HYPOXIA TOLERANCE IN FISHES:
CARDIORESPIRATORY PERFORMANCE AND METABOLISM

by

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B.Sc., The University of Guelph, 2003
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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(ZOOLOGY)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

August 2012

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ABSTRACT

Cardiac failure occurs in most vertebrates including humans following even short hypoxia exposure due to an inability to match cardiac energy demand to the limited energy supply. In contrast, hypoxia-tolerant ectothermic vertebrates show the remarkable ability to maintain cardiac energy balance and stable cardiac function during prolonged exposure to severe hypoxia (cardiac hypoxia tolerance, CHT). I investigated how CHT is achieved and its relationship to whole-animal hypoxia tolerance using measurements at multiple physiological levels in two study models: 1) tilapia, a hypoxia-tolerant teleost, and 2) a two-species comparison of elasmobranchs with different hypoxia tolerance.

I tested the hypothesis that CHT depends upon the depression of cardiac power output (PO) (i.e., cardiac energy demand) to a level lower than the cardiac maximum glycolytic potential (MGP). All species showed a hypoxic PO depression via bradycardia and my work generally supports this hypothesis. However, in tilapia, hypoxic PO depression is not necessarily required to maintain cardiac energy balance, contrary to previous suggestions, because of an exceptionally high MGP. Thus, in certain species, PO depression may primarily benefit CHT by minimizing fuel use and waste production.

I also tested the hypothesis that greater hypoxia tolerance is associated with enhanced hypoxic O₂ supply and consequently enhanced cardiovascular function (i.e., less PO depression and improved cardiac energy balance). My work on elasmobranchs supported this hypothesis and also suggested a role for strategic cardiac O₂ supply via O₂ sparing resulting from metabolic rate depression (MRD) in non-essential tissues. Finally, my work on elasmobranchs showed that critical oxygen tension (Pcrit) predicts hypoxic blood O₂ transport, supporting the use of Pcrit as an indicator of hypoxia tolerance.
Next, I tested the hypothesis that hypoxic $PO$ depression is associated with the depression of whole-animal $O_2$ consumption rate below $P_{\text{crit}}$. I found that this occurred in all species, suggesting that modulation of peripheral demand for blood flow (e.g., via MRD) may influence CHT.

Finally, my work on $in$ $vivo$ and $in$ $situ$ cardiac responses in tilapia provided little evidence for the hypothesis that hypoxic modulation of aerobic energy production pathways, including provision of aerobic fuels (specifically, fatty acids), contributes to CHT.
PREFACE

A version of Chapter 2 has been published as: Speers-Roesch, B., Sandblom, E., Lau, G. Y., Farrell, A. P., and Richards, J. G. 2010. Effects of environmental hypoxia on cardiac energy metabolism and performance in tilapia. *American Journal of Physiology* 298: R104-R119. I designed and conducted all experiments, carried out all analyses, and wrote the manuscript under the supervision of Dr. J. G. Richards. Dr. E. Sandblom and Ms. G.Y. Lau assisted in surgical instrumentation and AMPK assay, respectively. Dr. A. P. Farrell provided advice on experimental design. All authors provided editorial input.

Chapter 3 is based on work I carried out in collaboration with Ms. S. L. Lague and Drs. A. P. Farrell and J. G. Richards. I designed the experiments and wrote the manuscript under the supervision of Dr. J. G. Richards. Ms. S. L. Lague assisted me in conducting the experiments. Dr. A. P. Farrell provided advice on experimental design.

A version of Chapter 4 has been published as: Speers-Roesch, B., Richards, J. G., Brauner, C. J., Farrell, A. P., Hickey, A. J. R., Wang, Y. S., and Renshaw, G. M. C. 2012. Hypoxia tolerance in elasmobranchs. I. Critical oxygen tension as a measure of blood oxygen transport during hypoxia exposure. *Journal of Experimental Biology* 215: 93-102. All authors were involved in designing and conducting the experiments, but I played a primary role in this process. I carried out all analyses and wrote the manuscript under the supervision of Dr. J. G. Richards. All authors provided editorial input.

Biology 215: 103-114. All authors were involved in designing and conducting the experiments, but I played a primary role in the process. All authors were involved in the design and conduction of all experiments, but I played a primary role in this process. I carried out all analyses and wrote the manuscript under the supervision of Dr. J. G. Richards. All authors provided editorial input.

All experiments were approved by the UBC Animal Care Committee (A09-0611 and A09-0008-R003).
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\( \alpha O_2 \), solubility of O₂

ADP\textsubscript{free}, free adenosine diphosphate

AMP\textsubscript{free}, free adenosine monophosphate

AMPK, AMP-activated protein kinase

ATP, adenosine triphosphate

BSA, bovine serum albumin

\( C_aO_2 \), arterial blood oxygen content

CPT, carnitine palmitoyltransferase

Cr, creatine

CrP, creatine phosphate

EF-1\( \alpha \), elongation factor-1\( \alpha \)

\( f_{hi} \), heart rate

\( H^+ \), hydronium ion

Hb, hemoglobin

Hb-O₂ \( P_{50} \), hemoglobin-oxygen binding affinity

Hct, hematocrit

HIF, hypoxia inducible factor

\( \beta \)-HB, \( \beta \)-hydroxybutyrate

MCHC, mean cellular hemoglobin content

MS-222, tricaine methanesulfonate

MGP, maximum glycolytic potential
\( \dot{M}_{O_2} \), whole-animal oxygen consumption rate

MRD, metabolic rate depression

\( M_v \), ventricular mass

NEFA, non-esterified fatty acids

\( n_H \), hill coefficient

\( P_{\text{crit}} \), critical \( O_2 \) tension of whole-animal \( O_2 \) consumption rate

PDH, pyruvate dehydrogenase

PDH\(_a\), active fraction of pyruvate dehydrogenase

PDK, pyruvate dehydrogenase kinase

PPAR\( \alpha \), peroxisome proliferator-activated receptor \( \alpha \)

\( P_1 \), inorganic phosphate

\( P_{\text{in}} \), input pressure

\( P_{\text{out}} \), output pressure

\( P_{\text{DA}} \), dorsal aortic blood pressure

\( P_{\text{VA}} \), ventral aortic blood pressure

\( P_{\text{CO}_2} \), partial pressure of carbon dioxide

\( P_{\text{O}_2} \), partial pressure of oxygen

\( P_{a\text{O}_2} \), arterial blood \( P_{\text{O}_2} \)

\( P_{w\text{O}_2} \), water \( P_{\text{O}_2} \)

\( P_{a\text{CO}_2} \), arterial blood \( P_{\text{CO}_2} \)

\( pH_i \), intracellular pH

\( PO \), cardiac power output

\( PO_{\text{max}} \), maximum cardiac power output
$\dot{Q}$, cardiac output

$\dot{Q}_{\text{max}}$, maximum cardiac output

qRT-PCR, quantitative real-time PCR

$R$, peripheral resistance

$R_{\text{SYS}}$, systemic peripheral resistance

TES, 2-(Tris(hydroxymethyl)methylamino)ethane-1-sulphonic

$\dot{V}_{O_2}$, myocardial oxygen consumption rate

$V_{SH}$, stroke volume
ACKNOWLEDGEMENTS

No man is an island. I wish to thank, with a bare minimum of tired clichés, the many who have helped me along the way.

My supervisor Jeff Richards is a fantastic mentor who allowed me independence, afforded me many opportunities, and pushed me to always do better. His guidance has helped my intellectual development immeasurably, for which I express my gratitude. Although I can only hope that some of Jeff’s considerable scientific talents have rubbed off on me, I know that some of his catch phrases have (e.g., “Oh lord”; “Sure-sure”; “Data?”; “How’s the paper coming?”). I look forward to inflicting them upon my colleagues in the future.

Jeff has always assembled a remarkably awesome lab group. I consider myself fortunate to have shared my office and lab space with the following talented and fun individuals: Dave Allen, Rush Dhillon, Gina Galli, Derrick Groom, Patrik Henriksson, Lindsay Jibb, Gigi Lau, Milica Mandic, Matt Regan, Tammy Rodela, Mark Scott, Andrew Thompson, Travis van Leeuwen, and Lili Yao. In particular, I would like to thank Milica Mandic and Gigi Lau. Milica has been my lab and office mate throughout my PhD and we have shared many excellent times at Bamfield, UBC, and Library Square. I thank her for allowing me (and no one else) to use her micropipettes when required, for tolerating my imperialistic policy regarding her bench and desk space, for letting me sometimes drive the boat, and for being an expert air guitar partner. Gigi is another Richards lab fixture, dating to the old BioSci days, and she has always been a smiling face while running my AMPK assays, acting as my reluctant and long-suffering secretary, and keeping me sugared with unknown candies from the Far East.

The remarkable talents and collegiality of UBC Zoology made my PhD all the more enjoyable. Colin Brauner, Tony Farrell, and Trish Schulte served on my committee and I thank them for challenging me and for showing me the wisdom of thinking ever more broadly. Bill Milsom and Bob Shadwick similarly provided sage advice on my work. Agnes Lacombe was a great boss in 363 lab, helping me to improve my teaching and being tolerant of my occasional research- and diving-related absences. Beyond those already mentioned, I have been fortunate to count among my colleagues and friends at UBC the following individuals: Dan Baker, Carol Bucking, Jason Bystriansky, Matt Casselman, Tim Clark, Georgie Cox, Carla Crossman, Erica Dale, Anne Dalziel, Yvonne Dzal, Erika Eliason, Nann Fangue, Clarice Fu, Alezea Gerstein, Taylor Gibbons, Kristen Haakons, Linda Herbert (Hanson), Katie Huynh, Ken Jeffries, T. Todd Jones, Sabine Lague, Stella Lee, Jessica McKenzie, Jessica Meir, Leithen M’Gonigle, Jasmine Ono, Michelle Ou, Sheldon Pinto, Cosima Porteus, Brock Ramshaw, Catalina Reyes, Jodie Runner, Mike Sackville, Erik Sandblom, Brian Sardella, Graham Scott, Ryan Shartau, Matt Siegle, Dave Toews, Katelyn Tovey, Gerrit Velema, and Christine Verhille.

A special tip of the Scotch glass is reserved for Erica Dale, Rush Dhillon, Milica Mandic, Jessica Meir, Matt Regan, Mike Sackville, and Dave Toews, as well as Jason
Bystriansky, Leithen M’Gonigle, Sheldon Pinto, Graham Scott, and Gerrit Velema. You are great friends; thanks for all the laughs, intellectual dialogue, and good times.

I thank Pat Tamkee for his reliable and helpful aquatics management. Don Brandys, Bruce Gillespie, and Vince Grant provided expert and invaluable technical help. Barry Sjostrom at Redfish Ranch was always willing to sell me fish at a fair price.

Two chapters of my thesis arose from work done during the great Australian shark expedition of 2009. I was lucky to share this experience with Colin Brauner, Tony Farrell, Tony Hickey, Gillian Renshaw, Jeff Richards, and Yuxiang Wang. Learning in the trenches from such seasoned veterans was a formative and enjoyable experience for me, all the more because they covered my bar tab.

Other work in my thesis benefited from direct collaborations with Erik Sandblom and Sabine Lague. I thank both of them for enjoyable, educational, and productive times in the lab. I also thank Jay Treberg for fruitful on-going collaborations and many informative discussions. Finally, I thank Dan Lingwood for his friendship, enthusiastic support, and discussions of all things.

I have many fond memories of summer research trips to Bamfield Marine Sciences Centre with Milica Mandic, Patrik Henriksson and others. I wish to thank the BMSC staff and in particular Bruce Cameron for their expert support. My time at BMSC was greatly enriched by the presence of Will Duguid and members of the Chris Wood and Greg Goss labs.

My research and I have been funded generously by Natural Sciences and Engineering Research Council (Canada), UBC Zoology, IODE Canada, Company of Biologists, and Canadian Society of Zoologists.

Stephanie Avery-Gomm, thank you for your patience, love, and support. You are the greatest discovery I made during my PhD. Thank you for sharing this time with me. I also thank the Avery-Wilson and Gomm families for their support and for making me feel at home.

For as long as I can remember, I’ve wanted to be an academic scientist. My family has always unreservedly supported me in this goal. For their love and encouragement, I thank the Atkinson and Vogel families, Barry Speers and family, Lillian and Orval Speers, Marisa Graziosi and family, Alex Speers-Roesch, and especially my mother, Heather Speers. I also thank my late father, Otto Roesch, who encouraged my curious mind and who continues to inspire me. My parents were my first and most important mentors and it is difficult to overstate their significance in whatever success I have achieved.
CHAPTER 1: GENERAL INTRODUCTION

1.1 OXYGEN IS ESSENTIAL FOR VERTEBRATE LIFE

All cellular life depends upon the matching of energy (i.e., ATP) supply to energy demand such that metabolic homeostasis is maintained. For most animal life and all vertebrate life, there is a heavy reliance upon O$_2$-dependent (aerobic) energy production to sustain the metabolic energy demands associated with basal metabolism and metabolic scope (i.e., growth, locomotion, and reproduction). Aerobic energy production involves the oxidation of reduced organic compounds in the mitochondria to produce ATP via oxidative phosphorylation (Hochachka and Somero, 2002). The other major form of energy production in vertebrates is O$_2$-independent substrate-level phosphorylation, including creatine phosphate (CrP) hydrolysis and anaerobic glycolysis of glucose to lactate. However, CrP is a limited ATP buffer and anaerobic glycolysis yields about 14 times less ATP than oxidative phosphorylation (Brand, 2003). Thus, aerobic energy production is far more energetically efficient than anaerobic energy production. Indeed, the emergence of compartmentalized aerobic energy production via the endosymbiotic acquisition of mitochondria in the first eukaryote, followed by the consequent exploitation of rising atmospheric O$_2$ levels, likely enabled the remarkable evolutionary expansion of morphological complexity and metabolic scope seen in animals (Catling et al., 2005; Koch and Britton 2008; Lane and Martin, 2010). One major consequence of the dependence upon aerobic metabolism and the related increase in morphological complexity in vertebrates is the evolution of intricate respiratory and circulatory systems to take up O$_2$ and transport it within the body (Burghgren and Reiber, 2007). Another major consequence is that decreased levels of environmental O$_2$ (i.e., environmental hypoxia), which commonly occur in many habitats (e.g., high altitude,
subterranean, and aquatic), can impair metabolic scope and, if severe enough, can impair basal metabolism potentially leading to death.

Death from O$_2$ deprivation, which can occur within minutes in many vertebrates including humans, is thought to result from an inability to sustain the energy demands of vital tissues such as heart and brain in the face of impaired aerobic energy supply, leading to a precipitous decline in cellular ATP levels and consequently cell death and tissue necrosis (Boutilier, 2001a; Hochachka, 1986). At the onset of O$_2$ lack, cellular ATP levels initially are defended via CrP hydrolysis but these stores are rapidly depleted. The rate of anaerobic glycolysis is greatly increased in an attempt to make up for the shortfall in aerobic ATP production (the Pasteur effect). However, because of the limited ATP yield of anaerobic glycolysis, the increase in glycolytic flux required to maintain ATP production at normal aerobic rates may not be achievable by the cell’s maximum glycolytic potential. Even if it is, the inefficiency of anaerobic glycolysis (~14-fold lower ATP production per mol glucose vs. glucose oxidation) means that the finite stores of fermentable fuel (i.e., glycogen) are rapidly depleted leading to impaired ATP production. Thus, ATP levels fall. Vital ATP-dependent cellular processes such as ionoregulation and the maintenance of membrane potentials become starved of energy, leading to equilibration of cellular ion gradients (Boutilier, 2001a; Hochachka, 1986). Depolarization of cellular membranes leads to electrochemical and ionic imbalances that can trigger apoptotic or necrotic cell death (Boutilier, 2001a; Chen et al., 2007; Hochachka, 1986). Accumulation of anaerobic by-products such as H$^+$ or P$_i$ resulting from substrate-level phosphorylation can also exacerbate tissue injury and dysfunction (Allen and Orchard, 1987; Orchard and Kentish, 1990). Tissue damage resulting from this cascade
of deleterious cellular events can cause irreversible injury to the heart and brain, leading to circulatory and neural failure and death of the organism.

Because of the prevalence of hypoxia in certain environments, however, a number of vertebrate species have evolved the ability to survive extended periods of severe hypoxia and even anoxia, in large part by being able to balance energy supply and demand in vital organs during $O_2$ limitation thus averting hypoxic death (Boutilier, 2001a). The major objectives of the present thesis are to investigate:

1) the mechanisms by which cardiac hypoxia tolerance (i.e., maintenance of cardiac metabolic energy balance and continued cardiac function during prolonged hypoxia exposure) is achieved

2) the relationship between cardiac hypoxia tolerance and whole-animal hypoxia tolerance

In this General Introduction, I summarize what is known to date about the physiological strategies by which whole-animal and cardiac hypoxia tolerance is achieved, with a focus on aquatic ectothermic vertebrates (especially fishes).

1.2 DEFINITIONS: HYPOXIA & HYPOXIA TOLERANCE

Before continuing, we must define the terms hypoxia and hypoxia tolerance as they are used extensively in the present thesis and, often, are used in the literature without clear definition.
1.2.1 What is hypoxia?

Hypoxia is a shortage of O$_2$ that can be environmental or functional in origin (Farrell and Richards, 2009). Within the context of my thesis I will not discuss functional hypoxia, which occurs when tissue O$_2$ demand exceeds O$_2$ supply independently of any change in environmental O$_2$ levels, for example during strenuous exercise (Farrell and Richards, 2009).

Environmental hypoxia is common in aquatic habitats because of the low diffusion rate and concentration of O$_2$ in water and because many water bodies are closed or can become stagnant such that biomass respiration can deplete O$_2$ (Nilsson, 2010).

Environmental (aquatic) hypoxia, in its absolute definition, is any decrease in the partial pressure of O$_2$ in water ($P_{wO_2}$) below air saturation, which at sea level at 20°C is 20.7 kPa or 156 torr. I use $P_{wO_2}$ in defining hypoxia because it is the partial pressure gradient of a gas that drives diffusion and because $P_{wO_2}$ is a major determinant of the O$_2$ concentration of water (Farrell and Richards, 2009). In absolute terms, the range of possible $P_{wO_2}$ from air saturation to complete anoxia then can be arbitrarily divided approximately in three and environmental hypoxia simply described as mild (14 to ~20.7 kPa), moderate (7 to 14 kPa), or severe (1 to 7 kPa). One further division, profound hypoxia, is reserved for situations where the $P_{wO_2}$ is ≤1kPa and is useful when making an example of the champions of animal hypoxia tolerance.

Anoxia is the most extreme case of hypoxia: the total absence of O$_2$. However, this is difficult to achieve in experimental settings and anoxia is typically used in reference to situations where $P_{wO_2}$ approaches zero (i.e., <0.1 kPa). This absolute definition of environmental hypoxia is useful, for example, when contrasting the abilities of different species to respond to and tolerate variable environmental O$_2$ tensions.
Environmental hypoxia must also be defined relative to the animal to account for species-specific differences in the physiological response to hypoxia and to better understand and compare these responses. From the perspective of the animal, Farrell and Richards (2009) suggested that environmental hypoxia occurs when the animal itself experiences O₂ shortage, i.e. a decrease in P_wO₂ that compromises physiological function such that compensatory physiological responses are elicited. Following this, two major levels of relative environmental hypoxia can be defined based on specific physiological responses related to changes in whole-animal O₂ consumption rate (\( \dot{M}_{O_2} \)) as P_wO₂ is decreased (Fig. 1.1). Level 1 is the P_wO₂ at which the blood initially becomes hypoxemic (i.e., the P_wO₂ at which arterial O₂ content initially decreases due to a fall in Hb-O₂ saturation). This hypoxemia can be compensated for by responses at the various steps of the O₂ transport cascade (see section 1.4.1) such that tissue O₂ supply and routine \( \dot{M}_{O_2} \) (i.e., \( \dot{M}_{O_2} \) in a resting, post-absorptive state) is maintained, but this compensation limits the animal’s aerobic scope and thus its capacity for activity, growth, and reproduction (Farrell and Richards, 2009). As P_wO₂ decreases further, aerobic scope continues to decrease while routine \( \dot{M}_{O_2} \) is maintained by the compensatory mechanisms. Eventually routine \( \dot{M}_{O_2} \) cannot be maintained and begins to decrease with the fall in P_wO₂. This point (level 2) is termed the critical O₂ tension (P_{crit}) and indicates where routine \( \dot{M}_{O_2} \) transitions from being independent of P_wO₂ (oxyregulation) to being dependent upon P_wO₂ (oxyconformation) (see section 1.4.1), and below which the associated hypoxemia causes routine aerobic metabolism to be limited such that anaerobic energy production is increasingly relied upon and lactate accumulates (see below). Thus, relative environmental hypoxia generally may be defined as the range of P_wO₂ over which
aerobic scope gradually decreases to zero (Farrell and Richards, 2009). Following a similar line of reasoning as above for absolute levels of environmental hypoxia, we can describe relative environmental hypoxia as either moderate ($P_{wO_2}$ between level 1 and level 2: from initial hypoxemia and limitation of aerobic scope to $P_{crit}$) or severe ($P_{wO_2}$ below level 2: $P_{wO_2}$ below $P_{crit}$ where routine aerobic metabolism is limited and anaerobic glycolysis is stimulated). These two levels can also be referred to as causing moderate or severe hypoxemic stress (Fig. 1.1).

In practice, the best way to distinguish and reconcile the absolute and relative definitions of environmental hypoxia is to explicitly describe the level of environmental hypoxia used (including the $P_{wO_2}$) with reference to the corresponding level and type of hypoxemic stress it imposes on the study species. This is the approach taken in the present thesis, where $P_{crit}$ has been measured in all study species. My general use of “hypoxia” (e.g., general use of “hypoxia exposure” as well as “O$_2$ limitation”, “low O$_2$”, and “hypoxic conditions”), unless otherwise noted, refers to low $P_{wO_2}$ causing severe hypoxemic stress (i.e., $P_{wO_2}$ below $P_{cri}$), because of my interest in the most metabolically-challenging level of hypoxia. On the other hand, I have used “environmental hypoxia” when referring generally to absolute decreases in $P_{wO_2}$ (and a gradual decrease in $P_{wO_2}$ is referred to as “progressive hypoxia”). Hypoxia tolerance is defined separately in the following section 1.2.2.

Two additional physiological events that occur below $P_{crit}$ (stimulation of anaerobic glycolysis and metabolic rate depression) are worth discussing briefly with regard to the definition of environmental hypoxia from a relative, physiological perspective. Both relate to the major challenge of balancing energy supply and demand when aerobic ATP production is constrained below $P_{crit}$ (see section 1.4). The first is the $P_{wO_2}$ at which anaerobic glycolysis
becomes stimulated and lactate accumulates in the body. This $P_wO_2$ is important because it indicates a limitation of routine aerobic energy production and it has major consequences for energy production, acid-base balance, and thus homeostasis. From a theoretical standpoint, increased reliance on anaerobic energy production may occur at $P_{crit}$, when there is a mismatch between $O_2$ supply and demand. However, this hypothesis is much debated because available evidence is varied in its support, possibly because of the confounding effects of metabolic rate depression; in general, however, lactate accumulation does occur below $P_{crit}$ and here I consider it to be a component of severe hypoxemic stress as defined above (McKenzie et al., 2000; Nonnotte et al., 1993; Pörtner and Grieshaber, 1993; Tattersall and Ultsch, 2008; Ultsch et al. 2004). Metabolic rate depression (MRD), which is a profound and reversible decrease of basal cellular ATP turnover (Guppy and Withers, 1999) (see section 1.4.3), is the second physiological event potentially occurring at a certain $P_wO_2$ below $P_{crit}$ that may help define environmental hypoxia relative to the animal. However, the threshold $P_wO_2$ at which this occurs is not well defined and is complicated by the fact that the ability to undergo MRD (and the magnitude that can be achieved) is likely highly species-specific.

1.2.2 What is hypoxia tolerance?

Hypoxia tolerance describes the magnitude and duration of hypoxia exposure that an animal can survive. All animals are hypoxia-tolerant to an extent, but species with greater hypoxia tolerance can survive exposure to lower $P_O_2$ and greater hypoxemic stress for longer periods of time than species with lesser hypoxia tolerance (i.e., species that are comparatively hypoxia-sensitive) (see section 1.3) (Boutilier, 2001a). In general, I define a hypoxia-tolerant
animal as one that can survive exposure to profound environmental hypoxia causing severe hypoxemic stress for many hours (e.g. crucian carp, common carp *Cyprinus carpio*, tilapia *Oreochromis* spp., and epaulette shark *Hemiscyllium ocellatum*; Stecyk and Farrell, 2006; Stecyk et al., 2004; Chapter 2; Chapter 5) whereas a hypoxia-sensitive species is one that survives only minutes under the same conditions (e.g., rainbow trout *Oncorhynchus mykiss* and Atlantic cod *Gadus morhua*; Doudoroff and Shumway, 1970; Perry and Reid, 1992; Petersen and Gamperl, 2011), at a comparable environmental temperature.

Indeed, temperature has a profound effect upon hypoxia tolerance. For example, survival time of common carp exposed to profound hypoxia is ~25 h at 6°C, ~6 h at 10°C, and ~2 h at 15°C (Stecyk and Farrell, 2002). Similarly, survival time in anoxia-exposed crucian carp and turtles is greatly extended at lower environmental temperature (Farrell and Stecyk, 2007; Vornanen et al., 2009). The relationship between hypoxia tolerance and temperature is probably explained, in part, by slower rates of fuel use and waste accumulation at colder temperatures. Also, if there are differential Q_{10} effects on energy demand vs. energy supply pathways, temperature could effect the ability to maintain cellular energy balance. On the other hand, there are many tropical freshwater fishes such as oscar (*Astronotus ocellatus*) and tilapia that can survive P_{aw}O_{2} as low as 1 kPa for at least 24 hours at temperatures above 20°C (Richards et al., 2007; Chapter 2), indicating the evolution of impressive hypoxia tolerance even in fishes living at warm temperatures. This suggests that warm-water fishes represent excellent models to study mechanisms of hypoxia tolerance.

A distinction should be made between simply surviving environmental hypoxia exposure and continuing to exist normally (i.e., maintaining a normal metabolic scope for growth, reproduction, etc.) during environmental hypoxia exposure. An animal may be able
to survive indefinitely at a low $P_wO_2$ causing moderate hypoxemic stress, but growth or reproduction can be curtailed (Diaz and Breitburg, 2009; Jobling, 1994). However, it is reasonable to assume that greater hypoxia tolerance is generally correlated with the ability to maintain a normal metabolic scope to a lower $P_wO_2$, if the hypoxia tolerance is associated with enhanced $O_2$ extraction (see discussion of $P_{crit}$ in section 1.4.1) (Wang et al., 2009). For example, the $O_2$ level at which growth is retarded in the hypoxia-tolerant tilapia (3-4 mg $O_2$/L) is lower than that of the relatively hypoxia-sensitive rainbow trout (7 mg $O_2$/L) (Haug et al., 2008; Siddiqui et al., 1991; Jobling, 1994). Thus, a greater hypoxia tolerance confers the ability to survive potentially deadly severe hypoxemic stress but is also correlated with the ability to maintain aerobic scope to low $P_wO_2$.

1.3 EXAMPLES OF HYPOXIA TOLERANCE AMONG AQUATIC ECTOTHERMIC VERTEBRATES

The most impressive and numerous examples of organismal hypoxia tolerance occur in aquatic environments, likely because aquatic hypoxic events are more common and of greater severity and duration compared with most terrestrial environments, where $O_2$ concentration and diffusion are higher and stagnation of the medium is much less likely. Among the champions of vertebrate hypoxia tolerance are the crucian carp ($Carassius carassius$) and the freshwater turtles $Chrysemys picta$ and $Trachemys scripta$, which may experience anoxia in ice-covered freshwaters during winter and thus can survive the complete absence of $O_2$ for months at cold temperatures (Bickler and Buck, 2007; Jackson, 2000; Vornanen et al., 2009). The great diversity of fishes has given rise to the apparently independent evolution of hypoxia tolerance in multiple taxa (Hochachka and Somero, 2002).
Many of the most hypoxia-tolerant fishes, including many cichlids such as oscar and tilapia, live in tropical freshwater habitats where environmental hypoxia is common (Chapman et al., 1995; Chapman et al., 2002; Richards et al., 2007; Chapter 2). Hypoxia tolerance is also common among fishes living in certain marine environments with variable O\textsubscript{2} regimes including coral reefs, oxygen minimum zones, and the intertidal zone (Childress and Seibel, 1998; Mandic et al., 2009; Nilsson et al., 2007; Utne-Palm et al., 2010). However, probably because the occurrence of aquatic hypoxia varies in magnitude and spatio-temporal scale depending on the local habitat (Diaz and Breitburg, 2009), the severity and duration of environmental hypoxia that can be survived is highly species-specific among fishes and other aquatic vertebrates (Bickler and Buck, 2007). Many aquatic habitats such as fast flowing streams are rarely hypoxic and species living there, such as the highly active salmonids, typically possess limited hypoxia tolerance (Doudoroff and Shumway, 1970). Thus, hypoxia tolerance varies greatly among fishes and other aquatic vertebrates and depends upon the natural history of the species in question.

### 1.4 PHYSIOLOGICAL STRATEGIES OF HYPOXIA TOLERANCE

Hypoxia tolerance is thought to be dependent ultimately upon the ability to balance cellular energy supply with energy demand, and therefore maintain stable levels of cellular [ATP], despite low environmental O\textsubscript{2} levels (Boutilier, 2001a; Hochachka et al., 1996; Richards, 2009). Thus, hypoxia-tolerant animals maintain metabolic energy balance at lower P\textsubscript{ao}O\textsubscript{2} and for longer durations than comparatively hypoxia-sensitive animals (Fig. 1.2). Maintenance of metabolic energy balance is most important in organs such as brain and heart, which are particularly hypoxia-sensitive by virtue of their high ATP turnover rates.
A mismatch between energy supply and demand during environmental hypoxia exposure rapidly results in death (Fig. 1.2), as discussed in section 1.1.

There are three strategies by which metabolic energy balance can be maintained during exposure to environmental hypoxia. The first is to maintain $O_2$ supply to tissues as $P_{w}O_2$ decreases, which is thought to be reflected by the $P_{\text{crit}}$, the lowest $P_{w}O_2$ at which routine $\dot{M}_{O_2}$ can be maintained (Fig. 1.1; Fig. 1.2). At $P_{w}O_2$ below $P_{\text{crit}}$, hypoxia tolerance becomes dependent on maintenance of cellular energy balance in the face of decreases in aerobic energy supply (Richards, 2009). This may be achieved by an increased reliance on $O_2$-independent energy supply (e.g., anaerobic glycolysis) as well as a profound, reversible MRD in which large decreases in cellular energy demand occur (Richards, 2009) (Fig. 1.2). MRD also greatly extends hypoxic survival time, thus improving hypoxia tolerance, because it slows the rate of fuel use and the accumulation of deleterious wastes (e.g., $H^+$, lactate) associated with anaerobic energy production (Richards, 2009). Optimization of tissue $O_2$ supply at $P_{w}O_2$ below $P_{\text{crit}}$ may also be important in decreasing reliance on the relatively inefficient anaerobic glycolysis (Fig. 1.2). In this section, I will briefly discuss these strategies and their underlying mechanisms.

1.4.1 $P_{\text{crit}}$ and $O_2$ supply

The $P_{\text{crit}}$ is thought to be an indicator of the ability of an organism to extract $O_2$ from its environment. $P_{\text{crit}}$ is used specifically with regard to routine $\dot{M}_{O_2}$, but critical $O_2$ tensions also occur at different levels of $\dot{M}_{O_2}$ (e.g. during exercise, digestion) (Farrell and Richards, 2009; Thuy et al., 2010). Based on available evidence, virtually all fishes are thought to be
oxyregulators that possess a $P_{\text{crit}}$ (Steffensen, 2007; Ultsch et al., 1981). There may be a few exceptions that show oxyconformation of $\dot{M}_{O_2}$ at all $P_{wO_2}$ (Urbina et al., 2012).

Within the context of comparative physiology, the concept of $P_{\text{crit}}$ can be traced back to the seminal work of Fry (Fry, 1947; Fry and Hart, 1948), which was built upon by Hughes (1973), Ultsch (Ott et al., 1980; Ultsch et al., 1981), Pörtner (Pörtner and Grieshaber, 1993), and others. These and later authors recognized the obvious potential benefit for hypoxia tolerance conferred by the ability to maintain routine $\dot{M}_{O_2}$ at low $P_{wO_2}$, as indicated by $P_{\text{crit}}$.

It is now generally accepted that $P_{\text{crit}}$ is an indicator of hypoxia tolerance and that a lower $P_{\text{crit}}$ is associated with greater hypoxia tolerance, presumably because of improved $O_2$ uptake and transport to tissues at low $P_{wO_2}$ (Chapman et al., 2002; Childress and Seibel, 1998; Mandic et al., 2009; Nilsson and Östlund-Nilsson, 2008; Nilsson et al., 2007). However, this notion has rarely been directly tested nor has it been shown that $P_{\text{crit}}$ is associated with greater $O_2$ uptake or blood $O_2$ transport at $P_{wO_2}$ below $P_{\text{crit}}$.

$P_{\text{crit}}$ represents an integration of all the steps in the $O_2$ transport cascade, which is the diffusive and convective movement of $O_2$ down its partial pressure gradient from the water to the mitochondrion (Fig. 1.3) (Bouverot, 1985; Dejours, 1981; Weibel, 1984). Consequently, physiological modifications at any step in the $O_2$ transport cascade may affect $P_{\text{crit}}$, including changes in ventilation, diffusion of $O_2$ into the blood (e.g., gill surface area), blood $O_2$ capacity (including blood hemoglobin concentration [Hb] and Hb-$O_2$ binding affinity), circulation of $O_2$ (e.g., cardiac output), diffusion of $O_2$ into tissues, and mitochondrial $O_2$ turnover (Dejours, 1981; Farrell and Richards, 2009). These adjustments affecting $P_{\text{crit}}$ can occur over acclimatory or evolutionary time scales. For example, hypoxia-induced gill remodeling that increases gill surface area causes a decrease in $P_{\text{crit}}$ in crucian carp and
goldfish (Carassius auratus) (Sollid and Nilsson, 2006). On the other hand, there is great species-specific variation in $P_{crit}$ among fishes that reflects intrinsic, evolved differences in the steps of the $O_2$ transport cascade. There is a major interest in identifying which of these steps are most important in determining the interspecific variation in $P_{crit}$ in order to better understand the mechanisms and evolution of hypoxia tolerance.

A phylogenetically-independent comparison of $O_2$ transport in sculpins with differing $P_{crit}$ suggested that routine $\dot{M}_{O_2}$, gill surface area, and whole blood Hb-$O_2$ $P_{50}$ (i.e., Hb-$O_2$ binding affinity) are significant determinants of $P_{crit}$ (Mandic et al., 2009). Hb-$O_2$ $P_{50}$ plays a particularly important role in controlling blood $O_2$ content and $O_2$ uptake in animals (Brauner and Wang, 1997), so it is perhaps unsurprising that the strongest correlation was observed between $P_{crit}$ and Hb-$O_2$ $P_{50}$; sculpins with lower $P_{crit}$ had lower Hb-$O_2$ $P_{50}$ (Mandic et al., 2009). These findings support the long-held notion that a high Hb-$O_2$ binding affinity contributes to hypoxia tolerance in fishes (Nikinmaa, 2001; Powers, 1980), which derived from independent studies showing that species with a low Hb-$O_2$ $P_{50}$ usually also have a low $P_{crit}$, have greater blood $O_2$ content at low $P_{wO_2}$, and are relatively hypoxia-tolerant (e.g., goldfish: Burggren, 1982; tench Tinca tinca: Jensen and Weber, 1982; eels Anguilla spp.: Cruz-Neto and Steffensen, 1997; Laursen et al., 1985; Perry and Reid, 1992; crucian carp: Sollid et al., 2005; common carp: Takeda, 1990; Ott et al., 1980). In contrast, species with relatively high Hb-$O_2$ $P_{50}$, such as rainbow trout and Atlantic cod, usually have higher $P_{crit}$, show greater hypoxemia during hypoxia exposure, and are comparatively hypoxia-sensitive (Milligan and Wood, 1987; Ott et al., 1980; Perry and Reid, 1992; Petersen and Gamperl, 2011). Nonetheless, the relationship between $P_{crit}$, Hb-$O_2$ $P_{50}$, blood $O_2$ transport, and
hypoxia tolerance has not been directly investigated under controlled conditions within a single study and this is one of the major goals of the present thesis.

1.4.2 Fuel selection and energy supply during hypoxia exposure

The three common metabolic fuels used for ATP production under normoxic conditions in fishes and other vertebrates are carbohydrates (e.g., glucose and lactate), lipids (specifically, fatty acids), and amino acids (Weber and Haman, 1996). During hypoxia exposure below \( P_{\text{crit}} \), however, use of fatty acids and amino acids is greatly restricted because, generally, they must undergo oxidative phosphorylation to produce ATP. Instead, the major source of ATP becomes the fermentation of glucose to lactate, via anaerobic glycolysis.

1.4.2.1 Anaerobic glycolysis during hypoxia exposure

At \( P_{\text{wO}_2} \) below \( P_{\text{crit}} \), where routine aerobic ATP production is constrained, \( \text{O}_2 \)-independent energy production (e.g. CrP hydrolysis and anaerobic glycolysis) is increasingly relied upon to meet energy demands (Richards, 2009) (Fig. 1.2). Because [CrP] represents a relatively small pool of high-energy phosphate that can be quickly depleted, anaerobic glycolysis plays the central role in \( \text{O}_2 \)-independent energy production and flux through this pathway can be increased (Omlin and Weber, 2010). It is well established that anaerobic glycolysis is a requisite for sustained hypoxic function of organ systems (e.g., brain and heart) and therefore hypoxia survival in animals (Gamperl and Driedzic, 2009; Lutz et al., 2002). However, there is little clear evidence of a link between the capacity for anaerobic glycolysis and hypoxia tolerance (Bailey et al., 1999; De Almeida-Val et al., 2006; Gesser
and Poupa, 1974; Nilsson and Östlund-Nilsson, 2008; West et al., 1999), possibly because anaerobic glycolytic capacity can be affected by confounding factors such as activity level and lifestyle, exercise capacity, and thermal history. One known exception may be glycogen stores, from which glucose is mobilized to fuel glycolysis; hypoxia-tolerant animals typically have greater glycogen levels in most tissues and especially in the liver, which is the main glycogen depot in vertebrates (Richards, 2009).

