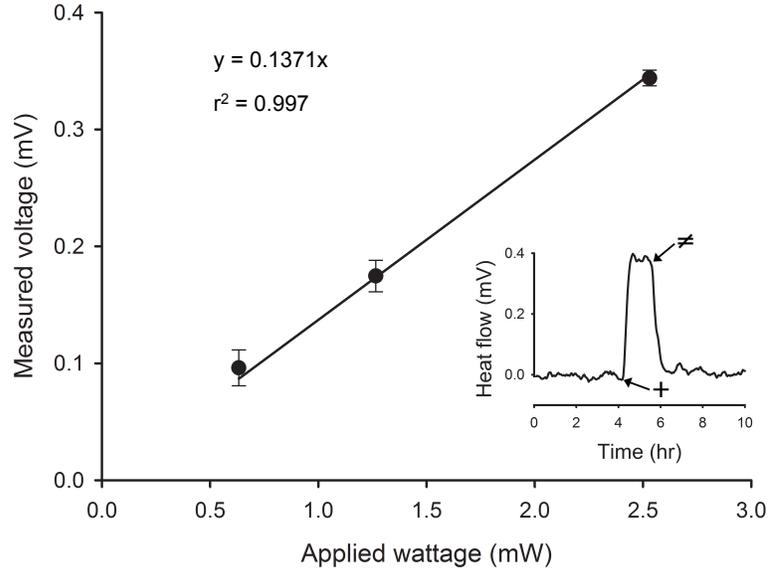


Figure 2-3. Average millivoltage measured by calorimeter in response to known quantities of heat liberated within the calorimeter’s fish chamber. Power supply was measured at 4.98 V, and resistors were measured at 9890 Ω , 19 980 Ω and 39 560 Ω , resulting in milliwattages of 2.528 mW, 1.251 mW and 0.632 mW, respectively. Sample size of 3 for each average value, with error bars representing s.e.m. Trendline is forced through zero. Inset, a rectangular heat pulse of 2.528 mW switched on at “+” and switched off at “≠”.

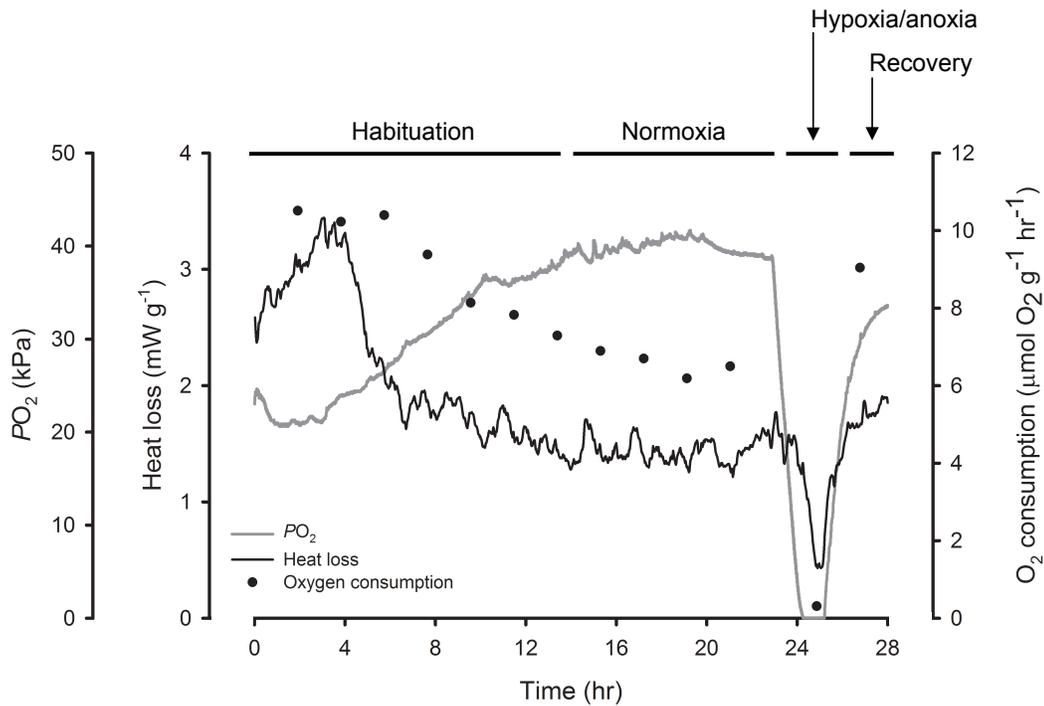


Figure 2-4. A representative trace showing a 28 h calorespirometry experiment on a single goldfish of 0.628 g. The fish chamber containing the fish was inserted into the calorespirometer at time zero. This particular run saw a habituation period of ~14 h, a more stable normoxic period of ~10 h, an anoxic exposure of ~1.5 h, and a recovery of ~2.5 h. Water temperature of 20°C throughout. PO_2 reading is from the PO_2 optode located within the fish chamber (see text for details).

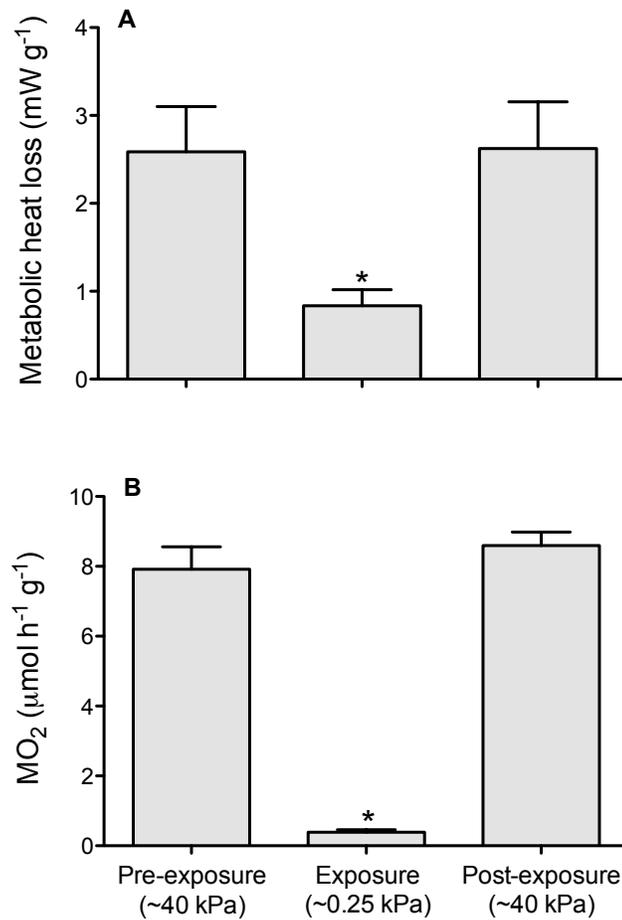


Figure 2-5. Mean measurements of metabolic heat loss (A) and O₂ consumption rate (B) in goldfish held at 40 kPa PO₂ before (pre) and after (post) exposure to severe hypoxia (0.25 kPa). Average wet body weight of fish was 0.756 ± 0.087 g, and water temperature was 20°C. Sample size of 5 for each, with error bars representing s.e.m. Asterisks denote statistically significant differences (P<0.001).

Table 2-1. Required components and costs for construction of the calorimeter described in the Materials and Methods section.

Calorimeter-specific components	Total cost
Aluminum blocks (8)	\$648
Brass rod (1)	\$125
Peltier units (4)	\$78
Ice chest (2)	\$140
Stainless steel tubing (for chambers)	\$47
Stainless steel tubing (for water lines)	\$60
Materials for PO ₂ optode chambers (2)	\$75
Plexiglas caps (2)	\$8
Silver conductive epoxy	\$5
Styrofoam insulation	\$80
	\$1,266
Additional components	
Voltmeter and copper lead (1)	-
Computer and data acquisition system	-
PO ₂ optodes, hardware, software (3)	-
Peristaltic pump and tubing (1)	-
Gas mixer (1)	-
Temperature controlled environment chamber	-
Machining costs	-

Prices in Canadian dollars; taxes not included.

Table 2-2. Percent increase in heat lost by goldfish when exposed to increasing concentrations of FCCP.

	Sham	4.0 $\mu\text{mol l}^{-1}$	8.0 $\mu\text{mol l}^{-1}$	12.0 $\mu\text{mol l}^{-1}$
FCCP	1.0 \pm 4.8	25.2 \pm 10.8*	47.9 \pm 3.5*	60.5 \pm 10.3*

All percent increases are relative to resting levels of metabolic heat loss. Water temperature of 20°C. Sample size of 3 for each, with values representing mean \pm s.e.m. Asterisks denote statistically significant difference from resting.

Chapter 3

Calorespirometry reveals that goldfish prioritize aerobic metabolism over metabolic rate depression in all but near-anoxic environments

3.1 Summary

Metabolic rate depression (MRD) has long been proposed as the key metabolic strategy of hypoxic survival, but surprisingly, the effects of changes in hypoxic O_2 tensions (P_wO_2) on MRD are largely unexplored. I simultaneously measured the O_2 uptake rate ($\dot{M}O_2$) and metabolic heat of goldfish using calorespirometry to test the hypothesis that MRD is employed at hypoxic P_wO_2 s and initiated just below P_{crit} , the P_wO_2 below which $\dot{M}O_2$ is forced to progressively decline as the fish oxyconforms to decreasing P_wO_2 . Specifically, I used closed-chamber and flow-through calorespirometry together with terminal sampling experiments to examine the effects of P_wO_2 and time on $\dot{M}O_2$, metabolic heat and anaerobic metabolism (lactate and ethanol production). The closed-chamber and flow-through experiments yielded slightly different results. Under closed-chamber conditions with a continually decreasing P_wO_2 , goldfish showed a P_{crit} of 3.0 ± 0.3 kPa and metabolic heat production was only depressed at P_wO_2 between 0 and 0.67 kPa. Under flow-through conditions with P_wO_2 held at a variety of oxygen tensions for 1 and 4 h, goldfish also initiated MRD between 0 and 0.67 kPa, but maintained $\dot{M}O_2$ to 0.67 kPa, indicating that P_{crit} is at or below this P_wO_2 . Anaerobic metabolism was strongly activated at $P_wO_2 \leq 1.3$ kPa, but only used within the first hour at 1.3 and 0.67 kPa as anaerobic end-products did not accumulate between 1 and 4 h exposure. Taken together, it appears that goldfish reserve MRD for near-anoxia, supporting routine metabolic rate at sub- P_{crit} P_wO_2 s with the help of

anaerobic glycolysis in the closed-chamber experiments, and aerobically after an initial (<1 h) activation of anaerobic glycolysis in the flow-through experiments, even at 0.67 kPa P_{wO_2} .

3.2 Introduction

Aerobic pathways of ATP production yield ~18-times more ATP than anaerobic pathways (Hochachka and Somero, 2002). Consequently, environmental hypoxia and the corresponding shift to anaerobic metabolism seriously threaten energy balance by reducing an animal's ability to generate sufficient ATP to meet metabolic demands. Despite the critical importance of aerobic respiration to the maintenance of metabolic function, many organisms inhabit and thrive in various hypoxic and even anoxic environments (Bickler and Buck, 2007; Ramirez et al., 2007). Fishes are particularly adept at surviving low-oxygen environments, having independently evolved hypoxia tolerance numerous times (Hochachka and Lutz, 2001) due to the relatively high prevalence of hypoxia among aquatic habitats (Boesch, 2002; Diaz and Breitburg, 2009; Diaz and Rosenberg, 1995; Smith et al., 2006).

Metabolic rate depression (MRD) has been proposed as the hallmark response enabling hypoxic survival in hypoxia-tolerant animals (e.g., Boutilier and St-Pierre, 2000; Hochachka et al., 1996). MRD is achieved through reductions in whole animal (e.g., locomotion, reproduction, feeding) and cellular (e.g., growth, repair, protein synthesis) processes (Guppy and Withers, 1999; Richards, 2010), reducing ATP demand and rates of anaerobic fuel depletion (glycogen) and waste accumulation (lactate and protons). Although MRD is a well described response to anoxia exposure in a range of animals including fruit flies (Callier et al., 2015), goldfish (Addink et al., 1991 van Waversveld et al., 1989) and turtles (Jackson, 1968), it has been suggested that MRD would also enhance hypoxic survival (Boutilier and St-Pierre, 2000; Hochachka et al., 1996). Indirect (*i.e.*, non-calorimetric) measurements on common frogs (Donohoe and Boutilier, 1998) and direct (*i.e.*, calorimetric) measurements on goldfish (van Ginneken et al., 1994, 2004) and tilapia (van Ginneken et al., 1997) suggest that MRD may be employed at hypoxic O_2 tensions (P_{wO_2} for water). Indeed, goldfish reduced metabolic heat by ~31% at ~2.1 kPa P_{wO_2} compared with normoxia (~21 kPa) (van Ginneken et al., 2004), and tilapia reduced metabolic heat by ~40% at ~1.1 kPa P_{wO_2} (van Ginneken et al., 1997). However, it is still unknown how these changes in

metabolic heat correspond with changes in aerobic and anaerobic metabolism, and how metabolic heat may be affected by P_wO_2 .

MRD would be particularly important at P_wO_2 s below an animal's critical P_wO_2 for O_2 uptake rate ($\dot{M}O_2$), referred to as P_{crit} , which is the P_wO_2 at which $\dot{M}O_2$ becomes dependent on environmental PO_2 . P_{crit} is largely determined by the O_2 binding affinity of hemoglobin (Hb) (Mandic et al., 2009b), and at P_wO_2 s below P_{crit} the ability to extract environmental O_2 to saturate Hb is constrained and thus unable to support routine metabolic rate (MR) aerobically. The animal can attempt to sustain ATP production at routine levels through an up-regulation of anaerobic glycolysis, but this comes with the depletion of carbohydrate reserves and the accumulation of deleterious anaerobic end-products (Richards, 2009), ultimately limiting hypoxic survival time (Lague et al., 2012; Speers-Roesch et al., 2013). However, if the animal is capable of reducing its energy-consuming processes through a controlled, hypoxia-induced MRD, then it could simultaneously mitigate the negative consequences of reduced ATP production and increased rates of fuel depletion and waste accumulation. I therefore hypothesized that MRD is employed at hypoxic P_wO_2 s and is initiated just below P_{crit} , where the negative impacts of reduced aerobic capacity and increased anaerobic reliance begin to accrue.

I tested this hypothesis using closed-chamber and flow-through calorimetry to simultaneously measure $\dot{M}O_2$, MRD (*via* metabolic heat) and anaerobic glycolysis (*via* excretion rates of the anaerobic end-product ethanol) in goldfish held at P_wO_2 s ranging from normoxia to anoxia. I also performed terminal sampling experiments on goldfish exposed to the same P_wO_2 s as used in the calorimetry experiments to fully quantify whole-body anaerobic metabolism. Goldfish were chosen owing to their exceptional hypoxia tolerance and well documented ability to induce MRD (*e.g.*, Addink et al., 1991; van Ginneken et al., 2004; van Waversveld et al., 1989), something not all fish species are capable of (Stangl and Wegener, 1996), and I used calorimetry because it is the “gold standard” of MR measurements and the only way to accurately do so on hypoxemic animals in real time (see Kaiyala and Ramsay, 2011; Nelson, 2016). Despite the superiority of calorimetry, only a few studies have measured the metabolic heat of fishes (Chapter 2; Addink et al., 1991; Stangl and Wegener, 1996; van Ginneken et al., 1994, 1997, 2004; van Waversveld et al., 1989) and only three of these (van Ginneken et al., 1994, 1997, 2004) have measured

metabolic heat at P_wO_2 other than normoxia and anoxia. While the data from these studies suggest MRD is employed at hypoxic P_wO_2 s, the authors exposed their organisms to progressive hypoxia over sometimes prolonged periods of time and they did not relate their measurements to P_{crit} nor directly assess the contributions of anaerobic metabolism at various hypoxic P_wO_2 s. Furthermore, other studies that have attempted to examine the role of MRD and other metabolic and respiratory responses to hypoxia have not attempted, to my knowledge, to directly assess the relative contributions of MRD, aerobic respiration, and anaerobic metabolism at different hypoxic P_wO_2 s over time. Consequently, we still do not have a comprehensive picture of the hypoxic survival strategies of fishes.

3.3 Materials and methods

3.3.1 Study organisms

I obtained adult goldfish (*Carassius auratus auratus*; 2.06 ± 0.39 g wet mass; $n=264$; sex unknown) from a commercial supplier (Delta Aquatics, Burnaby, BC, Canada) and held them under a 12 h:12 h light:dark cycle in a 76 L recirculating system of aerated, dechlorinated, 17°C water at the University of British Columbia (Vancouver, BC, Canada). Stocking density was <0.4 g L⁻¹ and water in the recirculating system was replaced weekly. I fed the fish to satiation daily (Nutrafin Max Goldfish Flakes) except for 24 h before transfer to the experimental apparatus, when feeding ceased. The University of British Columbia's Animal Care Committee approved all procedures (protocol A13-0309).

3.3.2 Calorespirometer

I used a differential calorespirometer to simultaneously measure metabolic heat and $\dot{M}O_2$ under closed and flow-through conditions. Details on the design and operation of the calorespirometer are described in Chapter 2. Briefly, the metabolic heat of a fish is detected as a voltage by a collection of Peltier units (Custom Thermoelectric Peltier module 12711-5L31-03CQ, Bishopville, MD, USA) *via* the Seebeck effect and converted to wattage using an empirically determined calibration coefficient (see Chapter 2). The design of the calorespirometer allows for the simultaneous measurements of metabolic heat and $\dot{M}O_2$ using PO_2 optodes (Ocean Optics OR125, Dunedin, FL, USA) placed on the inflowing and outflowing water lines as well as in the fish chamber. To determine $\dot{M}O_2$ under closed-

chamber conditions, a PO_2 optode within the fish chamber measured the change in P_wO_2 over sequential 5 min intervals and was then corrected for chamber volume and fish weight according to

$$\dot{M}O_2 = (\Delta CO_2 \cdot \Delta T^{-1} \cdot V) M^{-1}$$

where CO_2 is O_2 content of the water converted to $\mu\text{mol L}^{-1}$ from P_wO_2 using the solubility factor of $1.9312 \mu\text{mol L}^{-1} \text{ mmHg}^{-1}$ (Boutilier et al., 1984), T is the time period over which the change in CO_2 is calculated (5 min), V is the fish chamber volume (32 mL) minus the volume displaced by the fish itself, and M is the mass of the fish. To determine $\dot{M}O_2$ under flow-through conditions, the difference in P_wO_2 between inflowing and outflowing water lines supplying the fish chamber was measured using the same PO_2 optode and corrected for flow rate and fish weight according to

$$\dot{M}O_2 = ((C_iO_2 - C_oO_2) \cdot FR) M^{-1}$$

where C_iO_2 and C_oO_2 are O_2 content of inflowing and outflowing water, respectively converted from P_wO_2 as described above, FR is water flow rate (22 mL h^{-1}), and M is the mass of the fish. Under flow-through conditions, the chamber P_wO_2 could be held constant for extended time periods, allowing me to measure $\dot{M}O_2$ and metabolic heat at different time points at any desired P_wO_2 .

3.3.3 Hypoxic exposures

Individual fish were transferred to a flow-through calorimeter held at 17°C and a flow rate of 22 mL h^{-1} , and in this apparatus I performed both closed-chamber and flow-through calorimetry experiments following a 16 h normoxic habituation period. For the closed-chamber experiments ($n=8$), the trial began by stopping water flow and allowing the fish to reduce P_wO_2 from normoxia to anoxia over 60-90 min. The experiment was ended when the chamber P_wO_2 reached anoxia, at which point I introduced a lethal dose of anaesthetic (buffered MS-222, final chamber concentration of 150 mg L^{-1}) to determine the calorimeter's baseline heat signature. For the flow-through experiments, inflowing P_wO_2 was manually adjusted to yield one of four chamber P_wO_2 s over a ~60 min period (20, 1.3, 0.67 or 0 kPa; $n=3-6$ for each) and the animals were maintained at one of these P_wO_2 s for up to 4 h (referred to as the experimental period). I measured metabolic heat over the full 21 h period (16 h normoxia habituation, 1 h transition to exposure P_wO_2 , 4 h experimental

period) and collected effluent water samples either before (time 0) or at 1 and 4 h during the experimental period for measurements of ethanol (a glycolytic end-product excreted across goldfish gills). Following the experiment, I introduced a lethal dose of anaesthetic (buffered MS-222, see above) to determine the calorespirometer's baseline heat signature. At the end of each experiment, I recalibrated the PO_2 optodes to determine any drift that had occurred over the course of the experiment (up to ~10%) for the purpose of later correction, and then washed the calorespirometer and its water lines with a 10% bleach solution. The flow-through and closed-chamber calorespirometry experiments were performed in fall 2014 and winter 2015, respectively.

3.3.4 Comparison of closed-chamber and flow-through calorespirometry

In order to more directly compare the results of the closed-chamber and flow-through calorespirometry experiments, I conducted a back-to-back comparison of the two techniques using the same fish. This was required because my first experiments (presented in Figs. 3-1 and 3-2) using these techniques were conducted at different times of year and yielded different routine normoxic $\dot{M}O_2$ values, which could affect my determination of P_{crit} . I measured routine $\dot{M}O_2$ using both techniques and determined P_{crit} during closed-chamber respirometry (P_{crit} was not determined *via* flow-through calorespirometry because it would require the fish to undergo multiple runs at different P_wO_2 s). Briefly, fish were introduced to the calorespirometer and allowed to habituate under the same conditions as the calorespirometry experiments described above. Following the habituation period, I first measured the fish's routine $\dot{M}O_2$ under normoxia using flow-through respirometry (as described above), then immediately closed off the respirometer chamber and measured $\dot{M}O_2$ using closed-chamber respirometry (as described above). I repeated this three times for each of six fish, and the P_wO_2 was not allowed to drop below 16 kPa during these closed-chamber measurements. Following the final closed-chamber measurement, I allowed the fish to deplete the chamber's O_2 content so as to determine its P_{crit} . Metabolic heat was not measured during these back-to-back experiments.

3.3.5 Terminal sampling experiments

To better estimate the effects of P_wO_2 on anaerobic metabolism, I ran parallel hypoxia exposures where I euthanized animals to measure whole-body concentrations of lactate and ethanol. For each P_wO_2 , I exposed 24 goldfish spread across six 10 L tanks (four fish per tank) and sample two replicate tanks at each of 0, 1 and 4 h to match the experimental periods of the calorimetry experiments (n=8 per time point). I sampled fish by inconspicuously introducing a lethal dose of anaesthetic (buffered MS-222, see above), weighing the individual fish, freezing them immediately in liquid N_2 , then storing them at $-80^\circ C$ for later metabolite analyses. I ensured the conditions between these experiments and the calorimetry experiments were similar by including a 16 h habituation period followed by a 1 h transition period to the desired P_wO_2 , conducting exposures in the dark, and by preventing the fish in the tank from accessing the air-water interface (which was not available to the calorimetry fish). Thus, the main difference between this experiment and the calorimeter experiment was vessel size (calorimeter chamber was 32 mL and exposure tanks were 10 L), which could affect the ability of the fish to move during the hypoxia exposures and yield different levels of lactate and ethanol accumulation. I am however confident that fish movement is minimal in the calorimeter based on the relatively smooth heat traces observed over the habituation and experimental periods and periodic visual inspection of the fish in the 10 L tanks revealed little to no movement, especially during the hypoxia exposures. Thus, despite the differences in exposure regimes, the fish from both the calorimetry and the terminal sampling experiments likely responded to hypoxia in a similar manner.