The increased reliance on anaerobic glycolysis is reflected by an accumulation of tissue lactate, which is a key indicator of a mismatch between O\textsubscript{2} supply and O\textsubscript{2} demand during environmental hypoxia exposure. Accompanying the lactate is an equimolar production of H\textsuperscript{+} resulting from glycolytic ATP hydrolysis (Hochachka and Somero, 2002). The ensuing metabolic acidosis places formidable constraints on hypoxia tolerance in animals (Nilsson and Östlund-Nilsson, 2008), having been implicated in dysfunction of organs such as the heart (Driedzic and Gesser, 1994) as well as hypoxic death (DiAngelo and Heath, 1987; van Raaij et al., 1994). It is probably not coincidental that the vertebrates with the greatest known hypoxia tolerance, freshwater turtles and Carassius carps, have evolved novel strategies to mitigate the effects of acidosis, including the use of the shell as a buffer of H\textsuperscript{+} in turtles and the production of ethanol as the ultimate end-product of anaerobic glycolysis in Carassius spp. (Jackson, 2000; Vornanen et al., 2009). Other hypoxia-tolerant vertebrates must rely on conventional methods of acid-base regulation during hypoxia exposure but may also possess an improved ability to maintain intracellular pH and to tolerate acidosis at the cellular level (Driedzic and Gesser, 1994; Jackson, 2004; Stecyk et al., 2008).
Another major disadvantage of anaerobic glycolysis is that it is 14-fold less efficient than oxidative phosphorylation, resulting in faster rates of glucose utilization and glycogen depletion (and waste production) in order to sustain a given level of ATP demand. The cessation of feeding that often accompanies hypoxia exposure (Wang et al., 2009) compounds this greater rate of fuel use. Thus, even though hypoxia-tolerant species may store more glycogen, the availability of this fermentable fuel may eventually limit hypoxia survival time (Nilsson and Östlund-Nilsson, 2008; Richards, 2009; Vornanen et al., 2009).

Overall, anaerobic glycolysis is a double-edged sword, providing ATP independently of O\textsubscript{2} but with the costs of deleterious waste production and inefficiency. To better exploit the potential of anaerobic glycolysis, hypoxia-tolerant animals have evolved the ability to undergo hypoxia-induced MRD, which greatly slows fuel use and waste production by decreasing the demand for ATP (see section 1.4.3). In fact, MRD is so profound in hibernating freshwater turtles that there is a coordinated downregulation of the glycolytic pathway; this does not appear to be the case in hypoxia-exposed fishes where typically there is a relatively greater level of hypoxic activity and the magnitude of MRD is less and, in crucian carp, glycolysis is enhanced as part of its ethanol-producing strategy (Lutz and Nilsson, 1997; Nilsson and Östlund-Nilsson, 2008).

1.4.2.2 Aerobic fuels during hypoxia exposure

Fermentation of certain amino acids (e.g., aspartate, glutamate) may occur anaerobically in some fishes, although this appears to be quantitatively unimportant for ATP production compared with anaerobic glycolysis (Johnston, 1975). Alternatively, amino acids
can be deaminated during hypoxia exposure and their carbon skeletons used for gluconeogenesis (Jobling, 1994).

Fatty acids are poor metabolic fuels during O\textsubscript{2} limitation for three major reasons. First, fatty acid oxidation provides a low ATP yield per O\textsubscript{2} compared with glucose oxidation, which could be detrimental during hypoxia exposure when low rates of oxidative phosphorylation still contribute to ATP production (Hochachka and Somero, 2002). Second, studies on mammals have demonstrated that under hypoxic conditions, residual fatty acid oxidation in tissues such as the heart occurs at the expense of pyruvate oxidation, exacerbating cellular acidosis and leading to ionic disturbances (Lopaschuk et al., 2010). Third, studies on mammals also show that during severe hypoxia or anoxia exposure, impairment of fatty acid oxidation due to lack of O\textsubscript{2} can lead to intracellular accumulation of fatty acids, fatty acid intermediates resulting from incomplete \(\beta\)-oxidation, and other lipids (Corr et al., 1984; Lopaschuk et al., 2010; Moore et al., 1980; van der Vusse et al., 1992). This lipid accumulation can cause lipotoxicity, which has been implicated in metabolic dysfunction and cellular apoptosis in organs such as heart and kidney during O\textsubscript{2} limitation (Feldkamp et al., 2009; Lopaschuk et al., 2010; van der Vusse et al., 1992; Weinberg, 2006).

Consequently, it is reasonable to hypothesize that pathways of lipid metabolism and fatty acid oxidation are downregulated during hypoxia exposure in hypoxia-tolerant fishes and other ectothermic vertebrates. A limited number of available studies provide equivocal support for this hypothesis. Indirect calorimetry of goldfish suggests that fatty acid oxidation is decreased during anoxia (van Waversveld et al., 1989), but this would be expected based on a lack of O\textsubscript{2} and potential regulation was not studied. The fish heart, as in mammals, relies heavily upon fatty acids as aerobic fuels (Driedzic, 1992). Cardiac mRNA expression of
genes related to fatty acid oxidation decreased in zebrafish (*Danio rerio*) during chronic (weeks) environmental hypoxia exposure (~2 kPa) (Marques et al., 2008). On the other hand, chronic (weeks) exposure to milder environmental hypoxia (4.0-4.7 kPa) caused no change in the activity of an important enzyme of fatty acid oxidation in the heart of eelpout (*Zoarces viviparus*), although the level of hypoxemic stress achieved is unclear (Driedzic et al., 1985). Confusing matters further, fatty acids were mobilized from hepatic triglyceride stores in the goby *Gylichthys mirabilis* during several days of hypoxia exposure (~2 kPa; ~70% of this species’ P_{crit}) (Gracey et al., 2011), a response that could have been triggered by either hypoxia exposure or by the hypoxia-induced fasting that was observed. In contrast, anoxic goldfish accumulate lipid, possibly to regenerate reducing equivalents to allow for limited anaerobic tricarboxylic acid cycle activity (van Waversveld et al., 1989). Aside from Driedzic et al. (1985), there are virtually no studies on the cellular responses of the fatty acid oxidation pathway during acute hypoxia exposure in hypoxia-tolerant vertebrates.

In many species of fishes, hypoxia exposure causes a substantial (~50%) and rapid decrease in the plasma concentration of non-esterified fatty acids (NEFA), which are a metabolically dynamic fraction of lipid in blood that are heavily relied upon for fatty acid oxidation in peripheral tissues (Henderson and Tocher, 1987). Hypoxic depression of plasma [NEFA], which appears to be mediated by inhibition of lipolysis by noradrenaline (van Raaij et al., 1995; van Raaij et al., 1996b; Vianen et al., 2002), has been observed in a number of hypoxia-tolerant species including Mozambique tilapia (*Oreochromis mossambicus*), African sharptooth catfish (*Clarias gariepinus*), common carp, and oscar (Muusze et al., 1998; van Heeswijk et al., 2005; van Raaij et al., 1996b; Vianen et al., 2002). Hypoxic decreases in plasma [NEFA] are more variable in the hypoxia-sensitive rainbow trout, with two studies
showing no change (Haman et al., 1997; van Raaij et al., 1996b) but another study showing a 50% decrease (van den Thillart et al., 2002). NEFA turnover decreased in rainbow trout during hypoxia exposure (Haman et al., 1997). The significance of the hypoxic depression of plasma [NEFA] in fishes is unknown, but it has been hypothesized that it contributes to hypoxia tolerance by decreasing fatty acid supply to tissues such as the heart, thereby avoiding the potentially detrimental effects of impaired fatty acid oxidation and lipid accumulation during hypoxia exposure (van den Thillart et al., 2002).

1.4.3 Metabolic rate depression

MRD is a common survival strategy in animals exposed to environmental conditions that are associated with energy stress, such as drought, low temperature, and hypoxia (Bickler and Buck, 2007; Guppy and Withers, 1999; Staples and Buck, 2009). The first evidence of the involvement of MRD in low O₂ tolerance in vertebrates was Jackson’s (1968) classic measurements of depressed (>80%) heat production in anoxic turtles. However, the significance of MRD for hypoxia tolerance was not fully appreciated until the 1980s when Hochachka synthesized earlier findings in anoxia-exposed turtles, goldfish, and intertidal molluscs (Hochachka 1985, 1986; reviewed by Guppy, 2004). Later calorimetric measurements by van den Thillart and colleagues showed that MRD (of ~50-75%) is a common hypoxic response accompanying decreases in $\dot{M}_{O_2}$ (i.e., below $P_{crit}$) in many species of hypoxia-tolerant fishes, including goldfish (van Waversfeld et al., 1989), crucian carp (Nilsson et al., 1993), Mozambique tilapia (van Ginneken et al., 1997), and European eel (Anguilla anguilla) (van Ginneken et al., 2001) (reviewed by van Ginneken and van den Thillart, 2009). MRD is now generally recognized as a universal and crucial strategy of
hypoxia tolerance in which cellular energy demand is lowered to a level that matches the decreased energy supply available during O$_2$ limitation, thus maintaining metabolic energy balance (Hochachka et al., 1996; Storey and Storey, 2004a; Richards, 2009) (Fig. 1.2). In certain cases of hypoxic MRD (e.g., goldfish liver and turtle heart), cellular [ATP] may be reset at a lower, yet stable level (Jibb and Richards, 2008; Richards, 2009; Stecyk et al., 2009). Because MRD obviates a substantial stimulation of anaerobic glycolysis, it also prolongs hypoxia survival time by conserving fuel and slowing waste accumulation.

Depression of cellular energy demand during MRD is ultimately achieved largely by decreases in energy-intensive cellular processes such as ion pumping and protein synthesis (Hochachka and Somero, 2002). Depression of ion-motive ATPases is best characterized in brain and liver of anoxic turtles, including ‘channel arrest’ (decreased membrane ion permeability allowing a decrease in ion pumping while maintaining ion homeostasis) and ‘spike arrest’ (depression of synaptic firing rates), but evidence suggests it also occurs to a certain extent in various tissues of frogs and some fishes exposed to low O$_2$ (Bickler and Buck, 2007; Donohoe et al., 2000; Richards et al., 2007). Similarly, the hypoxic bradycardia observed in many hypoxia-tolerant ectothermic vertebrates is the cardiac analogue of spike arrest and it may help decrease the energy demand of the heart (Farrell and Stecyk, 2007) (see section 1.5). Depression of protein synthesis is a widely observed phenomenon among hypoxia-tolerant ectothermic vertebrates including many fishes, yet the magnitude and tissues affected vary: typically heart and liver show greater decreases than brain in fishes, whereas in turtles all tissues show similar large decreases, a finding consistent with the greater activity level of hypoxia-exposed fishes compared with turtles (Lewis et al., 2007; Smith et al., 1996; Storey and Storey, 2004a).
Depression of cellular ATP demand traditionally was seen as the major controlling factor underlying MRD, likely because respiratory control in a broader context was also traditionally viewed as demand-driven (Guppy, 2004). Metabolic control analyses have recently demonstrated, however, that ATP turnover rate also can be greatly affected by modulation of metabolic pathways involved in ATP supply such as mitochondrial substrate oxidation (Brand, 1997), including under hypoxic conditions and in metabolically-depressed animals (Staples and Buck, 2009; Wheaton and Chandel, 2011). For example, MRD in estivating snails appears to be controlled by substrate oxidation (Bishop et al., 2002). Similar results have been found for hypoxic hibernating frogs and hibernating mammals (Barger et al., 2003; Staples and Buck, 2009; St-Pierre et al., 2000). Furthermore, pharmacological or hypoxic inhibition of substrate oxidation in mammalian neonatal cardiomyocytes causes decreases in energy demand processes such as protein synthesis (Casey et al., 2002). Depression of the activity of pyruvate dehydrogenase (PDH), the key regulatory point of pyruvate entry into the TCA cycle and mitochondrial oxidation, has been suggested to be a mechanism by which substrate oxidation may be modulated in order to achieve MRD (Storey and Storey, 2004b). In fact, decreased PDH activity, which occurs via reversible phosphorylation of PDH, has been observed in metabolically depressed snails as well as in white skeletal muscle of two hypoxia-tolerant fishes, the oscar and the killifish (*Fundulus heteroclitus*), during hypoxia exposure (Brooks and Storey, 1992; Richards et al., 2007, 2008). PDH-mediated depression of mitochondrial function may not only contribute to MRD but also may improve hypoxia tolerance by slowing aerobic metabolism to prevent reactive oxygen species production, as has been shown in mammalian cells and tissues under hypoxic conditions (Aragonés et al., 2008; Semenza, 2007; Wheaton and Chandel, 2011). Little else
is known about the role of modulation of PDH and other aerobic energy supply pathways in MRD and other hypoxic responses in vertebrates and this is a topic that is investigated in this thesis.

1.5 HYPOXIC CARDIAC PERFORMANCE IN FISHES

The cardiovascular system, driven by the heart, is an essential component of the O$_2$ transport cascade and also is vitally important for the internal convection of fuels, hormones, and wastes. The level of cardiac performance is thus matched to the whole-animal metabolic demand for blood flow. In hypoxia-sensitive vertebrates exposed to low O$_2$, there can be an impairment of cardiac function resulting from an imbalance between cardiac energy supply and demand such that that whole-animal blood flow demand cannot be met, leading to death. Hypoxia-tolerant vertebrates, however, are able to avoid this fate, ensuring continued cardiovascular function. Internal convection during hypoxia exposure remains important for circulation of available O$_2$ as well as for fuel supply and waste removal, which likely becomes the primary role of the cardiovascular system during profound hypoxia and anoxia exposure as a result of the heavy reliance on anaerobic metabolism. Intrinsically, sustained hypoxic cardiovascular function is thought to be achieved by matching cardiac energy demand with energy supply, thus maintaining stable cardiac function for prolonged periods of severe hypoxia exposure (i.e., hypoxia-tolerant species show great cardiac hypoxia tolerance) (Farrell and Stecyk, 2007). As discussed generally in section 1.4, this can depend upon blood O$_2$ supply to the heart, cardiac anaerobic glycolytic capacity (and, possibly, a decreased reliance on poor hypoxic fuels such as fatty acids), and depression of cardiac energy demand (Fig. 1.4 and see section 1.5.2). Extrinsically, modulation of whole-animal
demand for blood flow, for example \textit{via} MRD, can also be expected to have an effect on hypoxic cardiac performance and tolerance (Jackson, 2000). Here I will provide an overview of what is known about the integrated cardiovascular responses to hypoxia exposure in fishes, including heart rate \((f_{hi})\), cardiac output \((\dot{Q})\), stroke volume \((V_{SH})\), arterial blood pressure, peripheral resistance \((R)\), and finally cardiac power output \((PO)\). Following this discussion, I provide an introduction to proposed strategies of cardiac hypoxia tolerance.

1.5.1 \textit{In vivo} cardiovascular responses to hypoxia exposure in fishes

The \textit{in vivo} cardiovascular responses to environmental hypoxia exposure in fishes vary interspecifically but relatively few species have been studied, complicating efforts to identify the responses that contribute to cardiac hypoxia tolerance (Gamperl and Driedzic, 2009). A slowing of \(f_{hi}\) (bradycardia) in hypoxic fishes, which contrasts with the tachycardia seen in hypoxic mammals, was first noted for spiny dogfish \((\textit{Squalus acanthias})\), tench, and rainbow trout in pioneering studies in the 1960s (Holeton and Randall, 1967a,b; Randall and Shelton, 1963; Satchell 1961). It is now recognized that bradycardia, typically mediated by vagal tone, is a common response among hypoxia-exposed fishes, with a few exceptions (e.g., some air-breathing fishes) (Farrell, 2007; Gamperl and Driedzic, 2009). The significance of hypoxia-induced bradycardia remains incompletely understood, but Farrell (2007) proposed four possibilities: 1) improved cardiac contractility \textit{via} a negative force frequency effect, 2) decreased myocardial \(O_2\) demand \textit{via} a decrease in the rate of pressure development, 3) enhanced myocardial \(O_2\) diffusion and thus \(O_2\) supply \textit{via} greater stretching of the myocardium as well as longer blood residence time in the heart lumen, and 4) in those species possessing a coronary circulation, greater myocardial \(O_2\) delivery \textit{via} greater
coronary blood flow resulting from the extended diastolic period, when most coronary blood flow occurs. Regarding the second point, bradycardia can also decrease myocardial $O_2$ demand when it is associated with a decrease in $PO$, which is analogous to cardiac energy demand (see below and section 1.5.2). The magnitude of bradycardia varies greatly depending on species and depth of hypoxia but up to a halving of normoxic values is not atypical (Farrell, 2007; Gamperl and Driedzic, 2009). The $P_wO_2$ at which bradycardia is initiated also is highly species specific and may coincide with $P_{crit}$, with decreasing $M_{O_2}$ below $P_{crit}$ being associated with decreasing $f_H$ (Gehrke and Fielder, 1988; Iversen et al., 2010; McKenzie et al., 2009; Rantin et al., 1993; present thesis, Figure 5.6). This relationship may explain why the onset of bradycardia is typically seen at lower $P_wO_2$ in hypoxia-tolerant fishes compared with more sensitive species (Gamperl and Driedzic, 2009). The correlation of $P_{crit}$ with the initiation of bradycardia apparently is not due to involvement of circulatory conductance in determining $P_{crit}$ itself, because abolition of hypoxic bradycardia via atropinization or vagotomy has no affect on $P_{crit}$ (Iversen et al., 2010; McKenzie et al., 2009). Instead, the potential benefits of bradycardia outlined by Farrell (2007) may become particularly useful at $P_wO_2$ below $P_{crit}$, where $O_2$ availability becomes limited. Furthermore, where whole-animal MRD occurs, bradycardia may in part reflect decreases in whole-animal demand for blood flow. However, there are relatively few data to inform these speculations and to fully assess the value of bradycardia, simultaneous measurements of other cardiovascular parameters are needed, especially $\dot{Q}$ and $PO$.

The response to environmental hypoxia of $\dot{Q}$, which is the product of $f_H$ and $V_{SH}$ and arguably the most important measure of integrated cardiovascular function, varies interspecifically among fishes even more than that of $f_H$. Gamperl and Driedzic (2009)
suggested that there are three general patterns of $\dot{Q}$ in fishes exposed to decreased $P_wO_2$. In the first pattern, bradycardia is initially compensated for by increased $V_{SH}$ such that $\dot{Q}$ is maintained at or even increased above resting normoxic levels, but if $P_wO_2$ continues to fall eventually $\dot{Q}$ also falls (e.g., cod [Petersen and Gamperl, 2011] and rainbow trout [Gamperl et al., 1994; Holeton and Randall, 1967b; Wood and Shelton, 1980]). In the second pattern, there is an increase in $V_{SH}$ but it is insufficient to fully compensate for the bradycardia so $\dot{Q}$ falls gradually as $P_wO_2$ decreases (e.g., common carp: Stecyk and Farrell, 2006; Japanese eel Anguilla japonica: Chan, 1986; lingcod Ophiodon elongatus: Farrell, 1982; sea bass Dicentrarchus labrax: Axelsson et al., 2002; smallmouth bass Micropterus dolomieu: Furimsky et al., 2003). In the third pattern, $V_{SH}$ is increased and fully compensates for the hypoxia-induced bradycardia such that $\dot{Q}$ is maintained (e.g. Adriatic sturgeon Acipenser naccarii: Agnisola et al., 1999; spotted catshark Scyllorhinus canicula: Butler and Taylor, 1975; winter flounder Pseudopleuronectes americanus: Mendonça and Gamperl, 2010). However, as Gamperl and Driedzic (2009) concede, the third pattern is somewhat questionable for these species because the cited studies did not achieve environmental hypoxia lower than a $P_wO_2$ of ~4.7 kPa; it is quite possible that had the study species been exposed to lower $P_wO_2$ they would have conformed with one of the first two patterns. For example, the $P_{crit}$ of the Adriatic sturgeon is 4.9 kPa (McKenzie et al., 2007) so it is unlikely that an exposure to a $P_wO_2$ of ~4.7 kPa accurately describes the hypoxic cardiovascular response of this species. Nevertheless, although not mentioned by Gamperl and Driedzic (2009), the crucian carp may be a true example of the third pattern, where $\dot{Q}$ is maintained and even elevated during prolonged anoxia exposure as a result of increased $V_{SH}$ (Stecyk et al., 2004b). Similarly, hagfishes (Eptatretus stoutii and Myxine glutinosa) show no change in
\( \dot{Q} \) or modest decreases during severe environmental hypoxia and anoxia exposure, respectively (Axelsson et al., 1990; Cox et al., 2010). The changes in \( V_{\text{SH}} \) in hypoxia-exposed fishes appear to be controlled by passive (i.e., increase in filling time and filling pressure resulting from bradycardia) as well as active factors (e.g., adrenergic control of venous tone that modulates cardiac filling) (Gamperl and Driedzic, 2009). How the aforementioned patterns of \( \dot{Q} \) relate to hypoxia tolerance in fishes is unclear. In \textit{Anguilla} spp. eels there appears to be a correlation between \( P_{\text{crit}} \) and decreases in \( \dot{Q} \) (Chan, 1986; Iversen et al., 2010). The decrease in \( \dot{Q} \) plays no role in determining \( P_{\text{crit}} \) (Iversen et al., 2010) but could reflect declining circulatory requirements resulting from whole-animal MRD below \( P_{\text{crit}} \), which is known to occur in hypoxic eels (van Ginneken et al., 2001). Alternatively, decreases in \( \dot{Q} \) could be related to depression of \( \text{PO} \), which is thought to be important for cardiac hypoxia tolerance (see below and section 1.5.2).

Unsurprisingly, hypoxic responses of arterial blood pressure (including ventral aortic, an important determinant of \( \text{PO} \), and dorsal aortic) and \( R \) are extremely varied in fishes. This is the result of interspecific differences as well as variation in experimental design including the level and duration of environmental hypoxia exposure, which in some studies has been too short to differentiate a hypoxic response from a general stress response (reviewed by Gamperl and Driedzic, 2009). Using data from longer or progressive environmental hypoxia exposures, it appears that in some species arterial blood pressure is increased (e.g., cod: Petersen and Gamperl, 2011; rainbow trout: Holeton and Randall, 1967a; Wood and Shelton, 1980) whereas a hypotension is seen in other species (e.g., common carp: Stecyk and Farrell, 2006; eel \textit{Anguilla} spp.: Chan, 1986; Peyraud-Waitzenegger and Soulier, 1989; lingcod: Farrell, 1982; spotted catshark: Butler and Taylor, 1975). Stecyk and Farrell (2006)
suggested that hypoxic hypotension might contribute to cardiac hypoxia tolerance by decreasing cardiac work at any given $\dot{Q}$. Increased $R$ has been observed in hypoxic teleosts (e.g., eel *Anguilla* spp.: Chan, 1986; Peyraud-Waitzenegger and Soulier 1989; lingcod: Farrell, 1982; rainbow trout: Holeton and Randall, 1967a) and has been attributed to a peripheral vasoconstriction that may prioritize blood flow to critical tissues with greater $O_2$ demands (Stecyk and Farrell, 2006). However, hypoxia exposure in elasmobranchs and sturgeons caused a decrease in $R$ so phylogenetic differences also may exist (Agnisola et al., 1999; Butler and Taylor, 1975; Gamperl and Driedzic, 2009).

The product of $\dot{Q}$ and ventral aortic blood pressure ($P_{VA}$) is $PO$. Because $PO$ is roughly analogous to cardiac energy demand (Farrell and Stecyk, 2007), its measurement is of great interest in considering how cardiac energy supply and demand is balanced during hypoxia exposure in fishes. Unfortunately, there are relatively few *in vivo* measurements of $PO$ in hypoxia-exposed fishes in part because $\dot{Q}$ and $P_{VA}$ rarely have been measured simultaneously. In hagfishes and crucian carp, $PO$ is largely unchanged even during anoxia exposure (Cox et al., 2010; Stecyk et al., 2004b). On the other hand, in the common carp, $PO$ is decreased by $\sim$80% from routine normoxic values to a new stable level during profound environmental hypoxia exposure (Stecyk and Farrell, 2006). Decreases in $PO$ likely also occurred in Japanese eel (Chan, 1986) and lingcod (Farrell, 1982) during progressive short-term environmental hypoxia exposure, based on the measurements of decreased $\dot{Q}$ and $P_{VA}$.

Overall, the paucity of available studies means that the relationship between hypoxic responses of $PO$, cardiac hypoxia tolerance, and whole-animal hypoxia tolerance remains poorly understood. However, in a synthesis integrating available studies on *in vivo* as well as *in situ* hypoxic cardiac performance, Farrell and Stecyk (2007) proposed a schema linking
PO with the maintenance of cardiac energy balance and function during hypoxia exposure in hypoxia-tolerant ectothermic vertebrates, as described in the next section.

1.5.2 Cardiac power output and cardiac hypoxia tolerance

In order for cardiac energy balance and sustained cardiac function to be achieved during hypoxia exposure below $P_{\text{crit}}$, the $PO$ must be at or below a level that can be met by the limited energy supply available (Farrell and Stecyk, 2007), which depends largely upon anaerobic glycolysis with a minor contribution from residual oxidative metabolism (Fig. 1.4). The capacity for anaerobic glycolysis to sustain $PO$ is set by the heart’s maximum glycolytic potential (MGP, i.e. the maximum glycolytic ATP turnover rate). If hypoxic $PO$ can be set to a level at or below that sustainable by the MGP, continued cardiac function is possible in the virtual absence of $O_2$ (Farrell and Stecyk, 2007). This has obvious benefits during anoxia exposure but it also benefits fishes exposed to severe or profound hypoxia, when large declines in blood $PO_2$ can greatly constrain cardiac $O_2$ supply, especially in species lacking coronary circulation where cardiac $O_2$ supply derives solely from venous blood. Based on a limited number of available studies of in situ hearts of both hypoxia-tolerant (e.g., hagfishes, freshwater turtles) and -sensitive species (e.g., rainbow trout), Farrell and Stecyk (2007) estimated the MGP of ectothermic vertebrate hearts to be $\sim 70$ nmol ATP $s^{-1} g^{-1}$ at $15^\circ C$, an unremarkable rate that sustains a $PO$ of only $\sim 0.7$ mW g$^{-1}$, which is lower than the routine $PO$ in most fishes and other ectothermic vertebrates (Farrell and Stecyk, 2007). Thus, Farrell and Stecyk (2007) suggested that there are two strategies by which hypoxia-tolerant animals match cardiac energy demand to limited hypoxic energy supply in order to maintain cardiac energy balance and function. The first strategy is to maintain an intrinsically low routine
level of $PO$ ($PO$ level “C” in Fig. 1.4) that can be sustained by their cardiac MGP, such that routine $PO$ can be maintained during hypoxia exposure (e.g., hagfishes: Hansen and Sidell, 1983; Axelsson et al., 1990; Cox et al., 2010; Forster, 1991; and crucian carp: Stecyk et al., 2004b). The second strategy is to downregulate $PO$ during hypoxia exposure, typically via a bradycardia, to a level that can be sustained by the MGP (e.g. common carp: Stecyk et al., 2006; red eared slider turtle: Arthur et al., 1997; Hicks and Farrell, 2000; Stecyk et al., 2004a) ($PO$ level from “A” to “C” in Fig. 1.4). A key corollary to this schema is that there is a species-specific lower limit on $PO$ that depends upon the minimum systemic requirement for internal convection as well as the basal limit of the heart’s pacemaker, such that in hypoxia-sensitive species, depression of $PO$ to a level sustainable by MGP may not be possible or, if it did occur, would compromise systemic blood flow resulting in organismal death (e.g., rainbow trout: Arthur et al., 1992).

Because Farrell and Stecyk’s (2007) schema and their estimated limits of MGP in ectothermic vertebrate hearts was derived from relatively few studies of a limited number of species, the question remains as to how widespread their two proposed strategies for cardiac hypoxia tolerance are among fishes and other ectothermic vertebrates. Furthermore, there have been very few direct tests of the hypothesis that in vivo depression of $PO$ is, in fact, required to match energy demand to energy supply in order to maintain cardiac energy balance and sustained cardiac function. An alternative hypothesis is that in vivo depression of $PO$ simply reflects decreased whole-animal demand for blood flow due to MRD. Similarly, there have been no direct comparative studies of the responses of $PO$ in species with different hypoxia tolerance. Finally, it is worth considering the possibility that in hypoxia-tolerant animals, an MGP greater than previously estimated or an increased $O_2$ supply may
be other mechanisms by which cardiac energy balance can be achieved during hypoxia exposure (Fig. 1.4).

1.6 Thesis Questions and Chapter Hypotheses

In the subsequent four data chapters of my thesis, I investigate two overarching questions. First, how is cardiac hypoxia tolerance achieved in fishes? Specifically, I was interested in illuminating the role (and the underlying mechanisms) of each of the three major strategies of hypoxia tolerance outlined previously, including \( \text{O}_2 \) supply, anaerobic glycolysis, and depression of energy demand (i.e., \( PO \) and related cardiovascular responses). Second, what is the relationship between cardiac hypoxia tolerance and whole-animal hypoxia tolerance? In other words, how does cardiac hypoxia tolerance differ between hypoxia-tolerant and -sensitive species, and how is hypoxic cardiac function associated with whole-animal hypoxic responses of \( \dot{M}_{\text{O}_2} \) (including \( P_{\text{crit}} \)), blood \( \text{O}_2 \) transport, and metabolism? I framed these questions to test several specific hypotheses using an integrative and comparative approach that combined measurements at the subcellular, organ, and whole-animal levels in two study models: 1) the tilapia (\( Oreochromis \) hybrid), a hypoxia-tolerant teleost, and 2) a two-species comparison of elasmobranchs that differ in their hypoxia tolerance. The types of \textit{in vivo} environmental hypoxia exposures employed were consistent across studies, including progressive hypoxia (that allowed simultaneous measurements of whole-animal \( \dot{M}_{\text{O}_2} \) and \( P_{\text{crit}} \) and provided a broad picture of responses to environmental hypoxia) as well as prolonged exposures at fixed levels of severe hypoxia.
1.6.1 Tilapia: Chapters 2 and 3

Tilapia (Tribe Tilapiini: genera *Oreochromis*, *Sarotherodon*, *Tilapia*) are cichlid fishes native to the tropical freshwaters of Africa and the Middle East (Nelson, 2006), but they now have a world-wide distribution because they are widely farmed for food. Many tilapia, including *Oreochromis* spp., are known to be tolerant of profound hypoxia exposure (Chapman et al., 1995; Chapman et al., 2002), although mechanistic studies of this hypoxia tolerance are surprisingly rare. The species that I used in my thesis, and which I refer to simply as tilapia, is a Nile tilapia hybrid (*Oreochromis niloticus* × *mossambicus* × *hornorum*) that was developed for aquaculture. My earliest studies on this particular hybrid showed that it survived at least 24 h of exposure to a $P_{\text{wO}_2}$ of 1 kPa at 22°C, which indicates a remarkable hypoxia tolerance. In comparison, the common carp exposed to a similar level of hypoxia at 15°C survives for only about 2 h (Stecyk and Farrell, 2002). The tilapia is therefore an excellent model species for studying the mechanisms of hypoxia tolerance in fishes.

In Chapter 2, I present the results of a detailed examination of *in vivo* hypoxic responses of pathways of energy supply and demand in the tilapia heart. Specifically, by examining cardiac performance and metabolism during progressive and prolonged hypoxia exposure, I tested three hypotheses: 1) cardiac hypoxia tolerance depends upon the depression of $P_O$ to a level lower than the estimated MGP for the ectothermic vertebrate heart (see section 1.5.2), 2) hypoxic depression of $P_O$ is associated with the depression of whole-animal $\dot{M}_{\text{O}_2}$ below $P_{\text{crit}}$ (see section 1.5.1), and 3) downregulation of aerobic ATP supply contributes to decreased ATP turnover and cardiac hypoxia tolerance during hypoxia exposure (see section 1.4.3). The results from this study also provided information on hypoxic cardiac fuel selection, allowing me to test the hypothesis that pathways of fatty acid
oxidation are downregulated during hypoxia exposure (see section 1.4.2.2). The findings of this study informed the development of the subsequent studies in my thesis.

In Chapter 3, I used an in situ perfused tilapia heart preparation, which I helped to develop (Lague et al., 2012), to test the hypothesis that hypoxic depression of plasma [NEFA] contributes to cardiac hypoxia tolerance by obviating lipotoxic perturbation of cardiac function (see section 1.4.2.2). Furthermore, the results of Lague et al. (2012), a study in which I was heavily involved, provided a direct test of the hypothesis that hypoxic depression of PO to a level sustainable by the MGP is required to maintain cardiac energy balance and function (i.e., a direct test of hypothesis 1 in Chapter 2) (see section 1.5.2). My results in Chapter 3 provided confirmation of our conclusions on this hypothesis.

1.6.2 Elasmobranchs: Chapters 4 and 5

The studies on the elasmobranchs provided a comparative model of hypoxia tolerance that allowed me to investigate how the mechanisms of cardiac hypoxia tolerance in fishes vary between hypoxia-tolerant and -sensitive species. These studies also allowed me to clarify the relationship between cardiac hypoxia tolerance and whole-animal hypoxia tolerance. The hypoxia-tolerant species was the epaulette shark (Hemiscyllium ocellatum), an inhabitant of shallow and often hypoxic coral reef environments that is capable of surviving hours of profound environmental hypoxia (<1.0 kPa) exposure and up to 45 min of anoxia exposure (Renshaw et al., 2002; Routley et al., 2002). The hypoxia-sensitive species was the eastern shovelnose ray (Aptychotrema rostrata), which does not frequent hypoxic environments and succumbs rapidly if held at or below a P_{O_2} of 2.0 kPa for more than
approximately 30 min (see Chapter 4). Both species are of similar size and activity level, facilitating comparison of metabolic and cardiovascular parameters.

In Chapter 4, I characterized the whole-animal hypoxia tolerance of the two species by measuring the \( P_{\text{crit}} \), blood \( O_2 \) transport, and metabolic responses during progressive environmental hypoxia in each species. This characterization informed my subsequent chapter (5) on cardiovascular responses and allowed me to test three hypotheses about whole-animal hypoxia tolerance in fishes: 1) a lower \( P_{\text{crit}} \) is associated with greater blood \( O_2 \) transport at low \( P_{wO_2} \), explaining the utility of \( P_{\text{crit}} \) as a measure of hypoxia tolerance (see section 1.4.1), 2) \( \text{Hb-O}_2 \) \( P_{50} \) is an important determinant of \( P_{\text{crit}} \) (see section 1.4.1), and 3) increased stimulation of anaerobic metabolism occurs at \( P_{\text{crit}} \) (see section 1.2.1).

In Chapter 5, I report on simultaneous measurements of cardiovascular function in the two species exposed to progressive hypoxia. Also, I present data on metabolic responses in heart and other tissues to up to 4 h of exposure to a level of environmental hypoxia that was standardized to \( P_{\text{crit}} \) in each species. These data, combined with the data from Chapter 4, allowed me to investigate two major questions (specific hypotheses are provided in the introduction to Chapter 5): 1) what is the role of blood \( O_2 \) transport in cardiac hypoxia tolerance?, and 2) how do cardiovascular (including \( PO \)) and metabolic hypoxic responses associated with cardiac hypoxia tolerance differ in hypoxia-tolerant and -sensitive species? (see section 1.5). The experiments of Chapter 5 also provided another test of hypotheses 1 and 2 from Chapter 2 (see above).
1.6.3 General discussion: Chapter 6

In the general discussion, I summarize my salient findings and discuss them in a broader context in an attempt to draw general conclusions about the mechanisms and interrelationship of cardiac and whole-animal hypoxia tolerance in ectothermic vertebrates. Finally, I consider the question of whether hypoxic depression of cardiac performance in hypoxia-tolerant fishes reflects the intrinsic needs of the heart to maintain cardiac metabolic energy balance and function, or whether it simply reflects the extrinsic requirements of the body for blood flow, which may be decreased as a result of MRD.
Figure 1.1. Metabolic responses of fishes to decreased water PO$_2$ (P$_w$O$_2$) (i.e., increased environmental hypoxia). Below the x-axis, normoxia, environmental hypoxia, and anoxia refer to the absolute range of P$_w$O$_2$ from air saturation to 0 kPa. The solid lines represent the whole-animal oxygen consumption rate (M$_{O_2}$) supporting the routine and maximum metabolic rates. The dotted lines indicate the two critical levels of environmental hypoxia relative to the animal. The zone of relative environmental hypoxia begins at “Level 1”, the P$_w$O$_2$ at which an initial hypoxemia occurs that can be physiologically compensated for, such that routine M$_{O_2}$ is maintained but aerobic scope becomes limited. Level 2 is the critical oxygen tension (P$_{crit}$), the P$_w$O$_2$ at which routine M$_{O_2}$ can no longer be maintained and transitions from being independent to being dependent upon environmental O$_2$. At P$_w$O$_2$ between Level 1 and Level 2, moderate hypoxemic stress (i.e., moderate relative environmental hypoxia) occurs where aerobic scope (locomotion, growth, and reproduction) is increasingly constrained as P$_w$O$_2$ is decreased. At P$_w$O$_2$ below P$_{crit}$ (i.e., below Level 2), severe hypoxemic stress (i.e., severe relative environmental hypoxia) occurs where routine aerobic energy production is constrained and anaerobic pathways of energy production and/or metabolic rate depression are necessary to maintain cellular metabolic energy balance (see Fig. 1.2). Species with a lower P$_{crit}$ are able to maintain routine M$_{O_2}$ (i.e., transfer rate of O$_2$) to a lower P$_w$O$_2$ by modifications that increase O$_2$ conductance and/or increase O$_2$ partial pressure differences at any step of the O$_2$ transport cascade. Modified from Richards (2011) and Farrell and Richards (2009).
Figure 1.2. Hypoxia tolerance at water PO₂ ($P_w$O₂) below $P_{\text{crit}}$ depends upon the ability to match ATP supply and demand and achieve metabolic energy balance (i.e., stable ATP levels) at a time when routine aerobic ATP production (solid line) is decreased. In a hypoxia-sensitive species (A), ATP demand (dotted line) is maintained at routine levels despite declining total ATP production (aerobic plus anaerobic ATP production; dashed line). Initially, total ATP production may sustain the ATP demand by a large stimulation of anaerobic ATP production (anaerobic glycolysis), but as $P_w$O₂ decreases further, ATP demand outstrips total ATP production, resulting in loss of metabolic energy balance and death. In a hypoxia-tolerant species (B), ATP demand can be decreased via metabolic rate depression (MRD) so that it is matched to the declining total ATP production, achieving metabolic energy balance and hypoxia tolerance. The level of hypoxia that is tolerable depends upon the ability to depress energy demand to match continually falling total ATP production: species with a greater capacity (+) to depress ATP demand can tolerate exposure to a lower $P_w$O₂ than species that are comparatively limited in their capacity for MRD (−). If at any $P_w$O₂ the total ATP production falls below the lower limit of depression of ATP demand, metabolic energy balance is perturbed. In an anoxia-tolerant species (C), ATP demand can be depressed to a level sustained by anaerobic ATP production alone. There are three strategies that can potentially improve the ability to achieve metabolic energy balance and tolerate any given hypoxic $P_w$O₂: (i) increased anaerobic glycolysis that increases total ATP production, (ii) increased $O_2$ supply that increases total ATP production or lessens reliance on anaerobic ATP production, and (iii) greater MRD that decreases ATP demand. Hypoxia tolerance is a function of the magnitude of hypoxia that can be tolerated, as described above, as well as the duration of hypoxia that can be tolerated, which depends upon tolerance of waste accumulation and the size of fermentable fuel stores. Thus, the strategies that minimize reliance on anaerobic ATP production (greater $O_2$ supply and greater MRD) may further improve hypoxia tolerance. Modified from Richards (2011) and Nilsson and Renshaw (2004).
Figure 1.3. Schematic diagram of the O₂ transport cascade in fishes. O₂ moves from the water to the mitochondria of tissue cells via several convective and diffusive steps, including counter-current ventilation of water across the gills, diffusion of O₂ from the water across the gill membrane into the blood, circulation of O₂ in the blood to the tissues by the cardiovascular system, diffusion of O₂ from blood into cells, and utilization of O₂ by mitochondria generating ATP via oxidative phosphorylation. Modification of any step in the O₂ transport cascade can affect O₂ uptake from the environment and thus affect the critical O₂ tension. Modified from Storz et al. (2010).
**Figure 1.4 (overleaf).** Schematic diagrams illustrating how cardiac metabolic energy balance and sustained cardiac function can be achieved during conditions of normoxia, hypoxia, and anoxia. The solid line indicates the relationship between cardiac power output ($PO$, i.e. cardiac ATP demand) and cardiac ATP turnover. The “$A$”, “$B$”, and “$C$” circles indicate three hypothetical levels of $PO$ (see below). The “Normoxia” dashed box indicates the maximum $PO$ sustained under normoxic, aerobic conditions. The “Anoxia” grey box indicates the maximum $PO$ sustained by maximum anaerobic ATP production alone (i.e. maximum glycolytic potential, MGP), which was estimated by Farrell and Stecyk (2007) to be $\sim70\text{ nmol ATP s}^{-1}\text{g}^{-1}$ at $15^\circ\text{C}$ sustaining a $PO$ of $\sim0.7\text{ mW g}^{-1}$. The “Hypoxia” dashed box indicates the maximum $PO$ sustained by maximum ATP supply at a given level of hypoxia, which depends upon the MGP plus residual aerobic ATP production. Assuming all else remains equal, the limits of the hypoxia ATP supply box will be determined by the level of blood $O_2$ supply, which is dependent in part upon water $PO_2$ ($P_wO_2$). For cardiac metabolic energy balance to occur at any given level of blood $O_2$ and corresponding $P_wO_2$, the operating $PO$ must be within the limits of the ATP supply box. In Panel I, #1 and #2 indicate the two strategies of achieving cardiac metabolic energy balance during low $O_2$ proposed by Farrell and Stecyk (2007). In strategy #1, routine $PO$ is low enough that it can be sustained by the MGP, meaning the heart can operate at routine levels in anoxia (i.e., $PO$ at all $P_wO_2$ is at level $C$). In strategy #2, $PO$ is depressed from the normoxic operating level ($A$) to a new lower level ($B$ or $C$) that allows ATP demand to be matched to decreased ATP supply. If $PO$ is depressed to level B, the heart can function in hypoxia. If $PO$ can be depressed to level C, the heart can function in anoxia. Panel II indicates how, aside from $PO$ depression, an increased cardiac $O_2$ supply at a given level of hypoxia or an increased MGP can improve the ability to match cardiac energy supply and demand during low $O_2$ conditions. Modified from Farrell and Stecyk (2007).
CHAPTER 2: EFFECTS OF ENVIRONMENTAL HYPOXIA ON CARDIAC ENERGY METABOLISM AND PERFORMANCE IN TILAPIA

2.1 SYNOPSIS

The ability of an animal to depress ATP turnover while maintaining metabolic energy balance is important for survival during hypoxia exposure. In the present study, I investigated the responses of cardiac energy metabolism and performance in the hypoxia-tolerant tilapia (Oreochromis hybrid sp.) during exposure to environmental hypoxia. Specifically, I tested three hypotheses: 1) cardiac hypoxia tolerance depends upon the depression of \( PO \) to a level lower than the estimated maximum glycolytic potential (MGP) for the ectothermic vertebrate heart, 2) hypoxic depression of \( PO \) is associated with the depression of whole-animal \( O_2 \) consumption rate (\( \dot{M}_{O_2} \)) below the critical \( O_2 \) tension (\( P_{crit} \)), and 3) downregulation of aerobic ATP supply contributes to decreased ATP turnover and cardiac hypoxia tolerance during hypoxia exposure. Exposure to progressive hypoxia (water \( P_{O_2} \) of \( \geq 19.0 \) kPa to 0.5 kPa over 3.6±0.2 h) followed by exposure to a water \( P_{O_2} \) of 1.0 kPa for 8 h caused a decrease in whole-animal \( \dot{M}_{O_2} \) below \( P_{crit} \) that was accompanied by parallel decreases in heart rate, cardiac output, and cardiac power output (\( PO \), analogous to ATP demand of the heart). These cardiac parameters remained depressed by 50-60% compared with normoxic values throughout the 8 h exposure. This hypoxic level of \( PO \) was lower than the estimated MGP of the ectothermic vertebrate heart. During a 24 h exposure to a water \( P_{O_2} \) of 1.0 kPa, cardiac [ATP] was unchanged compared with normoxia and anaerobic glycolysis contributed to ATP
supply as evidenced by considerable accumulation of lactate in the heart and plasma. Reductions in the provision of aerobic substrates were apparent from a large and rapid (in <1 h) decrease in plasma [non-esterified fatty acids] and a modest decrease in activity of pyruvate dehydrogenase (PDH). Depression of cardiac ATP demand via bradycardia and a consequent decrease in $PO$ appears to be associated with metabolic rate depression in tilapia and likely contributes to hypoxia tolerance.

2.2 INTRODUCTION

During periods of low $O_2$, hypoxia-tolerant animals undergo a profound, rapid and reversible metabolic rate depression (MRD) as shown by large decreases in whole-animal $O_2$ consumption rate ($\dot{M}_{O_2}$) and heat production (Richards et al., 2008; van Ginneken et al., 1997). This MRD reflects a downregulation of cellular ATP turnover to a level that can be sustained by $O_2$-independent ATP production. The ability to balance ATP demand with supply and thus maintain stable cellular [ATP] is a key response ensuring hypoxia survival in tolerant animals, including many species of fishes that regularly encounter environmental hypoxia (Boutilier, 2001a).

A major component of the hypoxia-induced depression of ATP turnover is a reduction of cellular ATP demand including the regulated arrest of ion pumping and anabolic pathways such as protein synthesis (Lewis et al., 2007; Richards et al., 2007; Staples and Buck, 2009). Metabolic control analyses demonstrate, however, that ATP turnover in both active and metabolically-depressed organisms can be controlled both by regulating ATP demand as well as via modulation of metabolic pathways involved in ATP supply such as mitochondrial substrate oxidation (Bishop et al., 2002; Staples and Buck, 2009). Our
knowledge is incomplete as to how processes of ATP demand and ATP supply respond during hypoxia exposure in order to achieve depressed ATP turnover and stable cellular [ATP], including how these responses depend on the species and tissue investigated (Staples and Buck, 2009).

The heart of hypoxia-tolerant fishes represents a good system to study the metabolic balance of processes involved in ATP supply and demand during hypoxia exposure because it is a vital, highly aerobic tissue and its ATP demand can be readily quantified by measuring cardiac power output (PO). Cardiac power output is the product of cardiac output ($\dot{Q}$) and ventral aortic blood pressure ($P_{VA}$) and represents the mechanical output of the heart, which is the main source of cardiac ATP consumption (Farrell and Stecyk, 2007). Depression of PO, primarily mediated by cholinergic bradycardia and an associated reduction in $\dot{Q}$, has been observed in the hypoxia-tolerant common carp (Cyprinus carpio) and the anoxia-tolerant turtle Trachemys scripta (Farrell and Stecyk, 2007) during exposure to hypoxia. The depression of PO is thought to decrease cardiac ATP demand to a level that can be sustained solely by the maximum glycolytic potential (MGP) (i.e., maximum glycolytic ATP production), which for the ectothermic vertebrate heart has been estimated to be ~70 nmol ATP s$^{-1}$ g$^{-1}$ at 15°C sustaining a PO of ~0.7 mW g$^{-1}$ (Farrell and Stecyk, 2007). This strategy for hypoxic depression of cardiac ATP turnover contrasts with what is seen in the anoxia-tolerant crucian carp (Carassius carassius), where routine PO is kept at levels below the MGP even under normoxic conditions, obviating the need to reduce PO during hypoxia exposure (Stecyk et al., 2004). The degree to which these schemes generally apply to hypoxia-tolerant fishes is uncertain because PO often is not measured and cardiovascular responses to environmental hypoxia exposure vary between species studied (Gamperl and
Driedzic, 2009). For example, hypoxia-induced cholinergic bradycardia is not found in all fishes and compensatory increases in stroke volume ($V_{SIH}$) and changes in $P_{VA}$ are species-dependent, resulting in variation in how $Q$ and $PO$ in different fishes respond to hypoxia exposure (Farrell, 2007; Gamperl and Driedzic, 2009). The degree to which changes in these parameters relate to whole-animal hypoxia-induced MRD is also unclear. Studies on certain teleosts show that the $O_2$ tension at the onset of hypoxic bradycardia corresponds well with the point where $M_{O_2}$ transitions from being independent of environmental $O_2$ tension to being dependent (quantified as $P_{crit}$) and the depression of $M_{O_2}$ and heart rate ($f_H$) are closely linked (Gehrke and Fielder, 1988; Rantin et al., 1993).

Our understanding of how ATP supply pathways in heart of hypoxia-tolerant animals respond during periods of low $O_2$ is incomplete. It is known that glycolysis is essential for hypoxic function of the fish heart (Gamperl and Driedzic, 2009). To what extent aerobic ATP supply pathways, including glucose oxidation and fatty acid oxidation, are modulated during hypoxia in heart of tolerant fishes is unknown, despite the potential importance of such responses in controlling depression of ATP turnover during hypoxia exposure (Bishop et al., 2002; Staples and Buck, 2009), as well as preventing the possible accumulation of harmful byproducts of inefficient mitochondrial respiration such as reactive oxygen species (ROS) and fatty acid intermediates (Chen et al., 2007; Corr et al., 1984). Richards et al. (2007; 2008) showed that the activity of pyruvate dehydrogenase (PDH), the key regulatory point of pyruvate entry into the TCA cycle and mitochondrial oxidation, decreased in white skeletal muscle of two hypoxia-tolerant teleosts, the oscar ($Astronotus ocellatus$) and the killifish ($Fundulus heteroclitus$), during hypoxia exposure. The decrease may be mediated by PDH kinase-2 (PDK-2; Richards et al., 2008), one of four PDKs found in mammals and fishes that.
phosphorylate and inactivate PDH. Normally this occurs when cellular energy status is high (e.g. high [acetyl CoA]/[CoA]; Holness et al., 2003), but hypoxia inducible factor (HIF)-mediated increases of PDKs during hypoxia exposure have been shown to decrease \( \text{O}_2 \) consumption and ROS formation and contribute to hypoxia tolerance in mammalian cells and tissues (Aragonés et al., 2008; Semenza, 2007). Less is known about the responses of fatty acid oxidation in hypoxia-tolerant fishes exposed to hypoxia, but a noradrenaline-mediated decrease in circulating non-esterified fatty acids (NEFA) appears to be a common response to \( \text{O}_2 \) deprivation that may limit fatty acid oxidation in hypoxia-tolerant fishes (van Heeswijk et al., 2005; van Raaij et al., 1996b; Vianen et al., 2002). A regulated decrease in fatty acid oxidation could contribute to reduced ATP turnover and possibly avert detrimental effects of fatty acid oxidation in hypoxia. These effects, which are well characterized in mammalian ischaemic heart models, include lipotoxicity and uncoupling of glycolysis from glucose oxidation that exacerbates metabolic acidosis and disturbs ion homeostasis (Dyck and Lopaschuk, 2006). Interestingly, this latter event is mediated by AMP activated protein kinase (AMPK), which is potentially important in decreasing \( \dot{M}_{\text{O}_2} \) and ATP demand in certain tissues in goldfish (Carassius auratus) and crucian carp during \( \text{O}_2 \) lack (Jibb and Richards, 2004; Stensløkken et al., 2008).

In this study I investigated how exposure to environmental hypoxia affects cardiac ATP demand and metabolic fuel selection in the hypoxia-tolerant tilapia (Oreochromis hybrid sp.). Specifically, I tested three hypotheses: 1) cardiac hypoxia tolerance depends upon the depression of \( PO \) to a level lower than the estimated MGP for the ectothermic vertebrate heart, 2) hypoxic depression of \( PO \) is associated with the depression of whole-animal \( \dot{M}_{\text{O}_2} \) below \( P_{\text{crit}} \), and 3) downregulation of aerobic ATP supply contributes to
decreased ATP turnover and cardiac hypoxia tolerance during hypoxia exposure. Cardiac ATP demand and whole-animal MRD were quantified by monitoring cardiovascular parameters and $\dot{M}_{O_2}$, respectively, during progressive hypoxia (water partial pressure of $O_2$ $[P_{wO_2}]$ of $\geq 19$ kPa to 0.5 kPa over 3.6±0.2 h; 100% air saturation = 155.1 torr = 20.7 kPa) followed by exposure to a $P_{wO_2}$ of 1.0 kPa for 8 h. In a separate 24 h hypoxia exposure ($P_{wO_2}$= 1.0 kPa), plasma and heart were sampled and components of certain ATP supply pathways assessed. In heart, I measured AMPK activity, markers of $O_2$-independent ATP production (lactate, CrP), and energetic status (ATP, ADP$_{free}$, AMP$_{free}$). Control of carbohydrate oxidation was assessed via analysis of PDH activity and expression of PDK2 and fatty acid oxidation was investigated by measuring concentrations of carnitine esters and mRNA levels of carnitine palmitoyltransferase-1 (CPT-1), the rate limiting step in mitochondrial fatty acid oxidation, and peroxisome proliferator-activated receptor $\alpha$ (PPAR$\alpha$), a key transcriptional regulator of genes related to fatty acid oxidation. Plasma was assayed for circulating levels of glucose and NEFA to indicate how substrate supply is altered during hypoxia exposure. Together, these experiments provide insight into how ATP supply and demand is modulated in heart of tilapia during environmental hypoxia exposure.

2.3 MATERIALS AND METHODS

2.3.1 Animals

Adult male tilapia (*Oreochromis niloticus* x *mossambicus* x *hornorum*; strain origin, Ace Developments, Bruneau, ID, USA) were purchased from Redfish Ranch (Courtenay, BC, Canada). Fish were kept at the University of British Columbia in well-aerated 400 L tanks supplied with recirculating freshwater (22°C). Fish were held for at least 2 months
before experimentation and fed daily to satiation with commercial trout pellets (Firstmate Taplow Aquaculture, North Vancouver, BC, Canada). All experiments were conducted following guidelines set out by the Canadian Council for Animal Care as administered by the University of British Columbia Animal Care Committee.

2.3.2 Experiments

Three experiments were performed to characterize the responses of tilapia hearts to environmental hypoxia exposure. First (series I), I assessed the hypoxia tolerance of tilapia by measuring $\dot{M}_{O_2}$ and critical $O_2$ tension ($P_{crit}$) via closed respirometry as well as whole blood hemoglobin-$O_2$ $P_{50}$. Second (series II), I measured cardiovascular responses during progressive hypoxia followed by exposure to profound hypoxia ($P_{wO_2} = 1.0$ kPa) for 8 h to evaluate the effects of hypoxia on ATP demand of the heart. Simultaneous measurement of $\dot{M}_{O_2}$ during progressive hypoxia allowed us to calculate $P_{crit}$ and investigate the relationship between depression of $\dot{M}_{O_2}$ and cardiovascular status. Third (series III), during a 24 h hypoxia exposure ($P_{wO_2} = 1.0$ kPa) I investigated pathways of ATP supply in the heart by measuring the products of $O_2$-independent ATP production as well as key biochemical and molecular parameters contributing to the regulation of fatty acid and carbohydrate oxidation.

2.3.2.1 Series I. Assessment of hypoxia tolerance

Routine whole-animal $O_2$ consumption rate ($\dot{M}_{O_2}$) and $P_{crit}$, the inflection point at which $\dot{M}_{O_2}$ ceases to be independent of $P_{wO_2}$, were determined at 22°C using closed respirometry following the methods of Henriksson et al. (2008). Briefly, individual fish ($n=7$, 46
185±2.9 g) were placed in a 10 L swim tunnel respirometer (Loligo Systems, Tjele, Denmark) and allowed to acclimate under flow-through conditions for 12 h, at which point the respirometer was sealed and $P_wO_2$ was continuously recorded using a fibre optic $O_2$ sensor (Foxy system; Ocean Optics, Dunedin, FL, USA) until 1.2 kPa was reached. The swim tunnel propeller was run slowly throughout the trial to provide for adequate water mixing within the respirometer without swimming being necessary. All fish remained quiescent throughout the trials, each of which lasted about 6 h. Oxygen consumption rate and $P_{crit}$ were calculated as described by Henriksson et al. (2008) and the values of $\dot{M}_{O_2}$ presented in Figure 2.1 are means of individual $\dot{M}_{O_2}$ calculated from 5 minute intervals that occurred at specific $P_wO_2 \pm 0.5$ kPa between approximately 14.5 kPa to 1.2 kPa. A blank was run without a fish in the chamber to calculate background $\dot{M}_{O_2}$ and this value was subtracted from fish measurements.

Tilapia whole blood hemoglobin-$O_2$ binding affinity ($P_{50}$) was measured on freshly sampled blood from normoxic anaesthetized fish at physiological temperature (22°C) using a custom-made PWee50 following the methods described by Henriksson et al. (2008).

2.3.2.2 Series II. Cardiac ATP demand

2.3.2.2.1 Surgical procedures

Fish (709.4±25.2 g, $n=6$) were netted from the holding tanks, anaesthetized in water containing NaHCO$_3$ buffered MS-222 (0.2 g L$^{-1}$ and 0.2 g L$^{-1}$, respectively) and moved to a surgery table where the gills were continuously irrigated via the mouth with chilled aerated water (13°C) containing NaHCO$_3$ buffered MS-222 (0.2 g L$^{-1}$ and 0.15 g L$^{-1}$, respectively).
To permit measurement of $P_{VA}$, the left third afferent branchial artery was occlusively cannulated (Axelsson and Fritsche, 1994) with a PE-50 or PE-30 catheter filled with heparinised (100 IU ml$^{-1}$) glucose-free Cortland saline. The tip of the catheter was advanced towards the ventral aorta and secured in place by tying the catheter to the gill arch with 2-0 silk suture and then suturing the free end of the catheter to the skin. Correct placement of the catheter was verified by withdrawal of blood and by post mortem dissection of several fish. To measure ventral aortic blood flow (i.e., $\dot{Q}$), the ventral aorta was exposed at the isthmus and a 2.5S Transonic transit-time blood flow probe (Transonic Systems, Ithaca, NY, USA) was positioned around the vessel. The lead from the flow probe was tied to the skin with 2-0 silk. Following surgery, fish were revived in fresh water at 22°C and placed in individual holding containers that were floating in a larger holding tank. Fish were allowed at least 24 h of recovery following instrumentation as this period of recovery was found to be sufficient to allow return of cardiovascular function to baseline levels (data not shown).

2.3.2.2.2 Experimental setup and protocol

The instrumented fish was moved to an acrylic glass respirometer (10 L, Loligo Systems, Tjele, Denmark) that was submersed in a larger outer glass aquarium fed with the recirculating water system (22°C) that ensured normoxic ($P_{\text{wO}_2} \geq 19.0$ kPa) conditions. A submersible pump created a continuous flow of water from the external tank to the respirometer that was sufficient to ensure complete mixing inside the respirometer but negated the need for active swimming movements to maintain position. The aquarium was covered with polystyrene foam to prevent visual disturbance of the fish. The respirometer could be closed by recirculating water inside the respirometer using the submersible pump.
The catheter and the flow probe lead from the fish was exteriorised through a hole in the respirometer fitted with a soft rubber stopper modified with a slit. The fish was allowed to habituate to the respirometer for at least 12 h prior to any experimental procedures.

Routine cardiovascular variables were continuously recorded in normoxia (≥19.0 kPa) for several hours to ensure stable baseline conditions. The respirometer was then closed and $\dot{M}_{O_2}$ was measured from the rate of depletion of $P_wO_2$ due to fish respiration. Once the nadir in $P_wO_2$ was reached (~0.5 kPa, which took 3.6±0.2 h), the $P_wO_2$ was increased to 1.0 kPa within <5 min by manually adjusting a siphon connected to the respirometer, which allowed a minimal flow of normoxic water to enter the respirometer to maintain $P_wO_2$. The fish was then held for 8 h at this profound level of hypoxia, which represented approximately 30% and 70% of $P_{crit}$ and hemoglobin-O$_2$ $P_{50}$, respectively (see results). Preliminary experiments showed that tilapia survived at least 24 h at 1.0 kPa at 22°C. Following the 8 h hypoxia exposure, normoxic water was reintroduced to the respirometer and measurements of cardiovascular variables were made for an additional 1.5 h of recovery in normoxic water. In one fish, blockage of the pressure cannula at approximately 7 h precluded further recordings of $P_{VA}$, so $n=5$ for 8 h and recovery points. Fish were killed at the end of the trial and the ventricle was excised, emptied of blood and blotted dry, and weighed.

Due to the use of closed respirometry, $\dot{M}_{O_2}$ was not measured at the initial normoxic $P_wO_2$ (~19.0 kPa) or the recovery period where flow through conditions were used, nor was $\dot{M}_{O_2}$ measured during the 8 h hypoxia exposure when $P_wO_2$ was kept at 1.0 kPa. The use of closed respirometry raises the question of whether changes in water parameters other than O$_2$ (e.g. pH, $P_{CO_2}$) could have had significant effects on measured parameters. This is unlikely for several reasons. Despite modest decreases in water pH and modest increases in water...
PCO$_2$ in closed respirometry trials of great sculpin (Myxocephalus polyacanthocephalus), P$_{crit}$ is not different from the same trials run under partial flow-through conditions where PCO$_2$ and pH were unchanged (J.G. Richards, unpublished observation). Additionally, in another hypoxia-tolerant cichlid the increases in water and blood PCO$_2$ are small during closed respirometry P$_{crit}$ trials (Scott et al., 2008). A similar increase in environmental PCO$_2$ has been shown to cause only a very modest change in heart rate and no change in Q in the sensitive rainbow trout (Oncorhynchus mykiss) (McKendry and Perry, 2001). Similarly, studies on other hypoxia-tolerant fishes show only modest effects of high CO$_2$ on heart rate (Reid et al., 2006). Thus, the small increases in PCO$_2$ and pH expected in the present study are unlikely to have contributed to the observed changes in cardiovascular parameters. During the 8 h exposure to 1.0 kPa (series II) further changes in water properties are unlikely because the fish metabolic rate was greatly depressed, fresh water was bled into the respirometer, and measured parameters were stable.

2.3.2.2.3 Data acquisition and calculation of cardiorespiratory variables

The ventral aortic catheter was connected to a pressure transducer (model DPT-6100, pvb Medizintechnik, Kirchseeon, Germany) that was calibrated against a static water column with the water surface in the experimental tank serving as zero pressure reference. The signal from the transducer was amplified with a 4ChAmp amplifier (Somedic, Hörby, Sweden). Blood pressure recordings made in the respirometer were compensated for the small change in pressure (~0.5 kPa) that occurred in the respirometer depending on if it was in an open or closed state. Cardiac output was recorded with a Transonic blood flow meter (Model T206, Transonic Systems, Ithaca, NY, USA). All flow probes used in the experiment were
calibrated according to manufacturer guidelines at 22°C following the experiment to compensate for the effect of calibration temperature on flow readings. Water PO$_2$ in the respirometer was measured using an Oxyguard probe (Mark IV, Point Four Systems, Richmond, BC, Canada), modified to give a ±1 V output signal, that was placed in a custom made plexiglass chamber connected in line with the circulation pump. All signals were fed into a Power Lab unit (ADInstruments, Castle Hill, Australia) and subsequently analysed using LabChart Pro software (v. 6.0; ADInstruments, Castle Hill, Australia).

Cardiovascular parameters were analyzed in LabChart Pro at discrete P$_w$O$_2$ from ~19.0 kPa to 0.5 kPa by averaging the data across the time span bracketed by each tension±0.5 kPa (2.5% air saturation) (i.e. the measurement of $\dot{Q}$ at 12.4 kPa [60% air saturation] represents an average of $\dot{Q}$ recorded from 12.9 kPa to 11.9 kPa [62.5% to 57.5% air saturation]). For measurements at 1.0 kPa and 0.5 kPa, data were averaged between approximately 1.4 kPa to 0.7 kPa and 0.7 kPa to 0.4 kPa, respectively. Whole-animal $\dot{M}_{O_2}$ was calculated from the rate of decline in P$_w$O$_2$ over the same discrete O$_2$ tensions where the cardiovascular parameters were collected (corrected for fish weight and respirometer volume), and $P_{crit}$ was calculated as previously described. Cardiac output was calculated directly from the flow trace in LabChart Pro. $P_{VA}$ was calculated using the blood pressure analysis module in LabChart Pro. Heart rate ($f_H$) was calculated from the pulsatile pressure or flow trace. Cardiac stroke volume ($V_{SH}$) was calculated as $\dot{Q}/f_H$ and total peripheral resistance ($R$; i.e., the sum of systemic and branchial resistance) was calculated as $P_{VA}/\dot{Q}$, with the assumption that central venous blood pressure is zero. Cardiac power output ($PO$, mW g$^{-1}$ wet ventricular mass) was calculated as the product of $P_{VA}$ (kPa) and $\dot{Q}$ (ml s$^{-1}$) divided by the wet ventricular mass (g). Cardiovascular parameters were plotted against P$_w$O$_2$ to identify
the inflection points where each parameter ceased to be independent of $P_wO_2$ (i.e. $P_{crit}$ of each cardiovascular parameter) as previously described for calculation of $P_{crit}$ of $M_{O_2}$.

2.3.2.3 Series III. Cardiac ATP supply

2.3.2.3.1 Gene identification, sequencing, and tissue distribution

Tilapia ($n=3$) were sampled directly from normoxic holding aquaria and euthanized with benzocaine solution (100 g/L ethanol). Samples of heart, liver, red muscle, white muscle, kidney, adipose tissue, intestine, blood, and brain, were dissected from the fish, immediately frozen in liquid nitrogen, and stored at -80°C. Total RNA was extracted from tilapia tissues using Tri Reagent (Sigma-Aldrich, St Louis, MO, USA) and then quantified spectrophotometrically and its integrity verified via electrophoresis on a 1.5% agarose gel containing ethidium bromide.

First strand cDNA was synthesized from 5 µg of total RNA isolated from the above tissues as described by Richards et al. (2008). cDNA from heart was screened to identify CPT-1, PPARα, and PDK-2 gene isoforms expressed in tilapia heart. Tilapia partial CPT-1 sequences were obtained using degenerate primers (Table 2.1) determined from conserved regions of CPT-1α from horse (Acc. No. AB188099), sheep (NM001009414), human (L39211), rat (NM031559), mouse (BC054791), chicken (AY675193), and rainbow trout (AF327058). A partial PPARα sequence was obtained using degenerate primers (Table 2.1) determined from conserved regions of PPARα from Salmo salar (AM230809.1), Sparus aurata (AY590299.1), rat (NM013196.1), and chicken (NM001001464.1). Tilapia partial PDK-2 sequences were obtained using degenerate primers (Table 2.1) determined from conserved regions of PDK-2 from zebrafish (NM200996.1), mouse (NM133667.1), rat
(NM030872.1), cow (BT025357.1), and *Xenopus laevis* (BC110980.1). Primers were designed with the assistance of GeneTool Lite software (www.biotool.com). Polymerase chain reactions (PCR) were carried out in a PTC-200 MJ Research thermocycler using Taq DNA polymerase (MBI Fermentas) and heart cDNA. Each PCR consisted of an initial 2 min at 94°C, followed by 40 cycles of: 0.5 min at 94°C, 0.5 min at 54°C (CPT-1) or 56°C (PPARα) or 47°C (PDK-2) and 1.5 min at 72°C. PCR products were electrophoresed on 1.5% agarose gels containing ethidium bromide to verify the amplification of product of the correct size.

Cloning of PCR products was carried out following the methods of Richards et al. (2008). Plasmids containing the PCR product were sequenced at the NAPS core facility at the University of British Columbia. Multiple clones of each cDNA fragment were sequenced in both directions at least twice and a majority-rule consensus for the cDNA transcript was developed for each isoform. The BLAST algorithm was used to compare the cDNA sequences with published sequences in GenBank and multiple alignments were produced with ClustalW. cDNA sequences have been deposited into GenBank (*CPT_Til-1 iso1*, Bankit 1241880; *CPT_Til-1 iso2*, Bankit 1241881; *PDK_Til-2*, Bankit 1241877; *PPAR_Tilα*, Bankit 1241879).

The relative tissue distribution of mRNA expression of the genes was ascertained by quantitative real-time PCR (qRT-PCR) using the cDNA obtained from the dissected tissues and isoform-specific primers. Primers were designed using Primer Express software (primer sequences in Table 2.1; Applied Biosystems Inc., Foster City, CA, USA). Quantitative real-time PCR was performed in duplicate on a ABI Prism 7000 sequence analysis system (Applied Biosystems) and all reactions contained 2 µl of cDNA, 4 pmoles of each primer,
double processed tissue culture water (Sigma-Aldrich) and Universal SYBR green master mix (Applied Biosystems Inc.) in a total volume of 22 µl. All qRT-PCR reactions were performed as follows: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Melt curve analysis was performed following each reaction to confirm the presence of only a single product of the reaction. Negative control reactions were performed for all samples using RNA that had not been reverse transcribed to control for the possible presence of genomic DNA contamination. Genomic contamination was less than 1 in 99 starting cDNA copies for all templates. Heart cDNA was used to develop a standard curve relating threshold cycle to cDNA amount for each primer set. All results were expressed relative to these heart standard curves and normalized to mRNA expression of EF-1α (primers in Table 2.1; designed from the known O. niloticus sequence, Accession No. AB075952.1). Tissue distributions were similar when expressed relative to total RNA or EF-1α (data not shown).

2.3.2.3.2 Hypoxia exposure

Tilapia were fasted for two days and then equal numbers (total=68, 100-400 g) were transferred to two separate 340 L aerated aquaria fed with recirculating filtered water at 22°C. Each fish was housed in a separate plastic container within the aquaria. Each container had a mesh top and elongate perforations on each side to allow adequate water flow. The plastic containers were stacked on top of one another and weighted inside with a few clean stones. To ensure good water flow several submersible aquarium pumps were placed at strategic positions in each of the tanks and a large air stone was present on either end of each tank.
The fish were allowed to acclimate for 24 h. Then, fish comprising the normoxic control group (~19.0 kPa) were gently removed from the aquaria in their individual containers using opaque plastic bags filled with the aquarium water. Four fish were removed from each aquarium for a total of eight fish. A benzocaine solution (100 g/L ethanol) was added to the bags to terminally anesthetize the fish (<30 s). Fish remained calm during this procedure. Immediately following loss of equilibrium, blood was sampled via caudal puncture and placed on ice until analysis of hematocrit (Hct) and hemoglobin (Hb).

Following blood sampling, the heart was quickly removed, emptied of blood and blotted dry, and frozen in liquid N\textsubscript{2}. Plasma was obtained by centrifuging whole blood (3000xg; 5 min) and then frozen in liquid N\textsubscript{2}. All samples were stored at -80°C until analyses.

Following the sampling of the normoxic fish, hypoxia was induced by bubbling N\textsubscript{2} into each aquarium, which were covered with plastic bubble wrap and polystyrene foam to prevent O\textsubscript{2} ingress. Water Po\textsubscript{2} decreased with N\textsubscript{2} bubbling from normoxia (~19.0 kPa) to profound hypoxia (1.0 kPa) over a 1.5 h period and was maintained at this level for a 24 h period, using dissolved O\textsubscript{2} controllers (alpha-DO2000W, Eutech Instruments, Singapore) connected to solenoid valves that controlled the flow of N\textsubscript{2} into the aquaria. This profound level of environmental hypoxia matched the level of hypoxia used in series II. Water Po\textsubscript{2} in both tanks was held at the same level and constantly monitored. Using the protocol described above, four fish were sampled from each tank (total=8 fish) after 1, 2, 4, 8, 12, and 24 h of the hypoxia exposure. After the 24 h hypoxia exposure, N\textsubscript{2} bubbling was ceased and the aquaria water was aerated, resulting in a rapid return to normoxia within 1 h. Twelve hours after the return to normoxia, three fish from each aquarium were sampled as above and are termed the recovery group (total=6 fish).
2.3.2.3.3 Analytical protocols

Blood [Hb] was measured spectrophotometrically (Richards et al., 2007). Haematocrit was determined by centrifugation at 5000 g in a sealed haematocrit capillary tube. Mean cellular [Hb] (MCHC) was calculated as [Hb]/Hct. Plasma [lactate] and [glucose] were measured on deproteinized and non-deproteinized plasma, respectively, using the spectrophotometric methods described in Bergmeyer (1983). Plasma [non-esterified fatty acids] was measured spectrophotometrically using a commercially available kit (NEFA-HR(2); Wako, Osaka, Japan).

Frozen heart was broken into small pieces under liquid N₂ using an insulated mortar and pestle. Ground tissue was aliquoted into pre-weighed 1.5 mL microcentrifuge tubes and stored at -80°C until analysis. For the extraction of metabolites, 1 mL of ice-cold 1 M HClO₄ was added to a microcentrifuge tube containing tissue and the mixture was immediately sonicated on ice for 20 s using a Kontes sonicator set to its highest setting. The homogenates were centrifuged at 10 000xg for 10 min at 4°C and the supernatant neutralized with 3 M K₂CO₃. The neutralized extracts were assayed spectrophotometrically for concentrations of ATP, creatine phosphate (CrP), creatine (Cr), and lactate following methods described in Bergmeyer (1983). Levels of acetyl CoA, and acetyl- and free carnitine were assayed radiometrically on neutralized extracts using the methods of Cederblad et al. (1990). Acetyl carnitine measurements were corrected for endogenous acetyl CoA. Intracellular pH (pHᵢ) was measured on a separate aliquot of heart tissue using the methods of Pörtner et al. (1991b) and a thermostatted (22°C) Radiometer BMS3 Mk2 capillary microelectrode with PHM84 pH meter (Radiometer, Copenhagen, Denmark).
Expression of mRNA in heart was ascertained using qRT-PCR (see Table 2.1 for qRT-PCR primer sequences). Using the methods outlined above, total RNA was extracted from heart tissue, first-strand cDNA was synthesized from 5 µg of total RNA, and qRT-PCR was performed in duplicate. One randomly selected control sample was used to create a standard curve relating threshold cycle to cDNA amount for each primer set. All results are expressed relative to these standard curves and mRNA amounts are normalized relative to EF-1α mRNA expression. Real time primers for EF-1α were designed using a GenBank sequence from Oreochromis niloticus (Accession No. AB075952.1). There was no effect of hypoxia exposure on the mRNA expression level of EF-1α (data not shown). Expression of mRNA was further normalized by setting the expression in normoxia to 1.

The active fraction of PDH (PDHₐ) was measured at 22°C in heart homogenates following the methods described by Richards et al. (2002a), except that reaction aliquots were sampled at 1, 2, and 3 min due to the relatively high PDHₐ activity in heart.

Total PDK-2 protein content was measured using immunoblot analysis according to the protocols described in Richards et al. (2007), with minor modifications. Briefly, aliquots of the homogenates used for measurement of PDHₐ were frozen at -80°C, thawed and centrifuged at 1000xg for 10 min at 4°C. The supernatant was diluted to 1 mg protein/mL, denatured by boiling for 3 min in Laemmli’s buffer, and loaded on denaturing SDS-polyacrylamide gels at a final concentration of 20 µg protein/lane. An identical control sample was included on each gel to control for gel-to-gel variation. Gel electrophoresis and protein transfer to nitrocellulose membranes were carried out as described by Richards et al. (2007). Blots were incubated overnight at 4°C with 1 µg/ml of primary antibody (raised against the COOH-terminal end of rabbit PDK-2; peptide sequence 5'
VPSTEPKNTSTYRVS-3; Abgent, San Diego, CA, USA), followed by incubation for 1 h with alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody (Sigma-Aldrich). Blots were developed and the band intensity measured as described by Richards et al. (2007). Individual samples were expressed relative to total protein and the gel-to-gel standard and then normalized to the mean of the normoxia control samples. A standard curve of select samples was run to confirm the binding efficiency of the antibody (not shown).

AMPK activity was measured by the modified SAMS peptide [\(^{32}\)P]-ATP method described in Jibb and Richards (2008).

2.3.2.3.4 Calculations of energetic status

Measured values of [ATP], [CrP], [Cr] and pH, were used to calculate free cytosolic [ADP] and [AMP], assuming equilibrium of the creatine kinase and adenylate kinase reactions and following the methods described by Jibb and Richards (2008).

2.3.3 Statistics

Changes in \( \dot{M}_{O_2} \) or cardiovascular parameters in series I and II were tested for statistical significance using a one-way repeated measures ANOVA followed by the Holm-Sidak post hoc test or, when data were non-normal and log transformation did not improve normality, a one-way repeated measures ANOVA on ranks followed by SNK post hoc test. Comparison of critical \( P_wO_2 \) of cardiovascular parameters and \( \dot{M}_{O_2} \) was carried out using a one-way ANOVA on ranks followed by SNK post hoc test. Data for series III was tested for statistical significance using a one way ANOVA followed by the Holm-Sidak post hoc test.
Statistical significance was accepted at p<0.05. All data means are presented as mean ± standard error.