3.3.6 Lactate and ethanol analyses

In order to link my whole body calorimetry measurements of MR to the activation of anaerobic metabolism, I measured whole body concentrations of lactate and ethanol. Entire goldfish from the 10 L tank exposures were ground into a fine powder using a liquid N_2 -chilled mortar and pestle. To extract the metabolites (lactate and ethanol) from the powder, an aliquot of powder was weighed and transferred to a 2 mL centrifuge tube containing 1 mL of ice cold 30% $HClO_4$ and immediately homogenized at $0^\circ C$ using a Polytron homogenizer set to the highest setting for 30 s. The resulting homogenate was then

centrifuged at 20 000 g for 5 min at 4°C and the supernatant was transferred to a new 1.5 mL centrifuge tube and neutralized using 3 M Tris base to avoid the volatilization of ethanol that occurs in association with vigorous CO₂ production when HClO₄ is neutralized with K₂CO₃. I confirmed that neutralization with Tris base does not affect my enzymatic analysis. I measured ethanol immediately following neutralization using a commercial kit designed for biological ethanol analysis (Diagnostic Chemical Ltd., PEI, Canada), and then froze the unused portion of the sample extract for later lactate analysis. Lactate concentration was measured using the LDH reaction according to the protocols outlined in Bergmeyer (1983).

3.3.7 *O₂ equilibrium curves*

To understand how goldfish's Hb-O₂ affinity related to P_{crit} and MRD, I constructed O₂ equilibrium curves for the whole blood of five normoxia-acclimated goldfish using the thin film spectrophotometric technique (Lilly et al., 2013). Blood was collected from the caudal artery of anaesthetized fish using 60 µl heparinized capillary tubes. I then centrifuged the tubes and resuspended the red blood cells in HEPES buffer (pH 7.8) to ensure a consistent blood pH across all samples. A Wosthoff gas mixing pump (H. Wösthoff Messtechnik GmbH, Bochum, Germany) mixed compressed O₂ and N₂ to each of seven PO_2 s for the construction of the O₂ equilibrium curves, and Hb P_{50} values (the PO_2 at which the blood is 50% saturated with O₂) were calculated using the equation of each sigmoidal curve as calculated by SigmaStat 11.0.

3.3.8 *P_{crit} calculation*

P_{crit} is defined as the P_{wO_2} at which an organism's routine $\dot{M}O_2$ transitions from being independent of, to being dependent upon, P_{wO_2} . I determined P_{crit} for each individual in the closed-chamber calorimetry experiments using the BASIC program (Yeager and Ultsch, 1989), which uses a two-segment linear regression model to determine P_{crit} as the P_{wO_2} at which the two linear trend lines intersect on a graph plotting $\dot{M}O_2$ as a function of P_{wO_2} . Some individuals' $\dot{M}O_2$ s increased above routine $\dot{M}O_2$ levels at hypoxic P_{wO_2} s close to P_{crit} , and including these $\dot{M}O_2$ values would overestimate P_{crit} . To prevent this, I excluded from my routine $\dot{M}O_2$ estimation any $\dot{M}O_2$ value that exceeded 1.5 times the standard deviation of an individual's average $\dot{M}O_2$ between 13 and 21 kPa P_{wO_2} .

3.3.9 Data analysis and statistics

$\dot{M}O_2$ and ethanol production rates were calculated at each time point, while metabolic heat was represented by averaging the continual heat measurements made over the 20 min straddling the time point (e.g., 50-70 min for 1 h time point). All data are presented as means \pm s.e.m. The effects of P_wO_2 on each variable were determined using one-way ANOVA (SigmaStat 11.0).

3.4 Results

3.4.1 Closed-chamber calorespirometry experiments

I used closed-chamber calorespirometry to measure P_{crit} and to characterize the effects of a progressive reduction in P_wO_2 on $\dot{M}O_2$ and metabolic heat. P_wO_2 in the closed-chamber experiments was decreased from normoxia to anoxia by the fish's own $\dot{M}O_2$ over 60-90 min (depending on the fish's $\dot{M}O_2$). P_{crit} was calculated to be 3.0 ± 0.3 kPa (Fig. 3-1). At P_wO_2 s above P_{crit} , there were no significant effects of changes in P_wO_2 on the average routine $\dot{M}O_2$, while at P_wO_2 s below P_{crit} (at which the fish spent ~ 30 min), $\dot{M}O_2$ progressively fell to zero as the goldfish depleted the available oxygen. Metabolic heat was maintained at routine levels at all P_wO_2 s between 20 and 0.5 kPa (Fig. 3-1) but was depressed upon reaching anoxia, eventually stabilizing at $\sim 21\%$ of routine normoxic values (a MRD of 79%; Fig. 3-1) after ~ 20 min.

3.4.2 Flow-through calorespirometry experiments

I used flow-through calorespirometry to characterize the effects of P_wO_2 and time on $\dot{M}O_2$, metabolic heat and excreted ethanol. I held individuals at one of four P_wO_2 s (20, 1.3, 0.67 or 0 kPa) for 1 and 4 h. For 1 h exposures, $\dot{M}O_2$ and metabolic heat were maintained at routine levels to P_wO_2 of 0.67 kPa, while at P_wO_2 s below this, $\dot{M}O_2$ fell to zero and metabolic heat fell to $\sim 32\%$ of routine levels (a MRD of 68%; Fig. 3-2A). Similarly, for 4 h exposures, $\dot{M}O_2$ and metabolic heat were maintained at routine levels to P_wO_2 of 0.67 kPa, while at P_wO_2 s below this, $\dot{M}O_2$ fell to zero and metabolic heat fell to $\sim 20\%$ of routine levels (a MRD of 80%; Fig. 3-2B).

Ethanol excretion rates were undetectable following 1 h exposure at all P_wO_2 s (Fig. 3-2A). These rates increased following 4 h exposure, and higher rates were generally detected at lower P_wO_2 s (Fig. 3-2B), but these increases were not statistically significant (Fig. 3-2B).

3.4.3 Whole body anaerobic end-product concentrations

Whole body concentrations of lactate significantly increased over time 0 values following 1 and 4 h at 1.3 kPa, 0.67 kPa and anoxia (Table 3-1). Whole body concentrations of ethanol significantly increased over time 0 values following 1 and 4 h of anoxia exposure (Table 3-1). The total anaerobic end-product concentrations at 1.3 and 0.67 kPa following 4 h were similar to those following 1 h, suggesting the rate of anaerobic end-product accumulation fell to near-zero levels after 1 h (Fig. 3-2A,B). A similar result was observed for the anoxia-exposed fish though to a lesser extent, with anaerobic end-product concentrations being ~1.8-fold higher following 4 h exposure than following 1 h exposure (Fig. 3-2A,B).

3.4.4 Closed-chamber versus flow-through calorimetry

Individual $\dot{M}O_2$ values determined in the same fish in a back-to-back comparison of closed-chamber and flow-through respirometry were positively correlated (Fig. 3-3A; $n=18$, $r=0.925$, $P<0.0001$) and yielded similar mean $\dot{M}O_2$ values (Fig. 3-3B; $t=0.423$, $P=0.678$). The closed-chamber portion of these experiments yielded a P_{crit} of 2.7 ± 0.2 kPa ($n=6$).

3.4.5 Hb-O₂ equilibrium curves

The Hb of goldfish displayed a very high affinity for O₂, resulting in a steep O₂ equilibrium curve and an average whole blood P_{50} of 0.49 ± 0.12 kPa (Fig. 3-4).

3.5 Discussion

I hypothesized that goldfish employ MRD at hypoxic P_wO_2 s and initiate it at P_wO_2 s just below P_{crit} . This hypothesis predicted that metabolic heat would decrease from routine levels at a P_wO_2 below P_{crit} , when the fish's ability to take up environmental O₂ to support a routine $\dot{M}O_2$ was compromised. The closed-chamber calorimetry experiments yielded a P_{crit} of 3.0 ± 0.3 kPa (Fig. 3-1), consistent with the P_{crit} values reported in other studies on

goldfish (Fry and Hart, 1948; Fu et al., 2011). However, contrary to my hypothesis that MRD is initiated at hypoxic P_{wO_2} s just below P_{crit} , metabolic heat was maintained at routine normoxic levels to a P_{wO_2} of 0.67 kPa and MRD was only evident in goldfish exposed to anoxia. The magnitude of the anoxia-induced MRD [79% depression in closed-chamber experiments; 68% (1 h) and 80% (4 h) in flow-through experiments; Figs. 3-1, 3-2] was very similar to what has been shown previously for anoxia-exposed goldfish using calorimetry (Addink et al., 1991; Stangl and Wegener, 1996; van Ginneken et al., 1994).

3.5.1 Metabolic responses to hypoxia

Goldfish maintained routine metabolic rate (MR) at severely hypoxic P_{wO_2} s under both closed-chamber and flow-through conditions (Figs. 3-1, 3-2), but appear to have used different strategies to do so. In the closed-chamber experiments, metabolic heat was maintained at routine normoxic levels to 0.67 kPa despite a decrease in $\dot{M}O_2$ at 3.0 kPa (Fig. 3-1), suggesting anaerobic glycolysis was up-regulated to support MR (though lactate and ethanol could not be measured in closed-chamber experiments as a function of P_{wO_2}). In the flow-through experiments, metabolic heat was similarly maintained at routine normoxic levels to 0.67 kPa at both 1 and 4 h, but unlike the closed-chamber experiments, $\dot{M}O_2$ was maintained at near-routine levels at all hypoxic P_{wO_2} s tested. This suggests that MR was supported aerobically even at severely hypoxic P_{wO_2} s and that MRD is reserved for all but severely hypoxic (<0.67 kPa) or near-anoxic environments. This is different than the results of van Ginneken and colleagues (1994, 2004) who showed moderate 27% and 33% decreases in heat production along with lower $\dot{M}O_2$ in goldfish exposed to 3.5 kPa and 2.1 kPa, respectively. These incongruent results are likely due to differences in experimental design and study goals. van Ginneken et al. (1994, 2004) exposed each fish in their studies to progressive hypoxia over prolonged periods of time (e.g., 8.4, 4.2, 2.1 and finally 0.63 kPa over a 16 h period in van Ginneken et al., 2004), which does not allow the authors to disentangle the effects of P_{wO_2} and time on metabolic heat and $\dot{M}O_2$. In contrast, my flow-through calorimetry experiments exposed goldfish to only a single hypoxic P_{wO_2} (after a 1 h adjustment period) for up to 4 h and I assessed the effects of varying hypoxic P_{wO_2} s using different individuals, allowing me to independently assess the effects of P_{wO_2} and time on metabolic responses. Using this approach, I clearly show that within 1 h exposure,

goldfish are capable of maintaining oxygen uptake under severely hypoxic conditions (0.67 kPa), obviating the need for hypoxia-induced MRD.

Elevated levels of lactate and ethanol at $P_wO_2 \leq 1.3$ kPa at 1 and 4 h indicate that anaerobic glycolysis also contributed to maintaining MR, though in slightly different ways in anoxia and hypoxia. In anoxia, lactate and ethanol levels continued to increase throughout the 4 h exposure but their rate of accumulation decreased from $5.81 \mu\text{mol h}^{-1} \text{g}^{-1}$ during the first hour to $1.73 \mu\text{mol h}^{-1} \text{g}^{-1}$ during the subsequent 3 h. These results are consistent with those observed in tissues from anoxia-exposed turtles (*Trachemys scripta elegans*), where lactate production rates were elevated during the first hour of anoxia exposure and subsequently decreased between 1 and 5 h anoxia in brain, liver and white muscle (Kelly and Storey, 1988). Combined, these results suggest that there is an initial reliance on anaerobic metabolism upon anoxia exposure that may compensate for the anoxia-induced limitations on aerobic ATP production while MRD is initiated. In hypoxia, the early reliance on anaerobic metabolism was temporally even more profound than in anoxia. Lactate and ethanol accumulation was confined entirely to the first hour of hypoxia exposure at 1.3 and 0.67 kPa, while $\dot{M}O_2$ was concurrently maintained at routine normoxic levels throughout the hypoxic exposures. Taken together, these data suggest that total ATP turnover is higher over the first hour of hypoxia exposure than in normoxia. Indeed, whole-body estimates of total ATP turnover during this period indicate that it increases from $\sim 10 \mu\text{mol h}^{-1} \text{g}^{-1}$ in normoxia to ~ 17 and $15 \mu\text{mol h}^{-1} \text{g}^{-1}$ at 1.3 and 0.67 kPa, respectively (assuming P:O₂ of 6 and ATP:lactate/ethanol of 1), while heat production does not change. These inconsistencies are likely a consequence of not being able to temporally match my measurements of anaerobic metabolism (taken as the delta accumulation of lactate and ethanol over the entire hour plus the P_wO_2 adjustment period) and $\dot{M}O_2$ and heat, which were taken at the end of the 1 h (between 50 and 70 min exposure). As such, it is possible there are temporal shifts in fuel selection within the first hour of hypoxia exposure, with lactate and/or ethanol accumulating during the initial descent *towards* the target P_wO_2 as $\dot{M}O_2$ -sustaining mechanisms are up-regulated. Finer scale studies are needed to confirm this idea.

The P_{crit} values derived from the closed-chamber and flow-through calorimetry experiments differed substantially, with P_{crit} shifting from 3.0 kPa in the closed-chamber experiments to somewhere between 0 and 0.67 kPa in the flow-through experiments (the

exact value cannot be determined). These technique-specific differences in P_{crit} are consistent with a recent study comparing closed and intermittent-flow respirometry (Snyder et al., 2016), which attributed the higher P_{crit} in closed respirometry to metabolic waste accumulation and a faster decline in P_{wO_2} . Similar factors may be at play in my closed-chamber calorerespirometry experiments resulting in an overestimation P_{crit} . Another possible explanation might be that the routine normoxic $\dot{M}\text{O}_2$ (and heat) in the closed-chamber experiments was ~2-fold higher than in the flow-through experiments (*c.f.* Figs. 3-1, 3-2). All else being equal, this would necessitate the fish from the closed-chamber experiments adopting an oxy-conforming strategy at a higher P_{wO_2} , yielding a higher P_{crit} . The back-to-back comparison of the calorerespirometry techniques suggests that time of year may influence measured $\dot{M}\text{O}_2$ values, causing upwards of a two-fold change in $\dot{M}\text{O}_2$ and heat production, but the differences in $\dot{M}\text{O}_2$ obtained in closed-chamber respirometry do not appear to affect P_{crit} and therefore do not explain why P_{crit} is higher in the closed-chamber experiment relative to the flow-through experiment.

Another factor possibly contributing to the lower P_{crit} values obtained from the flow-through experiments *versus* those from the closed-chamber experiments is time. Fishes possess many mechanisms that enhance O_2 uptake with decreasing P_{wO_2} , including increases to gill surface area (Sollid et al., 2003), Hb synthesis (Gracey et al., 2001) and concentration in the blood (Affonso et al., 2002), hematocrit (Lai et al., 2006; Turko et al., 2014), Hb- O_2 affinity (Turko et al., 2014), ventilation frequency and amplitude (Holeton and Randall, 1967; Itazawa and Takeda, 1978; Tzaneva et al., 2011; Vulesevic and Perry, 2006), as well as redistributed blood supply to critical tissues (Sundin et al., 1995). While these mechanisms effectively enhance the uptake of environmental O_2 and its distribution throughout the body, their induction takes time, varying from minutes to days depending on the physiological response examined. Because the fish in the flow-through experiments had spent 1 or 4 h at each P_{wO_2} when their $\dot{M}\text{O}_2$ was measured (in addition to the ~1 h required to reduce the P_{wO_2} from normoxia to the target P_{wO_2}), they may have had additional time to initiate some of these mechanisms of enhanced O_2 uptake compared to the closed-chamber fish that saw only ~30 min of continually decreasing sub- P_{crit} hypoxic conditions. If P_{crit} is in fact influenced by the rate and duration of hypoxia induction over relatively short time scales, then it becomes important to apply similar methodologies and time courses both within and

between studies (something that is not currently done; see Rogers et al., 2016) to ensure P_{crit} values are comparable. This is especially true when P_{crit} is used as a reference point for models that, for example, predict how climate change will reshape the distribution of fishes around the world (Deutsch et al., 2015).

3.5.2 *Hb-O₂ affinity and initiation of MRD*

Our results show that MRD is initiated in goldfish at a P_{wO_2} somewhere between 0 and 0.67 kPa. Interestingly, my analysis of whole blood Hb-O₂ affinity reveals a Hb P_{50} value of 0.49 ± 0.12 kPa (Fig. 3-4; consistent with Burggren, 1982), within the P_{wO_2} range that goldfish appear to reduce $\dot{M}O_2$ in the flow-through experiments and initiate MRD. It is therefore tempting to think of a causal link between the supply of O₂ to the tissues and the initiation of MRD. Considerable debate exists regarding the signal for MRD, with some data supporting signals residing on the energy production side of the cellular energy flux pathways (Bishop and Brand, 2000; Bishop et al., 2002; De Zwaan and Wijsman, 1976; Hochachka, 1982; Hochachka, 1985; Plaxton and Storey, 1984; Rees and Hand, 1991) and some data supporting signals on the energy consumption side (Caligiuri et al., 1981; Flanigan and Withers, 1991; Robin et al., 1979; Sick et al., 1982; see reviews by Guppy, 2004; Guppy and Withers, 1999; Storey and Storey, 1990). If Hb-O₂ affinity were in fact a signal for MRD, this would place the signal on the energy production side, consistent with some of the more recent views in the field (see Guppy, 2004). Similarly, Coulson (1977) postulated that metabolic rate was directly proportional to the circulatory system's ability to supply the tissues with O₂, and this idea gained empirical support when van Ginneken et al. (2004) showed a correlation between hypoxia-induced decreases in metabolic rate and heart rate. All told, it is not unreasonable to speculate that a signal for hypoxia-induced MRD involves the supply of O₂ to the tissue. The association between Hb P_{50} and the P_{wO_2} of MRD initiation is therefore enticing and worth further investigation.

3.5.3 *Ecological implications of MRD*

The fact that goldfish appear to initiate MRD only near anoxia and maintain $\dot{M}O_2$ without a long-term activation of anaerobic metabolism is well suited to the fish's (and the closely related crucian carp's, *Carassius carassius*) natural lake habitat. While these lakes

become ice-covered in winter and eventually anoxic, they are severely hypoxic (Vornanen, 2004) for most of the winter at P_wO_2 s that my study reveals goldfish remain aerobic. Goldfish can therefore maintain routine MR for most of the winter without relying on anaerobic glycolysis and/or MRD until it is entirely necessary. This strategy conserves the goldfish's finite anaerobic fuel stores (glycogen), reduces the accumulation of deleterious anaerobic end-products (lactate, protons and ethanol), and allows the goldfish to retain routine function and behaviour under most natural conditions.

Another benefit of a near-anoxic induced MRD is a delayed accumulation of MRD's inherent physiological and ecological costs (Humphries et al., 2003). These include oxidative stress resulting from the production of reactive O_2 species (Carey et al., 2000), impaired immunocompetence resulting from reduced lymphocyte production (Burton and Reichman, 1999), impaired cognitive and memory function resulting from reductions in synaptic contacts and dendritic branching (Popov et al., 1992), and significant reductions in sensory and motor activity (Choi et al., 1998) that increase predation susceptibility. Because the costs associated with each of these likely accumulate with time, a hypoxic survival strategy that involves an extended bout of MRD is likely to cause significant damage regardless of its effectiveness to balance cellular energy supply and demand. Goldfish's predominant reliance on aerobic respiration is therefore the ideal strategy for surviving long-term hypoxic bouts because it minimizes the time the fish is forced to rely on MRD and the physiological costs that come with it.

Taken together, goldfish's overall hypoxia tolerance strategy appears finely tuned to its particular hypoxic environment characterized by long, protracted descents into eventual anoxia. This may be the case with other species too; because hypoxic environments vary greatly in their severity, duration and rate of hypoxic induction, the hypoxia tolerance strategies employed by organisms native to these different environments are likely to be just as variable.

3.5.4 Conclusions

By demonstrating that goldfish prioritize O_2 uptake over MRD in all but near-anoxic environments, my results suggest two things. First, the exceptional hypoxia tolerance of goldfish owes more to its O_2 extraction abilities than to MRD. Second, MRD is not

necessarily a key mechanism of *hypoxic* survival as has been hypothesized (Hochachka et al., 1996), but of *anoxic* survival. While MRD is an effective means of balancing energy supply and demand, the potential costs associated with reducing cellular and whole-body processes may threaten organismal fitness and preclude its selection in all but the most extreme environments.

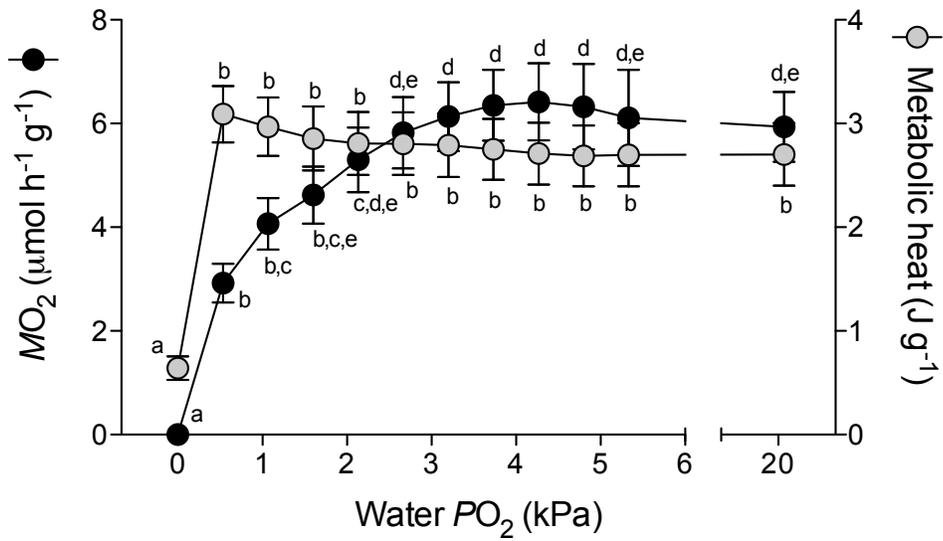


Figure 3-1. Closed-chamber calorespirometry measurements of $\dot{M}O_2$ and metabolic heat in goldfish. P_wO_2 was reduced from normoxia to anoxia over 60 to 90 min due to the fish's O_2 consumption. Data are mean \pm s.e.m, n=8. Data points sharing a letter are not significantly different (1-way ANOVA, $P>0.05$).

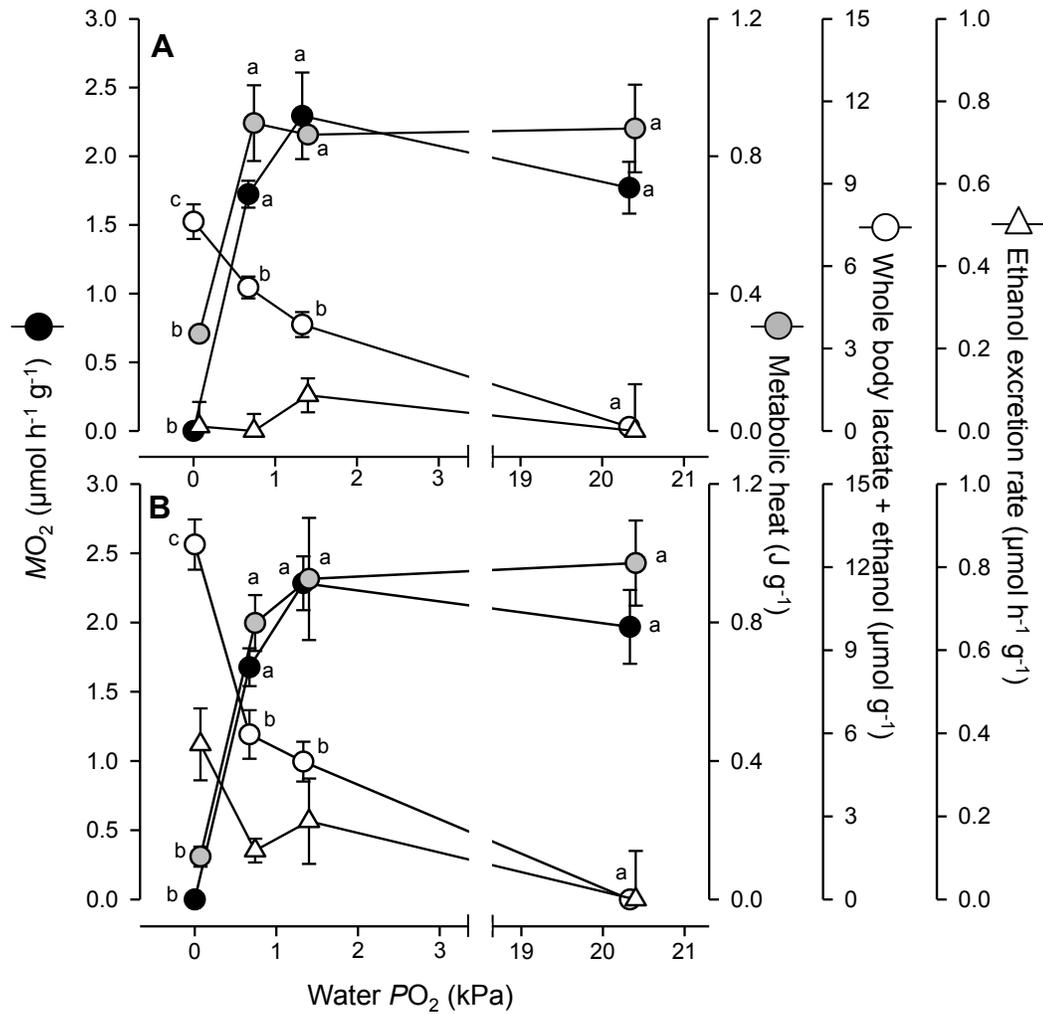


Figure 3-2. Flow-through calorimetry measurements of $\dot{M}O_2$, metabolic heat, and glycolytic end-products in goldfish held at different P_wO_2 s for 1 (A) and 4 (B) h. Data are mean \pm s.e.m, $n=3-6$, and data points are offset slightly on the X-axis for clarity. Data points sharing a letter are not significantly different (1-way ANOVA, $P>0.05$).