2.4 RESULTS

2.4.1 Series I. Assessment of hypoxia tolerance

Tilapia showed a typical relationship between $\dot{M}_{O_2}$ and $P_{wO_2}$. A zone of $O_2$-independent $\dot{M}_{O_2}$ occurred between $\geq 19.0$ kPa and the $P_{crit}$ of $3.73\pm0.31$ kPa, below which $\dot{M}_{O_2}$ became $O_2$-dependent and decreased with decreasing $P_{wO_2}$ (Fig. 2.1). The whole blood hemoglobin-$O_2$ $P_{50}$ was $1.46\pm0.11$ kPa.

2.4.2 Series II. Cardiac ATP demand

Our experimental protocol allowed for the simultaneous recording of whole-animal $\dot{M}_{O_2}$ and cardiovascular function during progressive depletion of $O_2$ from 16.5 kPa to 0.5 kPa; only cardiovascular function was followed during a subsequent 8 h exposure to hypoxia (1 kPa) and 1 h of normoxic recovery. Whole-animal $\dot{M}_{O_2}$ began to decrease significantly at 4.1 kPa reaching a minimum value at 0.5 kPa that represented a depression of $\dot{M}_{O_2}$ of more than 90% compared with normoxic $\dot{M}_{O_2}$ (Fig. 2.1). $P_{crit}$ was $3.69\pm0.34$ kPa, which was not significantly different from the $P_{crit}$ determined in series I even though routine normoxic $\dot{M}_{O_2}$ was lower in series II relative to series I fish (Fig. 2.1). The lower $\dot{M}_{O_2}$ in series II is probably due to the larger size of these fish.

Heart rate was unaffected by $P_{wO_2}$ between 19.2 kPa to 6.2 kPa. At 4.1 kPa there was a significant bradycardia and below this threshold $f_{fit}$ decreased steadily as $P_{wO_2}$ decreased,
reaching a minimum \( f_{H} \) at 0.5 kPa that was approximately 45-50% of the normoxic value (\( \geq 6.2 \) kPa) (Fig. 2.2A). Bradycardia persisted throughout a subsequent 8 h hypoxia exposure at 1.0 kPa (Fig. 2.2A,B). After 1 h of recovery, \( f_{H} \) was significantly elevated above the normoxic values (c.f. Fig. 2.2A and 2.2B).

The pattern of changes in \( \dot{Q} \) closely paralleled those for \( f_{H} \). Cardiac output was unaffected by decreases in \( P_{w}O_{2} \) from 19.2 kPa to 4.1 kPa, but decreased significantly at \( \leq 3.1 \) kPa (Fig. 2.2C). At 1.0 kPa and 0.5 kPa, \( \dot{Q} \) had decreased to a minimum value that was 40-45% of that seen in normoxia (\( \geq 6.2 \) kPa). During 8 h exposure at 1.0 kPa, \( \dot{Q} \) increased slightly and remained at about 55% of the normoxic value (Fig. 2.2D). After 1 h of recovery, \( \dot{Q} \) was significantly elevated above the normoxic value (c.f. Fig. 2.2C and 2.2D).

The pattern of changes in \( PO \) paralleled those for \( \dot{Q} \) and \( f_{H} \). Cardiac power output was constant at 1.30 mW g\(^{-1}\) between 19.2 kPa and 4.1 kPa, and then decreased significantly at and below 3.1 kPa, reaching a minimum value of approximately 0.5 mW g\(^{-1}\) at 1.0 kPa and 0.5 kPa (Fig. 2.2E). Cardiac power output remained depressed at approximately 0.7 mW g\(^{-1}\) throughout the 8 h hypoxia exposure at 1.0 kPa (Fig. 2.2F). After 1 h of recovery, \( PO \) returned to values that were similar to those seen during normoxia (c.f. Fig. 2.2E and 2.2F).

Stroke volume remained constant at all \( O_{2} \) tensions and throughout the 8 h hypoxia exposure (Fig. 2.3A,B). After 1 h of recovery, \( V_{SH} \) was significantly elevated compared with the normoxic value (c.f. Fig. 2.3A and 2.3B). Ventral aortic blood pressure was largely unaffected by decreasing \( P_{w}O_{2} \), but decreased significantly in recovery (Fig. 2.3C,D).

Peripheral resistance was unaffected by decreasing \( P_{w}O_{2} \) until 3.1 kPa, below which \( R \) steadily increased to a value at 0.5 kPa that was approximately double the normoxic level. Peripheral resistance remained significantly higher than the normoxic level throughout the 8
h hypoxia exposure at 1.0 kPa and then decreased significantly after 1 h of recovery (Fig. 2.3F).

Critical $\text{PO}_2$ for all the cardiovascular variables were within a 2 kPa range of $\text{P}_w$O$_2$: $\dot{Q} = 5.27 \pm 0.47$ kPa; $P_O = 4.75 \pm 0.59$ kPa; $f_H = 4.92 \pm 0.26$ kPa; and $R = 3.53 \pm 0.39$ kPa. The critical $\text{PO}_2$ for $\dot{Q}$, $PO$, and $f_H$ were not statistically different from one another, but all were significantly higher than the critical $\text{PO}_2$ for $R$ ($p<0.05$). Critical $\text{PO}_2$ of $M_O_2$ ($P_{crit}$) was significantly lower than the critical $\text{PO}_2$ for $f_H$, $\dot{Q}$, and $PO$ ($p<0.05$), but was not different from that of $R$ ($p>0.05$).

2.4.3 Series III. Cardiac ATP supply

2.4.3.1 Gene identification, sequencing, and tissue distribution

Partial cDNA sequences coding for PPAR$\alpha$ ($PPAR_{Til}\alpha$), PDK-2 ($PDK_{Til}\gamma$) and two isoforms of CPT-1 ($CPT_{Til}\gamma iso1$ and $CPT_{Til}\gamma iso2$) were identified in tilapia. Alignment of these sequences with those from other vertebrates showed, respectively, 75-86%, 70-75%, and 56-80% similarity to other available isoforms in fishes and mammals. The CPT-1 isoforms showed 66% sequence similarity to one another and neighbor-joining phylogenetic tree analysis shows that both grouped more closely with the mammalian CPT-1$\alpha$ rather than the mammalian CPT-1$\beta$ isoform (data not shown).

The two isoforms of CPT, $CPT_{Til}\gamma iso1$ and $CPT_{Til}\gamma iso2$, showed similar tissue mRNA distributions, except $CPT_{Til}\gamma iso1$ mRNA expression was higher in brain, liver, and red muscle compared with $CPT_{Til}\gamma iso2$, whereas $CPT_{Til}\gamma iso2$ was higher in white muscle (Fig. 2.4A). mRNA of $PPAR_{Til}\alpha$ was present in all tissues with the highest levels in heart, red muscle, and brain (Fig. 2.4B). $PDK_{Til}\gamma$ mRNA was also found in all tissues with the highest
levels in heart and red muscle (Fig. 2.4C). Relative tissue distributions of mRNA expression of the genes were similar whether mRNA was expressed relative to total RNA alone or normalized to EF-1α (data not shown for expression relative to total RNA).

2.4.3.2  
Response to hypoxia exposure

During the first minutes of hypoxia exposure, some of the fish appeared agitated whereas others remained quiescent. By the end of the first 1 h of hypoxia exposure, however, all fish were quiescent and rested quietly on the bottom of their container. Six fish died during the first 8 h of hypoxia exposure.

During hypoxia exposure, Hct did not change markedly from the normoxic value, but decreased during recovery (Table 2.2). Blood [Hb] decreased at 4 h and 8 h of hypoxia exposure as well as during recovery compared with the normoxic value. A decrease in MCHC was observed during hypoxia followed by a return to normoxic levels during recovery (Table 2.2).

Plasma [lactate] rapidly increased ~23-fold over the first 8 h of hypoxia exposure and then decreased to a new steady-state level at 12- and 24 h that was significantly elevated above normoxic levels (Fig. 2.5A). Plasma [glucose] increased significantly during the first 12 h of hypoxia exposure and then decreased significantly at 24 h but remained elevated compared with the normoxic value (Fig. 2.5B). Conversely, plasma [NEFA] dropped precipitously during the first 1 to 2 h of hypoxia exposure and remained depressed for the full 24 h exposure (Fig. 2.5C). [Lactate], [glucose], and [NEFA] in plasma all returned to normoxic levels following 12 h of recovery in normoxia.
Cardiac [ATP] was unaffected by 24 h of hypoxia exposure (Fig. 2.6A). Cardiac [CrP] also did not change markedly during hypoxia exposure, but increased following 12 h recovery (Table 2.3). I was initially concerned by the lack of an effect of hypoxia exposure on cardiac [CrP], so I exposed an additional group of tilapia to 1.0 kPa for 2 h and confirmed that cardiac [CrP] was not significantly affected by exposure to this level of hypoxia (unpublished observation). Free [Cr] in the heart was unaffected by hypoxia exposure and decreased during recovery (Table 2.3). Calculated [ADP_free] and [AMP_free] significantly decreased as the hypoxia exposure continued, reaching a relatively stable level by 8 h that continued in recovery (Table 2.3). During hypoxia exposure, [lactate] increased significantly in heart and returned to normoxic levels after 12 h of recovery (Fig. 2.6B). Similar to plasma [lactate], heart [lactate] peaked at 8 h of hypoxia exposure and then decreased by 24 h but remained significantly elevated above the normoxic value (Fig. 2.6B). Cardiac pH_i decreased significantly by 0.10-0.15 pH units during hypoxia exposure but returned to normoxic levels during recovery (Fig. 2.6C). Cardiac free [carnitine] and [acetyl carnitine] did not change in any consistent manner during hypoxia; levels stayed roughly the same as those in normoxia throughout the hypoxic and recovery periods (Table 2.3).

The activity of PDH_a in tilapia heart generally decreased during hypoxia exposure (Fig. 2.7A) with a 30-35% decrease in mean activity in the first 4 h and a significant 50-60% depression at 8 h and 24 h. At 12 h, however, PDH_a activity was not significantly different from the normoxic level. During recovery, cardiac PDH_a activity returned to the normoxic level. Heart [acetyl CoA], the product of the reaction catalysed by PDH as well as of fatty acid oxidation, decreased significantly by 30-40% during the first 1 to 4 h of hypoxia exposure and remained depressed during recovery (Fig. 2.7B). PDK_Til-2 transcript levels
increased by ~2-fold over the first 8 h of hypoxia exposure, followed by a decrease back to normoxic levels for the remainder of the hypoxia exposure and recovery (Fig. 2.7C). However, the amount of PDK-2 protein in heart of tilapia was unaffected during hypoxia exposure and recovery (Fig. 2.7D).

Levels of mRNA of CPT_Til-1 iso1 and CPT_Til-1 iso2 were unchanged in heart during hypoxia exposure and recovery (Table 2.4). The quantity of PPAR_Tilα transcript increased significantly in the first 8 h of hypoxia, followed by a decline to levels statistically indistinguishable from normoxic levels by 12 h exposure (Table 2.4).

The activity of AMPK was assessed in heart only during normoxia, and at 1 h, 2 h and 8 h during the hypoxia exposure, but no significant change in activity was observed (Table 2.5).

2.5 DISCUSSION

The ability to suppress cellular and whole-animal ATP turnover during O2 lack is a unifying strategy underlying hypoxia tolerance in many vertebrates (Boutilier, 2001a). In tilapia, exposure to hypoxia is marked by a large and rapid decrease in whole-animal $\dot{M}_{O_2}$ (Fig. 2.1). During exposure to progressive hypoxia, $\dot{M}_{O_2}$ decreased to ~23% and ~7% of normoxic values at 1.0 kPa and 0.5 kPa, respectively. This result is similar to the 70-80% reduction of $\dot{M}_{O_2}$ at 1.0 kPa measured by van Ginneken et al. (1997) in Mozambique tilapia (Oreochromis mossambicus) (where a ~50% decrease in heat production also occurred, indicating MRD). In the present study, depression of $\dot{M}_{O_2}$ occurred below the tilapia P_crit of approximately 3.7 kPa, which is also similar to that recorded previously for Nile tilapia (O. niloticus) (Chapman et al., 2002; Verheyen et al., 1994). Although P_crit is generally
considered to be a useful estimator of hypoxia tolerance (Chapman et al., 2002), tilapia possess a $P_{\text{crit}}$ that is similar to that of the intolerant rainbow trout at 20°C and higher than that of other hypoxia-tolerant tropical cichlids, which ranged from 8-16 torr (1.0 to 2.1 kPa) at 20°C (Chapman et al., 2002; Ott et al., 1980). However, a low whole blood hemoglobin-O$_2$ $P_{50}$ in tilapia (1.46 kPa in whole blood with pH 7.7-7.8 at 22°C), about half that seen in rainbow trout under similar conditions (Milligan and Wood, 1987), would facilitate O$_2$ extraction from the water during exposure to environmental hypoxia.

In most vertebrates, the heart is exquisitely sensitive to O$_2$ lack and susceptible to necrosis because of an inability to match ATP supply and demand. The maintenance of stable [ATP] in tilapia hearts during exposure to profound hypoxia (Fig. 2.6A) represents a hallmark measure of hypoxia tolerance (Boutilier, 2001a) and appears to be achieved through a combination of increased reliance on O$_2$-independent ATP product via anaerobic glycolysis (Fig. 2.5A; Fig. 2.6B) and a rapid, large and sustained reduction in PO (Fig. 2.2E,F). Depression of PO decreases cardiac ATP demand to levels that can be sustained by anaerobic glycolysis alone, based on the previous estimate of the MGP of the ectothermic vertebrate heart (Farrell and Stecyk, 2007). The ability of hypoxia-tolerant fishes such as tilapia to modulate PO during periods of low O$_2$ is therefore likely important in ensuring whole organism hypoxia survival.

2.5.1 Cardiac ATP demand

Few studies have examined how the cardiovascular status of hypoxia-tolerant fishes responds to progressive decreases in $P_{\text{a}}$O$_2$. During progressive decreases in environmental O$_2$, I showed that all cardiovascular parameters in tilapia remain at resting normoxic levels
down to a $P_{wO_2}$ of 6.2 kPa (Fig. 2.2A,C,E; Fig. 2.3A,C,E). The normoxic values compare well with previous studies. For example, $f_H$ is virtually identical to that measured in Nile tilapia by Thomaz et al. (2009), and resting values for $P_{VA}$ and $PO$ fall within the normal range observed in teleosts (2.9-4.9 kPa and 0.5-3.0 mW g$^{-1}$, respectively) (Stecyk and Farrell, 2006). At and below 4.1 kPa, a substantial bradycardia develops as $\dot{M}_{O_2}$ falls and the bradycardia is sustained at ~50% of normoxic $f_H$ for 8 h of hypoxia exposure at 1.0 kPa (Fig. 2.2A,B). Hypoxic bradycardia is observed in many, but not all fishes, and generally is mediated by cholinergic input via vagal innervation from gill chemosensors (Gamperl and Driedzic, 2009). Direct depressive effects of hypoxemia on $f_H$ also occur (Gamperl and Driedzic, 2009; McKenzie et al., 2009). The magnitude of bradycardia depends on numerous factors including temperature and the depth of hypoxia, but a halving of $f_H$ is not atypical in severe hypoxia exposure (Farrell, 2007; Gamperl and Driedzic, 2009). As seen in tilapia, development of bradycardia during progressive hypoxia below a certain, species-specific $P_{wO_2}$ is observed in many teleosts (e.g. lingcod ($Ophiodon elongatus$) (Farrell, 1982), $Hoplias$ spp. (Rantin et al., 1993), Atlantic cod ($Gadus morhua$) (Gamperl and Driedzic, 2009), Japanese eel ($Anguilla japonica$) (Chan, 1986)), and may be related to each species’ $P_{crit}$ and the depression of $\dot{M}_{O_2}$. In fact, $P_{crit}$ matches the $P_{wO_2}$ at initiation of bradycardia (i.e. $P_{crit}$ of $f_H$) in two $Hoplias$ spp. (Rantin et al., 1993), the spangled perch ($Leiopotherapon unicolor$) (Gehrke and Fielder, 1988), and cod (McKenzie et al., 2009), and parallel decreases in $f_H$ and $\dot{M}_{O_2}$ below $P_{crit}$ were observed in these species. The present measurements in tilapia show a similar result. The calculated critical $P_{wO_2}$ for $f_H$ was only slightly higher than for $\dot{M}_{O_2}$ and this may be explained by a steeper slope of the decrease in
compared with bradycardia as hypoxia progressed rather than by an intrinsically
different inflection point between these parameters (c.f. Fig. 2.1 and Fig. 2.2A). Instead, the
observation of initiation of bradycardia (i.e. first significant decrease in \( f_{Hi} \)) at the same \( P_wO_2 \)
(4.1 kPa) as \( \dot{M}_{O_2} \) first decreased significantly from normoxic values supports the contention
that these events occur more or less simultaneously in response to hypoxia. This may in part
be due to neural and humoral mechanisms responding to similar input from distinct or shared
O_2 chemosensors. The concurrent decreases of \( \dot{M}_{O_2} \) and heart rate as MRD progresses may
be similarly explained. Supporting a role for simultaneous regulation of \( \dot{M}_{O_2} \) and \( f_{Hi} \) by
distinct O_2 sensors, vagotomy and the resulting abolishment of reflex bradycardia due to gill
O_2 sensing in cod decreased the \( P_{crit} \) of \( f_{Hi} \) to below the unchanged \( P_{crit} \) of \( \dot{M}_{O_2} \), whereas in
sham-operated cod these values were the same (McKenzie et al., 2009). A similar result,
including measurements of \( \dot{Q} \), has been observed in atropinized eels (Iversen et al., 2010).
Decreased tissue O_2 demand caused by tissue-level MRD also may influence the decreases in
\( f_{Hi} \) (and \( \dot{Q} \); see below) via neural and humoral mechanisms. A close association between
\( \dot{M}_{O_2} \), \( \dot{Q} \), and \( f_{Hi} \) during routine and elevated activity is well established for fishes and largely
reflects changes in tissue O_2 demand (Webber et al., 1998).

Due to the lack of a significant compensatory increase in \( V_{SH} \) (Fig. 2.3A,B), the
observed bradycardia caused a substantial (~50%) decrease in \( \dot{Q} \) that also developed as
\( \dot{M}_{O_2} \) fell and was sustained during 8 h of hypoxia (Fig. 2.2C,D). This finding contrasts with
the typical observation of increases in \( V_{SH} \) in fishes during environmental hypoxia exposure
that help maintain \( \dot{Q} \) at least until a certain level of hypoxia is reached (Gamperl and
Driedzic, 2009). However, in several fishes, including short-horn sculpin (\textit{Myoxocephalus}
*scorpius* (MacCormack and Driedzic, 2004), common carp (Stecyk and Farrell, 2006), lingcod (Farrell, 1982), and Japanese eel (Chan, 1986), modest (or absent in the case of the short horn sculpin) increases in $V_{SH}$ coupled with large decreases in $f_{H}$ led to a reduction in $\dot{Q}$ during environmental hypoxia, similar to what I observed in tilapia. Direct comparisons across studies and species are complicated because of differences in the severity of environmental hypoxia used in each study, the usage of progressive vs. steady-state hypoxia, and the uncertain relative hypoxia tolerance of the species investigated. More clearly than in previous studies, I show that in the hypoxia-tolerant tilapia, $\dot{Q}$ is rapidly decreased via bradycardia (in the absence of compensatory changes in $V_{SH}$) as hypoxia and MRD develops below $P_{crit}$, and this depression is sustained throughout the hypoxia exposure.

The reductions of $f_{H}$ and $\dot{Q}$ might benefit cardiac hypoxia tolerance in tilapia because they facilitated the rapid decrease of $PO$ and thus ATP demand of the heart to below the level that can be sustained by the MGP alone (suggested to be ~0.7 mW g$^{-1}$ at 15°C in ectothermic vertebrates; Farrell, 2007; Farrell and Stecyk, 2007). This also appears to be the case for common carp, where the heart operates above this estimated $PO$ threshold in normoxia, but during $O_{2}$ deprivation $PO$ falls well below this value (Stecyk and Farrell, 2006). Conversely, the crucian carp maintains routine $PO$ low enough that further downregulation is unnecessary during hypoxia (Stecyk et al., 2004). Assuming that cardiac glycolytic capacity scales with temperature with a $Q_{10}$ of 2 (Overgaard et al., 2004), then the estimated $PO$ sustainable by glycolysis alone at 22°C for tilapia is ~1.1 mW g$^{-1}$ (based on the estimate of Farrell and Stecyk [2007]). Like the common carp, tilapia heart in normoxia operated slightly above this level (~1.3 mW g$^{-1}$, which is considerably lower than the maximum aerobic $PO$ of ~3.8 mW g$^{-1}$ observed after exhaustive exercise (B. Speers-Roesch, E. Sandblom, A. P. Farrell, and J. [68])
G. Richards, unpublished observations). Then, as hypoxia increased, $PO$ of tilapia heart decreased in the same fashion as $f_H$ and $\dot{Q}$, and remained at about 50% of the resting value for the duration of the hypoxic exposure (Fig. 2.2E,F), well below the estimated $PO$ sustainable by the MGP. Unlike cold anoxic turtles, but as in common carp, the depression is not great enough to prevent activation of a Pasteur effect (Farrell and Stecyk, 2007) and there was a rapid accumulation of lactate in tilapia during a similar hypoxia exposure (Fig. 2.6B). To my knowledge, the present measurements of $PO$ in tilapia are the first published for fish exposed to progressive hypoxia and demonstrate that in a hypoxia-tolerant teleost, ATP demand of the heart is rapidly downregulated as $O_2$ levels fall below $P_{crit}$ and whole-animal MRD develops. In hypoxic common carp, reductions in $PO$ appear to be caused by decreases in both $\dot{Q}$ and $P_{VA}$ (Stecyk and Farrell, 2006). In tilapia, however, $P_{VA}$ was unchanged (Fig. 2.3C,D) and thus arterial hypotension did not contribute to reduced $PO$. Instead, reductions in $f_H$ and $\dot{Q}$ appear to be the main determinants of decreased $PO$ in tilapia heart during hypoxia exposure, supporting the argument that a major benefit of hypoxia-induced bradycardia in fishes is to decrease ATP demand of the heart and depress cardiac ATP turnover (Farrell, 2007).

It has been suggested that a resetting of the barostatic reflex to facilitate arterial hypotension is a common response to exposure to low $O_2$ in hypoxia-tolerant but not hypoxia-sensitive teleosts (Stecyk and Farrell, 2006). The present results for the hypoxia-tolerant tilapia do not provide support for this hypothesis. Instead, it appears that in tilapia an elevation of $R$ in hypoxia, which occurs in many fishes including common carp (Stecyk and Farrell, 2006), helps conserve arterial blood pressure at normoxic levels in the face of large reductions in $\dot{Q}$ (Fig. 2.3E,F). The increased $R$ probably reflects a peripheral
vasoconstriction that shunts blood away from tissues with low O₂ demands such as white muscle and the gastrointestinal tract to those with higher O₂ demands such as the heart and brain (Stecyk and Farrell, 2006).

The cardiovascular status in tilapia recovering at 1 h post-hypoxia warrants brief mention. The rapid elevation of \( f_{\text{H}} \) and \( V_{\text{SH}} \) and thus \( \dot{Q} \), as well as a decrease in \( R \) (Fig. 2.2B,D; Fig. 2.3B,F), are probably important in recovering the O₂ debt of tissues, restoring acid-base status, and flushing accumulated metabolic wastes. Similar responses are observed in recovery from exhaustive exercise in fishes (e.g., Farrell, 1982). The observation of enhanced cardiovascular status following reoxygenation suggests that the tilapia heart does not sustain any significant irreversible damage or impairment after >8 h of hypoxia exposure.

### 2.5.2 Cardiac ATP supply

During O₂ limitation, ATP supply in hypoxia-tolerant animals is supported primarily by O₂-independent ATP production and to a much lesser extent by substrate oxidation (Boutilier, 2001a). In series III, I exposed tilapia for 24 h to profound hypoxia (\( P_{\text{aw}}O_2=1.0 \) kPa) representing approximately 30% and 70% of \( P_{\text{crit}} \) and hemoglobin-O₂ \( P_{50} \), respectively. Like many fishes, the tilapia ventricle lacks coronary arteries (Pieperhoff et al., 2009) so its O₂ supply is solely from venous blood (Gamperl and Driedzic, 2009). Haematological parameters related to O₂ transport were largely unaffected (Table 2.2) and inadequate O₂ delivery and resulting hypoxemia was evident from the large and rapid increase in plasma [lactate] (Fig. 2.5A). A significant increase in plasma [glucose], often seen in response to hypoxia exposure in fishes (e.g., Dunn et al., 1983), is thought to reflect mobilization of hepatic glycogen stores to fuel anaerobic glycolysis in other tissues, such as the heart. In fact,
heart of tilapia exposed to profound hypoxia accumulated lactate (Fig. 2.6B), probably partly due to an increase in plasma [lactate] but also demonstrating a reliance on anaerobic glycolysis in the heart because blood was blotted from the heart during sampling. In both heart and plasma, [lactate] peaked at 8 h followed by a significant decrease and stabilization at 12 h and 24 h (c.f. Fig 2.5A and Fig. 2.6B). This biphasic response in cardiac [lactate] may indicate that matching of ATP supply with demand and depression of ATP turnover was not optimized until 12 h to 24 h of hypoxia, at which point fully realized metabolic depression also may have allowed for the oxidation of some of the accumulated lactate. Activation of anaerobic glycolysis and associated ATP hydrolysis led to a significant decrease of pH_i in the heart (Fig. 2.6C). Similar reductions in pH_i (0.1-0.2 units) have been recorded from white skeletal and cardiac muscles of oscar and turtles, respectively (Richards et al., 2007; Wasser et al., 1990).

There was no indication that CrP was utilized for O_2-independent ATP production because cardiac [CrP] was not depleted following exposure to hypoxia (Table 2.3). Maintenance of cardiac [CrP] also has been observed in hearts of flounder (Platichthys flesus) and lungfish (Protopterus aethiopicus) but not rainbow trout during hypoxia exposure (Dunn and Hochachka, 1986; Dunn et al., 1983; Jorgensen and Mustafa, 1980) and contrasts with the depletion typically seen in other tissues of fishes during hypoxia, including Nile tilapia (Dunn et al., 1983; Jibb et al., 2008; Richards et al., 2008; Richards et al., 2007; van Ginneken et al., 1995). It is possible that the protection of [CrP] in heart of hypoxia-tolerant fishes may help maintain contractile function during periods of low O_2. Depletion of CrP and accumulation of P_i is associated with contractile dysfunction and cardiac failure during hypoxia in both mammals and fish (Allen and Orchard, 1987; Arthur et al., 1992).
Stable [CrP] in the heart of hypoxic tilapia also reflects adequate matching of ATP supply with demand, which avoids the need to dephosphorylate CrP to maintain [ATP] (Ingwall and Weiss, 2004). Indeed, cardiac [ATP] during the hypoxia exposure was unchanged from that in normoxia (Fig. 2.6A). Maintenance of stable cellular [ATP] in the face of severe hypoxemic stress is considered to be characteristic of hypoxia-tolerant animals (Boutilier, 2001a). Constant cardiac [ATP] has been observed in lungfish, flounder, and European eel (Anguilla anguilla) exposed to hypoxia (Dunn et al., 1983; Jorgensen and Mustafa, 1980; van Waarde et al., 1983), whereas in hypoxia-intolerant rainbow trout, cardiac [ATP] decreases significantly (Dunn and Hochachka, 1986). In all of these species, [ADP] and [AMP] remained unchanged, but the significance of this is unclear because these values are total (bound and free) adenylates and not the free adenylates that are of regulatory relevance (Schulte et al., 1992). In this study, estimated [ADP$_{free}$] and [AMP$_{free}$] decreased over the first few hours of hypoxia exposure, reaching a new, relatively stable level from 8 h to 24 h and remaining there in recovery (Table 2.3). These observations in heart contrast with the increase in [ADP$_{free}$] and [AMP$_{free}$] typically seen in white muscle and liver of teleosts exposed to hypoxia (Jibb and Richards, 2008; Richards et al., 2008; van Ginneken et al., 1995) and the difference appears mostly to be due to depletion of CrP in white muscle and liver whereas cardiac [CrP] is maintained. The absence of major perturbation of the energetic status of the heart of hypoxic tilapia (Fig. 2.6A; Table 2.3) in part may be explained by a rapid decrease in ATP demand and ATP turnover via depressed PO observed in the heart during hypoxia in series II (Fig. 2.2E,F). However, because PO reaches its minimum well before [ADP$_{free}$] and [AMP$_{free}$] stabilize, decreases in other pathways of ATP demand such as protein synthesis may also play a role (Lewis et al., 2007). Longer response times for
depression of certain ATP demand processes may also explain why [lactate] did not stabilize until 12 h of hypoxia exposure.

Understanding how aerobic ATP supply pathways are controlled in hypoxia-tolerant animals during periods of low O_2 is of interest because low levels of substrate oxidation occur during hypoxia (unlike that expected during anoxia exposure) and potentially deleterious effects of impaired substrate oxidation (e.g. ROS, lipotoxicity) must be avoided. Also, the initiation of MRD is thought to be under the control of pathways of aerobic ATP supply, such as mitochondrial substrate oxidation (Bishop et al., 2002). A potential mechanism for this downregulation is the modulation of PDH_a activity. Decreased PDH_a activity has been observed in metabolically depressed snails as well as in white muscle of fishes exposed to hypoxia (Brooks and Storey, 1992; Richards et al., 2008; Richards et al., 2007). The results of this study (series III) suggest that there is also a general depression of PDH_a activity in the heart of tilapia during hypoxia exposure (Fig 2.4A). Unlike the more rapid depression seen in white muscle of hypoxic fishes (Richards et al., 2008; Richards et al., 2007), however, a significant decrease in activity is not observed until 8 h of hypoxia exposure, although this partly may be due to the high variation in normoxic fish.

Furthermore, the activity decrease is not sustained: at 12 h, activity returned to a level similar to that in normoxia (Fig. 2.4C). Richards et al. (2008) observed the same result at 12 h of hypoxia in killifish white muscle, so some temporal fluctuation in PDH activity appears to be normal during hypoxia in fishes.

PDH_a activity is potentially regulated by modulators such as ATP and acetyl CoA acting on existing PDK protein and influenced by activity of the heart, as well as by regulation of PDK expression (Holness and Sugden, 2003). Cardiac [acetyl CoA] in tilapia
decreased significantly during hypoxia (Fig. 2.7B). Assuming the total CoA pool remained constant, this suggests that activation of PDH<sub>a</sub> activity via PDK would be favoured. PDH<sub>a</sub> activity generally decreased in this study, however, suggesting that significant modulation of PDH activity via acetyl CoA is not occurring. The decrease in acetyl CoA may instead simply reflect a slowing of cellular metabolic rate and the observed reduced work load (Fig. 2.2E,F) of the heart of hypoxia-exposed tilapia. The decrease in cardiac work also may in part explain the decrease in PDH<sub>a</sub> activity; in fish skeletal muscle, at least, PDH<sub>a</sub> activity is highly responsive to changes in muscular activity (Richards et al., 2002b). The effect on PDH<sub>a</sub> activity of manipulation of work load, and thus ATP demand, of isolated hearts operating at different PO<sub>2</sub> are needed to assess whether decreases in PDH<sub>a</sub> activity during hypoxia are due simply to reductions of PO or due to active downregulation.

Similar to white muscle of hypoxic killifish (Richards et al., 2008), there was a 2-fold increase in mRNA expression of a PDK-2 isoform (PDK<sub>Til-2</sub>) but no change in PDK-2 total protein in tilapia heart during hypoxia (Fig. 2.7C,D). The tissue distribution of PDK<sub>Til-2</sub> is comparable to that of a PDK-2 isoform in killifish (Richards et al., 2008) with relatively high levels in heart (Fig. 2.4C). A disconnect between mRNA and protein levels also has been observed in previous studies on hypoxia exposure in fishes (e.g. Richards et al., 2008; Richards et al., 2007). Relative increases of important mRNA transcripts may help ensure that the limited capacity for translation that occurs during hypoxia favors these transcripts to maintain protein level (Richards et al., 2008). Alternatively, the disconnect may be caused by poor specificity of mammalian antibody for the specific isoform measured via qPCR.

The hypoxic induction of PDK<sub>Til-2</sub> was transient, decreasing at 12 h and 24 h (Fig. 2.7C). Mitigation of tissue hypoxemia via improved O<sub>2</sub> delivery to the heart is not a likely
explanation because lactate load in plasma and heart remained greatly elevated at 12 h and 24 h (Fig. 2. 5A; Fig. 2.6B). The transient increase in $PDK_{Til}^{-2}$ mRNA instead may reflect an acute response to hypoxia, possibly contributing to downregulation of PDH$_a$ activity (although this remains unconfirmed), which ebbs during acclimation associated with longer-term exposure. Supporting this idea, heart of zebrafish ($Danio rerio$) exposed to chronic environmental hypoxia (~2 kPa for several weeks) show a >3-fold decrease of $PDK^{-2}$ mRNA expression (Marques et al., 2008). Marques et al. (2008) also observed decreases in mRNA expression of genes related to fatty acid oxidation suggesting a switch to carbohydrate rather than lipid oxidation in heart of zebrafish exposed to chronic environmental hypoxia. Here, there was no change in the mRNA expression of two CPT-1 isoforms ($CPT_{Til}^{-1}$ iso1, $CPT_{Til}^{-1}$ iso2) and a transient increase of $PPAR_{Til}^{-\alpha}$ in heart of tilapia exposed to hypoxia for 24 h (Table 2.4), so transcriptional downregulation of genes for enzymes involved in fatty acid oxidation may occur only after long-term acclimation to environmental hypoxia. Similarly, mammalian studies show that mRNA expression of fatty acid oxidation genes (including CPT-1 and $PPAR_{\alpha}$) in heart decreases after chronic but not acute in vivo environmental hypoxia exposure (Essop, 2007). The transient increase of $PPAR_{Til}^{-\alpha}$ (Table 2.4) was remarkably similar to that seen for $PDK_{Til}^{-2}$, suggesting related transcriptional control of these genes as seen in mouse hypoxic skeletal muscle (Aragonés et al., 2008).

Depression of fatty acid oxidation by other means during hypoxia exposure in tilapia is potentially important in contributing to MRD and avoiding the potential for harmful effects of lipotoxicity and uncoupling of glucose oxidation associated with uncontrolled fatty acid oxidation in hypoxic and reperfused mammalian myocardium (Corr et al., 1984; Dyck and
Lopaschuk, 2006). In particular, the large and rapid decrease of plasma [NEFA] (Fig. 2.5C) strongly implicates substrate supply as a major regulator of fatty acid oxidation in heart and other tissues of tilapia during hypoxia exposure. NEFA are a metabolically dynamic lipid fraction of the blood and are important for fatty acid oxidation (Henderson and Tocher, 1987). A ~80% decrease in NEFA supply to tissues, as seen in this study, could therefore significantly reduce substrate oxidation and $M_O$ of tissues, and as also suggested by Magnoni et al. (2008), may contribute to MRD. It may also lessen the potential effects of impaired fatty acid oxidation during hypoxia, including in the heart. Whatever the significance, several hypoxia-tolerant fishes including the Mozambique tilapia show a decrease in plasma [NEFA] during O$_2$ deprivation (van Heeswijk et al., 2005; van Raaij et al., 1996b; Vianen et al., 2002) that appears to be due to a noradrenaline-mediated inhibition of lipolysis (Vianen et al., 2002). Despite reductions in plasma [NEFA], measurements of carnitine esters in the heart provided no evidence of decreased fatty acid oxidation. However, metabolite concentrations are limited in their ability to explain flux (Haman et al., 1997), so further studies on fatty acid oxidation in hypoxic isolated hearts are warranted. Whether other cellular mechanisms, such as fatty acid import or inhibition of CPT-1 via malonyl CoA, are important in limiting fatty acid oxidation is unknown and should be investigated.

Activation of AMPK has recently been implicated in reducing ATP demand of certain tissues as well as whole-animal MRD in hypoxia-exposed goldfish and crucian carp (Jibb and Richards, 2008; Stensløkken et al., 2008). The present results do not suggest a role for AMPK in hypoxia tolerance of tilapia heart (Table 2.5) and the absence of an increase in [AMP$_{free}$] (Table 2.3) is consistent with the lack of AMPK activation. AMPK also was not hypoxia responsive in goldfish hearts (Jibb and Richards, 2008). Increases in cardiac AMPK
activity may not be necessary in hypoxic fishes because ATP demand can be greatly reduced via decreases in $\dot{Q}$, as observed in this study (Fig. 2.2C,D). AMPK also may be more important for anoxia tolerance rather than for hypoxia tolerance (Stensløkken et al., 2008).

### 2.5.3 Conclusions and perspectives

Hypoxic cardiac failure due to a mismatch between ATP supply and demand can quickly result in the death of hypoxia-sensitive animals, including most endotherms. Hypoxia-tolerant species, however, possess the ability to balance cardiac ATP supply with demand and downregulate cardiac ATP turnover to ensure survival during hypoxia exposure (Farrell and Stecyk, 2007). In the heart of the hypoxia-tolerant tilapia, my measurements indicate that hypoxia-induced MRD is associated with a rapid, sustained depression of $PO$ (i.e., cardiac ATP demand) that is achieved via a hypoxia-responsive bradycardia. The depression of $PO$ may bring the ATP demand of the tilapia heart below the estimated level sustainable by the MGP, in agreement with the schema of Farrell and Stecyk (2007). These findings support the argument that hypoxic bradycardia in fishes serves an important, potentially adaptive, role in hypoxia tolerance of the heart, as well as of the whole organism (Farrell, 2007).

Responses of cardiac ATP supply pathways during hypoxia exposure were usually slower and less well defined. These results suggest that depression of ATP demand, rather than ATP supply, is more important in controlling the downregulation of ATP turnover in the heart of hypoxia-tolerant fishes exposed to low $O_2$. Modulation of ATP supply may be important for other aspects of hypoxia tolerance, however. The PDH/PDK pathway, for example, may be important for mitigation of ROS damage at least during acute exposure.
(Aragonés et al., 2008; Chen et al., 2007). Finally, the marked reduction of circulating [NEFA] observed stands out as an ATP supply pathway that should be further investigated to ascertain its potentially major importance in contributing to MRD and preventing lipotoxicity in tissues such as the heart during hypoxia.

2.6 ACKNOWLEDGEMENTS

Milica Mandic and Lindsay Jibb assisted in sampling. Michael Axelsson kindly lent equipment needed for a portion of this work. Funding was provided by the Discovery Grant Program from NSERC to J.G. Richards. B. Speers-Roesch was the recipient of a Canada Graduate Scholarship from NSERC and a Pacific Century Graduate Scholarship from the University of British Columbia and the Province of British Columbia. E. Sandblom was supported by a NSERC Discovery Grant to A.P. Farrell.
Table 2.1. PCR primers used for the identification and quantification of CPT-1, PDK-2, PPARα, and EF-1α mRNA from tilapia tissues.

<table>
<thead>
<tr>
<th>Name</th>
<th>Purpose</th>
<th>Direction</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPT-1</td>
<td>Degenerate</td>
<td>Forward</td>
<td>5'-GA(C/T) TGG TGG GA(A/G) GA(A/G) TA(C/T) (A/G/T)TC TA-3'</td>
</tr>
<tr>
<td>CPT-1</td>
<td>Degenerate</td>
<td>Reverse</td>
<td>5'-GCC CA(A/C/G) GA(A/G) TG(C/T) TC(A/C/T) GC(A/G) TT-3'</td>
</tr>
<tr>
<td>PDK-2</td>
<td>Degenerate</td>
<td>Forward</td>
<td>5'-GGA AC(A/C) G(A/C/G)C ACA A(C/T)G A(C/T)G T-3'</td>
</tr>
<tr>
<td>PDK-2</td>
<td>Degenerate</td>
<td>Reverse</td>
<td>5'-GT(C/G) CC(A/G) (A/T)A(A/G) CCC TCC AT(A/C/G/T) G-3'</td>
</tr>
<tr>
<td>PPARα</td>
<td>Degenerate</td>
<td>Forward</td>
<td>5'-GAG GGC TGC AAG GGT TTC</td>
</tr>
<tr>
<td>PPARα</td>
<td>Degenerate</td>
<td>Reverse</td>
<td>5'-CGG AGG TC(A/G) GCC AGT TTC T-3'</td>
</tr>
<tr>
<td>CPT_Til-1 iso1</td>
<td>qRT-PCR</td>
<td>Forward</td>
<td>5'-GCC GCC TTC TTT GTG ACA CT-3'</td>
</tr>
<tr>
<td>CPT_Til-1 iso1</td>
<td>qRT-PCR</td>
<td>Reverse</td>
<td>5'-TCT AAA CTG GCT GCT GGG TCA T-3'</td>
</tr>
<tr>
<td>CPT_Til-1 iso2</td>
<td>qRT-PCR</td>
<td>Forward</td>
<td>5'-CAA ACC TCT CAT GCT CCT ACA CA-3'</td>
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<tr>
<td>PDK_Til-2</td>
<td>qRT-PCR</td>
<td>Forward</td>
<td>5'-CCG CGT AGA CAA TGG TCG TA-3'</td>
</tr>
<tr>
<td>PDK_Til-2</td>
<td>qRT-PCR</td>
<td>Reverse</td>
<td>5'-GAA ATG GGC AGG CCA TAG C-3'</td>
</tr>
<tr>
<td>PPAR_Tilα</td>
<td>qRT-PCR</td>
<td>Forward</td>
<td>5'-CAC GAC ATG GAG ACG TTC CA-3'</td>
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<tr>
<td>PPAR_Tilα</td>
<td>qRT-PCR</td>
<td>Reverse</td>
<td>5'-TCC GGA TAG TCG CTG TTT ATC A-3'</td>
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<tr>
<td>EF-1α</td>
<td>qRT-PCR</td>
<td>Forward</td>
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<tr>
<td>EF-1α</td>
<td>qRT-PCR</td>
<td>Reverse</td>
<td>5'-CTT GGA GAT ACC AGC CTC GAA-3'</td>
</tr>
</tbody>
</table>

Table 2.2. Blood haemoglobin (Hb), haematocrit (Hct), and mean cellular haemoglobin content (MCHC) in tilapia exposed to normoxia, up to 24 h profound hypoxia (water PO₂=1.0 kPa), and after 12 h recovery in normoxic water.

<table>
<thead>
<tr>
<th>Normoxia</th>
<th>1 h</th>
<th>2 h</th>
<th>4 h</th>
<th>8 h</th>
<th>12 h</th>
<th>24 h</th>
<th>Recovery</th>
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</thead>
<tbody>
<tr>
<td>Hb (mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>±0.04ac</td>
<td>1.30</td>
<td>1.20</td>
<td>1.12</td>
<td>1.00</td>
<td>0.96</td>
<td>1.21</td>
<td>1.31</td>
</tr>
<tr>
<td>Hct (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>±0.8a</td>
<td>31.5</td>
<td>33.6</td>
<td>32.3</td>
<td>30.9</td>
<td>26.9</td>
<td>31.5</td>
<td>34.1</td>
</tr>
<tr>
<td>MCHC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>±0.07ad</td>
<td>4.12</td>
<td>3.56</td>
<td>3.45</td>
<td>3.19</td>
<td>3.57</td>
<td>3.90</td>
<td>3.83</td>
</tr>
</tbody>
</table>

Data are means±s.e.m. (<i>n</i>=8, except 12 h recovery, <i>n</i>=6). Values with different letters are significantly different (p<0.05, one way ANOVA with Holm-Sidak test).
ANOVA with Holm

Data are means±s.e.m. (water PO₂=1.0 kPa), and after 12 h recovery in normoxic water.

Table 2.3. Heart [creatine phosphate], free [creatinine], [lactate], [ADPfree], [AMPfree], free [carnitine], and [acetyl carnitine] in tilapia exposed to normoxia, up to 24 h profound hypoxia (water PO₂=1.0 kPa), and after 12 h recovery in normoxic water.

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>1 h</th>
<th>2 h</th>
<th>4 h</th>
<th>8 h</th>
<th>12 h</th>
<th>24 h</th>
<th>24 h + 12 h recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>[CrP]</td>
<td>1.62</td>
<td>±0.28a</td>
<td>±0.16b</td>
<td>±0.31ab</td>
<td>±0.48ob</td>
<td>±0.60ab</td>
<td>±0.34ab</td>
<td>±0.37c</td>
</tr>
<tr>
<td></td>
<td>±0.24ab</td>
<td>±0.16a</td>
<td>±0.24b</td>
<td>±0.13bc</td>
<td>±0.17bc</td>
<td>±0.23bc</td>
<td>±0.21cd</td>
<td>±0.25d</td>
</tr>
<tr>
<td>[Cr]</td>
<td>1.86</td>
<td>±0.16abc</td>
<td>±0.08ab</td>
<td>±0.13bc</td>
<td>±0.17bc</td>
<td>±0.23bc</td>
<td>±0.21cd</td>
<td>±0.25d</td>
</tr>
<tr>
<td>[ADPfree]</td>
<td>12.3</td>
<td>±1.8a</td>
<td>±0.63ab</td>
<td>±1.28bcd</td>
<td>±0.71cd</td>
<td>±1.46bc</td>
<td>±0.32d</td>
<td>±0.82d</td>
</tr>
<tr>
<td>[AMPfree]</td>
<td>0.08</td>
<td>±0.033a</td>
<td>±0.02ab</td>
<td>±0.01ab</td>
<td>±0.02c</td>
<td>±0.002c</td>
<td>±0.002c</td>
<td>±0.002d</td>
</tr>
<tr>
<td>Free</td>
<td>84.9</td>
<td>±13.7a</td>
<td>±15.8a</td>
<td>±8.2a</td>
<td>±9.3a</td>
<td>±19.9a</td>
<td>±10.0a</td>
<td>±12.9a</td>
</tr>
<tr>
<td>[carnitine]</td>
<td>±4.27</td>
<td>±5.0bcde</td>
<td>±11.7e</td>
<td>±11.3e</td>
<td>±7.6e</td>
<td>±10.0e</td>
<td>±2.6e</td>
<td>±3.1e</td>
</tr>
<tr>
<td>[Acetyl carnitine]</td>
<td>±0.95</td>
<td>±0.002c</td>
<td>±0.002c</td>
<td>±0.002c</td>
<td>±0.002c</td>
<td>±0.002c</td>
<td>±0.002c</td>
<td>±0.002d</td>
</tr>
</tbody>
</table>

Data are means±s.e.m. (n=5-8). CrP, creatine phosphate; Cr, free creatine. All values are reported as nmol· g wet tissue⁻¹, except CrP and Cr, which are reported as μmol· g wet tissue⁻¹. Values that do not share a letter are significantly different (p<0.05, one way ANOVA with Holm-Sidak test).

Table 2.4. CPT₁α-1 iso1, CPT₁α-1 iso2, and PPARα mRNA in heart of tilapia exposed to normoxia, up to 24 h profound hypoxia (water PO₂=1.0 kPa), and after 12 h recovery in normoxic water.

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>1 h</th>
<th>2 h</th>
<th>4 h</th>
<th>8 h</th>
<th>12 h</th>
<th>24 h</th>
<th>24 h + 12 h recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPT₁α-1 iso1</td>
<td>1.00</td>
<td>±0.25a</td>
<td>±0.22a</td>
<td>±0.13a</td>
<td>±0.27a</td>
<td>±0.36a</td>
<td>±0.13a</td>
<td>±0.28a</td>
</tr>
<tr>
<td></td>
<td>±0.18a</td>
<td>±0.13a</td>
<td>±0.36a</td>
<td>±0.13a</td>
<td>±0.28a</td>
<td>±0.13a</td>
<td>±0.28a</td>
<td>±0.18a</td>
</tr>
<tr>
<td>CPT₁α-1 iso2</td>
<td>1.00</td>
<td>±0.8a</td>
<td>±0.38a</td>
<td>±1.03a</td>
<td>±0.13a</td>
<td>±0.39a</td>
<td>±0.20a</td>
<td>±0.09a</td>
</tr>
<tr>
<td></td>
<td>±0.31a</td>
<td>±0.13a</td>
<td>±0.39a</td>
<td>±0.20a</td>
<td>±0.09a</td>
<td>±0.31a</td>
<td>±0.13a</td>
<td>±0.31a</td>
</tr>
<tr>
<td>PPARα</td>
<td>1.00</td>
<td>±0.11a</td>
<td>±0.24ab</td>
<td>±0.24ab</td>
<td>±0.24ab</td>
<td>±0.24ab</td>
<td>±0.24ab</td>
<td>±0.24ab</td>
</tr>
<tr>
<td></td>
<td>±0.23ab</td>
<td>±0.24ab</td>
<td>±0.24ab</td>
<td>±0.24ab</td>
<td>±0.24ab</td>
<td>±0.24ab</td>
<td>±0.24ab</td>
<td>±0.24ab</td>
</tr>
</tbody>
</table>

Data are means±s.e.m. (n=7-8, except 12 h recovery, n=5). mRNA expression is reported relative to the mRNA expression of a control gene (EF-1α). Values with different letters are significantly different (p<0.05, one way ANOVA with Holm-Sidak test).

Table 2.5. AMP-activated protein kinase activity (pmol·min⁻¹·mg protein⁻¹) in heart of tilapia exposed to normoxia and up to 8 h profound hypoxia (water PO₂=1.0 kPa).

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>1 h</th>
<th>2 h</th>
<th>8 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPK activity</td>
<td>97.5±38.6a</td>
<td>58.4±16.9a</td>
<td>181.9±46.7a</td>
<td>63.8±23.8a</td>
</tr>
</tbody>
</table>

Data are means±s.e.m. (n=3-5). Values with different letters are significantly different (p<0.05, one way ANOVA with Holm-Sidak test).
Figure 2.1. Whole-animal oxygen consumption rate (\( \dot{M}_{O_2} \)) of tilapia during progressive decreases in water PO\(_2\) (\( P_{wO_2} \)) from 13.6 kPa to 2.1 kPa measured during initial assessment of hypoxia tolerance (series I) (unfilled squares, \( n=7 \)), or from 16.5 kPa to 0.5 kPa measured simultaneously with cardiovascular variables (series II) (filled circles, \( n=6 \)) (100% air saturation = 155.1 torr = 20.7 kPa). Data are means±s.e.m. Values that do not share a letter are significantly different from other values within each series (p<0.05) (letters for series I and series II are above and below data points/line, respectively).
Figure 2.2 (overleaf). Heart rate ($f_{hi}$) (A,B), cardiac output ($\dot{Q}$) (C,D), and cardiac power output ($PO$) (E,F) of tilapia (series II) during progressive decreases in water $PO_2$ ($P_{wO_2}$) from 19.0 kPa to 0.5 kPa (A, C, E) over 3.6±0.2 h, followed by up to 8 h at 1.0 kPa and 1 h of recovery (1 R) in normoxic water (B, D, F). Data are means±s.e.m. ($n=6; n=5$ for 8 h and recovery points). Values that do not share a letter are significantly different ($p<0.05$).
Figure 2.3. Stroke volume ($V_{SH}$) (A,B), ventral aortic pressure ($P_{VA}$) (C,D), and peripheral resistance ($R$) (E,F) of tilapia (series II) during progressive decreases in water PO$_2$ ($P_{wO_2}$) from 19.0 kPa to 0.5 kPa (A, C, E) over 3.6±0.2 h, followed by up to 8 h at 1.0 kPa and 1 h of recovery (1 R) in normoxic water (B, D, F). Data are means±s.e.m. ($n=6$; $n=5$ for 8 h and recovery points). Values that do not share a letter are significantly different (p<0.05).
Figure 2.4. Relative tissue distribution of mRNA of $CPT_{Til} - 1$ iso1 (A), $CPT_{Til} - 1$ iso2 (A), $PPAR_{Til} \alpha$ (B), and $PDK_{Til} - 2$ (C) genes in tissues from tilapia (means ± s.e.m., $n=3$). mRNA levels are normalized to $EF1\alpha$ mRNA expression, with the level in heart set to 1.
Figure 2.5. Plasma [lactate] (A), [glucose] (B), and [non-esterified fatty acids] (NEFA) (C) in tilapia exposed to normoxia (N), up to 24 h profound hypoxia (1.0 kPa), and after 12 h of recovery (12 R) in normoxic water. Data are means±s.e.m. (n=6-8). Values that do not share a letter are significantly different (p<0.05).
Figure 2.6. Heart [ATP] (A), [lactate] (B), and intracellular pH (C) in tilapia exposed to normoxia (N), up to 24 h profound hypoxia (1.0 kPa), and after 12 h of recovery (12 R) in normoxic water. Data are means±s.e.m. (n=5-8). Values that do not share a letter are significantly different (p<0.05).
Figure 2.7 (overleaf). Heart pyruvate dehydrogenase active form (PDH$_a$) activity (A), [acetyl CoA] (B), $PDK_{Til}$-2 mRNA (C), and PDK-2 total protein (D) in tilapia exposed to normoxia (N), up to 24 h profound hypoxia (1.0 kPa), and after 12 h of recovery (12 R) in normoxic water. Data are means±s.e.m. ($n=6-8$). Values that do not share a letter are significantly different (p<0.05).
CHAPTER 3: EFFECTS OF FATTY ACID PROVISION DURING PROFOUND HYPOXIA ON ROUTINE AND MAXIMAL PERFORMANCE OF THE IN SITU TILAPIA HEART

3.1 SYNOPSIS

The ability to maintain stable cardiac function during environmental hypoxia exposure is a crucial component of hypoxia tolerance in animals and depends upon the maintenance of cardiac energy balance as well as the state of the heart’s extracellular environment (e.g., availability of metabolic fuels). Hypoxic depression of plasma [non-esterified fatty acids] (NEFA), an important cardiac aerobic fuel, is a common response in many species of hypoxia-tolerant fishes, including tilapia (Chapter 2). I tested the hypothesis that this response contributes to cardiac hypoxia tolerance by obviating lipotoxic perturbation of cardiac function. I examined the effect of profound hypoxia exposure (PO$_2$<0.2 kPa) on routine and maximum performance of the in situ perfused tilapia heart under conditions of routine (400 µmol L$^{-1}$) and low (75 µmol L$^{-1}$) [palmitate], which mimicked the in vivo levels of plasma [NEFA] found in normoxic and hypoxic tilapia, respectively. Cardiac performance during and following profound hypoxia exposure was unaffected by the level of palmitate, suggesting that hypoxic depression of plasma [NEFA] is not important for protecting the heart from lipotoxicity. In fact, as observed previously in our experiments using saline without fatty acids (Lague et al., 2012), the in situ tilapia heart showed exceptional hypoxic performance under both concentrations of palmitate, with routine normoxic levels of PO being sustained for the duration of the profound hypoxia exposure (70 min). Furthermore, there was an appreciable scope for performance under profound hypoxia, with maximum PO
being 45% lower than that under normoxic conditions independent of the level of palmitate.
This impressive level of hypoxic cardiac ATP turnover is achieved via a high maximum glycolytic potential as indicated by measurements of cardiac lactate efflux. Thus, in agreement with our earlier results (Lague et al., 2012) but contrary to the conclusions in Chapter 2, in vivo $PO$ depression may not be needed to maintain cardiac energy balance but rather may have the benefit of minimizing fuel use and waste production.

3.2 INTRODUCTION

The ability to maintain stable cardiac function during environmental hypoxia exposure is a crucial component of hypoxia tolerance in animals and it depends upon the maintenance of cardiac energy balance at a time when aerobic energy production is constrained (Farrell and Stecyk, 2007). Under severe hypoxia or anoxia exposure, this cardiac hypoxia tolerance is achieved by attaining a cardiac power output ($PO$, analogous to cardiac energy demand) that is already below (e.g., hagfishes, crucian carp) or is depressed below (e.g., turtles) the level that can be sustained by the heart’s MGP, which was previously suggested to be $\sim$70 nmol ATP s$^{-1}$ g$^{-1}$ sustaining a $PO$ of $\sim$0.7 mW g$^{-1}$ at 15°C (Farrell and Stecyk, 2007) (see section 1.5.2). Recently, we have shown that the tilapia heart has a much higher MGP that can sustain routine $PO$ during profound hypoxia (Lague et al., 2012), indicating that the in vivo depression of $PO$ observed in tilapia (Chapter 2) is not apparently required to maintain cardiac energy balance. However, the impressive in situ hypoxia tolerance of the tilapia heart could be affected in vivo by the extracellular environment (e.g., levels of pH, hormones, and availability of fuels), which is known to have profound
consequences for continued cardiac function during hypoxia exposure (e.g., see Lague et al., 2012 for data on the substantial effects of low pH).

In particular, an exogenous supply of glucose, mobilized from the large hepatic glycogen stores, is essential for sustaining hypoxic cardiac performance in fishes, which is perhaps unsurprising given the relatively limited endogenous glycogen stores in the heart and the heavy reliance upon anaerobic energy production (Driedzic and Gesser, 1994; Bailey et al., 2000; Clow et al., 2004). Another extracellular change during hypoxia exposure in many species of hypoxia-tolerant fishes, including tilapia (Chapter 2), is a large decrease in the level of plasma non-esterified fatty acids (NEFA) (Muusze et al., 1998; van Heeswijk et al., 2005; van Raaij et al., 1996; van den Thillart et al., 2002; Vianen et al., 2002; Chapter 2), which are important fuels for the fish heart under aerobic conditions (Bailey and Driedzic, 1993; Driedzic, 1992; Driedzic and Hart, 1984). The significance of this decrease in plasma NEFA in hypoxia-exposed fishes remains unclear. It could represent a decrease in fatty acid supply to the heart and other tissues at a time when fatty acid oxidation is impaired due to lack of O\textsubscript{2}. In mammals, fatty acid supply is not depressed during O\textsubscript{2} deprivation and hypoxic or anoxic impairment of cardiac fatty acid oxidation can result in lipotoxicity, an intracellular accumulation of fatty acids and fatty acid intermediates of incomplete β-oxidation that may perturb cardiac function and initiate apoptosis (Chabowski et al., 2006; Corr et al., 1984; Lopaschuk et al., 2010; van der Vusse et al., 1992). Furthermore, under hypoxic conditions, residual fatty acid oxidation occurs in the heart at the expense of pyruvate oxidation, exacerbating cellular acidosis and leading to ionic disturbances. This situation can be aggravated by a stimulation of fatty acid oxidation via hypoxia-induced activation of AMP-activated protein kinase (AMPK) (Lopaschuk et al., 2010). Fatty acid oxidation may
similarly inhibit pyruvate oxidation during reperfusion, impairing cardiac recovery (Lopaschuk et al., 2010). A final downside to fatty acid oxidation under hypoxic conditions is that it is inefficient, producing fewer ATP per O\(_2\) consumed compared with oxidation of pyruvate (the so-called ‘oxygen wasting effect’ of fatty acids) (Lopaschuk et al., 2010; McClelland, 2004). Consequently, hypoxic depression of [NEFA] in hypoxia-tolerant fishes could be a cardioprotective response. The effects of fatty acid provision on hypoxic function of the fish heart have never been investigated.

Using an in situ perfused heart preparation from tilapia (*Oreochromis niloticus* x *mossambicus* x *hornorum*), I tested the hypothesis that hypoxic depression of plasma [NEFA] contributes to cardiac hypoxia tolerance by obviating lipotoxic perturbation of cardiac function. Specifically, I measured the effects of profound hypoxia exposure (P\(_{O_2}\)<0.2 kPa) on routine and maximal performance of the in situ perfused tilapia heart under conditions of routine (400 \(\mu\)mol L\(^{-1}\)) or low (75 \(\mu\)mol L\(^{-1}\)) [palmitate], which mimicked the in vivo levels of plasma [fatty acids] found in normoxic and hypoxic tilapia, respectively (Chapter 2). The in situ heart preparation we developed for tilapia generated routine and maximum cardiac performance that was comparable to in vivo levels (Lague et al., 2012). I predicted that hypoxic function would be perturbed under conditions of high [fatty acid] but not under conditions of low [fatty acid]. The results of the present study also allowed me to confirm our previous conclusions (Lague et al., 2012) on the hypothesis that hypoxic depression of PO to a level sustainable by the MGP is required to maintain cardiac energy balance and function.
3.3 MATERIALS AND METHODS

3.3.1 Animals

Adult tilapia (*Oreochromis niloticus* x *mossambicus* x *hornorum*; strain origin, Ace Developments, Bruneau, ID, USA) of mixed sexes were purchased from Redfish Ranch (Courtenay, BC, Canada). Fish (428.2±10.0 g, n=11) were held in a recirculating freshwater system (22°C) as described in Chapter 2. All experiments were conducted following guidelines set out by the Canadian Council for Animal Care as administered by the University of British Columbia Animal Care Committee.

3.3.2 Surgical procedures

Fish were netted from the holding tanks, anaesthetized in water containing NaHCO₃ buffered MS-222 (0.2 g L⁻¹ and 0.2 g L⁻¹, respectively), and moved to a surgery table where the gills were continuously irrigated via the mouth with chilled aerated water containing NaHCO₃ buffered MS-222 (0.15 g L⁻¹ and 0.15 g L⁻¹, respectively). An injection of 1 ml kg⁻¹ of heparinized saline (200 IU mL⁻¹) was made via the caudal vessels. The heart was perfused in situ using a preparation described by Lague et al. (2012), which is a minor modification of the protocol previously described for rainbow trout (Farrell et al., 1986; Farrell et al., 1988; Farrell et al., 1989). Briefly, an input cannula was inserted into the sinus venosus through the largest, continuous, ventrally-located hepatic vein (the other hepatic veins were ligated using 3/0 silk thread) and then secured by silk thread while the pericardium was left intact. Perfusion was commenced immediately from a temporary input reservoir with heparinized (10 IU mL⁻¹) saline (see below) containing adrenaline bitartrate salt (5 nmol L⁻¹; Sigma-Aldrich, Oakville, ON, Canada). To collect outflow from the heart, an output cannula was
inserted into the ventral aorta at a point confluent with the bulbus arteriosus and secured with two 0/0 silk threads. The perfused heart preparation was completed in approximately 30 min.

Following surgery, the fish were immersed in a physiological saline bath (0.7% NaCl) thermostatted at 22°C. The input cannula was attached to an adjustable constant-pressure reservoir and the output cannula was connected to a separate adjustable constant-pressure head, which was set at a routine output pressure of 2.5 kPa to simulate resting normoxic in vivo ventral aortic blood pressure (Chapter 2). The height of the input pressure reservoir was adjusted to obtain a routine cardiac output ($\dot{Q}$) of approximately 12 mL min$^{-1}$ kg$^{-1}$, simulating in vivo $\dot{Q}$ under resting normoxic conditions (Chapter 2). Under these conditions, routine power output of the heart was similar to that observed in vivo under resting normoxic conditions (Chapter 2). $\dot{Q}$ was measured by an in-line electromagnetic flow probe in the output line (SWF-4, Zepeda Instruments, Seattle, WA, USA). Input ($P_{in}$) and output ($P_{out}$) pressures were measured through saline-filled side arms (PE50 tubing) connected to pressure transducers (DPT 6100, Smiths Medical, Kirchsecon, Germany). Hearts were allowed to equilibrate for approximately 20 min under routine normoxic conditions before the experimental trials commenced.

### 3.3.3 Perfusion composition and $\text{PO}_2$

During surgery and initial equilibration of the heart preparation, the heart was perfused with a physiological saline containing (in mmol L$^{-1}$): 124.1 NaCl, 2.50 KCl, 0.93 MgSO$_4$·7H$_2$O, 2.52 CaCl$_2$·2H$_2$O, 5.55 glucose, 3.87 TES acid, 6.13 TES salt (Sigma-Aldrich), pH 7.75 at 22°C (Lague et al., 2012). The level of glucose provided is similar to that found in plasma of hypoxia-exposed tilapia (Chapter 2). During the experimental trials (see below),
the heart was perfused with the same saline containing 75 µmol L\(^{-1}\) (low, hypoxic level of [fatty acid]) or 400 µmol L\(^{-1}\) (routine, normoxic level of [fatty acid]) sodium palmitate (Sigma-Aldrich) bound to 3% fatty acid free bovine serum albumin (BSA) (BAH66, Equitech-Bio Inc., Kerrville, Texas, USA) (Bailey and Driedzic, 1993; Driedzic and Hart, 1984; Peters et al., 1998; Sidell et al., 1984). Briefly, palmitate was dissolved in 20 mL of warm 95% ethanol (55°C) and was added drop-wise to 3 L of warmed, stirring perfusate (37°C) containing 3% BSA and pH adjusted to 7.75 with 1 mol L\(^{-1}\) NaOH. The ethanol was evaporated by warming the perfusate to 45°C for 20 minutes. The concentration of palmitate was verified using a commercially available kit (NEFA-HR(2); Wako, Osaka, Japan).

Palmitate plays an important role in fatty acid metabolism in fishes; it is oxidized by fish hearts and comprises a large portion of plasma NEFA in fishes (Bailey and Driedzic, 1993; Ballantyne et al., 1993; Driedzic and Hart, 1984; Henderson and Tocher, 1987). The heart of tilapia has the capacity to oxidize palmitate, as indicated by an activity of carnitine palmitoyltransferase that is similar to that of other teleost hearts (~0.31 umol min\(^{-1}\) g\(^{-1}\) at 22°C, \(n=3\); B. Speers-Roesch, unpublished results; c.f. Sidell et al., 1987). The use of albumin-bound palmitate to study cardiac fatty acid metabolism is well established for fishes (Bailey and Driedzic, 1993; Driedzic and Hart, 1984; Septon et al., 1990; Sidell et al., 1984). My preliminary experiments under normoxic conditions showed that maximum in situ cardiac performance using saline containing albumin and palmitate was similar to performance when saline without albumin or palmitate was used.

BSA-containing salines cannot be directly gassed because of excessive foaming. Thus, the perfusates were aerated and deoxygenated using gas-permeable Silastic tubing through which was passed a constant flow of medical-grade compressed air or nitrogen,
respectively. To do this, I constructed plastic mesh cylinders around which was wrapped 2 m
of Silastic tubing (0.76 mm ID x 1.65 mm OD; Dow Corning, Midland, MI, USA). This
tubing apparatus was submerged in the perfusate reservoirs and the compressed gas flow was
introduced through the tubing via an attached clipped needle (23G, Becton-Dickinson,
Franklin Lakes, NJ). All portions of the Silastic tubing were contained within the perfusate
reservoir, except for the outflow end, which was vented from the top of the reservoir. The
opening of all reservoirs was wrapped with commercial plastic wrap to prevent oxygen
ingress. Because deoxygenation using Silastic is much slower than direct gassing, the
perfusates were gassed within the reservoirs for approximately 12 h prior to experimental
trials. During this period, the perfusates were kept at 6ºC to prevent bacterial growth but they
were warmed to 22ºC in advance of the experimental trials. During the profound hypoxia
exposure (see below), ingress of oxygen into the cardiac tissues was prevented in four ways:
1) by the surrounding tissues and pericardium; 2) by using glass connectors and tubes for the
input perfusate; 3) by bubbling the bath saline with nitrogen before (for 10 min) and during
exposure to profound hypoxia; and 4) by covering the saline bath with a plastic lid. This
procedure achieved a PO₂ of <0.20 kPa for the perfusate used for the profound hypoxia
exposure, which is well below the hemoglobin-O₂ P₅₀ of tilapia (1.45 kPa; Chapter 2) and
below the venous PO₂ of tilapia exposed in vivo to profound environmental hypoxia of 1 kPa
(PᵥO₂=~0.5 kPa; B. Speers-Roesch, unpublished results). Venous blood is the only source of
oxygen for the tilapia heart, which lacks a coronary circulation (Pieperhoff et al., 2009). The
level of profound hypoxia used in the present study has been previously demonstrated to
induce a state of virtual anoxia in the in situ tilapia heart (Lague et al., 2012). The PO₂ of the
normoxic perfusate remained at air saturation (>20 kPa). Previous studies on rainbow trout
indicate similar *in situ* cardiac performance between air-saturated and hyperoxic (95.5% O₂, 0.5% CO₂) perfusate (Hanson et al., 2006).

All perfusates contained a tonic level of 5 nmol L⁻¹ of adrenaline (Lague et al., 2012), which was replenished every 20 min to offset degradation.

### 3.3.4 Experimental trials

Following the 20-min normoxic equilibration under non-BSA physiological saline, the heart was perfused with the BSA saline containing either a low (75 µmol L⁻¹) \((n=6)\) or routine (400 µmol L⁻¹) \((n=5)\) level of palmitate and allowed a further 10 min to equilibrate at the routine work load. At this point, the experimental trial was commenced. For each trial, routine cardiac performance was monitored and maximum cardiac performance assessed once during each of the following sequence of exposures: normoxia, profound hypoxia, and recovery in normoxia (Fig. 3.1). Myocardial lactate efflux and oxygen consumption rate \((\dot{V}_{O_2})\) was measured at routine and maximum PO during each exposure (Fig. 3.1) to assess the effects of profound hypoxia and each fatty acid treatment on the relationship between cardiac work and aerobic or anaerobic ATP turnover. Each experimental trial began by first exposing the heart to 20 min of routine performance in normoxia, followed by a 10-min test of normoxic maximum cardiac performance, and 20 min of routine normoxic performance. The heart was then perfused with hypoxic saline, which caused a progressive decline in PO₂ from normoxia to profound hypoxia over a period of approximately 10 min, with profound hypoxia (PO₂ <0.20 kPa) being maintained for a further ~50 min before reassessing maximum cardiac performance. The total duration of profound hypoxia exposure was 70 min. Then, the bath lid was removed, the nitrogen bubbling of the bath ceased, and the hearts
were recovered at a routine work load in normoxic saline for 20 min. It took <2 min for the perfusate PO$_2$ to reach >20.0 kPa. Following a 10-min reassessment of maximum cardiac performance under normoxic recovery conditions, and a subsequent 10 min of routine performance in normoxia, the heart was quickly excised, blotted, and weighed (0.137±0.005 g, $n=11$).

3.3.5  **Assessment of cardiac performance**

3.3.5.1  **Maximum cardiac performance**

Maximum cardiac performance was quantified by measuring maximum cardiac output ($\dot{Q}_{\text{max}}$) and maximum cardiac power output ($PO_{\text{max}}$) via a “max test” (Farrell et al., 1988; Farrell et al., 1989). Briefly, input filling pressure ($P_{\text{in}}$) was raised in 0.05 kPa increments until $\dot{Q}_{\text{max}}$ was reached. Then, output pressure head ($P_{\text{out}}$) was increased in 0.3 kPa increments until $PO_{\text{max}}$ was ascertained. The duration of each assessment of maximum cardiac performance was approximately 10 min and subsequently $P_{\text{out}}$ and $P_{\text{in}}$ were restored to routine levels, which always achieved a return to routine levels of performance.

3.3.5.2  **Oxygen consumption rate and lactate efflux rate**

The PO$_2$ of perfusate entering and exiting the heart was monitored using in-line oxygen probes with temperature sensors (Microx TX3, PreSens, Regensburg, Germany). This allowed us to calculate myocardial oxygen consumption rate ($\dot{V}_O_2$, µl O$_2$ s$^{-1}$ g$^{-1}$ ventricular mass) at routine levels of cardiac performance immediately preceding the max test as well as at $PO_{\text{max}}$ during normoxia and normoxic recovery (Fig. 3.1), using the following equation:
\[ \dot{V}_{O_2} = \Delta P_{O_2} \times \alpha_{O_2} \times \dot{Q} / (M_v \times 60) \]

where \( \Delta P_{O_2} \) is the difference in \( P_{O_2} \) between the input and output perfusate (kPa), \( \alpha_{O_2} \) is the solubility of oxygen in the perfusate (0.2946 \( \mu \)L O\(_2\) mL perfusate\(^{-1}\) kPa\(^{-1}\) at 22°C; Graham, 1987), \( \dot{Q} \) is in mL min\(^{-1}\), \( M_v \) is ventricular mass (g), and 60 is the conversion factor from minutes to seconds. The effect of BSA on \( \alpha_{O_2} \) is considered to be negligible (Johnston et al., 1994).

Samples of outflow perfusate were collected during profound hypoxia for determination of lactate efflux rate at routine levels of cardiac performance immediately preceding the profound hypoxia max test (at 60 min of profound hypoxia) as well as at \( PO_{max} \) after 1 min of stabilization (Fig. 3.1). Outflow perfusate samples for lactate efflux rate calculation also were taken immediately following the measurements of \( \dot{V}_{O_2} \) during normoxic and normoxic recovery, in order to account for the possibility of a minor contribution of anaerobic ATP turnover under normoxic conditions. Samples were taken by clearing the sampling line for 30 s and then drawing 0.5 mL over a 30 s period. Perfusate samples were frozen and stored at -80°C until measurement of lactate.

Perfusate [lactate] was measured in duplicate spectrophotometrically (Bergmeyer, 1983). Lactate efflux rate (nmol lactate min\(^{-1}\) g\(^{-1}\) ventricular mass) was calculated using the following equation (Arthur et al., 1992):

\[ \text{Lactate efflux rate} = (\text{perfusate [lactate]}) \times \dot{Q} \times 1000 / M_v \]
where perfusate [lactate] is the lactate concentration in the outflow perfusate sample (mmol L\(^{-1}\)), \(\dot{Q}\) is in mL min\(^{-1}\), \(M_v\) is in g, and 1000 is a conversion factor. Inflow perfusate was periodically sampled during every experiment and contained very small amounts of lactate (originating from the BSA), the values for which were subtracted from the respective outflow perfusate [lactate] prior to calculation. The MGP was considered to be the lactate efflux rate when the heart was performing at \(PO_{max}\) during profound hypoxia exposure, when the \textit{in situ} tilapia heart is virtually anoxic (Lague et al., 2012).

Cardiac ATP turnover rate (nmol ATP s\(^{-1}\) g\(^{-1}\)) was estimated from \(\dot{V}O_2\) and lactate efflux rate. In normoxia and normoxic recovery, ATP turnover rate was dictated almost entirely by \(\dot{V}O_2\) (assuming that 1 mol O\(_2\) results in the formation of 6 mol ATP), with a minor anaerobic contribution in some cases reflected by lactate efflux (assuming that 1 mol lactate yields 1.5 mol ATP and that lactate is derived from either glucose or glycogen) (Arthur et al., 1997; Lague et al., 2012; Reeves, 1963). ATP turnover rate under profound hypoxia was calculated from lactate efflux rate alone because PO\(_2\) was too low to obtain reliable measurements of \(\dot{V}O_2\). Furthermore, the PO\(_2\) achieved in the profound hypoxia exposure has been shown previously to induce a state of virtual anoxia in the \textit{in situ} tilapia heart (Lague et al., 2012).

### 3.3.6 Data acquisition and analysis

Real-time measurements of heart rate (\(f_{\text{Ht}}\)), \(P_{\text{in}}\), \(P_{\text{out}}\), \(\dot{Q}\), and \(PO\) were recorded throughout each trial using data acquisition software (Labview version 5.1, National Instruments, Austin, TX, USA). The oxygen probes were calibrated at 0\% (1 g l\(^{-1}\) Na\(_2\)SO\(_3\); Sigma-Aldrich) and 21\% O\(_2\) (air-saturated water) before each experiment. Pressure
transducers were calibrated before each experiment and their signals were amplified with a 4ChAmp amplifier (Somedic, Hörby, Sweden). The $\dot{Q}$ signal from the in-line flow probe was amplified (Flowmeter SWF-4, Zepeda Instruments) and frequently calibrated volumetrically with known flow rates of perfusate. Cannulae resistances were accounted for in the measured input and output pressures so reported $P_{in}$ and $P_{out}$ values represent accurate pressures in the sinus venosus and in the bulbus arteriosus, respectively (Farrell et al., 1988).

The reported values for $f_H$, $P_{in}$, $P_{out}$, $\dot{Q}$, and $PO$ represent the average recording over a 30 sec interval preceding chosen time points. Routine values were analyzed during normoxia (2 minutes prior to the normoxic max test), at 30 min and 60 min of profound hypoxia, and at 10 and 20 min of normoxic recovery following profound hypoxia (Fig. 3.1). Values for $\dot{Q}_{\text{max}}$ and $PO_{\text{max}}$ were analysed at 1 min of stabilization at the respective level during each max test. Each $\dot{V}_O_2$ measurement was taken over the same interval for $PO_{\text{max}}$ in normoxia and normoxic recovery, as well as for routine performance in normoxia (2 minutes prior to the normoxic max test) and recovery (at 20 min) (Fig. 3.1). During profound hypoxia, the intervals for routine performance at 60 min and for maximum performance at $PO_{\text{max}}$ immediately precede the sampling of perfusate for measurement of routine and $PO_{\text{max}}$ lactate efflux rate. Data were analyzed using the LabView perfused heart data acquisition and analysis program written by Drs. M. Axelsson (University of Gothenburg) and J. Altimiras (Linköping University). $f_H$ was calculated from the pulsatile pressure trace. $PO$ (mW g$^{-1}$ ventricle) was calculated using the following equation (Overgaard et al., 2004):

$$PO = \dot{Q} \times (P_{\text{out}} - P_{\text{in}}) \times 0.0167/M_v$$
where \( \dot{Q} \) is in ml min\(^{-1} \), \( P_{\text{out}} \) and \( P_{\text{in}} \) is in kPa, \( M_v \) is in g, and 0.0167 is the conversion factor to mW (where 1 J = 1 kPa L).