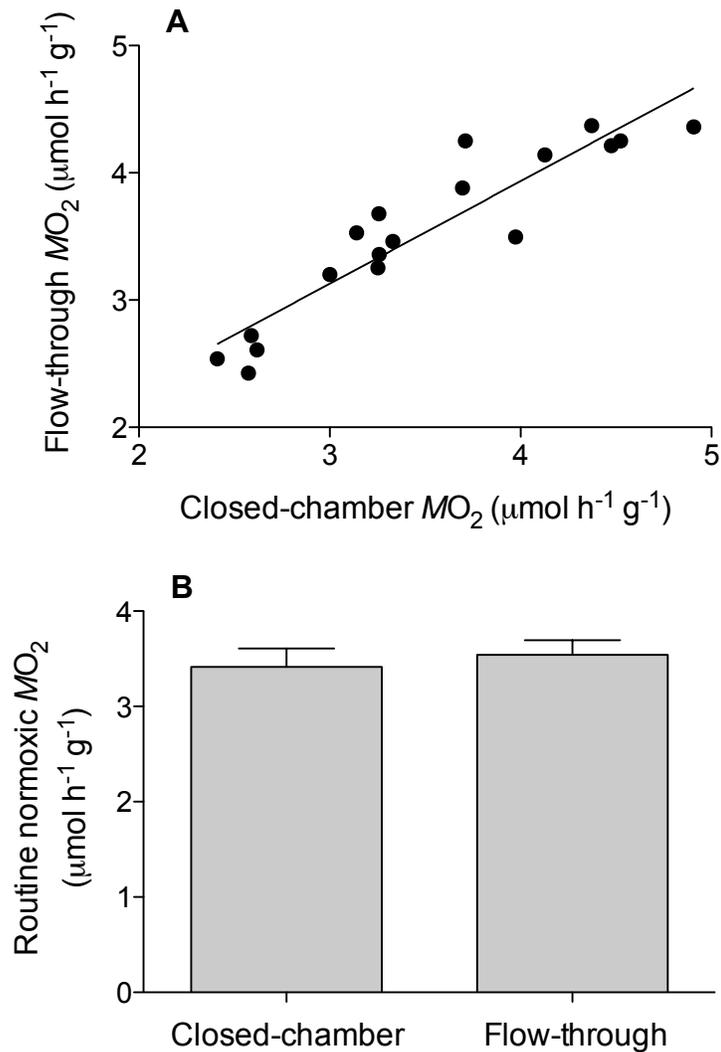


Figure 3-3. A comparison of $\dot{M}O_2$ measurements of goldfish made using closed-chamber and flow-through calorimetry. Both respirometric techniques were performed in the same apparatus following a ≥ 16 h habituation period, at the same time of day ($\sim 10:00$ am PST), and at $P_wO_2 \geq 16$ kPa. In (A), $\dot{M}O_2$ values resulting from closed and flow-through techniques, with measurements made back-to-back on the same fish ($n=18$, $r=0.925$, $P<0.0001$). In (B), average $\dot{M}O_2$ measurements for each technique, with error bars representing s.e.m. ($n=18$; $t=0.423$, $P=0.678$).

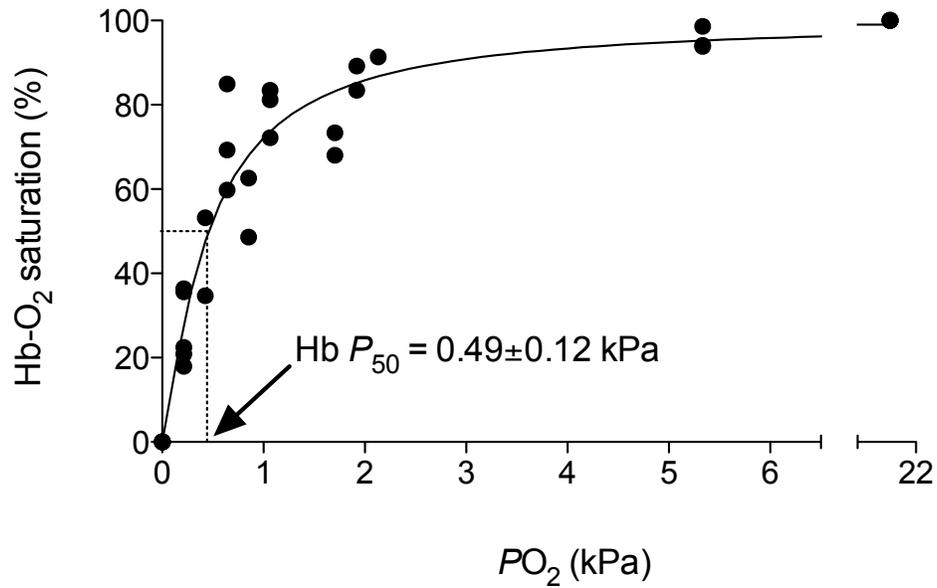


Figure 3-4. O₂ equilibrium curve for the whole blood of 5 normoxia-acclimated goldfish. Red blood cells were separated from plasma and resuspended in HEPES buffer (pH 7.8). O₂ levels were achieved using a Wösthoff gas mixing pump attached to cylinders of compressed O₂ and N₂.

Table 3-1. Whole body concentrations ($\mu\text{mol g}^{-1}$) of lactate and ethanol in goldfish exposed to different $P_w\text{O}_2$ s for different periods of time. Different letters indicate significant differences between time points within a $P_w\text{O}_2$ exposure ($P>0.05$). [Ethanol] measurements at 20 kPa were not taken.

		0 kPa	0.67 kPa	1.3 kPa	20 kPa
[Lactate]	0 h	1.20±0.40 ^a	0.22±0.18 ^a	0.53±0.47 ^a	0.21±0.20
	1 h	6.42±0.54 ^b	4.21±0.34 ^b	3.11±0.48 ^b	0.15±0.14
	4 h	10.6±0.93 ^c	4.89±0.86 ^b	4.52±0.72 ^b	0.11±0.15
[Ethanol]	0 h	0.61±0.11 ^a	0.74±0.14	0.87±0.20	-
	1 h	1.20±0.17 ^b	1.02±0.10	0.77±0.21	-
	4 h	2.24±0.20 ^c	1.07±0.10	0.45±0.08	-

Chapter 4

Rates of hypoxia induction alter mechanisms of O₂ uptake and the critical O₂ tension of goldfish

4.1 Summary

The rate of hypoxia induction (RHI) is an important but overlooked dimension of environmental hypoxia that may affect an organism's survival. I hypothesized that, compared with rapid RHI, gradual RHI will afford an organism more time to alter plastic phenotypes associated with O₂ uptake and subsequently reduce the critical O₂ tension (P_{crit}) of O₂ uptake rate ($\dot{M}O_2$). I investigated this by determining P_{crit} values for goldfish exposed to short (~24 min), typical (~84 min) and long (~480 min) duration P_{crit} trials to represent different RHIs. Consistent with my predictions, long duration P_{crit} trials yielded significantly lower P_{crit} values (1.0-1.4 kPa) than short and typical duration trials, which did not differ (2.6 ± 0.3 and 2.5 ± 0.2 kPa, respectively). Parallel experiments revealed these time-related shifts in P_{crit} were associated with changes in aspects of the O₂ transport cascade: gill surface areas and haemoglobin-O₂ binding affinities were significantly higher in fish exposed to gradual RHIs over 480 min than fish exposed to rapid RHIs over 60 min. My results also revealed that the choice of respirometric technique (*i.e.*, closed *versus* intermittent) did not affect P_{crit} or routine $\dot{M}O_2$, despite the significantly reduced water pH and elevated CO₂ and ammonia levels associated with closed-circuit P_{crit} trials of ~90 min. Together, these results demonstrate that gradual RHIs result in alterations to physiological parameters that enhance O₂ uptake in hypoxic environments. I therefore recommend rapid RHIs (<90 min) when determining an organism's innate P_{crit} so as to avoid the confounding effects of hypoxic acclimation.

4.2 Introduction

Environmental hypoxia is a common characteristic of many aquatic systems and is becoming increasingly prevalent, severe and long-lasting due to anthropogenic and climate change effects (Friedrich et al., 2014; IPCC, 2014; Schmidtko et al., 2017; Smith et al., 2006). Many studies have examined the physiological impacts of hypoxia exposure on a diverse array of fish species, but these studies have focused almost exclusively on either the severity of the hypoxic exposure (*i.e.*, water PO_2 , P_wO_2) or its duration. However, a third dimension of hypoxic exposure, the rate of hypoxia induction (RHI), has received very little attention and is rarely even controlled for (or at least reported) when environmental hypoxia is experimentally induced (Rogers et al., 2016). This is unlike other abiotic variables such as temperature, which are typically altered at consistent rates across studies (*e.g.*, $0.2\text{-}0.3^\circ\text{C min}^{-1}$ for the determination of critical thermal maxima; CTmax) due to the effects these rates have on organismal responses (*e.g.*, temperature tolerance in fishes; Mora and Maya, 2006). Similarly, RHIs may also influence the physiological responses of fishes to hypoxia, particularly time-dependent responses related to environmental O_2 extraction.

Most fishes possess mechanisms that enhance O_2 extraction and delivery to tissues as environmental PO_2 is reduced, such as increased haemoglobin (Hb) synthesis (Gracey et al., 2001) and concentration in the blood (Affonso et al., 2002), increased hematocrit (Lai et al., 2006; Turko et al., 2014), increased Hb- O_2 binding affinity (Turko et al., 2014), increased ventilation frequency and amplitude (Holeton and Randall, 1967; Itazawa and Takeda, 1978; Tzaneva et al., 2011; Vulesevic and Perry, 2006), and a redistribution of blood supply to critical tissues (Sundin et al., 1995). Some fishes, including the goldfish (*Carassius auratus*) and other species (Anttila et al., 2015; Borowiec et al., 2015; Crispo and Chapman, 2010; Dhillon et al., 2013; Ong et al., 2007; Turko et al., 2012), also have the ability to dramatically increase lamellar surface area in response to hypoxia exposure through apoptotic reductions to inter-lamellar cell mass (ILCM; Sollid et al., 2003). While these modifications to different parts of the O_2 transport cascade function to improve O_2 uptake at low P_wO_2 , the time-courses over which these modifications are enacted differ and may potentially impact the critical PO_2 (P_{crit}) of O_2 uptake rate ($\dot{M}O_2$).

P_{crit} is defined as the P_wO_2 at which a fish's $\dot{M}O_2$ transitions from being regulated at some stable level independent of P_wO_2 (*i.e.*, oxyregulation) to being dependent upon P_wO_2

(i.e., oxyconformation). The stable, oxyregulated $\dot{M}O_2$ typically represents the $\dot{M}O_2$ required to maintain the fish's standard metabolic rate (SMR) or routine metabolic rate (RMR). Standard metabolic rate is the $\dot{M}O_2$ of an awake, post-absorptive and entirely inactive ectothermic animal (Chabot et al., 2016), while RMR is $\dot{M}O_2$ under the same conditions, but also accounts for the small movements that are typical of fishes under experimental conditions (Chabot et al., 2016). At P_{crit} , the fish's aerobic scope is either at or near zero (for SMR- and RMR-based estimates, respectively), and at P_wO_2 s below P_{crit} , the fish's ability to generate ATP aerobically is limited (Farrell and Richards, 2009). P_{crit} therefore reflects a fish's ability to acquire and use environmental O_2 as a function of P_wO_2 , with a lower P_{crit} indicating a greater ability to extract O_2 to maintain aerobic metabolism in hypoxic environments. A low P_{crit} is beneficial because it allows the animal to maintain a routine level of function and activity in hypoxic environments while avoiding a reliance on anaerobic glycolysis and/or metabolic rate depression. Indeed, my results from Chapter 3 reveal that goldfish prioritize their use of aerobic metabolism in hypoxic environments over their exceptional ability to induce metabolic rate depression, which they reserve for anoxic environments. Goldfish also appear to enhance their ability to extract environmental O_2 over relatively short time periods in hypoxia, which in theory should result in a lowering of their P_{crit} value (Chapter 3). Because this ability is influenced by a suite of O_2 extraction mechanisms that are both plastic and time-dependent, I hypothesized that gradual RHIs would allow fish to induce plastic mechanisms that enhance O_2 extraction, resulting in lower P_{crit} values than those of fish exposed to rapid RHIs.

I tested this hypothesis by determining the P_{crit} values of goldfish exposed to progressive reductions in P_wO_2 (from normoxia to near-anoxia; referred to as P_{crit} trials) over different durations. P_{crit} is typically measured using closed-chamber respirometry, whereby P_wO_2 is decreased from normoxia to some hypoxic P_wO_2 by the fish's own respiration. The rate at which P_wO_2 decreases therefore depends on the fish's $\dot{M}O_2$ and the chamber volume, and these trials typically take 60 to 90 minutes to complete (Fry and Hart, 1948; Mandic et al., 2009; Rogers et al., 2016; Sollid et al., 2003; Speers-Roesch et al., 2011). In this study, I used different respirometric techniques to vary the duration of P_{crit} trials to determine the effects of rapid (~24 min), typical (~84 min) and gradual (~480 min) RHIs on the P_{crit} of goldfish. I supplemented my respirometry experiments with parallel hypoxic exposures of

different RHIs to investigate morphological and physiological traits of goldfish that might play causal roles in a time-related shift in P_{crit} . These analyses included: gill morphometrics to investigate changes in respiratory gas exchange surface area; whole blood [Hb] to investigate effects on O₂ carrying capacity; O₂ equilibrium curves (OECs) to investigate effects on Hb-O₂ binding affinity; red blood cell (RBC) organic phosphate (NTP; nucleoside triphosphates) concentrations to investigate effects on allosteric modulation of Hb-O₂ binding affinity; and lactate accumulation to investigate effects on anaerobic reliance. In addition to these biological assessments, my methods allowed me to address certain technical aspects of respirometry. Specifically, my use of different respirometric techniques allowed the effects of time and technique on the determination of P_{crit} to be disentangled, thus addressing a longstanding concern over the use of closed-chamber respirometry and its associated metabolic waste accumulation for the determination of P_{crit} (Keys, 1930; Rogers et al., 2016; Snyder et al., 2016; Steffensen, 1989). Finally, I chose goldfish as my study species because they have well-characterized responses to hypoxia exposure (Dhillon et al., 2013; Mitrovic et al., 2009), including a well-resolved P_{crit} as determined by closed-chamber respirometry (Chapter 3; Dhillon et al., 2013; Fry and Hart, 1948; Fu et al., 2011), that could aid my analysis of how RHI might influence the underlying physiology of P_{crit} .

4.3 Materials and methods

4.3.1 Animals

Goldfish (*Carassius auratus auratus*; 2.87±0.14 g wet mass; N=84; sex unknown) were purchased from a commercial supplier (The Little Fish Company, Surrey, BC, Canada) and held under a 12 h:12 h light:dark cycle in a series of 100 L recirculating systems of well-aerated, dechlorinated, 17°C water at the University of British Columbia (Vancouver, BC, Canada). Stocking density was <0.3 g L⁻¹ and water in the recirculating system was replaced weekly. Fish were fed to satiation daily (Nutrafin Max Goldfish Flakes) except for 24 h before transfer to the experimental apparatus, when feeding ceased. UBC's Animal Care Committee approved all procedures (protocol A13-0309).

4.3.2 Respirometry

I exposed goldfish to P_{crit} trials of short (~24 min), typical (~84 min, which represents the duration of a typical closed-chamber P_{crit} trial) and long (~480 min) durations to represent progressively reduced RHIs. These different RHIs were achieved using different respirometric techniques (details below), while the respirometer chambers, animal transfer protocol, habituation period, and mean fish mass remained consistent across all trials. Fish were only used once.

I used two 32 mL flow-through respirometer chambers made from stainless steel as described in Chapter 2. For each trial, I inserted a fish into the chamber and held them under flow-through conditions for at least 16 h prior to commencing the P_{crit} trial. The fish chamber was supplied with flow-through water at a rate of 190 mL h⁻¹ and maintained at 17°C. Inflowing water was drawn from a well-mixed reservoir held at ~26 kPa (manually controlled using compressed N₂ and O₂) and pumped to the respirometer chamber *via* a peristaltic pump (Gilson Minipuls 3, Middleton, WI, USA) through a combination of stainless steel tubing and gas-impermeable Tygon peristaltic tubing. The P_{wO_2} of the inflowing water was maintained slightly hyperoxic to ensure that the outflowing water was always at or slightly above normoxic P_{wO_2} . Following the habituation period, I conducted my respirometry experiments.

For the typical duration P_{crit} trials (84±8 min), I used closed-circuit respirometry. To start the trial, the inflowing and outflowing water supply lines were short-circuited so as to create a closed loop, with water recirculating through the chamber by the peristaltic pump at the same rate (190 mL h⁻¹) as during the habituation period to ensure minimal disturbance to the fish and good mixing of the chamber's water volume. Chamber P_{wO_2} was then allowed to decrease in proportion to the fish's $\dot{M}\text{O}_2$. An O₂ optode placed within the chamber (see Chapter 2) continuously measured P_{wO_2} , and $\dot{M}\text{O}_2$ was calculated according to

$$\dot{M}\text{O}_2 = (\Delta C_{\text{O}_2} \cdot \Delta T^{-1} \cdot V) M^{-1}$$

where C_{O_2} is O₂ content of the water converted to μmol L⁻¹ from P_{wO_2} using the solubility factor of 14.485 μmol L⁻¹ kPa⁻¹ (Boutilier et al., 1984), T is the time period over which the change in C_{O_2} is calculated (5 or 2 min; see below), V is the fish chamber volume (32 mL) plus the volume of the closed-circuit water lines minus the volume displaced by the fish itself, and M is the mass of the fish. The trials were ended when P_{wO_2} reached 0 kPa, at which point

the short-circuit was dismantled and flow-through conditions were reestablished to return chamber P_wO_2 to conditions similar to the habituation period.

For the short duration P_{crit} trials (24 ± 2 min), I again used closed-circuit respirometry as described for the typical P_{crit} trials. To shorten the trial and hasten the P_wO_2 decline, I made initial normoxic $\dot{M}O_2$ readings and then manually replaced the entire water volume of the respirometry chamber and its water supply lines over ~ 5 min with water equilibrated to 5.3 kPa P_wO_2 using a 60 mL syringe. Water P_wO_2 was therefore reduced from normoxia to ~ 5.3 kPa not by the fish's own $\dot{M}O_2$, but by the active replacement of the water volume. At this point, I attached the water supply lines to the peristaltic tubing, turned the pump back on to 190 mL h^{-1} , and allowed the fish to deplete the closed system's O_2 through its own respiration (typically over a ~ 20 min period). I chose 5.3 kPa as my replacement P_wO_2 for two reasons: first, it allowed reliable $\dot{M}O_2$ measurements to be made starting at ~ 4.8 kPa, which provided enough $\dot{M}O_2$ data points above P_{crit} to allow me to construct robust P_{crit} traces; second, the amount of time required for the fish to reduce P_wO_2 from 5.3 kPa to anoxia put the overall duration of these P_{crit} trials within my targeted duration of between 20 and 30 min. Although these procedures reduced the overall duration of the P_{crit} trial, I must point out that the RHI below 5.3 kPa was similar to that of the typical duration trials. If mechanisms of enhanced O_2 extraction are only induced a $P_wO_2 < 5.3$ kPa, then these two techniques could result in similar P_{crit} values.

Prior to actively replacing the water volume, I converted the system to closed-circuit and made a series of normoxic $\dot{M}O_2$ readings between 25 and 19 kPa to aid in my calculation of P_{crit} (see below). Upon reaching 19 kPa, I converted the system back to flow-through, reestablished a normoxic P_wO_2 of ~ 21 kPa, and then commenced the active water volume replacement.

For the long duration P_{crit} trials, I used three different respirometric techniques to ensure the mean P_{crit} values were the result of P_{crit} trial duration and not of the respirometric technique *per se*. These trials varied in average duration from 434 to 562 min depending on the technique used. I chose a time duration of ~ 480 min because it was significantly longer than the typical trial durations, but in line with some of the longer P_{crit} trial durations observed in the literature (see Rogers et al., 2016). For my first technique, I used closed-circuit respirometry where I added a 250 mL water reservoir to reduce the rate at which the

fish's respiration depleted the system's O₂. This reservoir was a glass bottle placed immediately after the peristaltic pump. Water leaving the respirometer chamber was pumped into the reservoir directly over a stir bar that mixed the water volume and prevented any O₂ stratification in the bottle. Water flowed out of the reservoir through a stainless steel line that punctured the bottle's rubber stopper and went directly into the stainless steel line supplying the respirometer chamber. All materials used were gas-impermeable glass or stainless steel. Attaching this reservoir to the closed-circuit system took ~2 min, after which the peristaltic pump was turned back on and the P_{crit} trial run according to the closed-circuit technique described for the typical duration P_{crit} trials. The average duration for these closed-circuit trials was 434±56 min.

Second, I used flow-through respirometry where $\dot{M}O_2$ was calculated according to

$$\dot{M}O_2 = ((C_{iO_2} - C_{oO_2}) \cdot FR) M^{-1}$$

where C_{iO_2} and C_{oO_2} are respectively O₂ content of inflowing and outflowing water converted from P_wO_2 as described above (I used a single PO_2 optode for these measurements), FR is water flow rate (190 mL h⁻¹), and M is the mass of the fish. I held each fish at 26, 16, 5.3, 2.7, 1.3, 0.7 and 0 kPa, each P_wO_2 in series, in that order and for 1 h, and at each P_wO_2 I measured $\dot{M}O_2$ at 10, 30 and 60 min (10 min was minimum time required to ensure P_wO_2 had equilibrated across the respirometer and the upstream and downstream P_wO_2 measurement chambers). Because the calculated $\dot{M}O_2$ at each P_wO_2 was nearly identical at each of the three time points, I averaged across the time points and calculated P_{crit} from those averaged $\dot{M}O_2$ values for each individual. The average duration for these flow-through trials was 562±19 min, including the time required to reach target P_wO_2 s.