### 3.3.7 Statistics

Two-way repeated measures ANOVA with Tukey’s post-hoc tests was used for each of four individual analyses of collected data with the individual fish being the repeated measure: 1) the effects of [fatty acid] (factor 1) and sequential exposure (normoxia, 30 and 60 min of profound hypoxia, and 5, 10, and 20 min of normoxic recovery) (factor 2) on routine \( f_{\text{Ht}} \), \( \dot{Q} \), or \( PO \); 2) the effects of [fatty acid] (factor 1) and sequential exposure (normoxia, profound hypoxia, and normoxic recovery) (factor 2) on \( \dot{Q}_{\text{max}} \) or \( PO_{\text{max}} \); 3) the effects of [fatty acid] (factor 1) and normoxia vs. recovery exposure (factor 2) on \( \dot{V}_{\text{O}_2} \) or aerobic ATP turnover rate; and 4) the effects of [fatty acid] (factor 1) and power level (routine \( PO \) vs. \( PO_{\text{max}} \)) (factor 2) on lactate efflux rate or anaerobic (profound hypoxia) ATP turnover rate. The relationship between power output and lactate efflux rate or anaerobic ATP turnover rate in the profound hypoxia exposure was further examined using linear regression analyses of all individual fish values at routine \( PO \) and \( PO_{\text{max}} \) for each [fatty acid]. The relationship between power output and \( \dot{V}_{\text{O}_2} \) or aerobic ATP turnover rate in the normoxic and recovery exposures was examined using linear regression analyses of all individual fish values at routine \( PO \) and \( PO_{\text{max}} \) for each [fatty acid] and exposure (normoxia and recovery). Comparison of slopes and y-intercepts of these relationships via linear regression analyses allowed us to test for effects of [fatty acid] or normoxic exposure vs. recovery exposure.
Statistical significance was accepted when p<0.05. Analyses were carried out using SigmaStat 3.0 or GraphPad Prism 5.0. Data were log or square root transformed prior to statistical analyses if assumptions of equal variance or normality were not met.

3.4 RESULTS

The concentration of palmitate had no effect on the routine values for heart rate, $\dot{Q}$, and $PO$ in normoxia, profound hypoxia, or normoxia recovery (Fig. 3.2A,B,C). Heart rate was approximately 60 beats min$^{-1}$ under normoxia and decreased significantly during profound hypoxia exposure, reaching a level that was ~22% lower than normoxia at 60 min (Fig. 3.2A). During normoxic recovery, heart rate rapidly returned to normoxic levels (Fig. 3.2A). During normoxia routine $\dot{Q}$ and routine $PO$ were approximately 13 mL min$^{-1}$ kg$^{-1}$ and 1.7 mW g$^{-1}$, respectively, and both remained unchanged from normoxic levels throughout the profound hypoxia and normoxic recovery exposures (Fig. 3.2B,C).

The concentration of palmitate had no effect on either $\dot{Q}_{\text{max}}$ or $PO_{\text{max}}$ during exposure to normoxia, profound hypoxia, or normoxic recovery (Fig. 3.3A,B). Under normoxia, $\dot{Q}_{\text{max}}$ was approximately 35 mL min$^{-1}$ kg$^{-1}$ and $PO_{\text{max}}$ was approximately 5.1 mW g$^{-1}$. Exposure to profound hypoxia caused a 31% decrease in $\dot{Q}_{\text{max}}$ and a 45% decrease in $PO_{\text{max}}$ compared with levels in normoxia, independent of palmitate concentration (Fig. 3.3A,B). During normoxic recovery, $\dot{Q}_{\text{max}}$ and $PO_{\text{max}}$ in both palmitate treatments were restored to levels that were not significantly different from those in normoxia (Fig. 3.3A,B), suggesting that neither a high palmitate concentration nor profound hypoxia caused lasting impairment of cardiac function.
The concentration of palmitate had no effect on either \( \dot{V}_O_2 \) or ATP turnover rate at routine or maximum \( PO \) during normoxia and normoxic recovery (Fig. 3.4A,B). At routine levels of \( PO (\sim 1.7 \text{ mW g}^{-1}) \) during normoxia, \( \dot{V}_O_2 \) and ATP turnover rate were approximately 0.66 \( \mu \text{L s}^{-1} \text{ g}^{-1} \) and 187 nmol s\(^{-1}\) g\(^{-1}\), respectively (Fig. 3.4A,B). There was a significant increase in \( \dot{V}_O_2 \) and ATP turnover rate with increasing \( PO \) in normoxia for each palmitate treatment, with \( PO_{\text{max}} (\sim 5.1 \text{ mW g}^{-1}) \) coinciding with a maximum \( \dot{V}_O_2 \) and maximum ATP turnover rate of approximately 1.29 \( \mu \text{L s}^{-1} \text{ g}^{-1} \) and 359 nmol s\(^{-1}\) g\(^{-1}\), respectively. A similar result was observed in normoxic recovery and the routine or maximum levels of \( \dot{V}_O_2 \) and ATP turnover rate were not significantly different between normoxia and normoxic recovery (Fig. 3.4A,B). Indeed, \( \dot{V}_O_2 \) and ATP turnover rate showed a significant positive relationship with \( PO \) in both normoxia and normoxic recovery independent of palmitate treatment and because the slopes and y-intercepts of these four relationships were not significantly different from one another, a single regression line could be used to represent all individual values across all conditions (Fig. 3.4A,B).

The concentration of palmitate had no effect on either lactate efflux rate or ATP turnover rate at routine or maximum \( PO \) during profound hypoxia exposure (Fig. 3.5A,B). At routine levels of \( PO (\sim 1.7 \text{ mW g}^{-1}) \), lactate efflux rate and ATP turnover rate were approximately 3900 nmol min\(^{-1}\) g\(^{-1}\) and 97 nmol s\(^{-1}\) g\(^{-1}\), respectively (Fig. 3.4A,B). There was a significant increase in lactate efflux rate and ATP turnover rate with increasing \( PO \) for each palmitate treatment, with \( PO_{\text{max}} (\sim 2.7 \text{ mW g}^{-1}) \) coinciding with a maximum lactate efflux rate and maximum ATP turnover rate of approximately 5900 nmol min\(^{-1}\) g\(^{-1}\) and 148 nmol s\(^{-1}\) g\(^{-1}\), respectively. The relationships (slopes and y-intercepts) of \( PO \) with lactate efflux rate or ATP turnover rate were not significantly different between the two palmitate treatments, so a
single regression line could be used to represent all individual values across both treatments (Fig. 3.5A,B).

3.5 DISCUSSION

The results of the present study suggest that the hypoxic depression of plasma [NEFA] observed in vivo in tilapia is not necessary to protect the heart from any lipotoxic dysfunction during oxygen limitation, at least in the time frame of the present study. The routine and maximum performance of the in situ tilapia heart during and following profound hypoxia exposure (P0₂<0.2 kPa) was similar whether perfused with 400 µmol L⁻¹ or 75 µmol L⁻¹ palmitate, which mimicked the physiological levels of plasma [NEFA] found in vivo in normoxic and hypoxic tilapia, respectively. Indeed, the in situ tilapia heart showed exceptional routine and maximum hypoxic performance under both levels of palmitate, which is consistent with and confirms our previous work using perfusate without BSA or fatty acid (Lague et al., 2012). In agreement with the findings of Lague et al. (2012), the impressive level of hypoxic cardiac ATP turnover is achieved via a high MGP as indicated by measurements of cardiac lactate efflux, which is also unaffected by provision with fatty acid. Thus, contrary to previous suggestions (Farrell and Stecyk, 2007; Chapter 2), in vivo PO depression may not be necessarily required for maintenance of cardiac energy balance and sustained cardiac performance during hypoxia exposure (Lague et al., 2012).

3.5.1 Effect of fatty acid concentration on normoxic cardiac performance

Normoxic performance of the in situ tilapia heart was similar at both concentrations of palmitate and also was similar to previous measurements using saline without BSA or
fatty acid (Lague et al., 2012). Routine $\dot{Q}$, $PO$, and heart rate were stable during normoxia at levels of approximately 13 mL min$^{-1}$ kg$^{-1}$, 1.7 mW g$^{-1}$, and 60 beats min$^{-1}$ (Fig. 3.2), respectively, compared with approximately 12 mL min$^{-1}$ kg$^{-1}$, 1.4 mW g$^{-1}$, and 55 beats min$^{-1}$ recorded by Lague et al. (2012). These values for $\dot{Q}$ and $PO$ are similar to the in vivo resting values in tilapia of approximately 11-13 mL min$^{-1}$ kg$^{-1}$ and 1.3-1.9 mW g$^{-1}$, respectively (Chapter 2; B. Speers-Roesch, E. Sandblom, A. P. Farrell, and J. G. Richards, unpublished observations). The intrinsic normoxic heart rate of the in situ tilapia heart is comparable to the maximum in vivo heart rate achieved after exhaustive exercise in tilapia (54 beats min$^{-1}$) (B. Speers-Roesch, E. Sandblom, A. P. Farrell, and J. G. Richards, unpublished observations), and is greater than in vivo resting values (~30-35 beats min$^{-1}$) (Chapter 2; B. Speers-Roesch, E. Sandblom, A. P. Farrell, and J. G. Richards, unpublished observations), suggesting a central role for vagal tone in setting heart rate. The values for $\dot{Q}$ max (~35 mL min$^{-1}$ kg$^{-1}$) and $PO$ max (~5.1 mW g$^{-1}$) (Fig. 3.3) are similar to those obtained previously in tilapia by Lague et al. (2012) (~39 mL min$^{-1}$ kg$^{-1}$ and ~5 mW g$^{-1}$, respectively). The values of $\dot{Q}$ max and $PO$ max of the in situ tilapia heart are intermediate between those of sluggish teleosts such as eels (~15 mL min$^{-1}$ kg$^{-1}$ and ~3.5 mW g$^{-1}$ at 15-20$^\circ$C; Davidson and Davie, 2001; Franklin and Davie, 1991) and more active teleosts such as cod (Gadus morhua) (50 mL min$^{-1}$ kg$^{-1}$ and 7 mW g$^{-1}$ at 10$^\circ$C; Petersen and Gamperl, 2010), rainbow trout (66 mL min$^{-1}$ kg$^{-1}$ and 8 mW g$^{-1}$ at 15$^\circ$C; Farrell et al., 1996), and sea bass (Dicentrarchus labrax) (91 mL min$^{-1}$ kg$^{-1}$ and 11 mW g$^{-1}$ at 18$^\circ$C; Farrell et al., 2007), which is consistent with the moderate activity level of tilapia. The maximum in situ cardiac performance of tilapia is substantially greater than the maximum in vivo cardiac performance observed following exhaustive exercise ($\dot{Q} = ~21$ mL min$^{-1}$ kg$^{-1}$; $PO = ~3.8$ mW g$^{-1}$) (B. Speers-Roesch, E. Sandblom, A. P.
Farrell, and J. G. Richards, unpublished observations), which is perhaps unsurprising because hypoxemia and acidosis associated with exhaustive exercise can have negative inotropic effects on the heart (Driedzic and Gesser, 1994).

In mammalian hearts, fatty acid provision and oxidation is typically associated with decreased cardiac efficiency, where the myocardial $\dot{V}O_2$ associated with a given work load is 25-50% greater than if glucose is provided as fuel (the ‘oxygen wasting effect’ of fatty acids) (Hutter et al., 1984; Korvald et al., 2000; Lopaschuk et al., 2010; McClelland, 2004; Simonsen and Kjekshus, 1978; Zhou et al., 2008). The ‘oxygen wasting effect’ of fatty acids occurs even if glucose is available at physiological levels (How et al., 2005; Hutter et al., 1984; Korvald et al., 2000; Simonsen and Kjekshus, 1978). In the tilapia heart, however, I found that the concentration of fatty acid in the perfusate had no effect on $\dot{V}O_2$ at either routine or maximum $PO$, with the routine normoxic value being $\sim 0.66$ $\mu$L $s^{-1}$ $g^{-1}$ (at $\sim 1.7$ mW $g^{-1}$) and the maximum normoxic value being $\sim 1.29$ $\mu$L $s^{-1}$ $g^{-1}$ (at $\sim 5.1$ mW $g^{-1}$) for both 75 and 400 $\mu$mol L$^{-1}$ palmitate (Fig. 3.4A). These $\dot{V}O_2$ values are marginally higher than those previously found by Lague et al. (2012) in the absence of fatty acid ($\sim 0.52$ $\mu$L $s^{-1}$ $g^{-1}$ at $\sim 1.4$ mW $g^{-1}$ and $\sim 1.16$ $\mu$L $s^{-1}$ $g^{-1}$ at $\sim 5$ mW $g^{-1}$), but these differences are probably largely explained by the correspondingly greater normoxic $PO$ values achieved in the present study. Similarly, the relationship between $\dot{V}O_2$ and $PO$, which indicates cardiac efficiency, was unaffected by palmitate concentration (Fig. 3.4A). Overall I found no evidence to support the presence of an ‘oxygen wasting effect’ of fatty acids in the tilapia heart exposed to physiological levels of fatty acid. Indeed, there is little evidence that such an effect is present in fish hearts, at least at warm temperatures. For example, in isolated hearts from sea raven (Hemitripterus americanus) acclimated to 5°C and perfused at 5°C and 15°C with media
containing glucose or glucose+palmitoleate, only at the colder experimental temperature did inclusion of fatty acid cause \( V_\text{O}_2 \) to be greater and cardiac efficiency to be decreased (Sephton et al., 1990). Similarly, in isolated hearts of rainbow trout (*Oncorhynchus mykiss*) perfused with either glucose or palmitate as fuel, perfusion with palmitate was associated with greater \( V_\text{O}_2 \) at a cold acclimation temperature (5°C; measured at 5°C) but not at a warm acclimation temperature (20°C; measured at 15°C and 5°C) (Bailey and Driedzic, 1993). In part, this may be related to the lower rates of fatty acid uptake and oxidation at the warm acclimation temperature compared with cold acclimation temperature (Bailey and Driedzic, 1993), which agrees with the concept that fatty acids are a preferred fuel source at cold temperature in fish muscles (Driedzic, 1992). Although the effects of temperature on cardiac fatty acid metabolism and myocardial \( V_\text{O}_2 \) are complex and remain incompletely understood (Driedzic, 1992), my data for warm-water tilapia (22°C) are consistent with the notion that the ‘oxygen wasting effect’ of fatty acids is minimal at warm temperatures in fish hearts.

Myocardial \( \dot{V}_\text{O}_2 \) of the *in situ* tilapia heart measured in the present study and by Lague et al. (2012) was similar to that of other teleosts at comparable PO and temperature (Davidson and Davie, 2001; Farrell et al., 1985; Graham and Farrell, 1990). The positive relationship of \( \dot{V}_\text{O}_2 \) with PO (Fig. 3.4A) had a slope (0.1861) that was similar to previous measurements in other teleosts (0.11-0.21; Davidson and Davie, 2001; Farrell et al., 1985; Graham and Farrell, 1990). The relationship between ATP turnover rate and PO was more or less equivalent to that for \( \dot{V}_\text{O}_2 \) and PO (Fig. 3.4A,B), because lactate efflux rate (i.e., anaerobic contribution to ATP turnover) was negligible during normoxic conditions, similar to previous findings for the *in situ* tilapia heart (Lague et al., 2012).
3.5.2 Effect of fatty acid concentration on cardiac performance during and following profound hypoxia

Provision of the *in situ* tilapia heart with a routine, *in vivo* normoxic level of fatty acid (400 $\mu$mol L$^{-1}$ palmitate) had no detrimental effect on cardiac function during and following recovery from profound hypoxia exposure compared with provision with a low, *in vivo* hypoxic level of fatty acid (75 $\mu$mol L$^{-1}$ palmitate). Furthermore, the hypoxic and recovery cardiac performance measured in the present study was generally similar to previous measurements for the *in situ* tilapia heart using saline without BSA or fatty acid (Lague et al., 2012) (see below). These data suggest that the hypoxic depression of plasma [NEFA] observed *in vivo* in tilapia (Chapter 2) is not required to protect the heart from the potential perturbations associated with fatty acid metabolism during oxygen limitation.

In contrast, deleterious effects of fatty acid provision and metabolism on cardiac function during hypoxic and reperfusion (i.e., recovery) events are well characterized for the mammalian heart (Corr et al., 1984; Lopaschuk et al., 2010; van der Vusse et al., 1992). The absence of similar effects in tilapia heart, despite a duration of profound hypoxia greatly exceeding that typically used in mammalian studies, may be explained by a number of factors. First, as discussed above, the provision of the tilapia heart with fatty acids does not appear to cause an ‘oxygen wasting effect’, which is a component of pathological effects of fatty acids in hypoxic and reperfused mammal hearts (Lopaschuk et al., 2010). Second, hypoxia-induced activation of AMPK appears to mediate some of the detrimental effects of fatty acid oxidation in mammalian hearts (Chabowski et al. 2006; Lopaschuk et al., 2010), but AMPK activity does not increase in heart of tilapia during *in vivo* hypoxia exposure (Chapter 2). Third, in mammalian studies, hearts typically are exposed to elevated levels of
fatty acid (e.g., double the routine level), which is physiologically relevant because the stress accompanying ischaemic events (e.g., acute myocardial infarction) is usually associated with catecholamine release that increases plasma [NEFA] (Lopaschuk et al., 2010). Tilapia, however, show a decrease in routine levels of plasma [NEFA] during *in vivo* hypoxia exposure (Chapter 2), so perfusion with elevated levels of fatty acid above the routine normoxic level would not be physiologically relevant. In any case, substantial accumulation of lipotoxic fatty acid intermediates and detrimental effects of fatty acid metabolism have been noted in ischaemic mammalian hearts even when exposed to routine levels of fatty acid (e.g., Liedtke et al. 1988; Moore et al., 1980). Although fatty acid uptake or accumulation in the hypoxic *in situ* tilapia heart was not measured in the present study, it is clear nonetheless that exposure to a high concentration of fatty acids, corresponding to *in vivo* normoxic levels, had no effect on *in situ* cardiac function during and following profound hypoxia exposure.

The tilapia heart may preferentially utilize glucose as metabolic fuel, in particular during hypoxia exposure, which could obviate the detrimental effects of fatty acid accumulation and metabolism during oxygen limitation. Glucose was freely available in perfusate saline at a physiological concentration intermediate between normoxic and maximum hypoxic *in vivo* levels (Chapter 2). Glucose transporter 1 expression and glycogen content are much greater in tilapia heart than in mammal (rat) heart (Wright et al., 1998). Furthermore, cardiac glucose uptake is increased during hypoxia exposure in fishes and exogenous glucose is required for hypoxic cardiac function (Bailey et al., 2000; Clow et al., 2004; Rodnick et al., 1997). Finally, in fishes acclimated to warm temperature (e.g., tilapia) fatty acids may be less preferred as a cardiac fuel source compared with carbohydrates.
(Bailey and Driedzic, 1993; Driedzic, 1992). Further studies of cardiac fuel selection in fishes during hypoxia exposure are warranted.

### 3.5.3 Effect of profound hypoxia on cardiac performance

Previously, Lague et al. (2012) demonstrated that the tilapia heart shows exceptional routine and maximum cardiac performance during profound hypoxia exposure and the results of the present study corroborate these findings. Although a small (~20%) decrease in heart rate occurred, likely due to direct depressive effects of hypoxia and/or H⁺ on the pacemaker (Driedzic and Gesser, 1994), routine levels of \( \dot{Q} \) and \( PO \) were maintained for the duration of profound hypoxia exposure (70 min) in both palmitate treatments, as observed by Lague et al. (2012) for performance in the absence of fatty acids. The ability of the \textit{in situ} tilapia heart to operate at routine \( PO \) during profound hypoxia contrasts with findings for \textit{in situ} hearts of less hypoxia-tolerant fish species, where impaired routine cardiac performance or cardiac failure is evident even at higher hypoxic \( PO_2 \) than used in the present study (e.g., \( \leq 4.0 \) kPa in sea raven [Farrell et al., 1985]; \( \leq 2.7-3.4 \) kPa in rainbow trout [Farrell et al., 1989; Hanson et al., 2006; Overgaard et al., 2004]; 0.6 kPa in cod [Petersen and Gamperl, 2010]). In fact, the only other vertebrates known to have hearts capable of functioning at a routine level under severe or profound hypoxia are the anoxia-tolerant hagfishes (Axelsson et al., 1990; Cox et al., 2010; Forster, 1991; Hansen and Sidell, 1983) and crucian carp (Stecyk et al., 2004b; J.A.W. Stecyk, K.O. Stensløkken, L.M. Hanson, A.P. Farrell, and G.E. Nilsson, unpublished results). These species, however, have a routine \( PO \) that is much lower than that found in tilapia (~0.1 to 0.2 mW g\(^{-1}\) for hagfishes at 10-18°C and ~0.44 mW g\(^{-1}\) for crucian carp at 8°C vs. ~1.7 mW g\(^{-1}\) for tilapia at 22°C). Thus, among ectothermic vertebrates studied to date, the
*in situ* tilapia heart shows an exceptional ability to sustain a comparatively high level of routine $PO$ during profound hypoxia exposure.

The *in situ* tilapia heart showed remarkable maximum performance in both palmitate treatments during profound hypoxia exposure, with $\dot{Q}_{\text{max}}$ and $PO_{\text{max}}$ respectively being approximately 31% and 45% lower than the level in normoxia, after 60 min of hypoxia exposure. These results are similar to those of Lague et al. (2012) using saline without BSA or palmitate, although in that study $PO_{\text{max}}$ decreased by approximately 30-35% rather than 45% as observed in the present study. The reason for this discrepancy is unclear. One possibility is intraspecific variation in hypoxic performance, since the individuals in the present study were from a different generation of tilapia than those in the previous study. Alternatively, there may be non-specific effects of BSA on hypoxic performance. Nonetheless, the maximum hypoxic performance measured in the present study remains impressive compared with that observed in ectothermic vertebrates renowned for their anoxia tolerance. For example, the *in situ* $PO_{\text{max}}$ of the hearts of crucian carp and red-eared slider turtle decreased 32% and 49%, respectively, during profound hypoxia exposure (Farrell et al., 1994; J.A.W. Stecyk, K.O. Stensløkken, L.M. Hanson, A.P. Farrell, and G.E. Nilsson, unpublished results). Furthermore, in absolute terms, the $PO_{\text{max}}$ of the tilapia heart is substantially greater than these species, although when temperature differences are considered the crucian carp may show similar performance (see Lague et al., 2012). In contrast, in comparatively hypoxia-sensitive species such as cod and rainbow trout, even brief exposure (15-20 min) to $PO_2$ of $\leq 0.6$ kPa causes a >70% decrease in maximum *in situ* cardiac performance that typically results in cardiac failure (Arthur et al., 1992; Hanson et al., 2006; Petersen and Gamperl, 2010).
Cardiac performance during profound hypoxia was sustained by an impressive glycolytic potential, confirming the results of Lague et al. (2012). Glycolytic ATP turnover rate, as indicated by lactate efflux rate (validated by Lague et al., 2012), increased with increasing $PO$ up to a maximum glycolytic ATP turnover rate (i.e., MGP) of ~148 nmol s$^{-1}$ g$^{-1}$ at the $PO_{\text{max}}$ of ~2.7 mW g$^{-1}$ (Fig. 3.5). Previously, Lague et al. (2012) found that the MGP of the tilapia heart was slightly higher (~172 nmol ATP s$^{-1}$ g$^{-1}$), but at a correspondingly greater $PO_{\text{max}}$ (~3.3 mW g$^{-1}$), as discussed above. Thus, the present study and Lague et al. (2012) demonstrate a similar relationship between glycolytic ATP turnover rate and $PO$ during profound hypoxia. The MGP of the tilapia heart is greater than the previous estimate of the MGP for the ectothermic vertebrate heart (~70 nmol ATP s$^{-1}$ g$^{-1}$ at 15°C, achieving a $PO$ of ~0.7 mW g$^{-1}$) (Farrell and Stecyk, 2007), even when the potential effect of temperature is considered (see Lague et al., 2012 for further discussion). Recent work on the anoxia-tolerant crucian carp shows that it may possess a similar cardiac MGP as tilapia when the effect of temperature is considered (see Lague et al. 2012; J.A.W. Stecyk, K.O. Stensløkken, L.M. Hanson, A.P. Farrell, and G.E. Nilsson, unpublished results). Clearly, there is greater interspecific variation in MGP than previously indicated by available evidence and further studies are needed to characterize the upper limits of cardiac glycolytic potential in hypoxia-tolerant vertebrates.

Statistical comparison of the relationships between $PO$ and ATP turnover rate under aerobic (normoxia and recovery) vs. anaerobic (profound hypoxia) conditions (i.e., Fig. 3.4B vs. Fig. 3.5B) demonstrated that although the slopes were not significantly different, the y-intercept was significantly (~49%) lower under conditions of profound hypoxia (linear regression, $p<0.0001$ for y-intercept, $p=0.2318$ for slope). These results held even if normoxic
recovery data were omitted from the analysis. Because the slopes of the lines were not significantly different, cardiac efficiency may be similar during normoxia and profound hypoxia. On the other hand, because the y-intercept provides an estimate of ATP turnover related to non-contractile processes (How et al., 2005), my data suggest that an endogenous metabolic depression of ATP turnover occurs in the tilapia heart during profound hypoxia exposure. A similar result was observed in the turtle in situ heart during anoxia exposure at cold temperature (5°C) (Arthur et al., 1997). Although not investigated in the present study, the metabolic depression of the tilapia in situ heart may involve downregulation of ATP demand pathways such as protein synthesis, which has been observed in the heart of other cichlids during hypoxia exposure in vivo (Lewis et al., 2007).

In cod and rainbow trout hearts, as little as 15 min of severe or profound hypoxia exposure followed by normoxic recovery can cause myocardial stunning (i.e., a post-hypoxic, reversible contractile dysfunction in the absence of necrosis [Bolli and Marbán, 1999]) whereby $\dot{Q}_{\text{max}}$ and $PO_{\text{max}}$ in recovery are lower than the levels under normoxia (Faust et al., 2004; Overgaard et al. 2004; Petersen and Gamperl, 2010). Following 70 min of profound hypoxia exposure and 20 min of normoxic recovery in both palmitate treatments, however, the in situ tilapia heart showed maximum cardiac performance during normoxic recovery that was not significantly different from normoxic values, which matches previous observations by Lague et al. (2012). The less pronounced myocardial stunning found in the in situ tilapia heart, despite operating at higher $PO$ for longer periods of hypoxia exposure compared with hearts of other fishes in previous studies, is consistent with the tilapia heart’s great hypoxia tolerance.
3.5.4 Conclusions and perspectives

The absence of any discernable effect of physiological levels of fatty acid on performance of the in situ tilapia heart during or following profound hypoxia exposure suggests that the hypoxic depression of plasma [NEFA] seen in tilapia and many other hypoxia-tolerant fishes does not play a role in cardiac hypoxia tolerance. Future studies should investigate the suggestion that depression of NEFA contributes to hypoxia-induced whole-animal metabolic rate depression (Magnoni et al., 2008; Chapter 2).

The present study confirms Lague et al.’s (2012) discovery that the tilapia heart has a MGP that exceeds previous estimates for the ectothermic vertebrate heart (Farrell and Stecyk, 2007), allowing it to operate at a comparatively high routine $PO$ for up to 70 min of profound hypoxia exposure and to achieve a comparatively great hypoxic $PO_{\text{max}}$. Tilapia exposed in vivo to profound hypoxia, however, depress $PO$ by ~50% (Chapter 2), in contrast with the maintenance of routine $PO$ seen in the only other ectothermic vertebrates known to possess a routine $PO$ sustainable by their cardiac MGP (crucian carp and hagfishes; Cox et al., 2010; Farrell and Stecyk, 2007; Stecyk et al., 2004b). Thus, as originally concluded by Lague et al. (2012), the in vivo hypoxic $PO$ depression in tilapia, and possibly in certain other hypoxia-tolerant ectothermic vertebrates that show $PO$ depression, may not occur primarily to balance cardiac energy demand with supply during hypoxia exposure, contrary to previous suggestions (Farrell and Stecyk, 2007; Chapter 2). Instead, hypoxic $PO$ depression may reflect decreased circulatory requirements accompanying hypoxia-induced whole-animal metabolic rate depression, which occurs in tilapia (van Ginneken et al., 1997). In fact, depression of $PO$ in tilapia closely tracks the decrease in whole-animal oxygen consumption rate that occurs during progressive hypoxia exposure (Chapter 2). Depression of $PO$ has the
added benefit of minimizing fuel use and waste production, which is important because acidosis and fermentable fuel stores are thought to place significant limitations upon hypoxia tolerance (Nilsson and Östlund-Nilsson, 2008). Finally, decreased PO also may be important in vivo because of the depressive effects of acidosis on cardiac function, which have been observed in tilapia (Driedzic and Gesser, 1994; Lague et al., 2012). Given the potential benefits of PO depression other than balancing energy supply and demand, it remains unclear why hypoxic PO depression is not observed in hagfishes or crucian carp. However, these species already possess a very low routine PO and, in crucian carp, the circulatory requirements for ethanol production and excretion may necessitate maintenance of cardiac function, which in turn may be facilitated by the mitigation of acidosis afforded by the crucian carp’s unique anaerobic metabolism (Farrell and Stecyk, 2007).

The impressive MGP and maximum hypoxic cardiac performance of the tilapia heart may represent a ‘safety factor’ that has evolved to allow a limited, yet significant, capacity for predator avoidance under the hypoxic conditions that are common to the natural environment of the tilapia (Chapman et al., 2002). In this regard, the tilapia may differ from anoxia-tolerant freshwater turtles, which appear to require a depression of PO to balance cardiac energy supply and demand because they have not evolved a remarkable MGP (Arthur et al., 1997; Farrell and Stecyk, 2007). Possibly, this is because turtles under natural winter anoxic conditions enter a state of comatose hibernation where higher levels of cardiac function are unnecessary. Future studies on the limits of hypoxic cardiac performance in ectothermic vertebrates should further consider the ecological conditions under which hypoxia is encountered. It is reasonable to hypothesize that the exceptional cardiac hypoxia
tolerance of the tilapia is not unique among tropical freshwater fishes that regularly encounter severe environmental hypoxia.

3.6 ACKNOWLEDGEMENTS

I wish to thank Sabine Lague for her expert assistance during the course of these experiments. The LabView perfused heart data acquisition and analysis program was written and kindly provided by Drs. M. Axelsson and J. Altimiras. L.M. Hanson provided experimental advice and J. A. W. Stecyk and coauthors kindly allowed the use of their unpublished crucian carp results. Funding was provided by the Discovery Grant Program from NSERC to J.G. Richards and A. P. Farrell. B. Speers-Roesch was the recipient of a War Memorial Scholarship from IODE Canada.
Figure 3.1. Graphical representation of the experimental protocol used to examine the effects of profound hypoxia and [palmitate] on cardiac performance of the in situ tilapia heart. Output pressure ($P_{\text{out}}$) is indicated by the solid line (routine level set at 2.5 kPa) and the three tests of maximum cardiac performance (‘max test’) are represented as the progressive increase in $P_{\text{out}}$. The interval times (and corresponding total time) and performance level during each interval (routine or max test) are provided. The 70-min exposure to profound hypoxia is shown as the grey bar and was preceded by 50 min of normoxia and followed by 40 min of normoxic recovery. Thus, max test 1 is during normoxia, max test 2 is near the end of profound hypoxia exposure, and max test 3 is during normoxic recovery. Arrows indicate where measurements of oxygen uptake rate (during normoxia and normoxic recovery) or lactate efflux rate (during normoxia, normoxic recovery, and profound hypoxia) were taken along with measurements of cardiac performance at routine (R) levels or $P_{\text{O}_{\text{max}}}$. Routine cardiac performance was also recorded at 30 min of profound hypoxia and at 10 min of normoxic recovery, and $\dot{Q}_{\text{max}}$ was recorded during all max tests.
Figure 3.2. Heart rate (A) and routine cardiac output ($\dot{Q}$) (B) and power output ($PO$) (C) of the in situ tilapia heart perfused with 75 or 400 µmol L$^{-1}$ palmitate during exposure to normoxia, up to 60 min of profound hypoxia (PO$_2$<0.20 kPa), and up to 20 min of normoxic recovery. The lower concentration of palmitate mimicks the in vivo hypoxic level of non-esterified fatty acids in tilapia (Chapter 2) and the higher concentration mimicks the in vivo normoxic level. Values that do not share a letter are significantly different from each other within the treatment of 75 or 400 µmol L$^{-1}$ palmitate (two-way ANOVA with Tukey’s test, p<0.05). Only one set of letters denoting significant differences is given because there was no significant difference between the values for 75 and 400 µmol L$^{-1}$ palmitate treatment at any sample point (two-way ANOVA with Tukey’s test, p<0.05). Data are means±s.e.m. ($n=6$ for 75 µmol L$^{-1}$ palmitate; $n=5$ for 400 µmol L$^{-1}$ palmitate).
Figure 3.3. Maximum cardiac output (\( \dot{Q}_{\text{max}} \)) (A) and maximum cardiac power output (\( PO_{\text{max}} \)) (B) of the in situ tilapia heart perfused with 75 or 400 µmol L\(^{-1}\) palmitate during exposure to normoxia, profound hypoxia (\( PO_2 < 0.20 \) kPa), and normoxic recovery. Values that do not share a letter are significantly different from each other within the treatment of 75 or 400 µmol L\(^{-1}\) palmitate (two-way ANOVA with Tukey’s test, \( p < 0.05 \)). For both \( \dot{Q}_{\text{max}} \) and \( PO_{\text{max}} \), there were no significant differences between values for 75 and 400 µmol L\(^{-1}\) palmitate treatment at any sample point (two-way ANOVA with Tukey’s test, \( p < 0.05 \)). Data are means±s.e.m. (\( n = 6 \) for 75 µmol L\(^{-1}\) palmitate; \( n = 5 \) for 400 µmol L\(^{-1}\) palmitate).
Figure 3.4. Myocardial oxygen consumption rate ($\dot{V}_O_2$) (A) and ATP turnover rate (B) of the \textit{in situ} tilapia heart perfused with 75 or 400 $\mu$mol L$^{-1}$ palmitate and functioning at routine power output (~1.7 mW g$^{-1}$) and maximum power output (i.e., $P_O_{max}$; ~5 mW g$^{-1}$) during normoxia and during normoxic recovery following 70-min exposure to profound hypoxia. The larger symbols represent means±s.e.m. ($n=6$ for 75 $\mu$mol L$^{-1}$ palmitate; $n=5$ for 400 $\mu$mol L$^{-1}$ palmitate). The smaller symbols represent the individual values for each fish at routine $PO$ and $P_O_{max}$. Linear regression analyses were performed using individual values within normoxia or recovery for each palmitate concentration. A significant positive relationship between $PO$ and $\dot{V}_O_2$ as well as between $PO$ and ATP turnover rate was observed in each case (data not shown); statistical comparison of the slopes and y-intercepts showed no significant differences between these relationships for either $\dot{V}_O_2$ or ATP turnover rate. Thus, the displayed linear regressions are based on pooled individual values across all conditions (total number of values=44). There was no significant difference between palmitate treatments nor between normoxia vs. recovery exposure for $\dot{V}_O_2$ or ATP turnover rate (statistics omitted for clarity) (two-way repeated measures ANOVA with Tukey’s, $p<0.05$).
Figure 3.5. Lactate efflux rate (A) and ATP turnover rate (B) of the in situ tilapia heart perfused with 75 or 400 µmol L\(^{-1}\) palmitate and functioning at routine power output (~1.7 mW g\(^{-1}\)) and maximum power output (i.e., \(PO_{\text{max}}\); ~2.7 mW g\(^{-1}\)) during exposure to profound hypoxia (\(P_{O_2}<0.20\) kPa). The larger symbols represent means±s.e.m. (\(n=6\) for 75 µmol L\(^{-1}\) palmitate; \(n=5\) for 400 µmol L\(^{-1}\) palmitate). The smaller symbols represent the individual values for each fish at routine \(PO\) and \(PO_{\text{max}}\). Linear regression analyses were performed using individual values within each palmitate concentration; a significant positive relationship between \(PO\) and lactate efflux rate as well as between \(PO\) and ATP turnover rate was observed for each palmitate concentration (data not shown). Statistical comparison of the slopes and \(y\)-intercepts showed that the relationships were not significantly different between the two palmitate concentrations for either lactate efflux rate or ATP turnover rate. Thus, the displayed linear regressions are based on pooled individual values across both palmitate concentrations (total number of values=22). There was no significant difference between palmitate treatments, but lactate efflux rate and ATP turnover rate at \(PO_{\text{max}}\) was significantly greater than that at routine \(PO\) (statistics omitted for clarity) (two-way repeated measures ANOVA with Tukey’s, \(p<0.05\)).
CHAPTER 4: HYPOXIA TOLERANCE IN ELASMOBRANCHS. I.
CRITICAL OXYGEN TENSION AS A MEASURE OF BLOOD
OXYGEN TRANSPORT DURING HYPOXIA EXPOSURE

4.1 SYNOPSIS

The critical $O_2$ tension of whole-animal $O_2$ consumption rate ($\dot{M}_{O_2}$), or $P_{\text{crit}}$, is the water $PO_2$ ($P_{wO_2}$) at which an animal transitions from an oxyregulator to an oxyconformer. Although $P_{\text{crit}}$ is a popular measure of hypoxia tolerance in fishes because it reflects the capacity for $O_2$ uptake from the environment at low $P_{wO_2}$, little is known about the interrelationships between $P_{\text{crit}}$ and blood $O_2$ transport characteristics and increased use of anaerobic metabolism during environmental hypoxia exposure in fishes, especially elasmobranchs. This knowledge gap was addressed using progressive hypoxia exposures of two elasmobranch species with differing hypoxia tolerance. Specifically, I hypothesized that: 1) a lower $P_{\text{crit}}$ is associated with greater blood $O_2$ transport at low $P_{wO_2}$, 2) hemoglobin (Hb)-$O_2$ binding affinity is an important determinant of $P_{\text{crit}}$, and 3) increased stimulation of anaerobic metabolism occurs at $P_{\text{crit}}$. The $P_{\text{crit}}$ of the hypoxia-tolerant epaulette shark (*Hemiscyllium ocellatum*) (5.10±0.37 kPa) was significantly lower than that of the comparatively hypoxia-sensitive shovelnose ray (*Aptychotrema rostrata*) (7.23±0.40 kPa). Plasma [lactate] was elevated above normoxic values at around $P_{\text{crit}}$ in epaulette sharks, but increased relative to normoxic values at $P_{wO_2}$ below $P_{\text{crit}}$ in shovelnose rays, providing equivocal support for the hypothesis that $P_{\text{crit}}$ is associated with increased anaerobic metabolism. The $\dot{M}_{O_2}$, arterial $PO_2$, and arterial blood $O_2$ content ($C_aO_2$) were similar between the two species under normoxia and decreased in both species with progressive
hypoxia, but as $P_{w_2}$ declined, epaulette sharks had a consistently higher $\dot{M}_{O_2}$ and $C_aO_2$ than shovelnose rays, probably due to their significantly greater in vivo Hb-O$_2$ binding affinity (in vivo Hb-O$_2$ $P_{50} = 4.27\pm0.57$ kPa for epaulette sharks vs. $6.35\pm0.34$ kPa for shovelnose rays). However, at $P_{w_2}$ values representing the same percentage of each species’ $P_{crit}$ (up to $\sim$175% of $P_{crit}$), Hb-O$_2$ saturation and $C_aO_2$ were similar between species. These data support the hypothesis that Hb-O$_2$ $P_{50}$ is an important determinant of $P_{crit}$ and suggest that $P_{crit}$ can predict Hb-O$_2$ saturation and $C_aO_2$ during environmental hypoxia exposure, with a lower $P_{crit}$ being associated with greater O$_2$ supply at a given $P_{w_2}$ and consequently better hypoxia tolerance. Thus, $P_{crit}$ is a valuable predictor of environmental hypoxia tolerance and hypoxia exposures standardized at a given percentage of $P_{crit}$ will yield comparable levels of arterial hypoxemia, facilitating cross-species comparisons of responses to environmental hypoxia.