Third, I used a variation on intermittent flow respirometry that combined flow-through and closed-circuit respirometry. I used flow-through conditions to manually reduce P_wO_2 from normoxia to ~2.8 kPa over ~430 min and then commenced a period of closed-circuit respirometry which took an additional ~15 min. I chose a target P_wO_2 of 2.8 kPa to start the closed-circuit portion of the trial based upon my earlier short-term P_{crit} trials (which used the same combined respirometric technique) that suggested I could reliably determine P_{crit} from this P_wO_2 . Upon reaching 2.8 kPa, I converted to the closed-circuit setup and allowed the fish's respiration to deplete the remaining O₂ in the closed system as described previously. This combination of techniques prevented metabolic waste accumulation. As

with the rapid RHI P_{crit} trials, I used closed-circuit respirometry to make a series of normoxic $\dot{M}O_2$ readings between 25 and 19 kPa prior to the active (but in this case gradual) reduction of P_wO_2 to aid in my calculation of P_{crit} (see below). Upon reaching 19 kPa, I converted the system back to flow-through, reestablished a P_wO_2 of ~21 kPa, and then commenced the active water volume replacement. The average duration of these combined flow-through and closed-circuit trials was 444 ± 12 min.

4.3.3 *Parallel hypoxic exposures for physiological measurements*

I ran two separate but identical parallel sets of hypoxic exposures to investigate potentially causal physiological and morphological factors in the time-dependent reduction in P_{crit} . These parallel exposures involved manually reducing P_wO_2 of aquaria from normoxia to anoxia over 60 and 480 min periods to represent rapid and gradual RHIs, respectively. I also ran normoxic control exposures during which P_wO_2 remained normoxic for 480 min following the habituation period. Each exposure was run in two 10 L aquaria housing four fish each, and each aquarium was fitted with a screen just below the water surface to prevent the fish from accessing the air-water interface. I mimicked the respirometric P_{crit} trials described above as closely as possible, with exposures being run at 17°C at the same time of day (each trial commenced at ~9:00am) following a >16 h habituation period, and the aquaria being covered with black plastic so the exposures were done in the dark. Fish from the first set of parallel exposures were sampled to assess gill morphology and haematological parameters, and fish from the second set of parallel exposures were sampled to measure plasma [lactate].

At the end of each exposure, fish were sacrificed by inconspicuously introducing anaesthetic (buffered MS-222, final concentration of 200 mg L^{-1}) to the water. Once the fish reached a surgical plane of anesthesia (~3 min, showed no response to tail pinching), they were individually removed, weighed, and then blood was sampled and gills dissected. To sample blood from the fish in the first set of parallel exposures, the fish's tail was severed and blood was collected from the caudal preduncle using a 60 μL heparinized capillary tube. Ten μL of blood was pipetted into 1 mL Drabkins reagent for determination of [Hb], 20 μL of blood was mixed with 10 μL of heparinized Cortland's saline plus 80 μL of 3% perchloric acid for determination of RBC [NTP], and 10 μL of blood was mixed with 5 μL of

heparinized Cortland's saline for determination of Hb-O₂ binding affinity. The entire right gill basket was then removed from the fish and immediately immersed in 1 mL of Karnovsky's fixative (25% glutaraldehyde, 16% formaldehyde, 0.15 mol L⁻¹ sodium cacodylate, pH 7.4). 24 h later, the gill basket was transferred to 0.15 mol L⁻¹ sodium cacodylate and stored at 4°C until use. This procedure was repeated for all four fish in each tank, and then duplicated for the second tank of four fish yielding $N=8$ for each treatment. For the second set of parallel exposures, fish were euthanized and blood was collected in the same manner as before, but the plasma was separated from the red blood cells by centrifugation and immediately assayed for plasma [lactate] (see below).

The goal of these parallel exposures was to allow me to assess the effects of RHI on morphological and physiological adjustments that may explain differences in P_{crit} , but there are differences between the P_{crit} trials and the parallel exposures that the reader should be made aware of. The main difference was vessel size (respirometer chamber was 32 mL and exposure aquaria were 10 L), which could have affected the ability of the fish to move throughout the exposure. However, observations of the fish in the 10 L aquaria suggest that goldfish do not increase activity during progressive hypoxia exposure. Furthermore, the parallel exposures were terminated when P_wO_2 reached 0 kPa. As the samples were taken at this point, the haematology and gill morphology measurements were not taken precisely at the point where I observed differences in P_{crit} , and this could affect my ability to relate the two studies. However, the fish used for the haematology and gill morphology analyses were only exposed to an additional ~7 to ~15 min of progressively deepening hypoxia (for rapid and gradual RHI, respectively) beyond what they had induced by the time P_{crit} had been reached. Thus I do not believe these relatively minor differences in time would affect my ability to directly relate these components of my study.

4.3.4 Gill morphometrics

Gill samples were randomly assigned an alphanumeric code by an independent party so analysis could be performed blindly. The second gill arch of each gill basket was isolated and its anterior hemibranch imaged using light microscopy (Olympus Stereomicroscope SZX10; 6.3× magnification, 10× zoom; image capture using cellSens Software). The images were used in combination with ImageJ v2.0.0 software to measure filament length and

number, and lamellar height (distance from base to the distal edge of the lamellae), length (distance lamellae runs along the filament) and frequency (number of lamellae per unit distance of filament). I made the lamellar measurements by dividing the length of the gill arch into five sections, then isolating a filament from each of these sections. Each filament was imaged from the top and the side, providing clear views of the height and length of its lamellae that I later measured. Specifically, I measured the height, length and width of three lamellae per filament (one from the filament's base, one from its middle and one from its tip), as well as the width of inter-lamellar channels in these three regions. I then estimated each filament's lamellar frequency (lamellae μm^{-1}) by dividing filament length by the sum of that filament's average channel and lamellar widths. Total lamellar surface area for each fish was then calculated according to,

$$\text{Total } L_{SA} = F_{SA} \cdot F_{\text{freq}} \cdot 16$$

where F_{SA} is the mean lamellar surface area of the five analyzed filaments, F_{freq} is the number of filaments per gill arch, and 16 is the product of 2 hemibranchs per gill arch, 4 gill arches per gill basket, and 2 gill baskets per individual fish (according to Wegner, 2011).

4.3.5 Blood analyses

Haemoglobin-O₂ binding affinity was determined within 60 min of blood sampling by constructing an OEC using the thin film spectrophotometric technique (Lilly et al., 2013) and a 96 well microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). A Wosthoff gas mixing pump (H. Wösthoff Messtechnik GmbH, Bochum, Germany) mixed compressed O₂ and N₂ to each of nine PO_2 s between 0 and 21 kPa PO_2 , always starting with 0 kPa and working toward 21 kPa, and each PO_2 was maintained for 20 min during which Hb-O₂ saturation was determined spectrophotometrically. A sigmoidal OEC was fit through the % Hb-O₂ saturation *versus* PO_2 data for each fish, and Hb P_{50} (the PO_2 at which Hb is 50% saturated with O₂) was determined using SigmaStat 11.0.

I measured whole blood [Hb] spectrophotometrically at 17°C and 540 nm after conversion to cyanomethemoglobin using Drabkin's reagent (Sigma-Aldrich). The measurements were made using a Shimadzu UV-160 spectrophotometer and a millimolar extinction coefficient of 11.

I measured red blood cell [NTP] spectrophotometrically at 17°C using the GAPDH- and PGK-catalyzed reactions converting glycerate 3-phosphate to glyceraldehyde 3-phosphate, where the oxidation of NADH to NAD⁺ was measured at 340 nm (Bergmeyer et al., 1983). The measurements were made using a 96 well microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Finally, I measured plasma [lactate] spectrophotometrically at 17°C using the LDH-catalyzed reaction converting lactate to pyruvate, where the reduction of NAD⁺ to NADH was measured at 340 nm (Bergmeyer, 1983). The measurements were made using a 96 well microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

4.3.6 *CO₂ and nitrogenous end-product measurements*

I ran a separate set of closed-circuit P_{crit} trials (91±10 min) to measure accumulated CO₂ and nitrogenous end-products (NH₃ + NH₄⁺). For each of four fish, I took water samples from the respirometer chamber at three time points: at the start of the habituation period, at the end of a 16 h habituation period immediately prior to starting the P_{crit} trial, and at the end of the P_{crit} trial as soon as the respirometer's P_{wO_2} reached 0 kPa. P_wCO_2 was determined using the Henderson-Hasselbalch equation and measurements of total CO₂ content in the water (CO₂ + HCO₃⁻; Corning 965 Carbon Dioxide Analyzer, Corning, NY, USA) and pH (probe: SaS gK2401C, Radiometer analytical, France; meter: VWR Symphony SB70P, VWR, Radnor, PA, USA). Total ammonia (NH₃ + NH₄⁺) was measured using an API ammonia test kit, and unionized ammonia (NH₃) was calculated from this value in combination with water pH and temperature (17°C).

4.3.7 *P_{crit} calculation*

P_{crit} is defined as the P_{wO_2} at which an organism's stable $\dot{M}O_2$ transitions from being independent of, to being dependent upon, P_{wO_2} . There are different methods to calculate P_{crit} , but analyses performed by Mueller and Seymour, (2011) suggest that most of the methods used yield comparable values. I therefore decided to use a variation on a two-segment linear regression model (details below) to identify P_{crit} as the P_{wO_2} at which the two linear trend lines (one representing the P_{wO_2} range of oxyregulation, the other of oxyconformation) intersect on a graph plotting $\dot{M}O_2$ as a function of P_{wO_2} (BASIC program of Yeager and

Ultsch, 1989). This method is employed widely throughout the literature (see Rogers et al., 2016) and has been used by myself and by others for goldfish (Chapter 3; Fu et al., 2011; Dhillon et al., 2013).

I calculated $\dot{M}O_2$ values by measuring the change in P_wO_2 over sequential time intervals, 5 min between 25 and 5.3 kPa and 2 min between 5.3 and 0 kPa. To standardize my estimates of a stable, oxyregulated $\dot{M}O_2$, I used the mean of each fish's calculated $\dot{M}O_2$ values between 21 and 18.7 kPa P_wO_2 . This represented a normoxic routine $\dot{M}O_2$ that was likely close to standard $\dot{M}O_2$ as a result of it being made following a ≥ 18 h habituation period. I then determined P_{crit} as the intersection of this horizontal line with a linear regression through the $\dot{M}O_2$ values that were $>15\%$ below the mean routine $\dot{M}O_2$ value. This technique was carried out according to McBryan et al. (2016).

4.3.8 Data analysis and statistics

I compared all average values of P_{crit} , normoxic $\dot{M}O_2$, blood properties, gill morphometrics and accumulated P_wCO_2 and nitrogenous end-products using one-way, two-tailed ANOVAs with a critical $\alpha=0.05$ (repeated measures for the water pH, P_wCO_2 and nitrogenous end-products comparisons). *Post hoc* Tukey tests were used to test for differences between treatment groups. Any data set that did not meet the assumptions of normality or equal variance was log-transformed prior to analysis. All analyses were performed using SigmaStat 11.0. Finally, values reported in the text are presented as means \pm sem.

4.4 Results

4.4.1 Respirometry

Long duration P_{crit} trials resulted in significantly lower P_{crit} values than typical and short duration P_{crit} trials (Fig. 4-1; Fig. 4-2A, ANOVA $P<0.001$). P_{crit} values determined by short and typical trial durations did not differ from one another, nor did the P_{crit} values determined by the three respirometric techniques used for the long duration trials (Fig. 4-2A). Each of the five respirometric techniques yielded statistically similar normoxic $\dot{M}O_2$ values (Fig. 4-2B; ANOVA $P=0.276$).

4.4.2 Effect of RHI on gill morphology

RHI had a significant effect on the mass-specific lamellar surface areas of goldfish (Fig. 4-3, ANOVA $P=0.004$), whereby fish exposed to gradual RHIs had significantly larger lamellar surface areas than fish exposed to rapid RHIs and normoxic controls, which did not differ.

4.4.3 Effect of RHI on Hb-O₂ affinity, [Hb] and RBC [NTP]

RHI had a significant effect on Hb-O₂ binding affinity (Fig. 4-4A,B; ANOVA $P=0.007$). Rapid RHI significantly reduced Hb-O₂ binding affinity relative to normoxic control values as determined by Hb P_{50} , while the gradual RHI had no significant effect.

RHI did not affect whole blood [Hb] (Fig. 4-5A; ANOVA $P=0.334$), but it did affect RBC [NTP] (Fig. 4-5B; ANOVA $P=0.001$), whereby gradual RHIs resulted in significantly lower RBC [NTP] than rapid RHIs and normoxic control exposures.

4.4.4 Effect of RHI on plasma lactate

Goldfish exposed to rapid and gradual RHIs both accumulated similar concentrations of plasma lactate to a level significantly higher than that observed in normoxic control fish (Fig. 4-5C; ANOVA $P=0.001$).

4.4.5 Metabolic end-product accumulation

Compared with the start of the habituation period, typical duration closed-circuit P_{crit} trials (91 ± 10 min) resulted in a ~2-fold increase in respirometer chamber P_wCO_2 over the course of the 16 h flow-through habituation period, and a ~13-fold increase over the course of the P_{crit} trial itself (Fig. 4-6A, ANOVA $P<0.001$). Water pH was concomitantly reduced from 7.61 to 6.93 over the course of the P_{crit} trial (Fig. 4-6B; ANOVA $P<0.001$). The concentration of total ammonia ($NH_3 + NH_4^+$) in the chamber also increased (Fig. 4-6C, ANOVA $P<0.001$). Unionized ammonia (NH_3) accumulated in a different way due to pH changes of the water, with $[NH_3]$ increasing ~3-fold over the 16 h habituation period, then falling to an intermediate value by the end of the P_{crit} trial habituation period (Fig. 4-6D, ANOVA $P<0.001$).

4.5 Discussion

I hypothesized that gradual RHIs would allow goldfish to induce time-dependent plastic phenotypes that enhance O₂ uptake. This hypothesis predicted that the P_{crit} of goldfish exposed to long-duration P_{crit} trials would be lower than those of goldfish exposed to short- or typical-duration P_{crit} trials, and my results agree with these predictions regardless of the respirometric technique used. Furthermore, my results suggest that this time-dependent shift in P_{crit} is the result of a greater lamellar surface area and a higher Hb-O₂ affinity in gradual RHI-exposed fish, leading to an enhanced ability to extract O₂ from hypoxic water. Taken together, these results suggest that time (more precisely, RHI) is a significant determinant of P_{crit} in goldfish.

The vast majority of P_{crit} measurements are made using closed-chamber respirometry over the course of 60 to 90 min (Rogers et al., 2016). Here, my representative closed-circuit P_{crit} trials lasted ~84 min and resulted in a P_{crit} of 2.5 ± 0.2 kPa (Fig. 4-2A). My values are in general agreement with the values previously reported for goldfish [~3.6 kPa (Fry and Hart, 1948); 3.0 kPa (Fu et al., 2011); 3.3 kPa (Dhillon et al., 2013); 3.0 kPa (Chapter 3)], though slightly lower owing to a possible combination of experimental temperature differences and the fact that my study used closed-circuit respirometry as opposed to static closed-chamber respirometry. Reducing the trial duration to ~24 min did not affect P_{crit} (Fig. 4-2A), which may not be surprising considering the RHI below a P_{wO_2} of 5.3 kPa was similar between the short and typical duration P_{crit} trials (see Materials & Methods for details). However, my results clearly indicate that increasing the trial duration from ~84 min (*i.e.*, reducing its RHI) to ~480 min resulted in significantly lower P_{crit} values (Fig. 4-2A). The reasons for this variation could be related to time, technique, or some combination of the two, and I will explore these possibilities below.

4.5.1 Effects of time on the physiology of O₂ uptake

Goldfish exposed to long duration P_{crit} trials displayed a greater ability to extract O₂ from hypoxic water than goldfish exposed to short and typical duration P_{crit} trials. In line with this, the physiological changes I observed in the fish from the parallel hypoxic exposures were consistent with the gradual RHI fish having improved O₂ extraction abilities compared with those of the rapid RHI fish.

Goldfish exposed to gradual RHIs had significantly larger lamellar surface areas than those of normoxic controls and goldfish exposed to rapid RHIs, which did not differ. Hypoxia-induced gill remodeling was first observed in goldfish and the closely-related crucian carp (*Carassius carassius*) 13 years ago (Sollid et al., 2003; Sollid et al., 2005) and in numerous fish species since [*e.g.*, mangrove killifish, *Kryptolebias marmoratus* (Ong et al., 2007; Turko et al., 2012); African cichlids (Crispo and Chapman, 2010); various carp species (Dhillon et al., 2013); Atlantic salmon *Salmo salar* (Anttila et al., 2015); Atlantic killifish *Fundulus heteroclitus* (Borowiec et al., 2015)]. Dhillon et al. (2013) observed a ~2-fold increase in the lamellar surface area of goldfish following 8 h acclimation to a constant P_wO_2 of 0.7 kPa, but to my knowledge my study is the first time gills have been shown to remodel over such short time scales under progressively decreasing P_wO_2 . Increases to lamellar surface area are typically the result of apoptotic reductions to the ILCM (Sollid et al., 2003). ILCM reductions *per se* also enhance the gill's diffusion capacity (Bindon et al., 1994; Greco et al., 1995) and contribute to a reduced P_{crit} in crucian carp (Sollid et al., 2003) and Atlantic killifish (Borowiec et al., 2015). However, in a study that examined (among other things) O_2 diffusion across the gills of two groups of goldfish with temperature-induced differences in gill surface area, the authors found that the differences in gill surface area had no effect on arterial PO_2 when acutely exposed to hypoxia (Tzaneva et al. 2011). While this seems to run counter to what Fick's first diffusion law would predict, the authors speculated that the goldfish that started hypoxia exposure with a smaller gill surface area may have been rapidly remodeling their gills to increase lamellar surface area over the course of the acute hypoxia exposure. My gill morphometric results lend empirical support to this speculation.

Goldfish exposed to gradual RHIs almost halved their Hb P_{50} values compared with goldfish exposed to rapid RHIs, and these values roughly correlated with P_{crit} : gradual RHI fish had an average Hb P_{50} of 1.6 kPa and an average P_{crit} of 1.2 kPa, while the rapid RHI fish had 2.4 and 2.5 kPa (for typical duration trials), respectively. This is consistent with the positive correlation of Hb P_{50} and P_{crit} shown across intertidal sculpin species by Mandic et al. (2009), and with the idea that the O_2 binding affinity of Hb is the main factor setting the environmental PO_2 when integrated physiological function begins to be lost (Farrell and Richards, 2009). But while the P_{50} values for the gradual RHI fish were lower than those for the rapid RHI fish, it was the rapid RHI fish that had significantly higher P_{50} values than both

the controls and gradual RHI fish. This suggests that rapid hypoxia induction reduces Hb-O₂ affinity, but gradual induction does not. The underlying mechanism(s) might involve RBC [NTP] and/or protons.

Nucleoside triphosphates (ATP and GTP, collectively NTPs) reduce Hb-O₂ binding affinity by binding to sites on the Hb tetramer that stabilize its deoxygenated conformation and consequently increase the P_{50} (Jensen et al., 1998; Wood and Johansen, 1972). The significantly lower [NTP]s of my gradual RHI fish (Fig. 4-5B) at least partly explain their lower Hb P_{50} values compared with those of the rapid RHI fish (Fig. 4-4), but the similar [NTP]s in the rapid RHI and normoxic control fish exclude this mechanism as the cause of the rapid RHI fish's elevated Hb P_{50} values. Another possibility is the goldfish's proton-sensitive Root effect Hbs (Rodewald and Braunitzer, 1984), whereby the higher P_{50} values in goldfish experiencing a rapid RHI might be the result of a more rapid accumulation of glycolytically-derived protons compared with goldfish experiencing a gradual RHI (as indicated by the similar plasma lactate accumulation following both the rapid and gradual RHI; Fig. 4-5C). However, if goldfish possess an adrenergically-activated RBC Na⁺/H⁺ exchanger like the closely related common carp (*Cyprinus carpio*) (Salama and Nikinmaa, 1988; Salama and Nikinmaa, 1989), then a reduced RBC pH may not explain the increased Hb P_{50} seen in my rapid RHI fish because such an exchanger would regulate RBC pH under hypoxic conditions. Regardless of the causal mechanism(s), the different Hb-O₂ binding affinities of the rapid and gradual RHI fish are likely to at least partly explain their different P_{crit} values.

Other factors besides lamellar surface area and Hb-O₂ binding affinity could potentially contribute to a time-dependent shift in P_{crit} , and one such factor may be reduced blood perfusion of the gills resulting in reduced O₂ uptake from the water. For example, common carp exposed to a rapid and progressive induction (<1 h) of severe hypoxia (to ~0.7 kPa) showed a 5-fold reduction in cardiac output with a nadir that coincided with the time and P_wO_2 of the fish's P_{crit} (Stecyk and Farrell, 2002). Similarly, dogfish (*Scyliorhinus canicula*) exposed to a rapid and progressive induction (rate/time domain not reported beyond "rapid") of hypoxia (to 4.0 kPa) showed an initial intense bradycardia that was not observed in dogfish exposed to a gradual induction of hypoxia, despite both groups stabilizing at the same heart rate following 30 min (Butler and Taylor, 1971). Finally, in two

elasmobranch species that differ in hypoxia tolerance [epaulette shark (*Hemiscyllium ocellatum*) and shovelnose ray (*Aptychotrema rostrata*)], the species-specific P_{crit} values coincided with the P_wO_2 s at which their cardiac output was reduced compared with normoxia-acclimated fish (Speers-Roesch et al., 2012a, 2012b). These authors also pooled literature values for various fish species and found a significant positive correlation between P_{crit} and the P_wO_2 s at the onset of hypoxic bradycardia (Speers-Roesch et al., 2012b). But while these authors highlight the dependence of cardiac function on P_{crit} of $\dot{M}O_2$, here I speculate the opposite; that is, P_{crit} may partially depend on cardiac function.

4.5.2 *Respirometric technique and waste accumulation*

Respirometric techniques can be broadly categorized as closed (closed-circuit or static closed-chamber), flow-through, or intermittent flow. Though none of these techniques are ideal for all experimental questions, intermittent flow respirometry is generally regarded as superior because it avoids the potential accumulation of metabolic end-products that can occur in closed respirometry and it has greater temporal resolution compared with flow-through respirometry (reviewed by Clark et al., 2013; Steffensen, 1989; Svendsen et al., 2016). It has been suggested that the choice of respirometric technique used to determine P_{crit} may influence the results, and indeed P_{crit} in shiner perch (*Cymatogaster aggregata*) shifted from ~9.9 kPa to ~6.1 kPa when using closed-chamber *versus* intermittent flow respirometry, respectively (Snyder et al., 2016). The authors attribute this to technique, but they also discuss the possibility that duration of the P_{crit} trials (~1 h for closed-chamber, ~5 h for intermittent flow) may play a role (Snyder et al., 2016). In the present study, I used modified versions of all three respirometric techniques for my long duration P_{crit} trials, and despite technique specific-differences and challenges [*e.g.*, flow-through trials demanded a step-wise reduction in P_wO_2 ; closed-circuit trials resulted in higher $\dot{M}O_2$ values in the mid- P_wO_2 range (Fig. 4-1)], each technique yielded nearly identical P_{crit} values, which were all lower than the typical or short duration P_{crit} trials. This suggests that the different P_{crit} values observed between my short and typical duration P_{crit} trials and those of the long duration trials are the result of RHI rather than technique, and this may also be the case with the results of Snyder et al. (2016). However, the fact remains that closed respirometry leads to end-product accumulation, which could still theoretically influence P_{crit} .

The buildup of metabolic wastes (CO₂ and ammonia) in closed-circuit respirometry has for many years been suggested to affect $\dot{M}O_2$ and consequently P_{crit} (Keys, 1930; Snyder et al., 2016; Steffensen, 1989). However, to my knowledge, ours are the first reported empirical measurements of these waste products following a closed-circuit P_{crit} trial. Chamber P_wCO_2 was determined to be 0.80 ± 0.02 kPa following typical RHI P_{crit} trials (Fig. 4-6A), and this value agreed with my original estimate of 0.71 kPa based on water volume, temperature, salinity and starting O₂ content, as well as fish size, average $\dot{M}O_2$, an RQ of 1 and a CO₂ solubility coefficient of $0.4224 \text{ mmol L}^{-1} \text{ kPa}^{-1}$ (from Boutilier et al., 1984). While this P_wCO_2 is certainly hypercapnic, it is much lower than the 5.3+ kPa typically tolerated by fishes (Baker et al., 2009; Baker et al., 2015; Brauner et al., 2004; Grøttum and Sigholt, 1996; Hayashi et al., 2004; McKenzie et al., 2002; Takeda and Itazawa, 1983). Furthermore, the three studies I know of that have measured P_{crit} as a function of hypercapnia present conflicting, but possibly P_wCO_2 -dependent, results. European eels (*Anguilla anguilla*) have shown both an increase in P_{crit} in hypercapnia (Cruz-Neto and Steffensen, 1997; P_wCO_2 of 2.7 and 4.0 kPa) and no effect (McKenzie et al., 2003; P_wCO_2 of 1.3 kPa), while Atlantic killifish and spot (*Leiostomus xanthurus*) have both shown no effect (Cochran and Burnett, 1996; max P_wCO_2 of 1.6 kPa). It therefore appears that P_{crit} in these species is impacted only by relatively high P_wCO_2 s. If this applies to goldfish too, then a P_wCO_2 of 0.80 kPa is unlikely to have a significant effect on P_{crit} . Finally, the most supportive piece of evidence for mild hypercapnia's limited effect on P_{crit} is the fact that my study's closed-circuit trials (where CO₂ accumulated) and combined flow-through/closed-circuit trials (where CO₂ did not accumulate) resulted in nearly identical P_{crit} values within the long duration P_{crit} trials (Fig. 4-2A).

I also measured ammonia accumulation following typical RHI closed-circuit P_{crit} trials. While the average total ammonia (NH₃ + NH₄⁺) concentration increased from 1.4 to 47 $\mu\text{mol L}^{-1}$ following the trial, the average concentration of NH₃ was not statistically different than the starting concentrations when water pH was taken into account (Fig. 4-6D). It is therefore unlikely that ammonia accumulation affected P_{crit} .

4.5.3 Implications and recommendations

The present study demonstrates that RHI influences the physiological responses of goldfish to hypoxia, which consequently influences their P_{crit} values. This influence can be substantial, and in my hands, reducing RHI with P_{crit} trials of ~480 min instead of ~84 min reduced the P_{crit} of goldfish by up to 2-fold. If this is broadly applicable to other species, then it could undermine recent P_{crit} -based modeling efforts that aim to understand how fishes will respond to an increasingly hypoxic world (Deutsch et al., 2015). This is in addition to already existing concerns regarding P_{crit} as an overall measure of hypoxia tolerance (Claireaux and Chabot, 2016; Rogers et al., 2016; Salin et al., 2015; Speers-Roesch et al., 2013).

The exceptional hypoxia tolerance of goldfish and the mechanisms that underlie it, particularly a highly plastic gill structure, may draw into question the general applicability of my results to other fish species. However, hypoxia-induced gill remodeling has now been described in at least eight species (see above for details), suggesting it may be a more widespread hypoxia-induced response of fishes than previously thought. In addition, and perhaps more importantly, many fish species (if not most or all) have the capacity to alter haematological parameters such as haematocrit and Hb-O₂ binding affinity over the course of hypoxia exposure (Wells, 2009), thus possibly affecting O₂ uptake and P_{crit} . The rates at which these physiological alterations are induced may vary among species, perhaps as a function of the RHI of their natural hypoxic habitats, but all species will likely acclimate to some extent. Thus, it is reasonable to suggest that RHI is an important factor to consider when examining the hypoxic responses of all fish species, but confirmation of this will require more work.

If my results are generally applicable across species, then it is important to consider the implications of RHI on the determination of P_{crit} . Because longer duration P_{crit} trials allow for some degree of acclimation that may consequently reduce P_{crit} , shorter duration P_{crit} trials likely best represent the innate abilities of a hypoxia-exposed fish to extract and use O₂ at the time of analysis. Thus, similar to the standardized rate of temperature change used when determining a fish's CT_{max}, an RHI should be chosen that is fast enough to avoid acclimation so as to capture the innate P_{crit} of the organism. Because my short (~24 min) and typical (~84 min) duration P_{crit} trials yielded nearly identical P_{crit} values for goldfish, P_{crit}

trials of <90 min are advised. In addition, my results also suggest that closed-chamber respirometry is appropriate for the determination of P_{crit} .

4.5.4 Conclusions

These results demonstrate that RHI significantly alters the P_{crit} of goldfish, whereby long duration P_{crit} trials (*i.e.*, gradual RHIs) yield lower P_{crit} values than short duration P_{crit} trials (*i.e.*, rapid RHIs). These reduced P_{crit} values are caused by time-dependent effects on mechanisms that enhance environmental O₂ extraction, including gill morphology and Hb-O₂ binding affinity. Thus, to determine the innate P_{crit} of a fish at a moment in time, I recommend shorter duration P_{crit} trials carried out using closed-chamber/circuit respirometry. This would avoid any effects of acclimation and remain consistent with the methods used for the majority of P_{crit} values in the literature.

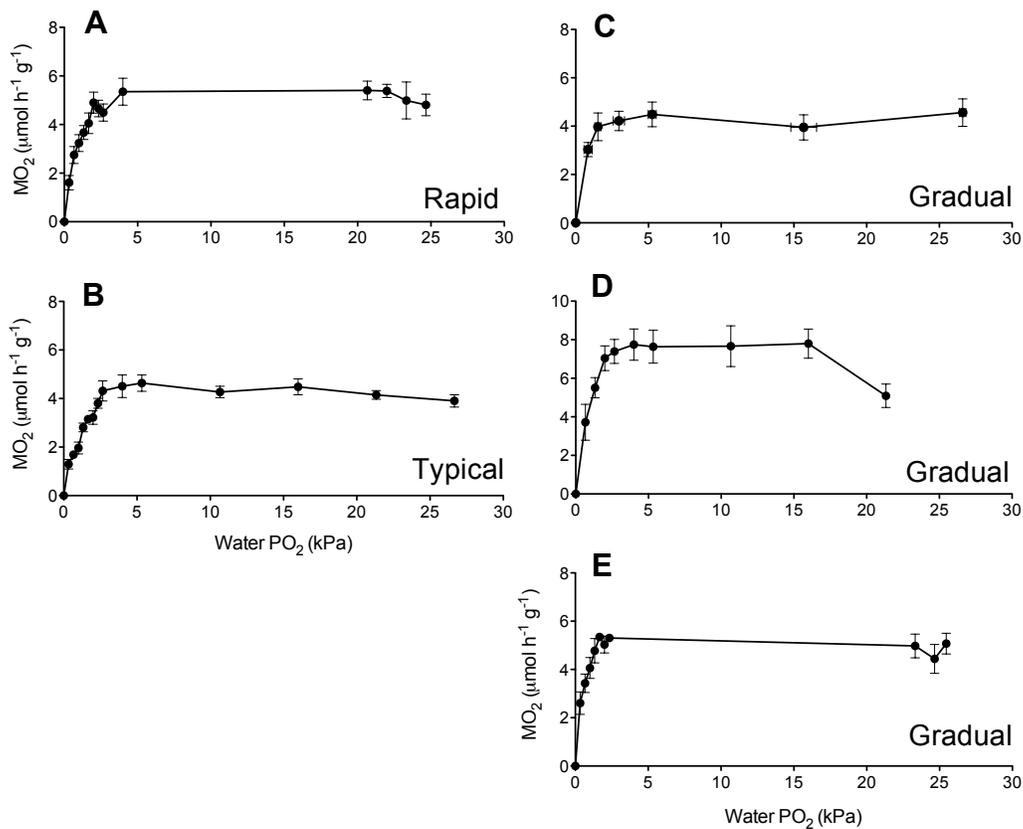


Figure 4-1. The effect of water PO_2 on the $\dot{M}O_2$ of goldfish exposed to rapid, typical and gradual rates of hypoxic induction. The different rates were achieved through different P_{crit} trial durations, and each of the five P_{crit} trails used a different respirometric technique to achieve its respective duration. (A) was achieved using a variation on intermittent flow respirometry ($N=5$; average duration 24 ± 2 min), (B) was achieved using closed-circuit respirometry ($N=6$; average duration 84 ± 8 min), (C) was achieved using flow-through respirometry ($N=6$; average duration 562 ± 19 min), (D) was achieved using closed-circuit respirometry with an additional water volume ($N=6$; average duration 434 ± 56 min), and (E) was achieved using a variation on intermittent flow respirometry ($N=4$; average duration 444 ± 12 min).

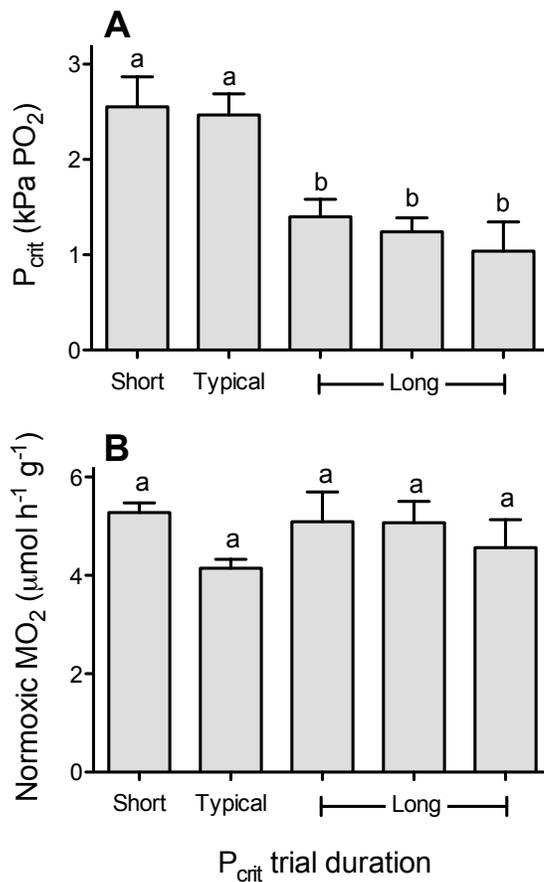


Figure 4-2. The effect of P_{crit} trial duration on the average P_{crit} and normoxic $\dot{M}O_2$ values of goldfish. (A) shows the average P_{crit} values of the individual fish comprising each set of respirometry experiments. (B) shows the average normoxic $\dot{M}O_2$ values of the individual fish comprising each set of respirometry experiments while those fish were exposed to normoxic P_wO_2 (18 to 26 kPa). Details on the respirometric techniques are included in the Materials and Methods, but briefly, “short” used combined flow-through/closed-circuit intermittent flow respirometry ($N=5$; 24 ± 2.2 min), “typical” used closed-circuit respirometry ($N=6$; 84 ± 8 min), and “long” from left to right used closed-circuit respirometry ($N=6$; 434 ± 56 min), combined flow-through/closed-circuit intermittent flow respirometry ($N=4$; 444 ± 12 min), and flow-through respirometry ($N=6$; 562 ± 19 min). Error bars are s.e.m., and bars that share a letter are not significantly different [1-way ANOVA, $P < 0.001$ for (A), and $P = 0.276$ for (B)].

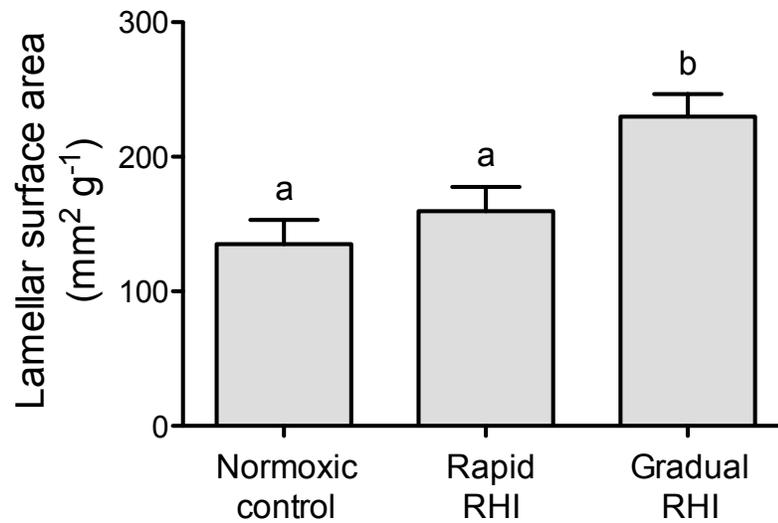


Figure 4-3. The effect of the rate of hypoxia induction on the mass specific lamellar surface area of goldfish. Mass-specific lamellar surface areas of goldfish exposed to rapid and gradual RHIs (normoxia-to-anoxia in 60 and 480 min, respectively), and normoxic controls ($N=8$ for each; 1-way ANOVA, $P=0.004$). Error bars are s.e.m., and bars that share a letter are not significantly different.

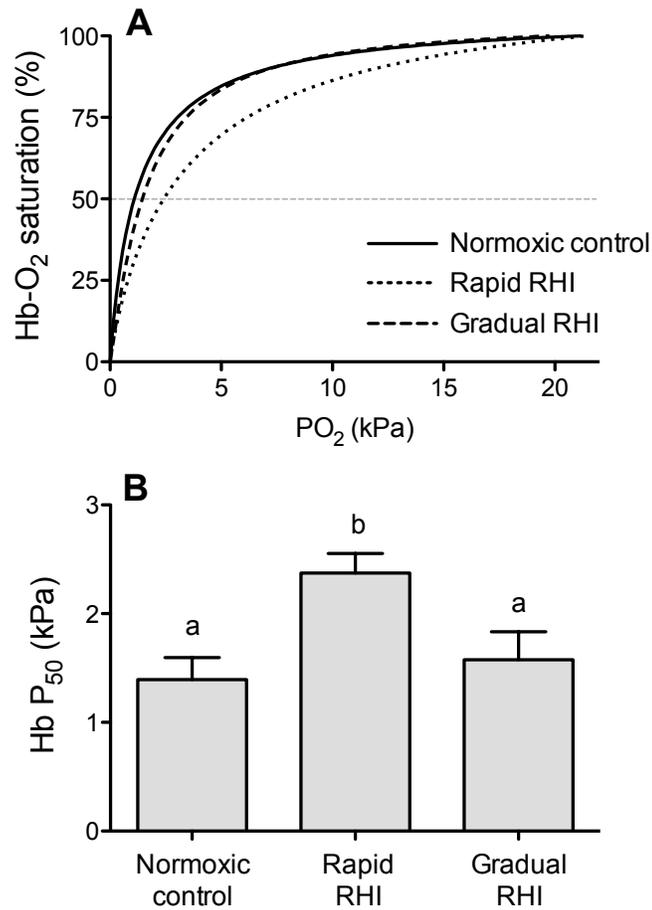


Figure 4-4. The effect of the rate of hypoxia induction on the O₂ equilibrium curve and Hb P₅₀ value of goldfish. . (A) shows the O₂ equilibrium curves (OECs) for the extracted whole blood of goldfish exposed rapid and gradual RHIs (normoxia-to-anoxia in 60 and 480 min, respectively), and normoxic controls. The blood was collected from the fish immediately upon P_wO_2 reaching ~0 kPa (for rapid and gradual RHIs), and the spectrophotometric determination of Hb-O₂ saturation was begun as soon thereafter as possible (<1 h). Each OEC is a trendline through the data points of eight blood samples exposed to nine P_wO_2 s between 0 and 21 kPa PO₂. Grey horizontal dashed line highlights the 50% Hb-O₂ saturation point. (B) shows the average Hb P₅₀ values (PO₂ at which Hb is 50% saturated with O₂) for each treatment group (1-way ANOVA, P=0.007). Error bars are s.e.m., and bars that share a letter are not significantly different.

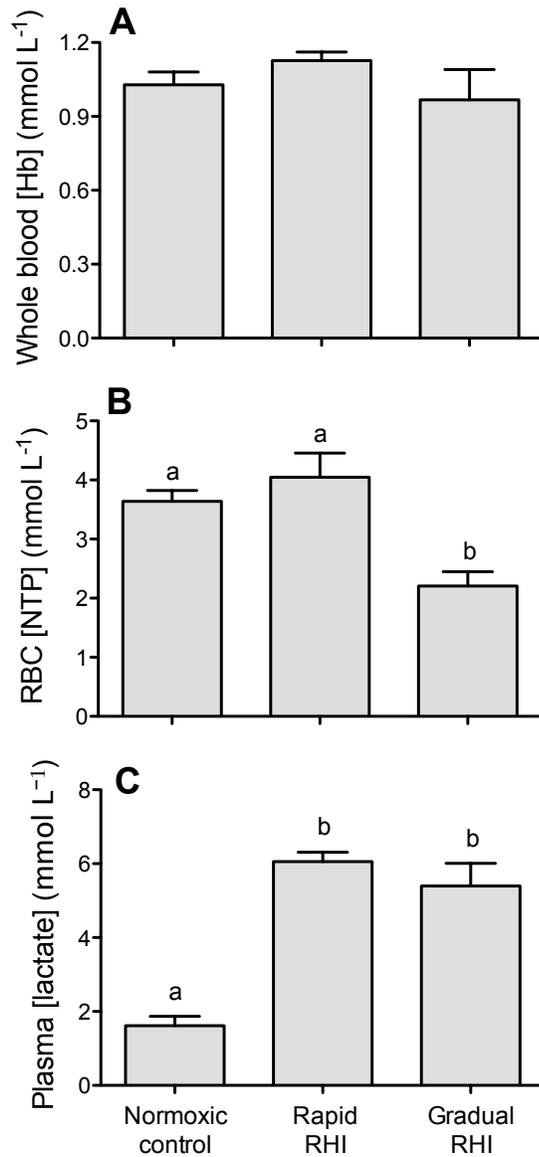


Figure 4-5. The effect of the rate of hypoxia induction on blood parameters of goldfish.

(A) shows average values for whole blood [Hb] measured spectrophotometrically using Drabkins reagent ($N=6-8$; 1-way ANOVA, $P=0.334$). (B) shows average values for RBC [NTP] ($N=6-8$; 1-way ANOVA, $P=0.001$). (C) shows average values for plasma [lactate] ($N=6-8$; 1-way ANOVA, $P=0.001$). Error bars are s.e.m., and bars that share a letter are not significantly different.

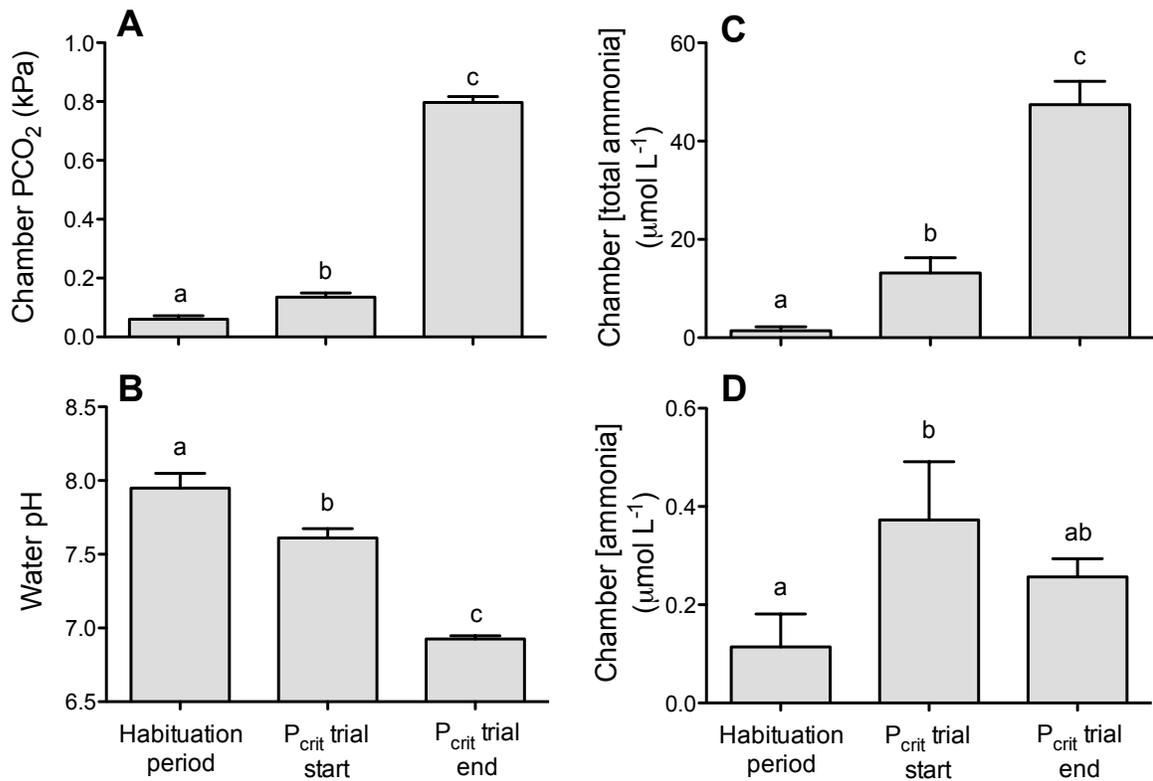


Figure 4-6. The effects of closed-circuit respirometry on water chemistry and the buildup of metabolic wastes. (A) shows chamber $P_w\text{CO}_2$, measured as total CO_2 and converted to $P_w\text{CO}_2$ using water pH and the Henderson-Hasselbalch equation ($N=4$; 1-way ANOVA, $P<0.001$). (B) shows water pH ($N=4$; 1-way ANOVA, $P<0.001$). (C) shows total ammonia concentration ($\text{NH}_3 + \text{NH}_4^+$; $N=4$; 1-way ANOVA, $P<0.001$) and (D) shows unionized ammonia concentration, calculated using water pH and temperature ($N=4$; 1-way ANOVA, $P<0.001$). Error bars are s.e.m., and bars that share a letter are not significantly different.

Chapter 5

Metabolic depression and the rapid evolution of hypoxia tolerance in threespine sticklebacks, *Gasterosteus aculeatus*

5.1 Summary

Anthropogenic increases in global temperature and agricultural runoff are increasing the prevalence of aquatic hypoxia throughout the world. If aquatic animals like fishes are to continue living where they are, they will need to enhance their hypoxia tolerances over relatively short timescales. I investigated the potential for a relatively rapid evolution of hypoxia tolerance using two isolated (for <11,000 years) populations of threespine sticklebacks, one from a lake that experiences long-term hypoxia (Alta Lake, British Columbia) and one from a lake that does not (Trout Lake, British Columbia). Loss-of-equilibrium experiments revealed the Alta Lake sticklebacks are significantly more tolerant of hypoxia than the Trout Lake sticklebacks, and the enhanced tolerance of Alta Lake sticklebacks is associated with an ability to employ metabolic rate depression (MRD) in hypoxia, something the Trout Lake fish do not do. The two populations do not differ in their capacities for O₂ extraction or anaerobic metabolism. These results reveal that significant intraspecific variation in hypoxia tolerance can evolve over relatively short timescales, as can MRD, a complex biochemical response that may be favoured in long-term hypoxic environments. Sticklebacks represent a powerful model to investigate the mechanisms and evolution of hypoxia tolerance in an increasingly hypoxic world.

5.2 Introduction

The world's aquatic environments are becoming increasingly hypoxic as a result of elevated water temperatures and increased agricultural runoff (IPCC, 2014; Schmidtko et al., 2017; Smith et al., 2006). Because animals rely on O₂ to supply sufficient ATP to meet their metabolic demands, environmental hypoxia threatens the animal's ability to maintain energy balance, homeostasis and, consequently, life. This threat is compounded in ectothermic animals like fishes by the concomitant increase in metabolic demand that necessarily accompanies rising temperatures. However, many fishes have evolved mechanisms of hypoxia tolerance as a result of adapting to environments that already experience hypoxia (Hochachka and Lutz, 2001), and we can look to the strategies of these species to better understand what might facilitate or hinder an animal's ability to adapt to a changing world.

Like all animals, fishes have three metabolic mechanisms for balancing energy supply and demand in hypoxia: aerobic metabolism, anaerobic metabolism (primarily in the form of anaerobic glycolysis) and metabolic rate depression (MRD). These mechanisms can be combined in different ways to maintain cellular energy balance, and because hypoxic environments vary in their hypoxic severity, duration and rate of induction, the combination a species uses to survive hypoxia likely stems from the unique hypoxic environment to which that species is adapted.