### 4.2 INTRODUCTION

Environmental hypoxia is a common abiotic stressor affecting the survival and distribution of aquatic species, but its occurrence varies in magnitude and spatio-temporal scale depending on habitat (Diaz and Breitburg, 2009). Consequently, many species of fish have evolved the ability to survive periods of low O$_2$ but the severity and duration of environmental hypoxia that can be tolerated is highly species-specific. Identification of the physiological responses contributing to hypoxia tolerance among fishes is an area of major research interest. The increasing occurrence worldwide of environmental hypoxia due to anthropogenic activities, in particular, has led to the desire for simple physiologic metrics of hypoxia tolerance in fishes and other aquatic organisms. Even so, relatively few comparative studies exist on this topic in fishes.
One simple metric that holds promise in this regard is the critical O₂ tension of whole-animal O₂ consumption rate (\(\dot{M}_{O_2}\)), or \(P_{\text{crit}}\), which is the water PO₂ (\(P_{wO_2}\)) at which the \(\dot{M}_{O_2}\) of an organism transitions from oxyregulation to oxyconformation. \(P_{\text{crit}}\) is thought to reflect the ability of an organism to extract O₂ from the environment to maintain routine \(\dot{M}_{O_2}\) as \(P_{wO_2}\) decreases, with a low \(P_{\text{crit}}\) being associated with greater hypoxia tolerance presumably because of improved O₂ uptake and transport to tissues at low \(P_{wO_2}\).

Consequently, \(P_{\text{crit}}\) has been employed routinely as an important measure of hypoxia tolerance in aquatic organisms including fishes (Chapman et al., 2002; Mandic et al., 2009; Nilsson and Östlund-Nilsson, 2008; Pörtner and Grieshaber, 1993; Routley et al., 2002). Physiological modifications at any step in the respiratory cascade may affect \(P_{\text{crit}}\), including changes in ventilation, gill surface area, blood O₂ capacity (including blood hemoglobin concentration [Hb] and Hb-O₂ binding affinity), circulation of O₂ (e.g., cardiac output), diffusion into tissues, and mitochondrial O₂ turnover (Dejours, 1981; Farrell and Richards, 2009). Whole blood Hb-O₂ \(P_{50}\) (i.e., Hb-O₂ binding affinity), in particular, has received attention as a determinant of \(P_{\text{crit}}\) as well as hypoxia tolerance in fishes because of its important role in controlling blood O₂ content and O₂ uptake (Brauner and Wang, 1997). A phylogenetically independent comparison of O₂ transport in sculpins showed that \(P_{\text{crit}}\) is strongly correlated with Hb-O₂ \(P_{50}\); species possessing a low \(P_{\text{crit}}\) typically also have a low Hb-O₂ \(P_{50}\) (Mandic et al., 2009). Other fishes known to have a high Hb-O₂ binding affinity typically also have a low \(P_{\text{crit}}\) and are often hypoxia-tolerant (Burggren, 1982; Jensen and Weber, 1982; Sollid et al., 2005). Overall, however, the relationship between \(P_{\text{crit}}\) and Hb-O₂ \(P_{50}\) rarely has been directly investigated and it remains incompletely defined. Also, it has not been unequivocally demonstrated that a low \(P_{\text{crit}}\) is associated with greater arterial blood O₂
transport during environmental hypoxia exposure in fishes. In fact, little is known in fishes about the responses of blood gas transport at and around $P_{\text{crit}}$.

At $P_{wO_2}$ below $P_{\text{crit}}$, O$_2$-independent energy production (e.g. anaerobic glycolysis) is increasingly relied upon to meet energy demands at a time when aerobic metabolism is constrained. Measurements of $P_{\text{crit}}$ and anaerobic end-product accumulation in certain animals provide evidence for the hypothesis that the increased activation of anaerobic metabolism coincides with $P_{\text{crit}}$ (Pörtner and Grieshaber, 1993), although support from other studies is equivocal (McKenzie et al., 2000; Nonnotte et al., 1993). The link between $P_{\text{crit}}$ and increased activation of anaerobic metabolism is thus uncertain, especially in fishes. Also, there are no direct comparisons of the onset of lactate accumulation in hypoxia-tolerant and -sensitive fishes that differ in $P_{\text{crit}}$.

In the present study, I investigated the relationship between $P_{\text{crit}}$ and arterial O$_2$ transport properties, including $in$ $vivo$ Hb-O$_2$ $P_{50}$ and arterial total O$_2$ content (C$_a$O$_2$), during progressive hypoxia exposure in two tropical elasmobranch species with similar lifestyles and activity levels, the epaulette shark (*Hemiscyllium ocellatum*) and the eastern shovelnose ray (*Aptychotrema rostrata*). Epaulette sharks inhabit shallow coral reef environments where nocturnal hypoxia occurs commonly and they can tolerate hours of profound hypoxia ($P_{wO_2}$$<$1.0 kPa) exposure and up to 45 min of anoxia exposure (Renshaw et al., 2002; Routley et al., 2002). In contrast, eastern shovelnose rays are found in generally well-oxygenated coastal sandy and muddy benthic habitats in eastern Australia, including the present study sampling site of Moreton Bay, where hypoxic events are rare and dissolved O$_2$ is usually close to air saturation at all depths (Dennison et al., 2004; Gabric et al., 1998; Kyne and Bennett, 2002). Indeed, preliminary observations from the present study showed that
shovelnose rays are relatively hypoxia-sensitive, succumbing rapidly if held at or below a
$P_wO_2$ of 2.0 kPa for more than approximately 30 min. I hypothesized that a lower $P_{crit}$ is
associated with greater blood $O_2$ transport at low $P_wO_2$ and that hemoglobin (Hb)-$O_2$ binding
affinity is an important determinant of $P_{crit}$. I predicted that the more hypoxia-tolerant
epaulette shark would have a lower $P_{crit}$ than the shovelnose ray and that the lower $P_{crit}$ in the
epaulette shark would be associated with a lower Hb-$O_2$ $P_{50}$ and a correspondingly greater
$C_aO_2$ at similar hypoxic $P_wO_2$. Furthermore, I predicted that at each species' $P_{crit}$ or at the
same percentage of $P_{crit}$, Hb-$O_2$ saturation and $C_aO_2$ would be similar between the species
despite being exposed to different $P_wO_2$ values. Finally, I measured arterial blood metabolic
status including pH, [lactate], and $CO_2$ status in order to further characterize the
physiological correlates of $P_{crit}$ in fishes and test the hypothesis that $P_{crit}$ is associated with
increased activation of anaerobic metabolism. Overall, the present study provides a
comprehensive picture of the physiological responses associated with $P_{crit}$ in two fishes,
elucidating for the first time the relationship between $P_{crit}$ and arterial blood $O_2$ transport
characteristics during environmental hypoxia exposure, and providing comparative insight
into the respiratory and metabolic attributes associated with hypoxia tolerance in fishes.

4.3 MATERIALS AND METHODS

4.3.1 Animals

Epaulette sharks and shovelnose rays of mixed sexes were supplied by Cairns Marine
(Cairns, QLD, Australia) or Seafish Aquarium Life (Dunwich, QLD, Australia), respectively.
The animals were collected under A1 level commercial harvest licenses granted by the
Department of Primary Industries, Australia. Epaulette sharks were caught on the Great
Barrier Reef and transported in flow-through seawater tanks to holding tanks at Cairns Marine, where they were kept for 2 days without feeding before being transported by air and automobile to Moreton Bay Research Station, North Stradbroke Island, Queensland, Australia. Shovelnose rays were caught in Moreton Bay, transferred to the mainland in flow-through seawater tanks, and transported to Moreton Bay Research Station by automobile. All fishes were held in a recirculating seawater system (28°C) for at least 5 days before experimentation. Animals were fed every other day and fasted for at least 24 h before experimentation. All experiments were conducted according to guidelines set out by the Canadian Council for Animal Care and protocols approved by the University of British Columbia Animal Care Committee and the Griffith University Animal Ethics Committee.

4.3.2 Experimental and analytical protocols

4.3.2.1 Surgical protocol

Epaulette sharks (1.29±0.04 kg, n=7) and shovelnose rays (1.54±0.06 kg, n=8) were netted from the holding tanks and anaesthetized in water containing a final concentration of 0.1 g L⁻¹ benzocaine (initially dissolved in 95% ethanol; 0.001% ethanol in anaesthetic bath). Fish were then moved to a surgery table where the gills were continuously irrigated with aerated seawater (28°C) containing 0.075 g L⁻¹ benzocaine.

To permit periodic sampling of blood, a PE50 (Clay-Adams, Parsippany, NJ, USA) cannula was fitted in the caudal artery via a lateral incision in the caudal peduncle, as described previously (de Boeck et al., 2001). The cannula was filled with heparinised (50 U mL⁻¹) elasmobranch saline (in mM: 257 NaCl, 7 Na₂SO₄, 6 NaHCO₃, 0.1 Na₂HPO₄, 4 KCl, 3 MgSO₄·H₂O, 2 CaCl₂·2H₂O, 300 urea, 100 trimethylamine oxide). The cannula was
exteriorized through a PE160 grommet and sutured to the skin. The incision was closed with silk sutures. The fishes were also fitted with ventral aorta flow probes as described in Chapter 5.

4.3.2.2 Experimental protocol

Following surgery, the instrumented fish was immediately moved to a cylindrical acrylic respirometer (18.2 L for epaulette sharks and 28.4 L for shovelnose rays) that was submersed inside an opaque aquarium that received seawater from the same recirculating system used for the fish holding tanks (28°C). A submersible pump inside the aquarium provided a continuous flow of water to the respirometer that ensured complete water mixing inside the respirometer and maintained $P_{w}O_2$. The respirometer was covered with black plastic to prevent visual disturbance of the fish. The cannula and the flow probe lead from the fish was exteriorised through a hole in the respirometer fitted with a soft rubber stopper modified with a slit. The fish was allowed to recover from surgery and habituate to the respirometer for at least 12 h before any experimental procedures were performed.

Stable baseline conditions were confirmed by monitoring routine cardiovascular variables (see Chapter 5) for 1-2 h at a normoxic $P_{w}O_2$ of between 15 and 16 kPa (=74-78% air saturation; 100% air saturation=20.4 kPa=153 torr). An aortic blood sample (1 mL) was taken at the end of this period for immediate measurement of normoxic resting levels of arterial whole blood pH, $P_{O_2}$, [hemoglobin] ([Hb]), hematocrit (Hct), and total $O_2$ content as described below. Plasma was separated by centrifugation (5000 g; 5 min), frozen in liquid nitrogen within 5 min of sampling, transported to Canada in a dry shipper and kept frozen at -80°C for several weeks until analyses of metabolites and [total $CO_2$] (see below). The red
blood cell pellet was re-suspended in elasmobranch saline to a final volume of 1 mL and this was injected via the cannula to replace the blood removed. The respirometer was then closed by connecting the inflow and outflow tubes of the respirometer via a submersible pump that re-circulated the water inside the respirometer. The fish was allowed to consume O_2 in the respirometer and the rate of depletion of P_wO_2 was used to calculate M_{O_2} as described below. The changes in water parameters (e.g. pH, PCO_2) potentially associated with the use of closed respirometry have been shown previously to have no effect on P_{crit} in fishes (Henriksson et al., 2008). Indeed, only modest increases in arterial PCO_2 (P_{aCO_2}) were observed in the present study (see results and discussion). Arterial blood samples were taken and treated as described above at regular intervals including P_wO_2 at approximately 11.8, 7.7, 5.8, 3.8 and 2.0 kPa in both species, as well as approximately 1.0 and 0.1 kPa in epaulette sharks (see Table 4.1). Fish were held in the closed respirometer until P_wO_2 reached ~0.1 kPa for epaulette sharks and ~1.6 kPa for the shovelnose rays, which took 135±8 min and 71±6 min, respectively, from the point at which the normoxic blood sample was taken and the respirometer was closed. These nadirs of P_wO_2 were chosen based on previous studies on epaulette sharks (e.g. Renshaw et al., 2002) and preliminary observations of hypoxia-exposed animals which clearly revealed that epaulette sharks tolerated prolonged bouts of profound environmental hypoxia whereas shovelnose rays showed distress or loss of equilibrium if held at or below 2.0 kPa for more than 30 min. The rate of O_2 depletion was similar between species (see Table 4.1). Once the nadir in P_wO_2 (~0.1 or ~1.6 kPa) was reached, normoxic water was reintroduced to the respirometer. A final blood sample was taken at 60 min of recovery in normoxic water and analyzed as previously described. In some cases, cannulae were damaged by the movements of the fishes during the overnight acclimation period or
during the experimental exposure and therefore sample sizes of measured parameters vary slightly (see figure captions for final *n* values). At the end of the trials fishes were terminally anesthetized in seawater containing benzocaine.

4.3.2.3  *Data acquisition and calculation of oxygen consumption rate and* P<sub>crit</sub>

Water PO<sub>2</sub> in the respirometer was measured using an Oxyguard probe (Mark IV, Point Four Systems, Richmond, BC, Canada), modified to give a ±1 V output signal, that was placed in a custom made plexiglass chamber connected in line with the circulation pump. The probe output was fed to a Power Lab unit (ADInstruments, Castle Hill, NSW, Australia) and subsequently analysed using LabChart Pro software (v. 6.0; ADInstruments).

Whole-animal \( \dot{M}_O_2 \) during the respirometry trials were calculated from the rate of decline in \( P_wO_2 \) over 10 min periods bracketing \( P_wO_2 \) at regular intervals from approximately 14.8 kPa to 0.67 kPa in epaulette sharks and approximately 13.7 kPa to 1.9 kPa in shovelnose rays, following the methods of Henriksson et al. (2008). Blanks without fish were run for both chambers and background \( \dot{M}_O_2 \) was subtracted from fish \( \dot{M}_O_2 \). The corrected \( \dot{M}_O_2 \) was plotted against \( P_wO_2 \) and the inflection point at which \( \dot{M}_O_2 \) transitions from being independent to being dependent on \( P_wO_2 \) (i.e., \( P_{crit} \)) was calculated using the BASIC program designed by Yeager and Ultsch (1989). At \( P_wO_2 \) above \( P_{crit} \), \( \dot{M}_O_2 \) values were constant and not significantly different from one another within a species, therefore confirming that \( \dot{M}_O_2 \) is independent of \( P_wO_2 \) above \( P_{crit} \). The \( \dot{M}_O_2 \) was not measured at the initial normoxic \( P_wO_2 \) (~15.3-16.0 kPa) or the normoxic recovery period because of the flow-through conditions.
4.3.2.4 Analytical protocols

Blood [Hb] was measured spectrophotometrically (Blaxhall and Daisley, 1973). Hematocrit was determined following centrifugation at 5000 g in a sealed capillary tube. Mean cellular Hb content (MCHC) was calculated as ([Hb]/Hct)100. Arterial blood PO$_2$ (P$_a$O$_2$) was measured with a Radiometer PO$_2$ (E-5046) electrode, thermostatically controlled in a D616 cell at 28°C, in conjunction with a PHM 71 acid-base analyzer (Radiometer, Copenhagen, Denmark). Whole blood C$_a$O$_2$ was measured following the method of Tucker (1967). In order to calculate the percentage O$_2$ saturation of Hb in arterial blood, the quantity of O$_2$ bound to Hb was calculated by subtracting physically dissolved O$_2$ in the blood (calculated from the measured P$_a$O$_2$ and published blood O$_2$ solubility coefficient from Christiforides and Hedley-Whyte, 1969) from the C$_a$O$_2$ measured in whole blood. The quantity of O$_2$ bound to Hb was then expressed as a percentage of the theoretical maximum quantity of O$_2$ bound to Hb, which was calculated using measured [Hb] values and assuming four O$_2$ bound per Hb tetramer at 100% saturation. In vivo blood P$_{50}$ and Hill coefficient values were then calculated from Hill plots based on data for arterial PO$_2$ and Hb-O$_2$ saturation.

Blood pH was measured using a Radiometer pH micro-electrode thermostatically held at 28°C and displayed on a Radiometer PHM 71 acid-base analyzer. True plasma [total CO$_2$] was measured according to Cameron (1971), immediately following thawing of the plasma on ice. To confirm that freezing of plasma and storage at -80°C has no effect on [total CO$_2$], I compared [total CO$_2$] measured in freshly sampled plasma from normocarbic, normoxic tilapia (Oreochromis hybrid sp.) with an aliquot of the same plasma that was frozen in liquid nitrogen and stored at -80°C for 4 weeks. [Total CO$_2$] was 7.1±0.7 mM in the
fresh samples vs. 7.0±0.8 mM in the frozen samples (n=6; paired t-test, p>0.05; B.S.-R., unpublished); thus, freeze-thaw of plasma does not appear to affect [total CO\textsubscript{2}]. Similar results have been observed in hagfish (*Eptatretus stoutii*) (D.W. Baker and C.J.B, unpublished). Arterial P\textsubscript{CO\textsubscript{2}} and [HCO\textsubscript{3}\textsuperscript{-}] were calculated from measured values of pH and [total CO\textsubscript{2}] through manipulation of the Henderson-Hasselbalch equation (Brauner et al., 2000) with appropriate constants for elasmobranchs (Boutilier et al., 1984). Plasma [lactate] and [glucose] were measured on deproteinized and untreated plasma, respectively, according to the protocols outlined by Bergmeyer (1983). Plasma [\(\beta\)-hydroxybutyrate] ([\(\beta\)-HB]) was measured on deproteinized plasma following the protocol of McMurray et al. (1984).

**4.3.3 Statistics**

The effects of species and P\textsubscript{ow}O\textsubscript{2} on blood gas, acid-base and metabolite parameters were tested for samples 1-6 (at overlapping P\textsubscript{ow}O\textsubscript{2} between species; see Table 4.1) using a two-way ANOVA followed by Holm-Sidak post hoc (H-S) tests against species or the normoxic resting values measured at ≥15.3 kPa (P\textsubscript{ow}O\textsubscript{2} were not statistically different between species at each sample point, allowing this two-way design; Student’s t-test, p>0.05). Samples 7 and 8 in epaulette sharks (see Table 4.1) were tested separately for significance against the normoxic resting sample 1 (16.0 kPa) using a one-way ANOVA with H-S tests. Species differences in the *in vivo* blood P\textsubscript{50} and Hill coefficient (\(n_H\)) values were examined using a Student’s t-test and the effect of O\textsubscript{2} on Hb-O\textsubscript{2} saturation was compared within species using a one-way ANOVA with H-S tests (comparisons between species were not made because arterial PO\textsubscript{2} at sample points were not always comparable). The relationship between Hb-O\textsubscript{2} saturation and P\textsubscript{crit} was examined *via* linear regression analysis of Hb-O\textsubscript{2} saturation.
against \( P_{wO_2} \) expressed as the percentage of \( P_{crit} \) (using values from individual animals). This analysis was carried out up to a Hb-O\(_2\) saturation of \( \sim 75\% \) because at higher values Hb saturation curves are asymptotic. Recovery values were compared between species and to the normoxic resting values at \( \geq 15.3 \) kPa within species using a two-way ANOVA with H-S test.

The critical \( P_{wO_2} \)’s of \( \dot{M}_{O_2} \) were compared between species using a Student’s t-test. The effects of species and \( P_{wO_2} \) on \( \dot{M}_{O_2} \) were tested using a two-way ANOVA with H-S tests using data from eight sampling points of overlapping \( P_{wO_2} \) at approximately 13.6, 12.3, 10.3, 6.1, 4.2, 3.4, 2.5, and 1.9 kPa and the points of statistical comparison are denoted by horizontal “\{"” on the figures. Overlapping \( P_{wO_2} \) values were not statistically different between species (Student’s t-test, \( p>0.05 \)). Data from other sampling points were omitted from these analyses. However, in order to fully assess the effect of \( P_{wO_2} \) on \( \dot{M}_{O_2} \) in each species, one-way ANOVA’s were run across all sampling points within each species, with H-S comparisons against the first normoxic resting value. The effect of \( P_{wO_2} \) on measured parameters was found to be similar for both two-way and one-way ANOVA designs.

Statistical significance was accepted when \( p<0.05 \) and analyses were carried out using SigmaStat 3.0 or GraphPad Prism 5.0. Data were log or square root transformed prior to statistical analyses if assumptions of equal variance or normality were not met. Repeated measures ANOVA could not be carried out because experimental constraints negated the use of data from the same animal at every single sample period. In any case, the standard ANOVA procedures utilized here results in a conservative statistical assessment of the data.

4.4 RESULTS

Under normoxic conditions, individuals of both species were generally quiescent. As
PawO₂ decreased, there was a modest and temporary increase in activity level associated with exploratory behaviour in some individuals of each species. As PawO₂ decreased further, the fishes again became quiescent. In a couple instances in each species, severe agitation occurred temporarily (<10 sec) at low PawO₂, causing the cannula to be dislodged and negating further blood sampling.

A typical relationship between ṀO₂ and PawO₂ was observed in both species, each with a zone of O₂-independent ṀO₂ occurring at higher PawO₂ followed by a zone of O₂-dependence below Pcrit, where ṀO₂ decreased with decreasing PawO₂ (Fig. 4.1). The Pcrit was significantly lower in epaulette sharks than in shovelnose rays (Fig. 4.1). When PawO₂ was above ~5 kPa, ṀO₂ was similar between species. However, when PawO₂ was below ~5 kPa, ṀO₂ was consistently greater for epaulette sharks than for shovelnose rays at any similar PawO₂ (Fig. 4.1).

Arterial PO₂ decreased linearly with decreasing PawO₂ and was not different between species, except at the two highest PawO₂ where epaulette sharks had higher PaO₂ compared with shovelnose rays (Fig. 4.2A). Total CaO₂ decreased with decreasing PawO₂ with the exception of between 16.01±0.43 and 11.80±0.06 kPa in epaulette sharks. Total CaO₂ was the same in both species at the highest PawO₂, but at all lower PawO₂ it was greater in epaulette sharks than in shovelnose rays (Fig. 4.2B). Recovery in normoxic water returned PaO₂ and CaO₂ to values similar to those measured at the beginning of the progressive hypoxia trial (Fig. 4.2A,B). Hb-O₂ saturation decreased as PaO₂ fell below approximately 9.0 kPa and 6.0 kPa in shovelnose rays and epaulette sharks, respectively (Fig. 4.3). The in vivo Hb-O₂ binding affinity was significantly higher (=lower P50) in the epaulette sharks than in
shovelnose rays (Fig. 4.3). The Hill coefficient was similar between species (Fig. 4.3). At each species’ P\textsubscript{50}, arterial pH was \textasciitilde7.82 in epaulette sharks and \textasciitilde7.81 in shovelnose rays and P\textsubscript{a}CO\textsubscript{2} was \textasciitilde0.23 kPa in both species (extrapolated from pH and P\textsubscript{a}CO\textsubscript{2} data at the extrapolated P\textsubscript{w}O\textsubscript{2} at P\textsubscript{50}).

A significant linear relationship was found for each species when Hb-O\textsubscript{2} saturation was regressed against P\textsubscript{w}O\textsubscript{2} expressed as a percentage of P\text{crit} and because the slopes and y-intercepts of these regression lines were not significantly different between species, one line of best fit was applied to the pooled data for both species (Fig. 4.4). Above approximately 175% of P\text{crit}, the Hb-O\textsubscript{2} saturation became asymptotic so these data were excluded from the linear regression. Thus, Hb-O\textsubscript{2} saturation was similar between species at P\textsubscript{w}O\textsubscript{2} values representing the same percentage of each species’ P\text{crit} (up to \textasciitilde175% of P\text{crit}). Because there were no major differences in [Hb] between species (see below), the same relationship existed for C\textsubscript{a}O\textsubscript{2} (data not shown). Overall, these data show that P\text{crit} is predictive of Hb-O\textsubscript{2} saturation and C\textsubscript{a}O\textsubscript{2} at P\textsubscript{w}O\textsubscript{2} lower than about 175% of P\text{crit}.

In both species progressive hypoxia had no effect on Hct, [Hb], or MCHC (Table 4.1). Hematocrit and [Hb] were significantly higher in epaulette sharks at sampling points 4 (P\textsubscript{w}O\textsubscript{2}=5.73±0.02 kPa in epaulette sharks and 5.83±0.09 kPa in shovelnose rays) and 6 (P\textsubscript{w}O\textsubscript{2}=1.85±0.03 kPa in epaulette sharks and 2.07±0.03 kPa in shovelnose rays) and [Hb] was also higher at sampling point 5 (P\textsubscript{w}O\textsubscript{2}=3.81±0.12 kPa in epaulette sharks and 3.82±0.07 kPa in shovelnose rays) (Table 4.1). Recovery [Hb] and MCHC were similar to normoxic resting values but Hct was higher in epaulette sharks during recovery (Fig. 4.2A,B; Table 4.1).
Plasma [lactate] was low at $P_{w}O_{2}$ above 8 kPa, then significantly increased above normoxic resting levels by ~3.7 kPa in both species and increased further with decreasing $P_{w}O_{2}$. Plasma [lactate] remained elevated after 60 min of recovery in normoxic water (Fig. 4.5A). Plasma [lactate] was generally similar between species at all $P_{w}O_{2}$ during progressive hypoxia but was greater in epaulette sharks during recovery (Fig. 4.5A). A significant decrease in blood pH first occurred at approximately 3.8 kPa in both species, decreasing further with declining $P_{w}O_{2}$ and remaining low after 60 min of recovery in normoxic water (Fig. 4.5B). Blood pH was similar between species but was significantly lower in the shovelnose rays at the final sampling point for this species at ~2.0 kPa (Fig. 4.5B). Arterial [HCO$_3$⁻] and [total CO$_2$] were unchanged during progressive hypoxia exposure in both species (Table 4.1). An increase in $P_aCO_2$ occurred in both species and it remained after 60 min of recovery in normoxic water (Table 4.1). In epaulette sharks only, normoxic recovery was associated with significantly lower [HCO$_3$⁻] and [total CO$_2$] compared with normoxic resting levels as well as compared with the same parameters in shovelnose rays in recovery (Table 4.1). Plasma [glucose] or [$\beta$-HB] were similar between species and were unaffected by either progressive hypoxia or recovery in normoxic water (Table 4.1).

4.5 DISCUSSION

The present study demonstrates a link between $P_{crit}$ and arterial blood $O_2$ transport characteristics during environmental hypoxia exposure in two elasmobranch species, adding significantly to a growing body of evidence showing that $P_{crit}$ is an important indicator of hypoxia tolerance in fishes (Chapman et al., 2002; Mandic et al., 2009; Nilsson and Östlund-Nilsson, 2008). The present study also provides the first comparative evidence that a lower
$P_{\text{crit}}$ is associated with maintenance of greater $C_aO_2$ during environmental hypoxia exposure, which benefits hypoxia tolerance. Similarly, I show that $P_{\text{crit}}$ is predictive of Hb-O$_2$ saturation and $C_aO_2$ (in the observed absence of changes in [Hb]) during environmental hypoxia exposure (Fig. 4.4), supporting the notion that differences in Hb-O$_2$ binding affinity determine differences in $P_{\text{crit}}$. Indeed, the *in vivo* Hb-O$_2$ $P_{50}$ of the epaulette shark was lower than that of the shovelnose ray and this is the likely explanation for the greater $C_aO_2$ observed in the former species at low $P_wO_2$. The impressive hypoxia tolerance of the epaulette shark is likely attributable, in part, to its enhanced O$_2$ transport characteristics compared with those of the less tolerant shovelnose ray.

### 4.5.1 Oxygen uptake and blood oxygen transport properties

Under normoxic resting conditions (i.e., $P_wO_2 > P_{\text{crit}}$) the $\dot{M}_{O_2}$ of epaulette sharks matches closely with that measured for this species by Routley et al. (2002). Epaulette sharks and shovelnose rays had similar resting $\dot{M}_{O_2}$, which fell within the range of temperature-corrected $\dot{M}_{O_2}$ for elasmobranchs of a similar activity level (0.9-3.5 µmol g$^{-1}$ h$^{-1}$; Butler and Metcalfe, 1988). In both species, exposure to progressive hypoxia caused a pronounced reduction of $\dot{M}_{O_2}$ below $P_{\text{crit}}$ (Fig. 4.1), similar to many teleosts (Mandic et al., 2009; Chapter 2) as well as at least one other elasmobranch, the spotted catshark (*Scyliorhinus canicula*) (Butler and Taylor, 1975). The $P_{\text{crit}}$ for epaulette sharks (Fig. 4.1) matches the lower range of $P_{\text{crit}}$ measured for this species by Routley et al. (2002). The shovelnose ray had a significantly higher $P_{\text{crit}}$ compared with the epaulette shark (Fig. 4.1), but it was lower than that of other previously studied elasmobranchs (see Routley et al., 2002). Although the level of O$_2$ demand can affect $P_{\text{crit}}$ (Thuy et al., 2010), this likely does not explain the
observed difference in $P_{crit}$ because $\dot{M}_{O_2}$ under normoxic conditions was the same in each species (Fig. 4.1). The difference in $P_{crit}$ between the hypoxia-tolerant epaulette shark and the comparatively hypoxia-sensitive shovelnose ray is consistent with the notion that a lower $P_{crit}$ is associated with greater hypoxia tolerance in fishes (Mandic et al., 2009; Nilsson and Östlund-Nilsson, 2008). The $P_{crit}$ of the epaulette shark is generally similar to those of hypoxia-tolerant teleosts and it is the lowest known $P_{crit}$ among elasmobranchs (Routley et al., 2002; Chapter 2). The lower $P_{crit}$ of epaulette sharks was associated not only with maintenance of routine $\dot{M}_{O_2}$ to a lower $P_{w O_2}$ compared with shovelnose rays, but also with maintenance of greater $\dot{M}_{O_2}$ at all comparable hypoxic $P_{w O_2}$ (i.e. below $P_{crit}$) where depression of $\dot{M}_{O_2}$ occurred in both species (Fig. 4.1). Thus, a low $P_{crit}$ allows a fish to maintain $\dot{M}_{O_2}$ as high as possible during hypoxia exposure, minimizing reliance on inefficient anaerobic metabolism.

Haematological (i.e., Hct, [Hb], MCHC) and $O_2$ transport properties (i.e. $P_{a O_2}$, $C_{a O_2}$, and Hb-$O_2$ saturation) in arterial blood of epaulette sharks and shovelnose rays under normoxic resting conditions (Fig. 4.2A,B and Fig. 4.3, values at highest $P_{w O_2}$; Table 4.1, sample 1) were generally similar between species and the values were typical for elasmobranchs (e.g. Hct, 10-20%; [Hb], 0.46-0.62 mM; MCHC, ~3.3; $P_{a O_2}$, 8-15 kPa; $C_{a O_2}$, 3-5 vol%; Hb-$O_2$ saturation, 75-100%; Butler and Metcalfe, 1988; Butler and Taylor, 1975; De Boeck et al., 2001; Lai et al., 1990; Perry and Gilmour, 1996; Routley et al., 2002). The lack of 100% Hb-$O_2$ saturation in normoxia is consistent with other studies on elasmobranchs (e.g. Lai et al., 1990) and the presence of up to 27% methemoglobin in fishes has been put forward as an explanation as to why some fish hemoglobins are not saturated to their
theoretical maximum \textit{in vivo} (Graham and Fletcher, 1986). The \textit{in vivo} P_{50} values for epaulette sharks and shovelnose rays are higher than previously measured in other elasmobranchs (1.9-2.7 kPa; Butler and Taylor, 1975; Cooper and Morris, 2004; Lai et al., 1990), but in the previous studies these were measured at lower environmental temperatures compared with the present study and environmental temperature appears to be positively correlated with P_{50} in marine fishes (Wells, 2005). The Hill coefficients were similar between species and relatively low, which is typical of elasmobranchs (Butler and Metcalfe, 1988).

The changes in P_{aO2} and C_{aO2} during progressive hypoxia (Fig. 4.2A,B) were similar to those seen in other elasmobranchs (Butler and Taylor, 1975; Perry and Gilmour, 1996; Routley et al., 2002). The roughly linear decrease in P_{aO2} was of similar magnitude in epaulette sharks and shovelnose rays, suggesting ventilatory responses to environmental hypoxia are comparable between species (Fig. 4.2A). However, at all matched P_{wO2} below the normoxic resting level, C_{aO2} was greater in epaulette sharks than in shovelnose rays, which appears to be due to greater Hb-O2 saturation resulting from the epaulette shark’s higher \textit{in vivo} Hb-O2 binding affinity (i.e., lower P_{50}) (Fig. 4.2B; Fig. 4.3). This result is consistent with findings suggesting that Hb-O2 P_{50} is an important component of hypoxia tolerance in fishes, with the most tolerant species possessing the lowest P_{50}, which result in greater blood O2 loading at low P_{wO2} (Jensen and Weber, 1982; Mandic et al., 2009; Perry and Reid, 1992).

Interestingly, the species differences in Hb-O2 saturation and C_{aO2} disappeared when these parameters were plotted against P_{wO2} expressed as a percentage of P_{crit} (i.e., the relationships overlapped between species) (Fig. 4.4). Thus, when measured at a P_{wO2} of the same percentage of each species’ P_{crit}, values of Hb-O2 saturation or C_{aO2} (because changes
in [Hb] were not apparent) were the same in epaulette sharks and shovelnose rays. These results suggest that $P_{\text{crit}}$ is predictive of Hb-O$_2$ saturation and $C_a$O$_2$ during environmental hypoxia exposure, with species with lower $P_{\text{crit}}$ having a greater capacity for arterial blood O$_2$ transport at similar hypoxic P$_w$O$_2$. These results also suggest that the differences in arterial Hb-O$_2$ saturation between epaulette sharks and shovelnose rays may represent the basis of species differences in $P_{\text{crit}}$. This observation, as well as the fact that at $P_{\text{crit}}$ the P$_a$O$_2$ in both species was similar to their respective in vivo Hb-O$_2$ P$_{50}$ (Fig. 4.2A; Fig. 4.3), agrees with Mandic et al.’s (2009) discovery of a close relationship between $P_{\text{crit}}$ and Hb-O$_2$ P$_{50}$ in sculpins and support the idea that P$_{50}$ is an important determinant of $P_{\text{crit}}$ in fishes.

The lack of a change in Hct or [Hb] during progressive hypoxia (Table 4.1) is consistent with previous studies on epaulette sharks and other elasmobranchs (Perry and Gilmour, 1996; Routley et al., 2002; Short et al., 1979) and thus adjustments of these parameters may have a minimal role in improving O$_2$ transport in elasmobranchs exposed to environmental hypoxia. Nonetheless, Hct and [Hb] were approximately 25% higher at some hypoxic P$_w$O$_2$ in epaulette sharks compared with shovelnose rays (Table 4.1), likely contributing to the higher $C_a$O$_2$ seen in the former species. However, the difference of $C_a$O$_2$ between species was greater (40-100% higher in epaulette sharks) at these hypoxic P$_w$O$_2$, affirming an important role for Hb-O$_2$ P$_{50}$ in enhancing O$_2$ supply in the epaulette shark.

### 4.5.2 Metabolites and acid-base status

An increased reliance on anaerobic glycolysis and a consequent metabolic acidosis during environmental hypoxia exposure was indicated by a large increase in plasma [lactate] and a decrease in blood pH at or below $P_{\text{crit}}$ in both species (Fig. 4.5A,B), similar to results
observed in other hypoxia-exposed fishes (Routley et al., 2002; Scott et al., 2008). At the lowest \(P_{w}O_2\) exposure of shovelnose rays, blood pH was significantly lower compared with epaulette sharks (Fig. 4.5B), suggesting that the latter species may possess more effective acid-base regulation or less reliance on anaerobic energy production, potentially due to superior \(O_2\) supply. Nevertheless, at lower \(P_{w}O_2\) epaulette shark blood pH dropped precipitously and like other hypoxia-tolerant vertebrates this species must be able to tolerate severe metabolic acidosis during hypoxia exposure (Driedzic and Gesser, 1994). The \(P_{w}O_2\) at which epaulette sharks first showed a significant increase in plasma [lactate] was approximately the same as found previously for this species (Routley et al., 2002) and coincided with its \(P_{crit}\) (Fig. 4.5A). In shovelnose rays, however, the increase in plasma [lactate] did not coincide with its \(P_{crit}\) but rather occurred at a similar \(P_{w}O_2\) as the epaulette sharks and accumulated at a similar rate (Fig. 4.5A). Although these data do not provide solid evidence for the hypothesis that \(P_{crit}\) correlates with increased activation of anaerobic metabolism in fishes (Pörtner and Grieshaber, 1993), they are consistent with this activation not occurring above \(P_{crit}\). It is also possible that a non-equilibrium state during early progressive hypoxia exposure including interspecific differences in blood volume or lactate handling may have obscured my ability to detect the onset of an increase of anaerobic metabolism at \(P_{crit}\) in shovelnose rays. This may also explain why the rate of plasma lactate accumulation was similar between species despite lower blood \(O_2\) content in shovelnose rays. The effect of changes in fish activity (see results) on plasma [lactate] are considered to be negligible because of the low measurement variability and the consistently low values at \(P_{w}O_2\) above \(P_{crit}\) (Fig. 4.1). Also, most fish activity was moderate and in the few instances of
excessive activity, cannula dislodgement meant that plasma [lactate] was no longer measured.