Fish species that are native to environments that regularly become hypoxic tend to be more tolerant of hypoxia than fish species that are native to less hypoxic environments (Chapman et al., 2002; Mandic et al., 2009b). This relationship may also exist among different populations of the same species as it does for other stressors like temperature (*e.g.*, Fanguie et al., 2006), and in fact two subspecies of Atlantic killifish (*Fundulus heteroclitus*) have recently been shown to differ in hypoxia tolerance (McBryan et al., 2016). If intraspecific variation in hypoxia tolerance exists, defining the underlying mechanisms in the context of the different populations' natural environments could reveal much about how the hypoxic environment shapes the hypoxic survival strategy.

To address this, I collected threespine sticklebacks (*Gasterosteus aculeatus*) from isolated populations native to two British Columbia lakes: Alta Lake, which experiences long-term hypoxic bouts due to overwinter freezing (Dunnington et al., 2016; Jacques Whitford/AXYS, 2007), and Trout Lake, which does not experience long-term hypoxia. I

predicted that Alta Lake sticklebacks would be more hypoxia tolerant than Trout Lake sticklebacks, and that this difference would result from an increased reliance on MRD in the Alta Lake fish. I predicted MRD to be the causal mechanism because of its effectiveness at maintaining cellular energy balance during long, deep hypoxic bouts (Hochachka et al., 1996) and its prevalence of use among ectothermic vertebrate species that inhabit similar winter environments (Chapter 3; Jackson, 1968; Johansson et al., 1995). To test this hypothesis, I used time-to-loss of equilibrium (LOE) experiments to assess the hypoxia tolerances of the two stickleback populations. I also used calorimetry to simultaneously measure the fish's use of aerobic metabolism and MRD at severely hypoxic O_2 tensions (P_wO_2), and then ran parallel hypoxic exposures to measure the anaerobic contributions to metabolic rate. I chose threespine sticklebacks as my model species because they are a well-studied example of local ecological adaptation, having invaded coastal British Columbia freshwater habitats ~11 000 years ago following the retreat of the Pleistocene glaciers (Bell and Foster, 1994; Jones et al., 2012; Mathews and Fyles, 1970; McPhail, 1994). As the land rebounded, the populations became isolated from one another in separate freshwater systems and consequently evolved, both in parallel and divergently, at physiological, morphological and behavioural levels (Boughman, 2001; Colosimo et al., 2005; Dalziel et al., 2012b; DeFaveri and Merila, 2014; Jones et al., 2012). In my study, I exploited the different O_2 regimes of Alta Lake and Trout Lake to investigate variation in hypoxia tolerance and the mechanisms that underlie it.

5.3 Materials and methods

5.3.1 Lakes

Alta Lake (Whistler, BC, Canada; 50°11'42"N 122°98'11"W) and Trout Lake (Sechelt, BC, Canada; 49°50'82"N 123°87'64"W) are similar oligotrophic water bodies (Table 5-1) that differ in elevation and subsequently the number of days each lake is covered in ice each year. Alta Lake is surface-frozen for $128 \pm 3.64 \text{ d y}^{-1}$, while Trout Lake does not freeze.

5.3.2 Field collection and husbandry

I used minnow traps to collect sticklebacks from both lakes. Traps were placed on the lake bottom at 1 to 2 m depth and 3 to 5 m offshore, far from tributaries. Fish for the calorespirometry experiments were collected in October 2015, and fish for the parallel hypoxic exposures and LOE trials were collected in May 2016.

I transported the fish to The University of British Columbia and held them in 100 L recirculating aquaria at a density of $<0.3 \text{ g L}^{-1}$ and under 12h:12h light:dark. Dechlorinated Vancouver tap water was aerated and held at 17°C , and 25% water changes were carried out every two weeks. Fish were fed bloodworms (Hikari Bio-Pure) daily to satiation and were held for 3 weeks prior to running experiments to allow adjustment to laboratory conditions of diet and light cycle. I held the water and laboratory temperature at 17°C because I had previously determined this to be the best temperature at which to run calorespirometry experiments (see Chapter 2).

5.3.3 Hypoxic exposures, calorespirometry and time-to-LOE

I withheld food from fish for 24 h and then transferred them to a custom designed calorespirometer (Chapter 2). Fish were allowed to habituate for 18 h after which measurements of normoxic routine $\dot{M}\text{O}_2$ and metabolic heat were measured as in Chapter 3. The $P_{\text{w}}\text{O}_2$ in the calorespirometer chamber was then reduced from normoxia to 2.8 kPa over 1 h using compressed N_2 , and measurements of metabolic heat and flow-through $\dot{M}\text{O}_2$ were made. I chose a hypoxic $P_{\text{w}}\text{O}_2$ of 2.8 kPa based on preliminary O_2 LC_{50} experiments that revealed 2.8 kPa to be the lowest $P_{\text{w}}\text{O}_2$ at which $\geq 75\%$ of sticklebacks survived 8 h of exposure. The hypoxia exposure was maintained for 4 h and then normoxia was re-established for a period of 2 h to allow for recovery in the calorespirometer. Following the recovery period, I then performed closed-chamber respirometry to determine the fish's critical O_2 tension (P_{crit}), which is the hypoxic $P_{\text{w}}\text{O}_2$ below which the fish is unable to extract sufficient environmental O_2 to support a stable $\dot{M}\text{O}_2$. This stable $\dot{M}\text{O}_2$ can represent either standard metabolic rate (*i.e.*, the $\dot{M}\text{O}_2$ of an awake, post-absorptive and entirely inactive ectothermic animal in the thermoneutral zone; Chabot et al., 2016) or routine metabolic rate (*i.e.*, $\dot{M}\text{O}_2$ under the same conditions as standard metabolic rate but accounting for the small movements that are typical of fishes under experimental conditions). In this

chapter, I represented this stable range of $\dot{M}O_2$ as routine $\dot{M}O_2$, and calculated it (and P_{crit}) according to Chapter 4.

A parallel set of experiments in which fish were held in opaque 10 L tanks was then carried out for the measurement of whole body concentrations of glycogen (glycolytic fuel) and lactate (glycolytic end-product). Each tank held four fish and, following an 18 h normoxic habituation period, P_wO_2 was adjusted in two tanks to 2.8 kPa ($N=8$) over 1 h and the other two remained normoxic to serve as controls ($N=8$). I repeated this for Alta Lake and Trout Lake fish. At 4 h, I inconspicuously introduced a lethal dose of anaesthetic (buffered MS-222, 0.3 g L^{-1}) and once the fish were unresponsive (~ 4 min), I removed, weighed and froze each fish in liquid N_2 for later metabolite analyses.

I measured hypoxia tolerance of the two populations by determining the time-to-LOE at a P_wO_2 of 1.3 kPa. Initial experiments to determine time-to-LOE were conducted at 2.8 kPa, but because Alta Lake fish survived for >72 h (Trout Lake fish survived <12 h), I decided to run the trials at 1.3 kPa instead. I ran these trials for each population by placing four fish in each of four tanks ($N=16$). Following an 18 h normoxic habituation period, I reduced P_wO_2 to 1.3 kPa over 1 h. I defined time-to-LOE as the time it took after the tank had reached 1.3 kPa for the fish to lose dorsal-ventral equilibrium and become unresponsive to gentle tail prods using a blunt dissection probe. When this point was reached, I removed the fish from the aquarium using a small dipnet, weighed the fish, and transferred it to a well-aerated recovery aquarium.

5.3.4 Metabolite assays

I measured changes in glycogen and lactate concentrations at the whole body level to be consistent with my whole body measurements of metabolic heat and $\dot{M}O_2$. I prepared whole bodies for metabolite extraction according to Chapter 3 and measured glycogen and lactate according to (Bergmeyer et al., 1983).

5.3.5 Statistical analyses

Statistical analyses were performed using SigmaStat 11.0. I used 2-way ANOVAs to analyze anaerobic metabolite concentrations and normoxic $\dot{M}O_2$ values, and 2-way repeated measures ANOVAs to analyze calorespirometry measurements of $\dot{M}O_2$ and metabolic heat.

The latter were also analyzed within each population (so as to identify potential use of MRD) using 1-way repeated measures ANOVAs with Tukey *post hoc* tests. P_{crit} and time-to-LOE were analyzed using two-tailed t-tests. Any data set that did not meet the assumption of homogeneity of variance was log transformed prior to analysis. Differences were considered to be significant at $P < 0.05$. All values are presented as means \pm sem.

5.4 Results

Time-to-LOE at a P_{wO_2} of 1.3 kPa was twice as long in Alta Lake sticklebacks than Trout Lake sticklebacks (t-test, $P < 0.001$, Fig. 5-1).

Closed-chamber respirometry revealed that both populations displayed a typical two-phase response in $\dot{M}O_2$ as P_{wO_2} was decreased. A stable routine $\dot{M}O_2$ was regulated and maintained at P_{wO_2} between 21 and 3.4 kPa in the Alta Lake sticklebacks, and between 21 and 4.1 kPa in the Trout Lake sticklebacks (Fig. 5-2A). At P_{wO_2} s below these values, $\dot{M}O_2$ then decreased with decreasing P_{wO_2} (Fig. 5-2A). Routine $\dot{M}O_2$ assessed under normoxic conditions (between 18 and 21 kPa) did not differ as a function of population or respirometric technique (2-way ANOVA, $P = 0.075$ and $P = 0.135$, respectively; interaction $P = 0.337$; Fig. 5-2B). Similarly, P_{crit} did not differ significantly between the two populations (t-test, $P = 0.245$, Fig. 5-2C), though these negative P_{crit} results should be interpreted with caution due to the small sample size and resulting low statistical power (0.100).

Analysis of the calorimetry results revealed that the time spent at 2.8 kPa P_{wO_2} had a significant effect on both metabolic heat ($P = 0.029$) and $\dot{M}O_2$ ($P = 0.007$), but the lake of origin did not ($P = 0.128$ and 0.285 for metabolic heat and $\dot{M}O_2$, respectively; interactions $P = 0.146$ and $P = 0.204$, respectively). Exploring each population's capacity for MRD using 1-way ANOVAs revealed that the Trout Lake fish showed no significant change in metabolic heat compared with normoxic levels ($P = 0.9634$, Fig. 5-3A), and a modest reduction in $\dot{M}O_2$ at 1 h of hypoxic exposure that returned to normoxic levels for the subsequent 3 h ($P = 0.009$, Fig. 5-3B). However, the Alta Lake fish showed significant reductions in both heat ($P < 0.001$, Fig. 5-3A) and $\dot{M}O_2$ ($P = 0.003$, Fig. 5-3B), with a $\sim 33\%$ reduction in metabolic heat relative to normoxic levels by 4 h exposure.

Alta Lake sticklebacks had significantly higher whole body concentrations of glycogen ($P = 0.044$, Table 5-2) than Trout Lake sticklebacks, but these concentrations were

unaffected by hypoxic exposure ($P=0.355$; interaction $P=0.292$; Table 5-2). Consistent with this, whole body concentrations of lactate did not differ as a function of population or hypoxic exposure ($P=0.461$ and $P=0.068$, respectively; interaction $P=0.878$; Table 5-2).

5.5 Discussion

I predicted that sticklebacks native to an environment that experiences long-term hypoxia (Alta Lake) would be more tolerant of hypoxia than sticklebacks native to an environment that does not experience long-term hypoxia (Trout Lake), and that this difference would result from an increased reliance on MRD in the Alta Lake fish. The results agree with these predictions. The time-to-LOE results reveal that Alta Lake sticklebacks are approximately twice as tolerant of hypoxia than Trout Lake sticklebacks (Fig. 5-1), and the calorimetry results suggest that this difference arises from the Alta Lake sticklebacks' ability to employ MRD in hypoxic environments, something the Trout Lake sticklebacks do not do (Fig. 5-3). Furthermore, the P_{crit} analyses suggest that the two populations do not differ significantly in the P_wO_2 at which their $\dot{M}O_2$ s start to decline (Fig. 5-2C), and the metabolite analyses suggest that, apart from slightly higher fuel reserves in the Alta Lake fish, the two populations show a similar capacity for and reliance on anaerobic glycolysis (Table 5-2).

5.5.1 Metabolic rate depression and hypoxia tolerance

Alta Lake sticklebacks reduced metabolic rate by ~33% relative to normoxic routine values by 4 h exposure to 2.8 kPa P_wO_2 (Fig. 5-3A). At this same time point and P_wO_2 , Trout Lake sticklebacks had a total metabolic rate approximately twice that of the Alta Lake fish (Fig. 5-3A), and this difference agrees well with the 2-fold longer tolerance time of the Alta Lake fish in the LOE experiment (Fig. 5-1). An MRD of 33% is similar in magnitude to the MRD measured in tilapia (*Oreochromis mossambicus*) at 3.1 kPa P_wO_2 (19% reduction; van Ginneken et al., 1997), and is greater than that measured in goldfish (*Carassius auratus*) at similar P_wO_2 s (Chapter 3; van Ginneken et al., 2004), but is modest compared to the ~80% MRD measured in anoxic goldfish (Chapters 2, 3; Addink et al., 1991; van Ginneken et al., 2004). An MRD of 33% would still greatly benefit hypoxic survival by reducing rates of anaerobic fuel use and deleterious end-product accumulation, and is a magnitude beyond

what other hypoxia-native fish species like zebrafish (*Danio rerio*) are capable of (Stangl and Wegener, 1996). Furthermore, because my analyses were carried out at 17°C (to maximize calorimetric signal:noise) instead of the near-freezing water temperature that would occur in Alta Lake in the winter (see Table 5-1), the 33% MRD measured in my study likely underestimates the total metabolic savings accrued by the Alta Lake fish under long-term hypoxic conditions in the wild. For example, if we assume a 10°C reduction in temperature and a typical Q_{10} for metabolic rate of 2.5 [though $\dot{M}O_2$ measurements on brook stickleback (*Culaea inconstans*) at 5°C and 15°C reveal a Q_{10} of ~4.5 (Klinger et al., 1982)], the total metabolic savings accrued by Alta Lake stickleback during winterfreeze would be ~75%. Larger temperature reductions and Q_{10} values, both plausible, would result in greater savings.

To my knowledge, these results represent the first time MRD use has been shown to vary significantly among geographically isolated populations of the same species. While MRD is believed to be a complex biochemical phenotype requiring a reorganization of cellular processes, the fact that the Alta Lake and Trout Lake populations have been isolated for a maximum of 11 000 y (Bell and Foster, 1994; Mathews and Fyles, 1970) suggests that it is a phenotype that can evolve rapidly. This is consistent with the rapidity with which marine sticklebacks have been shown to naturally evolve their freshwater-distinctive, genetically based morphological features *de novo* (<50 y; Lescak et al., 2015). However, an intriguing alternative hypothesis might be that MRD is a developmentally plastic neonatal characteristic of the Alta Lake fish, one that is developmentally lost in the Trout Lake fish. Similar developmental plasticity has been shown in the hypoxic ventilatory responses (HVR) of mammals, where exposure to hypoxia during a critical time window following birth can alter the HVR in adulthood [see Teppema and Dahan (2010) for review]. Support for this applying to MRD in fishes is provided by results for zebrafish, where embryos are capable of hypoxia-induced MRD (Padilla and Roth, 2001) but adults are not (Stangl and Wegener, 1996). Hypoxic exposure during a critical window of development might therefore preserve the ability to induce MRD in adulthood. For the stickleback in my study, the long-term hypoxia experienced by the Alta Lake fish could possibly provide this cue for developmental plasticity. This is worth further investigation.

If MRD enhances hypoxia tolerance, then why wouldn't all fish employ it in hypoxia? The answer may involve environmentally induced developmental plasticity as

described above, but could also involve the potential for reduced fitness when MRD is employed in certain environments. Physiological and ecological costs of MRD include oxidative damage (Carey et al., 2000), reduced growth, repair and immunocompetence (Burton and Reichman, 1999), cognitive impairments stemming from neuronal damage (Popov et al., 1992), ceased reproduction (Humphries et al., 2003), and increased susceptibility to predation by aquatic and aerial predators stemming from significantly reduced motor activity (Humphries et al., 2003). For the Trout Lake sticklebacks that do not naturally see long-term hypoxia, these costs, particularly the ecological ones, may outweigh any selection for MRD. But for the Alta Lake sticklebacks that do see long-term hypoxia, the costs of not employing MRD may outweigh the potential costs that come with MRD *per se*, especially because some of these costs could be mitigated during the wintertime when the sticklebacks were in their metabolically depressed states. For example, the surface layer of ice would eliminate the risk of aerial predation, and the migrations and/or reduced appetites of the resident trout (*Oncorhynchus clarkii*, *O. mykiss*) would reduce the risk of aquatic predation (Klemetsen et al., 2003). Moreover, the relatively low hypoxia tolerances of trout (Wagner et al., 2001) tend to restrict them to the uppermost water layers in winter, where O₂ levels are highest (Brown et al., 2011; Ultsch, 1989). This could enable the sticklebacks to use the deeper, more hypoxic water layers as refugia from the trout similar to how other species exploit hypoxic environments such as swamps (Chapman et al., 2002; Chapman et al., 1995), coral reef crevices (Nilsson and Östlund-Nilsson, 2004) and oceanic O₂ minimum zones (Brill, 1994; Nasby-Lucas et al., 2009; Seibel, 2011) to escape being eaten by their less-tolerant predators. Physiological costs of MRD may still accrue if these sticklebacks have not evolved mechanisms to mitigate them (Humphries et al., 2003), but these costs are generally sub-lethal and may be outweighed by the survival risks that come with not employing MRD during winter freeze. Indeed, long-term deprivation of some essential abiotic factor such as food, water or heat is the common theme underlying diverse animal taxa's use of hibernation, aestivation and torpor; it is therefore not unreasonable to speculate that long-term deprivations of O₂ have driven the evolution of MRD use in fishes.

5.5.2 *Intraspecific variation in hypoxia tolerance*

The isolation of these two populations and the variation in their respective natural habitats make for a powerful comparative system with which to study physiological adaptation. A similar approach has been taken with the Atlantic killifish, with various research groups using comparisons of northern and southern populations (subspecies *F. h. macrolepidotus* and *F. h. heteroclitus*, respectively) to test a wide range of hypotheses related to environmentally induced physiological adaptation (see reviews by Burnett et al., 2007; Schulte, 2014; Schulte, 2007). Incidentally, to my knowledge, Atlantic killifish are the only other fish species within which significant variation in hypoxia tolerance has been shown to exist. In that recent study, the southern subspecies maintained dorsal-ventral equilibrium for ~1.7-fold longer at 0.4 kPa P_wO_2 (at 15°C) than the northern subspecies (McBryan et al., 2016). The authors attribute the enhanced tolerance of the southern subspecies to their lower routine normoxic metabolic demand, and this strategy may also contribute to the enhanced tolerance of the Alta Lake stickleback (albeit non-significant; $P=0.075$, Fig. 5-2B). In any case, comparisons of isolated threespine stickleback populations have revealed variation in the physiology underlying environmentally induced differences in thermal tolerance (Barrett et al., 2011; Gibbons et al., 2016), osmoregulation (Gibbons et al., 2016; McCairns and Bernatchez, 2010; Schaarschmidt et al., 1999) and aerobic performance (Dalziel et al., 2012a; Dalziel et al., 2012b); my results here suggest that these comparisons could also be used to address questions regarding the mechanisms and evolution of hypoxia tolerance.

5.5.3 *Conclusions*

These results demonstrate two novel findings: first, hypoxia tolerance can vary within a species and evolve over relatively short timescales; and second, MRD, a complex biochemical phenotype, can improve hypoxia tolerance and evolve relatively quickly, particularly in long-term hypoxic environments. Investigating the hypoxia tolerance strategies of other stickleback populations that have independently colonized winterfreeze and non-winterfreeze lakes could shed light on the adaptive value of the responses measured in this study. Furthermore, my results demonstrate that the threespine stickleback, with its isolated populations native to different hypoxia environments, presents a powerful model system with which to investigate the mechanisms and evolution of hypoxia tolerance. Such

knowledge will improve our understanding of hypoxia tolerance in general, and importantly, benefit predictive models and conservation efforts that help identify and protect potentially vulnerable species in an increasingly hypoxic world.

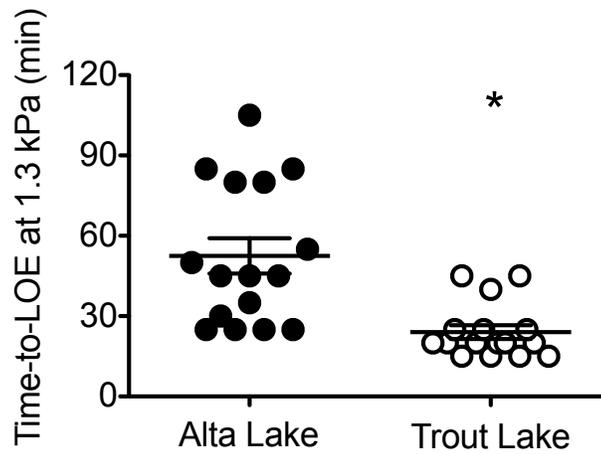


Figure 5-1. Time taken for two populations of threespine sticklebacks to lose dorsal-ventral equilibrium and become unresponsive to gentle tail prods when exposed to severely hypoxic water (1.3 kPa P_wO_2). Water was reduced from ~21 kPa to 1.3 kPa over 1 h following an 18 h normoxic habituation period. Experiments were run separately for each population and in four separate 10 L aquaria housing four fish each ($N=16$ for each population). Points represent individual values, horizontal line indicates mean value, error bars indicate s.e.m., and asterisk indicates significant difference (t-test, $P<0.001$).

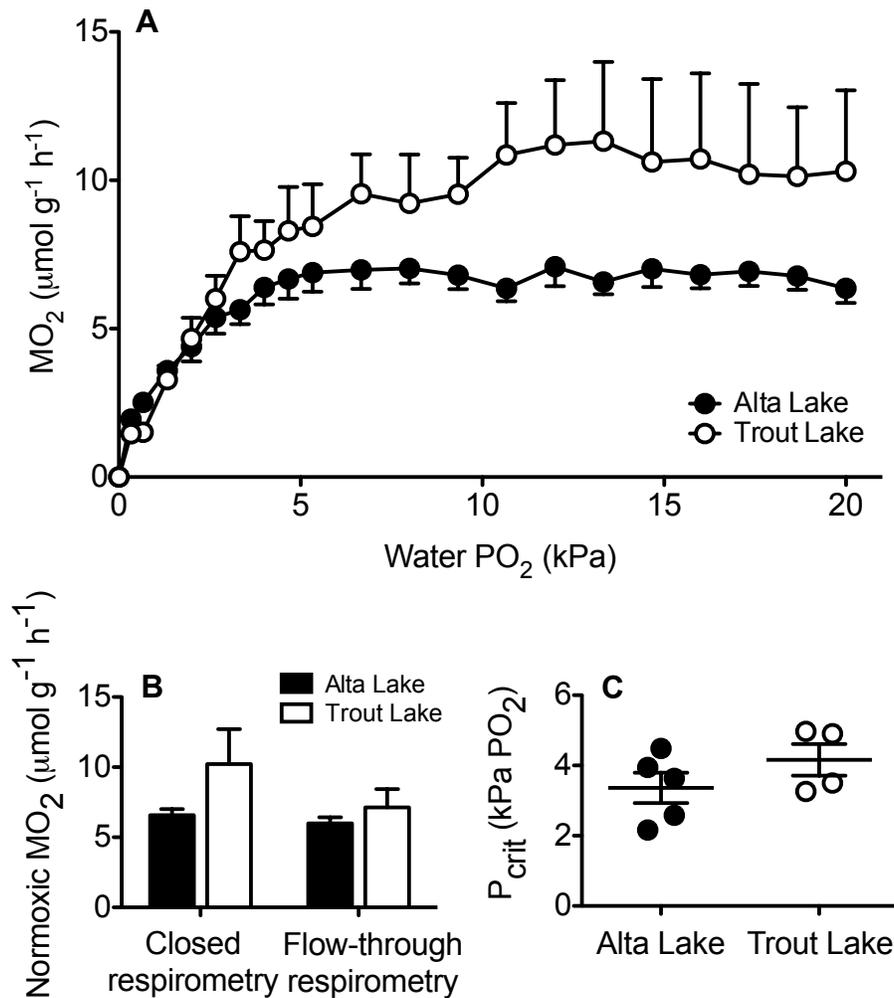


Figure 5-2. Closed-chamber $\dot{M}O_2$ measurements as a function of P_wO_2 (A), normoxic $\dot{M}O_2$ (B), and P_{crit} s (C) for two populations of threespine sticklebacks. P_wO_2 was reduced from ~20 to 0 kPa by the fish's own respiration over the course of ~90 min. (B) Average $\dot{M}O_2$ values at normoxic P_wO_2 for two populations of threespine sticklebacks as determined using closed-chamber and flow-through respirometry. Normoxic $\dot{M}O_2$ is not significantly affected by population or technique (2-way ANOVA, $P=0.075$ and $P=0.135$, respectively; interaction $P=0.337$). (C) Average P_{crit} values for two populations of threespine sticklebacks, calculated for each fish from its closed-chamber $\dot{M}O_2$ trace (t-test, $P=0.245$). Points represent individual values, horizontal line indicates mean value, and error bars indicate s.e.m. $N=5$ for Alta Lake and $N=4$ for Trout Lake.

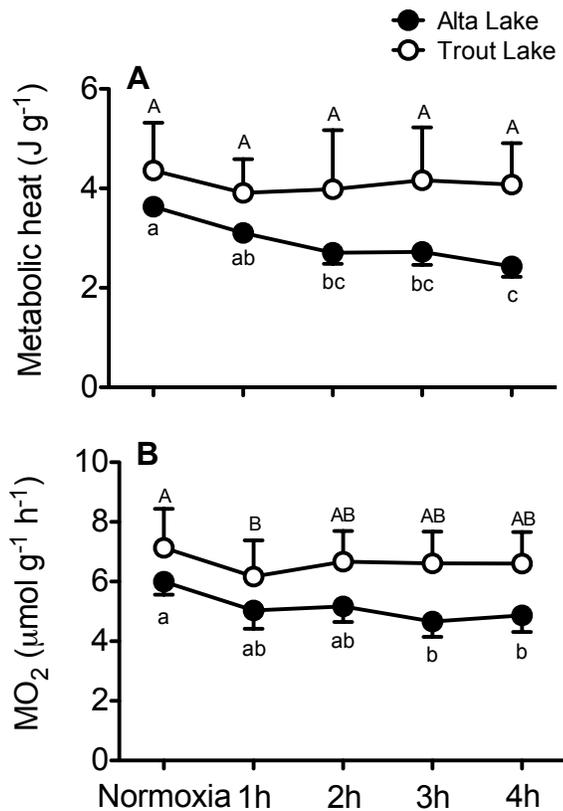


Figure 5-3. Flow-through calorimetric measurements of metabolic heat (A) and $\dot{M}O_2$ (B) as a function of time in severe hypoxia (2.8 kPa P_{wO_2}) for two populations of threespine sticklebacks. Normoxic measurements were made following an 18 h habituation period, after which P_{wO_2} was reduced from ~21 kPa to 2.8 kPa over 1 h. Subsequent measurements were made at 1, 2, 3 and 4 h following the point when P_{wO_2} reached 2.8 kPa. Points indicate average values and error bars indicate s.e.m. Statistical tests (1-way ANOVAs) were run within populations and values sharing a letter are not significantly different (Alta Lake: heat $P < 0.001$, $\dot{M}O_2$ $P = 0.003$, $N = 7$; Trout Lake: heat $P = 0.963$, $\dot{M}O_2$ $P = 0.000$, $N = 4$).

Table 5-1. Characteristics of Alta Lake and Trout Lake. Data provided by ^aWhistler Museum, ^bBritish Columbia Ministry of Environment, ^cJacques Whitford/AXYS Environmental Consultants, and ^dSeth Rudman.

	Days frozen	Elevation	Surface area	Average depth	Maximum depth	Surface pH	Surface conductivity	Fish species
Alta Lake	128 ^a	602 ^b	995600 ^b	9.5 ^b	24.4 ^b	6.5 – 7.7 ^c	89 ^c	<i>O. clarkii</i> ^b <i>O. mykiss</i> ^b <i>C. asper</i> ^b
Trout Lake	0	157 ^b	75600 ^b	5.8 ^b	17.4 ^b	-	75 ^d	<i>O. clarkii</i> ^b

Days frozen in mean $d\ y^{-1}$ since 1942; elevation in m; surface area in m^2 ; depth in m; conductivity in Siemens m^{-1} ; fish species (*Oncorhynchus clarkii*, cutthroat trout; *Oncorhynchus mykiss*, rainbow trout; *Cottus asper*, prickly sculpin) in addition to stickleback observed over past ten years.

Table 5-2. Whole body concentrations of glycogen and lactate in two populations of threespine sticklebacks. Measurements were made following a 4 h exposure to normoxia or hypoxia (2.8 kPa P_wO_2). $N=8$ for each treatment.

	Alta Lake		Trout Lake		2-way ANOVA P values		
	Normoxia	Hypoxia	Normoxia	Hypoxia	Pop.	PO_2	Interaction
[Glycogen]	1.37±0.19	1.32±0.11	0.89±0.17	1.19±0.10	0.044	0.355	0.292
[Lactate]	0.15±0.05	0.30±0.06	0.21±0.07	0.35±0.11	0.461	0.068	0.878

Concentrations in $\mu\text{mol g}^{-1}$ tissue; values are mean±s.e.m.

Chapter 6

General discussion

The objective of my thesis was to determine how hypoxia-exposed fishes combine aerobic metabolism, anaerobic metabolism and MRD as a total HMR, and how this HMR varies in different types of hypoxic environments. I addressed this using the goldfish (*Carassius auratus*) and the threespine stickleback (*Gasterosteus aculeatus*). The results of Chapters 3 and 4 show that the HMR of goldfish is affected by the severity (*i.e.*, P_wO_2), duration and RHI of the hypoxic exposure, while the results of Chapter 5 show that variation in naturally occurring environmental hypoxia results in variation in the HMRs of two populations of threespine sticklebacks. These results together suggest that the HMR is dynamic, changing as a function of both the biology of the fish and the abiotic aspects of its hypoxic environment. Because natural hypoxic environments vary with respect to P_wO_2 and time, it is therefore likely that the HMR of a given species (or population) is finely tuned to characteristics of its hypoxic environment.

This Discussion will explore how the naturally occurring O_2 dynamics of different hypoxic environments shape the HMRs of their resident species. I will explore this idea using a two-dimensional matrix of environmental hypoxia (Fig. 6-1), and by the end, I hope to identify patterns in the HMRs favoured in different hypoxic environments. But first, I will highlight what my thesis work has contributed to our understanding of hypoxic survival mechanisms and the methods I use to elucidate them.

6.1 Thesis highlights and main contributions

6.1.1 Calorespirometry: easier than might be expected

Our calorespirometer is one of only two instruments that have been used to measure the metabolic heat of adult fishes over the past 40 years. The other instrument, belonging to Guido van den Thillart's group in Leiden, was used for a series of six studies between 1989 and 2004. This is negligible compared to the countless number of studies that have used respirometry over that same time. Given direct calorimetry's widely recognized superiority over respirometry as a technique of metabolic rate measurement (Kaiyala and Ramsay, 2011; Lighton, 2008; Nelson, 2016; Richards, 2009; van Ginneken and van den Thillart, 2009), it is surprising that so few studies have used it. The reason typically put forward for this involves the difficulty of the technique, an idea originally espoused by leaders in the field (Fry, 1971; Brett and Groves, 1979) and one that has since become accepted as fact. This has prevented many otherwise interested investigators from pursuing calorimetry (or calorespirometry), and perhaps because of the small market for fish-accommodating calorimeters, the one company that offers a turnkey model charges \$168,000 for it. If the technique's perceived difficulty does not prove inhibitory, its known cost very well might!

However, having co-designed, built and used a calorespirometer myself, I can say that the perceived hurdles are not nearly as high as they are purported to be. Building the apparatus is relatively straightforward so long as one has access to machining tools (an experienced machinist helps), as is assembling it with the necessary accessories (*e.g.*, peristaltic pump, O₂ equilibration chamber, insulated ice chest). The most difficult step might be optimizing the calorespirometer's function with respect to its particular environment and the conditions of the desired experiments, entirely the result of the apparatus's (necessary) thermal sensitivity. I discussed some of these difficulties in Chapter 2, and by and large they can be overcome through trial and error. So long as the appropriate steps are taken to optimize the apparatus's heat-detecting function, running calorespirometry experiments is no more involved than running typical respirometry experiments. And finally, the same design remains effective when scaled to different sizes, allowing experiments to be performed on animals of various sizes. The only major limitation of our apparatus is a signal-to-noise ratio that precludes precise measurements on ectothermic animals at low temperatures, but this

could be mitigated by arranging the inflowing and outflowing waterlines counter-currently (to maximize signal) and/or improving the system's insulation (to reduce noise).

Combined with its low cost of construction (\$1300) and a published paper detailing its construction and function, it was my hope to dispel some of calorespirometry's discouraging aspects and make it more accessible to investigators. Encouragingly, there are at least three groups around the world who have built (or are in the process of building) calorespirometers based on my design to address their own questions.

6.1.2 Hypoxia-induced MRD: a response reserved for extreme environments

My results from Chapters 3 and 5 suggest that, at least in goldfish and sticklebacks, MRD is a hypoxic response that is reserved for extreme environments. This could have only been shown with calorespirometry. 'Extreme' hypoxic environments are those that experience particularly low P_wO_2 s and/or particular long duration hypoxic events. Chapters 3 and 4 reveal that goldfish prioritize maintaining routine metabolic rate through different plastic and time-dependent mechanisms (aerobic + anaerobic over short durations and rapid RHIs; aerobic over long durations and gradual RHIs), and reserve their pronounced MRD for near-anoxia. Chapter 5 reveals that sticklebacks native to an environment that experiences deep, long-term hypoxia (a winterfreeze lake) have evolved the ability to use MRD, while sticklebacks native to an environment that does not experience deep, long-term hypoxia have not evolved (or at least do not express) this ability. This pattern of MRD use may be related to the physiological and ecological costs that come with using MRD, and I elaborate on this idea in section 6.2.

Finally, the use of calorespirometry allowed me to determine the P_wO_2 at which goldfish induce MRD. This P_wO_2 (~0.5 kPa) coincides with goldfish's Hb P_{50} value, and so I hypothesized that tissue O_2 delivery *via* Hb might be an upstream signal for hypoxia-induced MRD in goldfish. With the help of a recently acquired drug (efaproxiral) that significantly increases goldfish's Hb P_{50} value, this hypothesis makes some clear predictions that I am now in the process of testing experimentally using cannulated goldfish and a larger version of the calorespirometer.

6.1.3 P_{crit} : musings on biology and methodology

My results from Chapter 4 show that the RHI used in a P_{crit} experiment can significantly alter the P_{crit} of the animal in the experiment (goldfish, in my case). Though this idea has been speculated on before (*e.g.*, Rogers et al., 2016; Snyder et al., 2016), my Chapter 4 experiments are the first to provide empirical support for it. The underlying mechanisms involve rapid alterations to steps along the O₂ transport cascade, including lamellar surface area and Hb-O₂ binding affinity. These alterations may occur more quickly in goldfish than in other species, but because all species have some capacity for environmental acclimation, it is probably true that RHI will affect all species to a corresponding degree and should therefore be controlled for when running hypoxic exposure experiments. Furthermore, because the results showed no change in P_{crit} with respirometric method *per se*, closed-chamber respirometry is a viable and effective method of P_{crit} determination despite the modest accumulation of CO₂ and ammonia that comes with it.

Were anyone to ask, my recommended method for P_{crit} determination would be closed-chamber/circuit respirometry over a ‘typical’ time duration of ~60 to 90 min. This time scale captures a fish’s P_{crit} in the present, before hypoxic acclimation is able to exert a significant effect on O₂ extraction mechanisms and subsequently P_{crit} . While this may underestimate the fish’s full ability to take up and use O₂ in hypoxia (*i.e.*, overestimate the fish’s P_{crit}), it at least captures the fish’s ability at a known point in the acclimation process (*i.e.*, pre-acclimation). Slowing the RHI by extending the P_{crit} trial duration will result in the fish acclimating to hypoxia to some degree, and because this degree will vary among species and individuals, knowing where in the acclimation process a given RHI will place a particular fish is difficult. This jeopardizes the investigator’s ability to standardize among species and individuals, and subsequently, to compare P_{crit} values among these groups. And because the majority of studies have determined P_{crit} s of fishes using closed-chamber respirometry (Rogers et al., 2016), sticking with this method will allow for the most relevant comparisons to literature values.

6.1.4 Threespine stickleback: a potentially powerful model for hypoxia research

My results from Chapter 5 reveal that different populations of threespine sticklebacks vary significantly in their hypoxia tolerances and abilities to use MRD. As far as I know, this

is only the second time such intraspecific variation in hypoxia tolerance has been shown to exist in fishes (the other instance being two subspecies of Atlantic killifish; McBryan et al., 2016), and the only time such intraspecific variation in MRD has been shown to exist. The threespine stickleback is therefore an excellent system with which to investigate the mechanisms and evolution of these traits, similar to how intraspecific comparisons of Atlantic killifish subspecies have been used to investigate the mechanisms and evolution of thermal tolerance (see reviews by Schulte, 2007; Schulte, 2014). Adding to the stickleback's potential is the vast research field that surrounds the species, arising first from the species' evolutionary history that lends itself to the study of local ecological adaptation, and pushed forward by methodological resources (*e.g.*, a sequenced genome) and an ever-growing body of stickleback literature. Combined with their prevalence on the west coast of North America, their ease of capture, and their adaptability to laboratory conditions, threespine sticklebacks represent an exciting new model system with which to probe the proximate and ultimate causes of hypoxia tolerance.

6.2 The hypoxic environment as sculptor of the HMR

Ultimately, life in hypoxia is about matching ATP supply and demand (Boutilier, 2001; Hochachka and McClelland, 1997). This balance is possible through different combinations of aerobic metabolism, anaerobic metabolism and MRD, and hypoxia tolerance involves any combination of these metabolic strategies. A 'grand unified theory' of hypoxia tolerance is therefore unlikely to exist. Rather, the strategy adopted by a species (or even a population or individual) is more likely shaped by its life history and the particular hypoxic environment to which it has become adapted. This hypothesis predicts that different species native to environments with similar O₂ characteristics (in terms of severity, RHI and/or duration) will employ similar hypoxic survival strategies, while those native to environments with different O₂ characteristics will employ different hypoxic survival strategies.

With the help of a two-dimensional matrix of environmental hypoxia (Fig. 6-1), I will explore how time- and P_wO_2 -related O₂ characteristics affect the HMRs of species adapted to three representative natural hypoxic habitats: severe P_wO_2 + short duration (Q2 in Fig. 6-1), moderate P_wO_2 + long duration (Q3 in Fig. 6-1), and severe P_wO_2 + long duration (Q4 in Fig. 6-1). For the sake of simplicity, I have incorporated RHI into the time axis of Fig. 6-1

because, though exceptions exist, it is generally the case that the short-duration hypoxic habitats (Q1 and Q2) have rapid RHIs, while the long-duration hypoxic habitats (Q3 and Q4) have gradual RHIs.

6.2.1 Q2: Severe P_wO_2 + short duration

Tidepools in the intertidal zone become isolated from the ocean as a result of tidal ebbs and flows. Pools located high in the intertidal zone relative to sea level at low tide can become isolated for hours to days. Their small water volumes, combined with their often-dense biota, result in enormous fluctuations in P_wO_2 , reaching anoxia at night and up to 80 kPa in the day and typically taking place over just 6 to 12 h (Richards, 2011; Truchot and Duhamel-Jouve, 1980). These tidepool habitats are therefore typified by hypoxia that is severe (P_wO_2) and short (duration).

Numerous animal species make their home in these tidepools, and a well-studied example is the tidepool sculpin (*Oligocottus maculosus*). The tidepool sculpin uses a variety of mechanisms to achieve a very high tolerance of hypoxia, and I can use a series of studies by Mandic and colleagues to put together how it employs some of these mechanisms as environmental P_wO_2 decreases. First, the sculpin uses a high Hb- O_2 binding affinity (P_{50} of 3.1 kPa) to regulate routine $\dot{M}O_2$ from normoxic P_wO_2 s to 3.5 kPa (P_{crit} of 3.5 kPa; Mandic et al., 2009a). As P_wO_2 falls below this value, the sculpin engages behavioural responses that increase its access to O_2 , including aquatic surface respiration (ASR) starting at 2.8 kPa, and then aerial emergence starting at 1.2 kPa (Mandic et al., 2009a). Aerial emergence allows fishes that are capable of it to support routine $\dot{M}O_2$ levels amid severely hypoxic (*i.e.*, sub- P_{crit}) water (Martin, 1996; Yoshiyama and Cech, 1994), and indeed for tidepool sculpins in particular, 72 h of aerial emergence has been shown to result in no significant accumulation of lactate (Sloman et al., 2008). These responses therefore uncouple the sculpin from its aquatic habitat and allow it to maintain routine $\dot{M}O_2$ levels at sub- P_{crit} P_wO_2 values. However, they also significantly increase the risk of aerial predation (Kramer and Manley, 1983). A perceived threat of predation from above will send the sculpin back into the tidepool's severely hypoxic water (or delay its emergence from it; Hugie et al., 1991; Shingles et al., 2005; Sloman et al., 2008), and so their survival depends on anaerobic metabolism and/or MRD until the predation threat subsides. The tidepool sculpin's capacity for hypoxia-induced

MRD has never been investigated, but recent work by Mandic et al. suggests that the sculpin's capacity for anaerobic glycolysis is highly adapted to its hypoxic environment. Specifically, their glycogen reserves are large (Mandic et al., 2013), their glycolytic enzyme activity levels are significantly higher than those of closely-related sculpin species native to less hypoxic sub-tidal environments (Mandic et al., 2013), and the expression of genes involved in anaerobic glycolysis do not change over the course of an 8 h hypoxia exposure, suggesting their high anaerobic capacity is a fixed trait that is capable of being immediately and maximally induced (Mandic et al., 2014). A reliance on anaerobic glycolysis over MRD is practical in this environment because it allows the sculpin to maintain cellular energy balance without impairing predator avoidance behaviour through reduced responsiveness and locomotor abilities. However, there are costs that come with anaerobic reliance. These costs (fuel depletion, end-product accumulation) are positively correlated with time spent in the hypoxic environment, but for the sculpin, this would typically be short; either the threat of predation will subside and allow the fish to reemerge and/or perform ASR, or the tidepool's water will be replenished with O₂ by photosynthesis and/or the rising tide. The costs accrued with anaerobic reliance would therefore be low.

The tidepool sculpin prioritizes aerobic metabolism under all possible hypoxic conditions, and likely relies on anaerobic glycolysis (not MRD) when forced to spend time in severely hypoxic water. If this HMR is a product of the tidepool sculpin's natural hypoxic environment, then I would predict that different species that are native to similar hypoxic habitats (*i.e.*, severe and short) would exhibit similar HMRs. One such example is the zebrafish (*Danio rerio*).

Zebrafish are native to rice paddies and stagnant water bodies in India that become severely hypoxic as a result of high eutrophication and plant respiration (Cruz et al., 2000; Magneschi and Perata, 2009; Spence et al., 2008). An abundance of aerial predators are typical of these habitats and are believed to be the reason why zebrafish generally avoid the water's surface (Blaser and Goldsteinholm, 2012; Spence et al., 2008). In many ways, this is a similar hypoxic habitat to the tidepools described above, and perhaps because of this, the HMR of zebrafish is similar to that of the tidepool sculpin. Zebrafish have a low P_{crit} value of ~2.7 kPa (Barrionuevo et al., 2010) and will employ ASR to supplement O₂ uptake starting at 4 kPa (Abdallah et al., 2015). However, a threat of predation from above would likely cause

the zebrafish to retreat to deeper, more hypoxic waters. This zone could theoretically be used as a refuge from aerial predators, but like the sculpin retreating into the tidepool, residency here would require a reliance on anaerobic glycolysis and/or MRD. Calorimetric measurements of hypoxia-exposed zebrafish by myself (unpublished) and others (Stangl and Wegener, 1996) clearly show that zebrafish do not depress metabolic rate at either 2.0 kPa P_wO_2 or near-anoxia, and this maintenance of metabolic heat at sub- P_{crit} P_wO_2 s suggests a strong activation of anaerobic glycolysis. Indeed, glycolysis-related gene expression has been shown to increase in sub- P_{crit} -exposed zebrafish (Ton et al., 2003; van der Meer, 2005). Furthermore, anaerobic metabolism (as represented by glycolytic enzyme activities and metabolite concentrations) is believed to be the metabolic strategy underlying variation in hypoxia tolerance among nine related *Danio* and *Davario* species (Yao, 2012). Buffering routine metabolic rate with anaerobic glycolysis while in deeper, hypoxic waters would allow the zebrafish to retain some level of activity, and this would be advantageous considering the abundance of air-breathing predatory fishes (e.g. *Notopterus notopterus*, *Xenentodon cancila*, *Channa* spp.) that inhabit this environment (Engeszer et al., 2007a; Engeszer et al., 2007b; Spence et al., 2008). All told, the zebrafish, like the tidepool sculpin, appears to prioritize the use of aerobic metabolism and resort to anaerobic glycolysis instead of MRD at sub- P_{crit} P_wO_2 s. This also appears to be the case with Atlantic killifish (*Fundulus heteroclitus*), whose estuarine habitats are similar to the sculpin's and zebrafish's in terms of O_2 regime and predation (Burnett et al., 2007; Kneib, 1982; Schulte, 2007); killifish acclimated to intermittent hypoxia up-regulate mechanisms that enhance glycolytic capacity and the processing of glycolytic end-products, while killifish acclimated to chronic hypoxia do not (Borowiec et al., 2015). That the zebrafish's and killifish's natural hypoxic environments are similar to the tidepool sculpin's suggests that this HMR is well suited—and perhaps selected for—in predator-rich environments experiencing rapid and severe fluctuations in PO_2 . But what happens to the HMR if predation pressure is removed from these short and severe hypoxic habitats? I can address this question with the help of migratory species of oceanic O_2 minimum zones.

Oceanic O_2 minimum zones (OMZs) occur throughout the world's oceans at depths between 200 and 1000 m, where certain biological and physical processes combine to reduce dissolved O_2 levels to P_wO_2 values <6.4 kPa around the OMZ's periphery and often as low as

0.5 kPa in its center. Biologically, a high density of aerobic bacteria reduce the OMZ's O_2 levels as they feed upon the organic matter falling from the mixed layer above, while physically, a lack of atmospheric contact and low levels of convective mixing keep these waters low in O_2 .

There are two types of OMZ resident: permanent and migratory. Permanent residents, which will be discussed later, spend their entire lives in the OMZ and therefore experience chronic moderate-to-severe P_wO_2 s (Q3 and Q4 in Fig. 6-1). Migratory residents on the other hand spend their days in the center of the OMZ and migrate vertically into well-oxygenated surface waters each night to feed in the cover of darkness (Seibel, 2011). This migratory pattern exposes these animals to progressively changing P_wO_2 s, becoming normoxic with upwards migration and hypoxic with downwards migration. The hypoxic exposures experienced by these animals are therefore severe (P_wO_2) and short (duration; Q2 in Fig. 6-1), similar to those of the sculpins and zebrafish described above. Despite this similarity, migratory OMZ residents use a different HMR. While they tend to possess traits that enhance O_2 extraction (*e.g.*, Seibel, 2013; Trueblood and Seibel, 2013) and glycolytic capacity (*e.g.*, Gonzalez and Quiñones, 2002; Torres et al., 2012), migratory OMZ residents rely primarily on MRD while in the deeply hypoxic OMZ during the day (Seibel, 2011; Seibel et al., 2016). For example, the jumbo (or Humboldt) squid (*Dosidicus gigas*) depresses metabolic rate by 87% when held at 0.6 kPa, the P_wO_2 at which it typically spends the daytime in the OMZ (Rosa and Seibel, 2010; Trueblood and Seibel, 2013). Migratory krill (*Euphausia eximia*) from this same OMZ region also employ MRD at this P_wO_2 (Seibel, 2011; Seibel et al., 2016). These are different HMRs than those employed by tidepool sculpins and zebrafish despite similar environmental O_2 characteristics, and the reason might involve predation risk. As discussed, predation risk in the hypoxic habitats of the sculpin and zebrafish is high, and so employing MRD, with its concomitant reduction in locomotor activity and responsiveness (Humphries et al., 2003), would likely increase the chances of being eaten. However, predation risk in the OMZ is relatively low owing to low levels of light and activity, a diffuse distribution of animals (see Childress, 1995; Drazen and Seibel, 2007; Seibel and Drazen, 2007; Seibel et al., 2000), and low O_2 levels that tend to keep top ocean predators like sharks, tunas and billfishes out (Brill, 1994; Nasby-Lucas et al., 2009; Vetter et al., 2008). Consequently, animals living in the OMZ—particularly those that migrate into oxygenated

surface waters to complete necessary behaviours like feeding and mating—can employ MRD with a relatively low risk of being eaten.

6.2.2 Q4: Severe P_wO_2 + long duration

The Amazon basin floods each year when the Amazon River, which drains the Andes, overflows. When this happens, water spills over the banks of the river and into the surrounding forests and floodplains, bringing with it many of the Amazon's 5600+ species of fish (Albert and Reis, 2011). At the peak of the wet season, all of the flooded areas are interconnected, allowing fish to move among them. But as the season wears on, the water levels recede and leave behind smaller, isolated water bodies that become hypoxic (even anoxic) as a result of plant and animal respiration, organic decomposition, and a lack of light penetration, remaining deeply hypoxic for months at a time, even chronically. These habitats are therefore typified by hypoxia that is severe (P_wO_2) and long (duration).

Perhaps because of the seasonality (*i.e.*, predictability) of these flood pulses, many of the Amazon's fish species have independently evolved a high tolerance to hypoxia (Almeida-Val and Val, 1993). Most of these species achieve this by using various behavioural and/or morphological features that maximize their abilities to acquire O_2 in their O_2 -depleted habitats, the prime examples being ASR and air breathing. Many Amazonian fish families have independently evolved morphological features to optimize ASR and air breathing, including extensible lower lips to syphon O_2 -rich water directly across the gills, and a wide variety of air breathing organs ranging from modified buccal cavities to lungs (Val and Almeida-Val, 1998). These morphological features, and the behaviours they optimize, are believed to have evolved in response to aquatic hypoxia (Graham, 1997; Kramer and McClure, 1982). It is no surprise then that they are widely used among the Amazon's hypoxia-dwelling fish species. One study collected the resident species of an isolated Amazonian lake that had become severely hypoxic (Camaleao Lake) and determined the primary hypoxic adaptation used by each species to survive in the lake. Of the 11 families caught (numerous species for most), seven used air-breathing as their means of tolerating hypoxia, two used ASR, one used Hb- O_2 binding affinity modulation, and one used MRD (Junk et al., 1983). In a similar study in which 20 species were caught in a hypoxic Amazonian lake, ten species used ASR as a primary means of tolerating hypoxia, four used

air-breathing, four positioned themselves directly adjacent to O₂-secreting plant roots, one combined a large gill surface area with a high Hb-O₂ binding affinity, and one used MRD (*Astronotus ocellatus*, the most tolerant of the group; Soares et al., 2006). Furthermore, while air-breathing and/or ASR behaviours increase the susceptibility of aerial predation (Kramer and Manley, 1983), some of these fishes have evolved complex group behaviours to mitigate this risk (Sloman et al., 2009).

In addition to ASR and air breathing, hypoxia-adapted Amazonian fishes tend to possess a suite of physiological characteristics that enhance O₂ extraction and delivery, including increased ventilation, blood-O₂ carrying capacity, and Hb-O₂ binding affinity *via* decreased allosteric interactions (reviewed by Val and Almeida-Val, 1995; Val and Almeida-Val, 1998). The obvious trend here is for hypoxia-adapted Amazonian species to prioritize aerobic metabolism over anaerobic metabolism and/or MRD, perhaps unsurprising given the benefits of maintaining routine metabolic rate aerobically and the fact that these species typically have constant access to air. But what about long-term, severely hypoxic habitats that do not allow access to air?

Winterfreeze lakes generally occur at high elevations or at far northern or southern latitudes, where wintertime atmospheric temperatures stay below 0°C for sufficient time periods to freeze the lake's surface layer. The breakdown of organic matter by aerobic microorganisms depletes the liquid water's dissolved O₂ levels, and a lack of water-atmosphere interface and photosynthesis (due to the ice layer reducing or eliminating the passage of light to aquatic plants) prevents them from being replenished until spring thaw (Ultsch, 1989). The hypoxic severity therefore depends on the lake's depth, biological activity, flowing water supply, and ice cover dynamics and duration, with severe lakes becoming anoxic for months at a time (Barica and Mathias, 1979; Mathias and Barica, 1980). The rate at which O₂ is depleted will depend on these same parameters, but even for a severe lake, the descent from normoxia to anoxia typically takes 2+ months (Vornanen et al., 2004). These winterfreeze lakes are thus typified by hypoxia that is severe (P_wO_2), long (duration) and slow developing.

Various species overwinter beneath the ice of frozen lakes, including reptiles (snakes and turtles), amphibians (frogs and salamanders) and numerous species of fishes (Ultsch, 1989). The reptiles and amphibians tend to bury themselves in the sediment and effectively

isolate themselves from the water environment, while the fishes, which I will focus on, tend to maintain some degree of activity and environmental engagement (but see Crawshaw et al., 1982; Loeb, 1964). Species assemblages in winterfreeze lakes are often determined by the magnitude of the winter hypoxic event. The limited tolerances of predatory fishes (e.g., Esocidae, Centrarchidae) restrict their ranges and preclude them from severe winterfreeze lakes, allowing more tolerant fishes (e.g., Cyprinidae, Umbridae) to colonize these lakes with a reduced risk of predation (Magnuson et al., 1989). An excellent example of such a species is the crucian carp (*Carassius carassius*), a cyprinid native to small lakes in northern Europe and Asia that is “probably the most anoxia-tolerant fish there is” (Vornanen et al., 2009). Similarly tolerant, though slightly less so, is the crucian carp’s cogener, the goldfish (*Carassius auratus*). The goldfish is native to the same type of habitat as the crucian carp and employs the same suite of anoxia-tolerance traits (though perhaps to a lesser degree; Ultsch, 1989). It too is therefore an excellent species with which to explore adaptations to winterfreeze hypoxia, and I will use the data collected in Chapters 3 and 4 to do so.

The HMR of goldfish (described above) is well suited to its native winterfreeze lakes. Exceptional O₂ uptake abilities—the plasticity of which reduces osmo-respiratory and O₂ extraction-delivery tradeoffs—allow the goldfish to maintain routine metabolic rate at all but near-anoxic P_wO₂s, preserving some level of routine function for most of the winter hypoxia exposure while simultaneously conserving finite anaerobic fuels until their use is absolutely necessary. As the winter progresses and near-anoxic P_wO₂ values are encountered, the goldfish induces a significant MRD that reduces the rate at which these fuels are used while simultaneously reducing the rate at which deleterious anaerobic end-products accumulate. However, if the exposure lasts sufficiently long for those end-products to accumulate, they are converted to ethanol and excreted across the gills, thus preventing a metabolic acidosis while delaying the loss of carbon until it is absolutely necessary.

Metabolic rate depression is a critical component of this HMR, one that on its own can extend the goldfish’s anoxic survival time by approximately 25-fold when temperature is accounted for (assuming Q₁₀ of $\dot{M}O_2$ of 4.6; Fry and Hart, 1948). It is probably true that MRD is the only mechanism by which a fish can survive anoxia for any appreciable amount of time, regardless of how large their glycogen reserves are or how they may deal with their anaerobic end-products (Hochachka et al., 1996). Even for a species like goldfish, with its

exceptionally large glycogen reserves and its ability to avoid a glycolytically-derived acidosis, anoxic survival time would only be ~5 h at routine metabolic rates [assuming P:O₂ of 6, routine $\dot{M}O_2$ of 3.5 $\mu\text{mol g}^{-1} \text{h}^{-1}$, P:glycogen of 3 (Hochachka and Somero, 2002), and estimated total body glycogen reserves of 35 $\mu\text{mol g}^{-1}$ (Mandic et al., 2008)]. With MRD and a reduced water temperature, this time would be extended to approximately 5 days. The combination of goldfish's native environment becoming anoxic in the winter, the requirement of MRD to survive appreciable periods of time in anoxia, and a low predation risk in these anoxic environments therefore probably explains why MRD was selected for in these animals. So can MRD be expected to play a role in the HMRs of all fish species native to winterfreeze lakes? The results of Chapter 5 suggest it may depend on the type of winterfreeze lake.

6.2.3 Q3: Moderate P_wO_2 + long duration

The winterfreeze lakes described above are eutrophic lakes, but oligotrophic lakes freeze during winter as well. Oxygen levels in these lakes are reduced and kept low for the same reasons as in eutrophic winterfreeze lakes, but the lower productivity levels inherent to oligotrophic lakes tend to result in less severe P_wO_2 s being reached by winter's end (Ultsch, 1989). These environments are therefore typified by hypoxia that is moderate (P_wO_2) and long (duration). Alta Lake in British Columbia is one such lake, and I can use the data collected in Chapter 5 to explore how this hypoxic environment has shaped the HMR of its resident sticklebacks.

The greater hypoxia tolerance of the Alta Lake sticklebacks compared to the Trout Lake sticklebacks appears to result from their ability to employ MRD at sub- P_{crit} P_wO_2 s. This is similar to goldfish. But despite both goldfish and the Alta Lake sticklebacks employing a hypoxia-induced MRD, there are notable differences between their respective MRDs and how they are used. In short, goldfish depress metabolic rate by 80% and wait until near-anoxia to initiate it, while Alta Lake sticklebacks depress metabolic rate by 33% and do so at 2.8 kPa P_wO_2 . These differences in MRD might relate to differences in each species' hypoxic environment. While the native lakes of goldfish likely become anoxic during wintertime (like the native lakes of crucian carps; Vornanen et al., 2004), apart from at the water-sediment interface (Dunnington et al., 2016), Alta Lake only becomes hypoxic (Jacques

Whitford/AXYS, 2007). These hypoxic conditions mean two things for the stickleback inhabitants and their use of MRD. First, while 2.8 kPa P_wO_2 is not ‘severe’ in the context of the goldfish’s natural anoxic habitat, it probably is in the context of Alta Lake. Therefore, selection may be acting on hypoxic survival strategies at higher P_wO_2 s in the Alta Lake environment than in the more severe goldfish environment, and this could be why Alta Lake sticklebacks induce MRD at a higher P_wO_2 than goldfish. Second, the presence of O_2 makes for a less extreme environment that can support other, less tolerant, species during wintertime, including predatory trout. Salmonid species are adept at locating and exploiting microhabitats in frozen lakes and streams (Brown et al., 2011), and can survive in these habitats so long as they can locate an O_2 source. For the salmonids that live in Alta Lake (cutthroat and rainbow trout), some move into the small creeks that drain into the Lake while others stay in the lake itself (Tara Schaufele, Resort Municipality of Whistler’s Environmental Supervisor; personal communication). Because salmonids continue to eat during wintertime (albeit at reduced rates; Klemetsen et al., 2003), the stickleback population remains under some level of predation pressure, and this could possibly limit the degree to which the sticklebacks depress their metabolic rates during wintertime (see Chapter 5 for a discussion on the possibility of hypoxic water layers serving as refugia for sticklebacks). This appears to be the case in the OMZ as well, where species that are more likely to be eaten depress their metabolic rates to lesser extents (*e.g.*, MRD for krill of ~40 to 50%) than species that are less likely to be eaten (*e.g.*, MRD for jumbo squid of ~80 to 90%; Seibel, 2011). And in my experience, it is certainly true that metabolically depressed sticklebacks are more responsive to stimuli than metabolically depressed goldfish, which are largely unresponsive. In any case, regardless of whether the hypoxic (as opposed to anoxic) conditions of Alta Lake explain the Alta Lake stickleback’s relatively modest MRD, it very likely explains their lesser tolerance when compared to goldfish.

Another environment that is typified by moderate P_wO_2 s and long (chronic) durations is the OMZ from the perspective of its permanent residents. Permanent residents, which include many fish and invertebrate species, tend to live towards the OMZ’s periphery where P_wO_2 s are not as severe as in its center (Childress and Seibel, 1998). Probably owing to the detrimental effects of long-term reliance on anaerobic glycolysis and/or MRD, these animals rely primarily on aerobic metabolism through a suite of highly effective O_2 extraction

adaptations. Compared with related species from less hypoxic waters, permanent OMZ residents tend to have enhanced ventilatory abilities, larger respiratory surfaces, thinner blood-water diffusion barriers, and higher affinity respiratory pigments. An example is the giant red mysid (*Gnathophausia ingens*), a permanent OMZ resident that is particularly well studied. The red mysid has a high ventilatory capacity (Childress, 1971), a large mass-specific gill surface area (Childress, 1971), a small blood-water diffusion distance across the gills (Seibel, 2011), a high circulatory capacity (Belman and Childress, 1976), and a haemocyanin with an extremely high affinity for O₂ and a large Bohr effect to facilitate tissue O₂ delivery (Sanders and Childress, 1990a; Sanders and Childress, 1990b). Combined with an exceptionally low routine metabolic rate (a common trait of permanent OMZ residents; see Childress, 1995), this results in a P_{crit} value of 0.8 kPa (Seibel, 2011), coincident with the minimum P_wO_2 that the mysid typically encounters in the OMZ (Childress and Seibel, 1998). In fact, across a wide range of OMZ residents, P_{crit} has been shown to correlate at near unity with the minimum P_wO_2 s each of these animals experience in the wild (Childress, 1975; Cowles et al., 1991; Donnelly and Torres, 1988; Torres et al., 1994). This finely tuned aerobic capacity may preclude a significant reliance on anaerobic glycolysis, and may subsequently explain why permanent OMZ residents tend to have limited anaerobic abilities (Childress and Seibel, 1998).

6.3 Summary and perspectives

We can combine these seven case studies with the environmental hypoxia matrix in Fig. 6-1 to draw some general conclusions on the HMRs used in different hypoxic environments (Fig. 6-2). Figure 6-2 uses a series of circular pie charts to represent HMR. The diameter of a pie chart is proportional to its metabolic rate; therefore, a smaller-diameter pie chart represents a depressed metabolic rate. The diameters of the pie charts are arbitrary; rather, they are qualitative representations to be compared to the normoxic routine metabolic rate shown in Q1. The black-filled portion of a pie chart represents the aerobic contribution to its metabolic rate, and the grey-filled portion represents the anaerobic contribution. All-black and all-grey therefore represent fully aerobic and anaerobic, respectively. The ratio of aerobic:anaerobic within a single pie chart is arbitrary and meant only to portray that aerobic

and anaerobic pathways are simultaneously contributing to total metabolic rate at some approximate ratio.

For short and severe hypoxic exposures (Q2), the HMR varies as a function of aerial/surface access and predators. If the air-water interface is accessible (*e.g.*, tidepools, rice paddies), fishes living in these environments tend to prioritize aerobic metabolism by using ASR and/or air breathing; if an aerial predator presents itself, the fish tend to dive into the hypoxic water and buffer routine metabolic rate using anaerobic glycolysis. If the air-water interface is inaccessible (*e.g.*, OMZ), the animals living in these environments tend to use MRD and save their routine activities such as feeding and mating for their daily migration into oxygenated surface waters. These animals use deep MRD if predation risk is low-to-absent (*e.g.*, jumbo squid), and moderate MRD if predation risk is moderate-to-low (*e.g.*, krill).

For long and moderate hypoxic exposures (Q3), the HMR varies as a function of exposure duration. Species that live in seasonally moderate hypoxia (*e.g.*, oligotrophic winterfreeze lakes) tend to use MRD, perhaps because they can accomplish their routine activities during the oxygenated months of the year. My particular example species (threespine stickleback from Alta Lake) employs a relatively modest MRD with only a small anaerobic contribution, possibly due to the presence of predatory trout. Species that live in chronically moderate hypoxia (*e.g.*, OMZ periphery) tend to rely on enhanced O₂ extraction abilities so as to support routine metabolic rate aerobically. Because these species never enter fully oxygenated waters, they need to support routine activities like feeding and mating in chronic hypoxia, and this makes an aerobic metabolism-based HMR ideal.

Finally, for long and severe hypoxic exposures (Q4), the HMR varies as a function of aerial/surface access. If it is accessible (*e.g.*, Amazon basin), then the fishes living in these environments tend to exploit its high O₂ content using ASR and/or air breathing, effectively uncoupling themselves from their severely hypoxic aquatic environment. If the air-water interface is inaccessible (*e.g.*, eutrophic winterfreeze lakes), then aerobic metabolism is not an option and the fishes living here tend to employ deep MRD instead so as to conserve anaerobic fuel reserves. A general lack of predators in these environments allows these fishes to surrender locomotor performance with minimal threat of being eaten, while the return of

O₂ with spring thaw allows them to complete routine activities such as feeding and mating in oxygenated water.

Together, these trends suggest that no matter what the hypoxic environment, fishes use a wide variety of adaptive mechanisms to do what they can to preserve aerobic metabolism. If this becomes impossible (as is the case with Q2 and Q4 hypoxic environments), fishes appear to use one of two approaches depending on the duration of the exposure: anaerobic glycolysis is used to buffer routine metabolic rate if the exposure is short, and MRD is used if the exposure is long. The presence of predators and/or aerial access complicates this, as will co-varying abiotic factors like temperature, $P_w\text{CO}_2$ and pH. But by and large, there appear to be patterns in the HMRs of unrelated species that depend on their native hypoxic environments. Searching for and understanding these patterns is important. The prevalence and severity of hypoxia among the world's aquatic environments is increasing (IPCC, 2014; Schmidtko et al., 2017; Smith et al., 2006), and so understanding the hypoxic metabolic responses of fishes will help us to better identify potentially vulnerable species, and better predict the ways they may redistribute themselves in an increasingly hypoxic world.

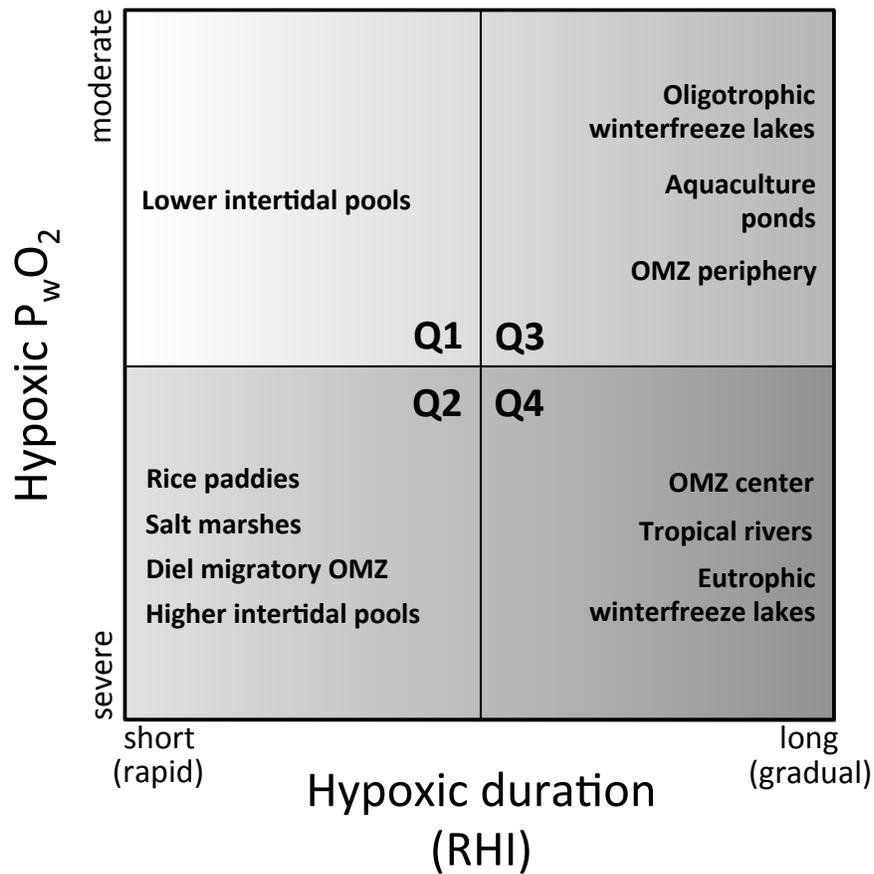


Figure 6-1. A matrix of environmental hypoxia portraying various natural hypoxic environments according to their hypoxic severities ($P_w O_2$) and hypoxic durations (time). Quadrant (Q) 1 portrays moderate ($P_w O_2$) + short (duration) environments, Q2 portrays severe + short environments, Q3 portrays moderate + long environments, and Q4 portrays severe + long environments. The environments listed within each quadrant will vary from one another in severity and duration. For the sake of simplicity, rate of hypoxia induction (RHI) is incorporated along the time duration axis whereby short-duration environments tend to experience rapid RHIs, and long-duration environments tend to experience gradual RHIs. See text for details.

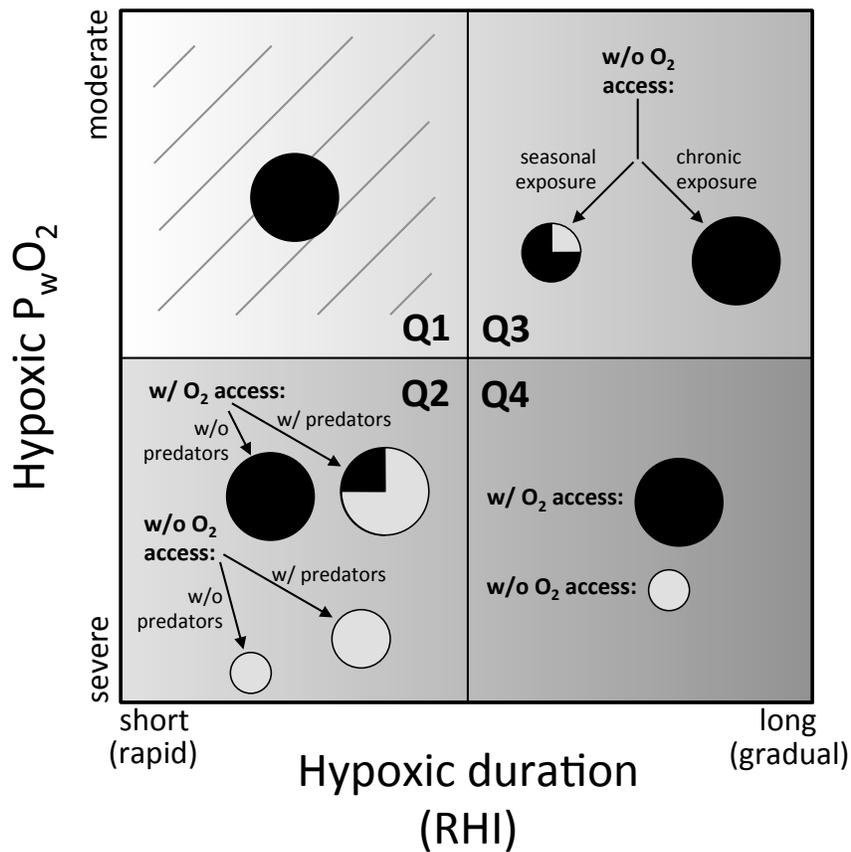


Figure 6-2. The hypoxic metabolic responses (HMRs) of species adapted to natural aquatic hypoxic environments listed in Fig. 6-1 and detailed in the text. The circular pie charts represent HMR and are built according to the multi-species analyses described in the text. Each pie chart's diameter is a qualitative representation of its total metabolic rate relative to the size of the pie chart in Q1 representing normoxic routine metabolic rate. Therefore, a smaller-diameter pie chart than that shown in Q1 represents a depressed metabolic rate. The black-filled portion of a pie chart represents the aerobic contribution to that HMR's metabolic rate, and the grey-filled portion represents the anaerobic contribution. The ratios of aerobic:anaerobic are estimations. The low hypoxic magnitudes of the Q1 habitats precluded their analysis (indicated by diagonal grey lines).

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