The metabolic acidosis seen in epaulette sharks and shovelnose rays during progressive hypoxia resulted in a downward trend in [HCO$_3^-$] and [total CO$_2$] (Table 4.1). In both species P$_a$CO$_2$ increased, possibly due to a build-up of CO$_2$ in the closed respirometer. This elevation in CO$_2$ probably had no major effect on other measured parameters, because the magnitude of the P$_a$CO$_2$ increase was not large until the final sample point and no major differences were observed between the final sample point and the previous sample point for any measured parameters. In any case, hypercarbia commonly accompanies environmental hypoxia so in this regard the present exposures are more ecologically relevant. The responses of blood CO$_2$ parameters to progressive hypoxia in the present study differ from the respiratory alkaloses seen in hypoxic spiny dogfish (*Squalus acanthias*) (Perry and Gilmour, 1996) or spotted catshark (Butler et al., 1979), but these other studies utilized less severe hypoxia that likely mitigated metabolic acidosis (Butler et al., 1979, found no increase in plasma [lactate]). The relatively low normoxic resting levels of arterial P$_a$CO$_2$, [HCO$_3^-$], and [total CO$_2$] in epaulette sharks and shovelnose rays are typical of elasmobranchs (Butler and Metcalfe, 1988; De Boeck et al., 2001; Lai et al., 1990).

Plasma [glucose] was unaffected by progressive hypoxia (Table 4.1), which is unlike most teleosts but mirrors previous findings in hypoxia-exposed epaulette sharks and spiny dogfish (Routley et al., 2002; Speers-Roesch and Treberg, 2010). In some hypoxia-tolerant teleosts, large decreases in plasma [non-esterified fatty acids], an important aerobic fuel, occur during hypoxia exposure (see Chapter 1 and Chapter 2). There was no similar response in epaulette sharks or shovelnose rays for plasma concentration of β-HB, which in
elasmobranchs serves an important role as an alternative lipid-derived aerobic fuel (Speers-Roesch and Treberg, 2010).

4.5.3 Recovery from progressive hypoxia exposure

Epaulette sharks and shovelnose rays showed comparable recovery of respiratory parameters after progressive hypoxia and followed a trajectory similar to other fishes (Hughes and Johnston, 1978; van Raaij et al., 1996a). Following 60 min of recovery in normoxic water, normoxic arterial O$_2$ parameters were restored in both species (Fig 4.2A,B; Fig. 4.3); however, recovery from metabolic acidosis and high [lactate] was incomplete, particularly in epaulette sharks which had experienced greater hypoxia and hypoxia-induced acidosis and lactate accumulation (Fig. 4.5A,B). Arterial [HCO$_3^-$] in recovery remained lower than normoxic levels in epaulette sharks, probably as a consequence of the persistence of acidosis, whereas shovelnose rays had recovered (Table 4.1).

4.5.4 Conclusions and perspectives

The present study on the hypoxia-tolerant epaulette shark and the hypoxia-sensitive shovelnose ray provides the first evidence that P$_{crit}$ is predictive of arterial Hb-O$_2$ saturation and C$_a$O$_2$ during environmental hypoxia exposure in fishes. At the same level of hypoxia, fishes with a low P$_{crit}$ can maintain higher C$_a$O$_2$ than fishes with a higher P$_{crit}$ and this appears to be due to the presence of a lower in vivo Hb-O$_2$ P$_{50}$ that allows greater Hb-O$_2$ saturation (Fig. 4.2B; Fig. 4.3). Additionally, at P$_{wO_2}$ of the same % of P$_{crit}$, Hb-O$_2$ saturation (and C$_a$O$_2$) is the same between species (Fig. 4.4). These results suggest that the interspecific differences in Hb-O$_2$ saturation may represent the basis of species differences in P$_{crit}$, providing strong
support for the hypothesis that Hb-O₂ P₅₀ is a major determinant of Pₐ is as well as hypoxia
tolerance in fishes (Mandic et al., 2009).

Our finding of a link between Pₐ, Hb-O₂ saturation, and CₐO₂ provides a mechanistic
explanation for the argument that Pₐ is a good indicator of hypoxia tolerance in fishes. A
low Pₐ is associated with greater CₐO₂ and therefore improved O₂ delivery to tissues during
environmental hypoxia exposure, which presumably enhances energy supply and reduces the
accumulation of deleterious anaerobic end-products thus improving hypoxia tolerance.
Importantly, my findings also show that hypoxia exposures that are standardized to Pₐ will
yield comparable levels of arterial hypoxemia, facilitating cross-species comparative
analyses. However, the results of the accompanying study (Chapter 5) warn that even when
hypoxia exposures are scaled to Pₐ, tissue-specific differences may occur in the metabolic
response to the same amount of circulating O₂.

Although my measurements of plasma [lactate] showed no difference in the onset
PₐO₂ or the rate of accumulation of lactate between species despite differing Pₐs, the ability
to detect such a difference may have been obscured by non-steady state lactate dynamics
during initial hypoxia exposure. Accumulation of lactate in each species did occur at or
below Pₐ providing equivocal support for the hypothesis that Pₐ is associated with
increased activation of anaerobic metabolism (Pörtner and Grieshaber, 1993). Investigations
of lactate turnover in hypoxia-exposed fishes, including consideration of the potential effect
of metabolic depression (which could blunt lactate accumulation), are needed to further test
this hypothesis.

The superior O₂ uptake and blood O₂ capacity during environmental hypoxia
exposure in epaulette sharks likely partly explains the renowned hypoxia tolerance of this
elasmobranch (Nilsson and Renshaw, 2004). Epaulette sharks also show enhanced hypoxic cardiovascular function compared with shovelnose rays (Chapter 5), which further improves O₂ delivery to tissues. Maintenance rather than depression of O₂ supply and aerobic metabolism at low levels of PₜO₂ may be an important component of hypoxia tolerance in fishes.

4.6 ACKNOWLEDGEMENTS

Kevin and Kathy Townsend and their staff at the Moreton Bay Research Station provided exemplary logistical and technical assistance. Craig Franklin kindly lent us equipment needed for a portion of this study. Miles Gray assisted with hypoxia exposures and sampling. Funding was provided by the Discovery Grant Program from NSERC to J.G. Richards, C.J. Brauner, A. P. Farrell, and Y. X. Wang. Funding was provided from Sea World Research and Rescue Foundation to G. M. C. Renshaw. A. J. R. Hickey was supported by the University of Auckland Early Career Research Excellence award. C.J.B. was supported by a Killam Faculty Research Fellowship. B. Speers-Roesch was supported by a Pacific Century Graduate Scholarship from the University of British Columbia and the Province of British Columbia, a War Memorial Scholarship from IODE Canada, a Journal of Experimental Biology Travelling Fellowship from the Company of Biologists, a Comparative Physiology and Biochemistry Student Research Grant from the Canadian Society of Zoologists, and a Graduate Travel Award from the Department of Zoology, University of British Columbia.
Table 4.1. Arterial hematocrit, [hemoglobin], mean cellular hemoglobin content, plasma [glucose], plasma [β-hydroxybutyrate], [HCO₃⁻], P<sub>CO</sub>₂, and [total CO<sub>2</sub>] of epaulette sharks and shovelnose rays exposed to progressive decreases in water P<sub>O</sub>₂, and after a subsequent 60 min of recovery in normoxic water.

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<tr>
<th>Sample</th>
<th>Time (min)</th>
<th>P&lt;sub&gt;O&lt;/sub&gt;₂ (kPa)</th>
<th>Hct (%)</th>
<th>[Hb] (mM)</th>
<th>MCHC ([Hb]/Hct)</th>
<th>[Glucose] (mM)</th>
<th>[β-HB] (mM)</th>
<th>[HCO₃⁻] (mM)</th>
<th>P&lt;sub&gt;CO&lt;/sub&gt;₂ (kPa)</th>
<th>[Total CO&lt;sub&gt;2&lt;/sub&gt;] (mM)</th>
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Data are means±s.e.m. (n=4–6 for epaulette sharks except for recovery where n=3; n=5–6 for shovelnose rays). Total water P<sub>O</sub>₂ ranges of exposures: approximately 16.0 kPa and 15.3 kPa (normoxia) to approximately 0.1 kPa and 1.6 kPa, in epaulette sharks and shovelnose rays, respectively; total durations of exposures from initial blood sample 1 and closing of respirometer to P<sub>O</sub>₂ nadir: 135±8 min and 71±6 min, respectively; see Materials and methods for further details. P<sub>CO</sub>₂, water P<sub>O</sub>₂; Hct, hematocrit; Hb, hemoglobin; MCHC, mean cellular hemoglobin content; β-HB, β-hydroxybutyrate; P<sub>CO</sub>₂, arterial PCO₂. * denotes value is significantly different from the resting value (sample 1) within species (sample 2-6 for both species: two-way ANOVA with Holm-Sidak test; sample 7-8 for epaulette sharks: one-way ANOVA with Holm-Sidak test; recovery values for both species: two-way ANOVA with Holm-Sidak test; p<0.05); † denotes that the shovelnose ray value is significantly different from that of the epaulette shark at the same sample point, for samples 1-6 or recovery (two-way ANOVA’s with Holm-Sidak tests, p<0.05). Time and P<sub>a</sub>O₂ were excluded from statistical analyses.
Figure 4.1. Whole-animal oxygen consumption rate ($\dot{M}_{O_2}$) and critical O$_2$ tension ($P_{\text{crit}}$) values of epaulette sharks (closed circles) and shovelnose rays (open circles) exposed to progressive decreases in water PO$_2$ ($P_\text{wO}_2$). See Table 1 and Materials and methods for further information on time course and starting and ending $P_\text{wO}_2$ of the exposures. Data are means±s.e.m. ($n=7$ for epaulette sharks, $n=8$ for shovelnose rays). * denotes statistically significant difference from the first normoxic resting value within species. $\Psi$ denotes statistically significant difference between species for $P_{\text{crit}}$, and denotes a statistically significant difference between the epaulette shark value and the shovelnose ray value bracketed by a horizontal { (where the two species values were taken at statistically similar $P_\text{wO}_2$); in the absence of $\Psi$ the two bracketed species values are not statistically different from each other. See Materials and methods for details on statistical methods.
Figure 4.2. Arterial PO$_2$ (P$_a$O$_2$) (A) and arterial O$_2$ content (C$_a$O$_2$) (B) of epaulette sharks (closed circles) and shovelnose rays (open circles) exposed to progressive decreases in water PO$_2$ (P$_w$O$_2$), and after a subsequent 60 min of recovery in normoxic water. See Table 1 and Materials and methods for further information on time course and starting and ending P$_w$O$_2$ of the exposures. Data are means±s.e.m. (n=4-6 for epaulette sharks except for recovery where n=3; n=5-6 for shovelnose rays). Recovery values are offset for clarity. * denotes statistically significant difference from the first, normoxic resting value at ≥15.3 kPa within species. Ψ denotes that the shovelnose ray value is significantly different from that of the epaulette shark at the same sample point. See Materials and methods for details on statistical methods. Recovery values were not significantly different between species or when compared to resting values in both species.
Figure 4.3. Arterial hemoglobin-O\textsubscript{2} (Hb-O\textsubscript{2}) saturation of epaulette sharks (closed circles) and shovelnose rays (open circles) exposed to progressive decreases in water PO\textsubscript{2} (P\textsubscript{w}O\textsubscript{2}), and after a subsequent 60 min of recovery in normoxic water. In vivo Hb-O\textsubscript{2} P\textsubscript{50}'s and Hill coefficients (n\textsubscript{H}) were calculated from Hill plots (see Materials and methods) and are presented in the figure panel. At each species’ P\textsubscript{50}, arterial pH was ~7.82 in epaulette sharks and ~7.81 in shovelnose rays and P\textsubscript{a}CO\textsubscript{2} was ~0.23 kPa in both species (extrapolated from pH and P\textsubscript{a}CO\textsubscript{2} data at the extrapolated P\textsubscript{w}O\textsubscript{2} at P\textsubscript{50}). See Materials and methods for details on calculation of Hb-O\textsubscript{2} saturation. See Table 1 and Materials and methods for further information on time course and starting and ending P\textsubscript{w}O\textsubscript{2} of the exposures. Data are means±s.e.m. (n=4-6 for epaulette sharks except for recovery where n=3; n=5-6 for shovelnose rays). Recovery values are offset for clarity. * denotes statistically significant difference from the first, normoxic resting value at ≥15.3 kPa within species. ′′ denotes that the shovelnose ray value for Hb-O\textsubscript{2} P\textsubscript{50} is significantly different from that of the epaulette shark. The Hill coefficients were not significantly different between species. See Materials and methods for details on statistical methods. Recovery values were not significantly different between species or when compared to resting values in both species.
Figure 4.4. Hemoglobin-O₂ (Hb-O₂) saturation as a function of water PO₂ (P₇O₂) represented as percentage of P₇_cri in epaulette sharks (closed circles) and shovelnose rays (open circles) exposed to progressive decreases in P₇O₂ (i.e., P₇_cri of each species occurs at 100%). Data are means±s.e.m. (n=4-6 for epaulette sharks; n=5-6 for shovelnose rays). The linear regression is statistically significant (p<0.0001, linear regression).
Figure 4.5. Arterial plasma lactate (A) and arterial blood pH (B) of epaulette sharks (closed circles) and shovelnose rays (open circles) exposed to progressive decreases in water PO$_2$ (P$_{wO_2}$), and after a subsequent 60 min of recovery in normoxic water. See Table 1 and Materials and methods for further information on time course and starting and ending P$_{wO_2}$ of the exposures. Data are means±s.e.m. (n=4-6 for epaulette sharks except for recovery where n=3; n=5-6 for shovelnose rays). Recovery values are offset for clarity. * denotes statistically significant difference from the first, normoxic resting value at ≥15.3 kPa within species. ψ denotes that the shovelnose ray value is significantly different from that of the epaulette shark at the same sample point.
CHAPTER 5: HYPOXIA TOLERANCE IN ELASMOBRANCHS. II.
CARDIOVASCULAR FUNCTION AND TISSUE METABOLIC
RESPONSES DURING PROGRESSIVE AND RELATIVE HYPOXIA
EXPOSURES

5.1 SYNOPSIS

Cardiovascular function and metabolic responses of the heart and other tissues during environmental hypoxia exposure were compared between the hypoxia-tolerant epaulette shark (*Hemiscyllium ocellatum*) and the hypoxia-sensitive shovelnose ray (*Aptychotrema rostrata*). The goals of this study were to investigate 1) if the enhanced blood O$_2$ transport in the epaulette shark (Chapter 4) was associated with enhanced hypoxic cardiovascular function, and 2) how cardiovascular and metabolic hypoxic responses associated with cardiac hypoxia tolerance differ in hypoxia-tolerant and -sensitive species. The results of this study also provided a test of the hypothesis that hypoxic depression of cardiac power output ($PO$, an assessment of cardiac energy demand) is associated with the depression of whole-animal O$_2$ consumption rate ($\dot{M}_{O_2}$) below the critical water PO$_2$ for $\dot{M}_{O_2}$ ($P_{crit}$). In both species, progressive hypoxia exposure caused increases in stroke volume and decreases in heart rate, cardiac output, $PO$, and dorsal aortic blood pressure, all of which occurred at or below each species’ $P_{crit}$. In epaulette sharks, which have a lower $P_{crit}$ than shovelnose rays, routine levels of cardiovascular function were maintained to lower water PO$_2$ levels and the changes from routine levels during hypoxia exposure were smaller compared with those for the shovelnose ray. The maintenance rather than depression of cardiovascular function during environmental hypoxia exposure may contribute to the superior hypoxia tolerance of the epaulette shark,
presumably by improving $O_2$ delivery and waste removal. Compared with shovelnose rays, epaulette sharks were also better able to maintain a stable cardiac high-energy phosphate pool and to minimize metabolic acidosis and lactate accumulation in heart (despite higher $PO$) and other tissues during a 4 h exposure to 40% of their respective $P_{crit}$ (referred to as a relative hypoxia exposure), which results in similar hypoxemia in the two species (~16% Hb-$O_2$ saturation). These different metabolic responses to relative hypoxia exposure suggest that variation in hypoxia tolerance among species is not solely dictated by differences in $O_2$ uptake and transport, but also by tissue-specific metabolic responses. In particular, lower tissue [lactate] accumulation in epaulette sharks compared with shovelnose rays during relative hypoxia exposure suggests that enhanced extra-cardiac metabolic depression occurs in the former species. This could facilitate strategic utilization of available $O_2$ for vital organs such as the heart, potentially explaining the greater hypoxic cardiovascular function of epaulette sharks.

5.2 INTRODUCTION

The ability to tolerate environmental hypoxia varies greatly among fishes and the physiological attributes that underlie this variation in hypoxia tolerance remain incompletely understood. A growing body of evidence from comparative studies suggests that one important determinant of hypoxia tolerance in fishes is the ability to maintain $O_2$ uptake at low water $PO_2$ ($P_{wO_2}$), which is reflected by the $P_wO_2$ at which whole-animal $O_2$ consumption rate ($\dot{M}_{O_2}$) transitions from being independent to dependent on environmental $O_2$; this inflection point is termed $P_{crit}$ (Mandic et al., 2009; Chapter 4). At $P_wO_2$ below $P_{crit}$, hypoxic survival becomes dependent on maintenance of cellular energy balance in the face of
decreases in aerobic energy supply (Richards, 2009). This may be achieved by an increased reliance on O$_2$-independent energy supply (e.g. anaerobic glycolysis) as well as a profound, reversible metabolic rate depression (MRD) in which large decreases in cellular energy demand occur (Richards, 2009).

The cardiovascular system is an essential component of the respiratory cascade and is driven by the heart, a hypoxia-sensitive organ with high-energy demands. In fishes exposed to environmental hypoxia the cardiovascular system is vital for transport of available O$_2$ as well as in the distribution of fermentable fuel and the removal of waste. However, maintenance of cardiac function may be constrained by the limited energy supply during hypoxia exposure and in hypoxia-sensitive species catastrophic cardiac failure may occur as a result of a mismatch between energy supply and demand in the heart that leads to perturbed cardiac energy status (i.e., the levels of high-energy phosphate compounds) (Farrell and Stecyk, 2007). Hypoxia-tolerant fishes are able to avoid this fate, but the cardiovascular responses during environmental hypoxia exposure (including P$_w$O$_2$ at and below P$_{crit}$) that are associated with hypoxia tolerance remain unclear, in part because the responses reported are varied and because of the relatively few species studied. In most fishes exposed to hypoxia, bradycardia is observed and this generally occurs when P$_w$O$_2$ reaches P$_{crit}$, suggesting that hypoxia-tolerant fishes with lower P$_{crit}$ may be able to maintain heart rate ($f_h$) to lower P$_w$O$_2$ (Farrell, 2007; Chapter 2). However, responses of other cardiovascular parameters at and below P$_{crit}$ are more varied, confusing interpretation of their role in hypoxia tolerance. In some fishes, routine cardiac output ($\dot{Q}$) is defended to an extent by increases in stroke volume ($V_{SH}$), even as P$_w$O$_2$ falls below P$_{crit}$ (Butler and Taylor, 1975; Gamperl and Driedzic, 2009; Gamperl et al., 1994; Petersen and Gamperl, 2011). In other fishes, $V_{SH}$ is unchanged.
and \( \dot{Q} \) falls due to the decrease in \( f_{II} \) below \( P_{\text{crit}} \) (Iversen et al., 2010; Chapter 2; Stecyk and Farrell, 2006). Studies on fish species that allow \( \dot{Q} \) to fall during environmental hypoxia exposure, including tilapia (\textit{Oreochromis} hybrid) and common carp (\textit{Cyprinus carpio}), suggest that the reductions in \( f_{II} \) and \( \dot{Q} \) below \( P_{\text{crit}} \) enable a decrease in cardiac power output \( (PO) \), which represents a lowering of cardiac energy demand (Chapter 2; Stecyk and Farrell, 2006). Depression of \( PO \) may be an important component of hypoxia tolerance because it allows cardiac energy demand to be matched to reduced energy supply at \( P_wO_2 \) below \( P_{\text{crit}} \) where aerobic metabolism is limited, thus facilitating the maintenance of stable cardiac energy status and function (Farrell and Stecyk, 2007). The crucian carp (\textit{Carassius carassius}), in contrast, has a low routine \( PO \) that apparently can be sustained anaerobically, probably explaining how its \( \dot{Q} \) can remain relatively unchanged during hypoxia exposure (Stecyk et al., 2004). Overall, there is uncertainty about the degree to which depression or maintenance of cardiovascular function including \( PO \) is associated with hypoxia tolerance and \( P_{\text{crit}} \) in fishes.

Here, I carried out two series of experiments to assess cardiovascular and heart metabolic responses to progressive and relative hypoxia exposure in two species of elasmobranchs, the hypoxia-tolerant epaulette shark (\textit{Hemiscyllium ocellatum}) and the comparatively hypoxia-sensitive Eastern shovelray (\textit{Aptychotrema rostrata}). In one series of experiments, I report on cardiovascular function in epaulette sharks and shovelrays during progressive hypoxia exposure to examine how cardiovascular responses at and below \( P_{\text{crit}} \) compare between a hypoxia-tolerant and a hypoxia-sensitive elasmobranch given the lack of previous direct comparisons of the hypoxic responses of \( PO \) between hypoxia-tolerant and -sensitive fishes. I hypothesized that routine cardiovascular function is
maintained to a lower $P_{wO_2}$ in the epaulette shark due to its greater hypoxia tolerance and lower $P_{crit}$ compared with the shovelnose ray (see Chapter 4). At progressively lower $P_{wO_2}$ below $P_{crit}$, I hypothesized that there would be less depression of cardiovascular function including $PO$ in the epaulette shark compared with the shovelnose ray because of the enhanced $O_2$ transport at low $P_{wO_2}$ (Chapter 4). Finally, I hypothesized that hypoxic depression of $PO$ would be associated with the decreases in whole-animal $\dot{M}_{O_2}$ below $P_{crit}$, as seen in tilapia (Chapter 2). In a second series of experiments, I assessed metabolic status (i.e., pH and levels of metabolites of energy metabolism, e.g., high-energy phosphates and lactate) in cardiac and other tissues of hypoxic epaulette sharks and shovelnose rays held for $\leq 4$ h at a $P_{wO_2}$ representing the same percentage of each species’ $P_{crit}$. In Chapter 4, it was revealed that exposure to the same percent of $P_{crit}$ in these two species would yield a similar level of hypoxemia. Therefore, the present experiment allowed us to assess whether the greater hypoxia tolerance of epaulette sharks is associated with more stable hypoxic cardiac and extra-cardiac metabolic status compared with the shovelnose ray, even when variation in interspecific $O_2$ supply is controlled for. In turn, this allowed us to test the hypothesis that the ability to take up and transport $O_2$ at low $P_{wO_2}$, as indicated by $P_{crit}$, dictates tissue-level hypoxia tolerance. In this case, I predicted that tissues of the two species during relative hypoxia exposure would have similar energy status, lactate accumulation, and pH levels. Overall, these experiments and those in Chapter 4 provide insight into the cardiorespiratory and metabolic responses that contribute to hypoxia tolerance in elasmobranchs and other fishes.
5.3 MATERIALS AND METHODS

5.3.1 Animals

Epaulette sharks and shovelnose rays of mixed sexes were collected and held in a recirculating seawater system (28°C) at Moreton Bay Research Station, North Stradbroke Island, QLD, Australia, as described in Chapter 4.

5.3.2 Experimental series I: Cardiovascular responses to progressive hypoxia

5.3.2.1 Surgical protocol

Experimental series I is the same experiment as that described in Chapter 4 and the preparation of animals for surgery is fully described therein. In brief, cardiovascular responses to progressive hypoxia were monitored in epaulette sharks (1.29±0.04 kg, n=7) and shovelnose rays (1.54±0.06 kg, n=8) simultaneously with the measurements of \( \dot{M}_{O_2} \) and blood \( O_2 \) transport properties. Measurement of dorsal aortic blood pressure (\( P_{DA} \)) was via the caudal artery cannula that also allowed periodic blood sampling. To measure ventral aortic blood flow (i.e., cardiac output or \( \dot{Q} \)), an ultrasonic flow probe was fitted around the ventral aorta via a midline ventral incision made through the skin and overlying muscle anterior from the fifth gill slit. The connective tissue surrounding the ventral aorta was inspected for superficial vessels before being cut to expose the ventral aorta. Where present, vessels were ligatured with 4-0 silk to prevent bleeding. A 2.5 mm ultrasonic SB-type flow probe (Transonic Systems, Ithaca, NY, USA) filled with acoustic gel (Transonic Systems) was then fitted around the exposed ventral aorta distal to the third, fourth, and fifth afferent branchial arteries. This flow probe placement allows measurement of approximately 37% of \( \dot{Q} \) in elasmobranchs and this value does not change during hypoxia exposure (Taylor et al., 1977;
Lai et al., 1989 and references therein). *In vivo* measurement of total $\dot{Q}$ is not possible in elasmobranchs because the afferent branchial arteries for the posterior gill arches arise as the conus arteriosus exits the pericardium and therefore no portion of the ventral aorta outside of the pericardium carries the entire cardiac output. Entering the pericardium is not an option in elasmobranchs because of its importance for cardiovascular function (Franklin and Davie, 1993; Stensløkken et al., 2004). After placement, the flow probe was secured with two 4-0 silk sutures tied to the surrounding muscle. The muscle incision was closed with interrupted 4-0 silk sutures and then the skin incision was closed with interrupted 1-0 silk sutures. The lead of the flow probe was secured to the skin and tied to the arterial cannula to prevent entanglement.

5.3.2.2 *Experimental protocol*

The experimental protocol and other details for the progressive hypoxia exposure are described in the “Experimental protocol” section of Chapter 4. Routine cardiovascular variables ($f_H, \dot{Q}, P_{DA}$) were continuously recorded at a normoxic $P_{aO_2}$ of approximately 16.0 kPa or 15.3 kPa for epaulette sharks and shovelnose rays, respectively, (=75-78% air saturation; 100% air saturation=20.4 kPa=153 torr) for 1-2 h to ensure stable baseline conditions. After initial blood sampling and closing of the respirometer in which animals were exposed to progressive hypoxia, cardiovascular parameters were continuously monitored as $P_{aO_2}$ was depleted as a consequence of fish respiration. The effects on cardiovascular function of changes in water parameters (e.g. pH, $P_{CO_2}$) potentially associated with utilization of closed respirometry are considered to be negligible (see Chapter 2). In fact, in both species increases in arterial $P_{CO_2}$ were minor and apparently had no effect on
other measured parameters during progressive hypoxia exposure, as described in Chapter 4. The \( P_{\text{w}O_2} \) end points, rate of \( O_2 \) depletion, durations of the progressive hypoxia exposures, and recovery in normoxic water for 60 min are described in Speers-Roesch et al. (submitted). In some cases, flow probes became damaged by water exposure or by fish movements (typically during the overnight acclimation period) and therefore samples sizes of measured parameters vary slightly (see figure captions for final \( n \) values). At the end of the trials, fishes were terminally anesthetized in seawater containing benzocaine and the ventricle was excised, emptied of blood, blotted dry, and weighed.

5.3.2.3 Data acquisition and calculation of cardiovascular variables

The dorsal aortic cannula was connected to a pressure transducer (Capto SP844 model MLT844, MEMSCAP AS, Skoppum, Norway) calibrated against a static water column with the water surface in the experimental tank serving as zero pressure reference. The transducer signal was amplified with a ML221 bridge amplifier (ADInstruments, Castle Hill, NSW, Australia). Dorsal aortic blood pressure recordings made in the respirometer were compensated for the small pressure change (~0.5 kPa) that occurred depending on if the respirometer was open or closed. The cannula was temporarily disconnected to allow for the periodic blood sampling described in Speers-Roesch et al. (submitted). Cardiac output was recorded with a Transonic blood flow meter (Model T206, Transonic Systems, Ithaca, NY, USA). Flow probes were calibrated according to manufacturer guidelines at 28°C following the experiment to compensate for the effect of calibration temperature on flow readings. Water \( \text{PO}_2 \) in the respirometer was monitored as described in Chapter 4. Signal integration
and analysis was carried out using a Power Lab unit (ADInstruments, Castle Hill, NSW, Australia) and LabChart Pro software (v. 6.0; ADInstruments), respectively.

Cardiovascular parameters were analyzed over 5-10 min sampling periods bracketing \( P_{\text{w}O_2} \) values at regular intervals that were similar to those used for \( \dot{M}_{O_2} \) calculation, from approximately 16.0 kPa to 0.1 kPa in epaulette sharks and approximately 16.0 kPa to 1.9 kPa in shovelnose rays. Due to periodic blood sampling and routine variability in individual traces, it was not possible to analyze data at exactly the same \( P_{\text{w}O_2} \) values in each fish, so \( P_{\text{w}O_2} \) values are provided with standard errors. Cardiovascular function during 60 min of recovery in normoxic water was analyzed over 5-10 min intervals bracketing each time point (5, 15, 30, 45, 60 min).

Cardiac output was calculated directly from the flow trace in LabChart Pro and corrected for the estimated loss of flow (63%) due to the location of the flow probes on the ventral aorta, as discussed previously (Lai et al., 1989; Taylor et al., 1977). \( P_{\text{DA}} \) was calculated using the blood pressure analysis module in LabChart Pro. Due to limited animal numbers, it was not possible to directly measure ventral aortic blood pressure (\( P_{\text{VA}} \)). Therefore, \( P_{\text{VA}} \) was estimated from the \( P_{\text{DA}} \) measurements, using a percentage correction (\( P_{\text{VA}} = 1.3 \times P_{\text{DA}} \)) based on previous simultaneous measurements of \( P_{\text{VA}} \) and \( P_{\text{DA}} \) in normoxia and hypoxia in epaulette sharks and spotted catsharks (\textit{Scylliorhinus canicula}) (Short et al., 1979; Stensløkken et al., 2004; Taylor et al., 1977). Cardiac power output (\( P_O \), mW g\(^{-1}\) wet ventricular mass) was calculated as the product of \( P_{\text{VA}} \) (kPa) and \( \dot{Q} \) (mL s\(^{-1}\)) divided by the wet ventricular mass (g), where 1 J = 1 kPa L. Heart rate (\( f_H \)) was calculated from the pulsatile pressure or flow trace. Cardiac stroke volume (\( V_{SH} \)) was calculated as \( \dot{Q} / f_H \) and systemic peripheral resistance (\( R_{SYS} \)) was calculated as \( P_{\text{DA}} / \dot{Q} \), with the assumption that
central venous blood pressure is zero. Cardiovascular parameters were plotted against $P_wO_2$ to identify the inflection points where each parameter ceased to be independent of $P_wO_2$ (i.e., the critical $P_wO_2$ of each cardiovascular parameter) as previously described for calculation of $P_{crit}$ of whole-animal $\dot{M}_O_2$ (see Chapter 4).

5.3.3 Experimental series II: Tissue metabolic status during relative hypoxia exposure

5.3.3.1 Experimental protocol

Epaulette sharks (0.388±0.048 kg) and shovelnose rays (1.07±0.110 kg) were transferred from holding tanks to aquaria (~300 L) supplied with aerated recirculating filtered seawater (28°C). Six to ten epaulette sharks were distributed equally between two aquaria and the same was done for shovelnose rays in two separate aquaria. The fishes were allowed to acclimate for 12 h under well-aerated conditions. Then, one or two fish from the normoxic control group were gently removed from each aquarium and quickly immersed in a bucket of aerated seawater containing benzocaine (0.2 g L$^{-1}$ benzocaine, initially dissolved in 95% ethanol). Animals struggled minimally during this procedure. Following anaesthesia (<1 min), mixed arterial-venous blood was sampled via caudal puncture and placed on ice until measurement of whole blood pH as described in Speers-Roesch et al. (submitted). Following blood sampling, the fish was sacrificed by severing the spinal cord posterior to the head. The heart was quickly removed, emptied of blood, blotted dry, and frozen in liquid N$_2$. White muscle from the caudal peduncle, liver, and plasma obtained by centrifuging whole blood were also sampled. Frozen samples were transported to Canada in a dry shipper and kept at -80°C until analysis.
Following the sampling of the normoxic fishes, hypoxia was induced by bubbling N\textsubscript{2} into each aquarium, which was covered with plastic bubble wrap to prevent O\textsubscript{2} ingress. Epaulette sharks were exposed to a P\textsubscript{w}O\textsubscript{2} of 2.0 kPa (10\% air saturation) and shovelnose rays were exposed to 3.1 kPa (15\% air saturation), both of which represented 40\% of each species’ P\textsubscript{crit} and thus resulted in a similar level of physiological hypoxia (~16\% Hb-O\textsubscript{2} saturation; ~0.6 vol \% arterial O\textsubscript{2} content; see Chapter 4). These exposures are referred to henceforth as relative hypoxia. The hypoxic levels were reached after approximately 30 min of N\textsubscript{2} bubbling. Levels of O\textsubscript{2} were monitored using handheld O\textsubscript{2} meters and manually adjusted as needed by N\textsubscript{2} bubbling. At 2 h and 4 h of hypoxia exposure, fishes were sampled as previously described. This entire protocol was then repeated on subsequent days to achieve a total sample size of 4 to 7 fish per species and time point.

In a separate trial, the same protocol was repeated except epaulette sharks were exposed to a P\textsubscript{w}O\textsubscript{2} of 1.0 kPa (5\% air saturation) and shovelnose rays to 2.0 kPa (10\% air saturation) in order to investigate the effects of deeper hypoxia in both species and to compare the effect of a similar level of environmental hypoxia (2.0 kPa) between species. At 2.0 kPa, however, shovelnose rays succumbed to hypoxia in <30 min, negating sampling, but epaulette sharks tolerated 1.0 kPa, allowing sampling at 2 h and 4 h as described previously.

\textit{5.3.3.2 Analytical protocols}

Frozen tissue was broken into small pieces under liquid N\textsubscript{2} using an insulated mortar and pestle. For extraction of metabolites, 1.0 mL of ice-cold 1 M HClO\textsubscript{4} was added to a microcentrifuge tube containing 50-100 mg of tissue and the mixture was immediately sonicated on ice for three bursts of 10 s using a Kontes sonicator on its highest setting. An
aliquot was frozen at -80°C for measurement of [glycogen] (Bergmeyer, 1983). The remaining homogenate was centrifuged (10000 g; 10 min; 4°C) and the supernatant neutralized with 3 M K₂CO₃. Neutralized extracts were assayed spectrophotometrically for [adenosine triphosphate] ([ATP]), [CrP], [creatine] (heart only), [lactate], and [glucose] following methods described elsewhere (Bergmeyer, 1983). [Glycogen] was corrected for measured endogenous glucose levels. Intracellular pH (pHᵢ) was measured in frozen heart tissue using the methods of Pörtner et al. (1991b), as validated by Baker et al. (2009), and a thermostatically controlled (28°C) BMS3 Mk2 capillary microelectrode with PHM84 pH meter (Radiometer, Copenhagen, Denmark). Levels of cardiac adenosine diphosphate and adenosine monophosphate (ADP_free and AMP_free) were calculated as described in Speers-Roesch et al. (2010). Blood pH and plasma [lactate], [glucose], and [β-HB] were measured as described in Speers-Roesch et al. (submitted).

5.3.4 Statistics

The effects of species and O₂ on cardiovascular measurements (experimental series I) were tested via a two-way ANOVA with Holm-Sidak (H-S) post-hoc tests using data from eleven sampling points of overlapping P_wO₂ at approximately 16.0, 13.3, 11.7, 10.4, 8.3, 6.1, 5.1, 4.3, 3.1, 2.4, and 1.9 kPa and the points of statistical comparison are denoted by horizontal “{” on the figures. Overlapping P_wO₂’s were not statistically different between species (Student’s t-test, p>0.05). Data from other sampling points were omitted from these analyses. In order to fully assess the effect of O₂ on cardiovascular parameters in each species, one-way ANOVA’s were run across all sampling points within each species, with H-S comparisons against the first normoxic resting value. The effect of O₂ on measured
parameters was found to be similar for both two-way and one-way ANOVA designs. The critical $P_{wO_2}$’s of cardiovascular variables were compared to each other and between species using a two-way ANOVA with H-S tests. The critical $P_{wO_2}$ of $\dot{M}_{O_2}$ ($P_{crit}$) measured in Chapter 4 were included in this analysis to determine if critical $P_{wO_2}$’s of cardiovascular variables coincided with $P_{crit}$. Cardiovascular recovery values were compared between species and to the normoxic resting values measured at ~16.0 kPa using a two-way ANOVA with H-S tests.

The effects of species and hypoxia exposure on physiological parameters in fishes exposed to relative hypoxia (experimental series II) were tested using a two-way ANOVA with H-S tests. The data for epaulette sharks exposed to a $P_{wO_2}$ of 1.0 kPa for 2 h were omitted from these analyses, but these were compared to the normoxic control using a Student’s t-test.

Statistical significance was accepted when $p<0.05$. Analyses were carried out using SigmaStat 3.0. Data were log or square root transformed prior to statistical analyses if assumptions of equal variance or normality were not met. Repeated measures ANOVA was not used because experimental constraints negated the use of data from the same animal at every sample period. In any case, the standard ANOVA procedures utilized here results in a conservative statistical assessment of the data.

5.4 RESULTS

5.4.1 Experimental series I: Cardiovascular responses to progressive hypoxia

A hypoxia-induced bradycardia was observed in both species, with the onset (i.e., the critical $P_{wO_2}$ of heart rate) occurring at a significantly lower $P_{wO_2}$ in the epaulette sharks
compared with the shovelnose rays (Fig. 5.1A; Table 5.1). At P_{w}O_{2} higher than \sim 7.3 kPa, f_{H} was similar in both species, but at lower P_{w}O_{2}, f_{H} was greater in epaulette sharks, even after bradycardia commenced in both species (Fig. 5.1A). Heart rate showed a plateau below \sim 3.3 kPa in shovelnose rays and both species showed a maximal f_{H} depression of approximately 65% compared with normoxic resting values (Fig. 5.1A).

The responses of \dot{Q} and PO to progressive hypoxia exposure paralleled that of f_{H}, with the decreases commencing at a lower P_{w}O_{2} in the epaulette sharks compared with the shovelnose rays (Table 5.1; Fig. 5.1B,C). Above \sim 6.0 kPa, \dot{Q} was similar in both species, but it was significantly greater in epaulette sharks at lower P_{w}O_{2} (Fig. 5.1B). A maximal depression of \dot{Q} of approximately 50% and 60% was observed in shovelnose rays and epaulette sharks, respectively (Fig. 5.1B). Cardiac power output was significantly higher in epaulette sharks at all P_{w}O_{2}, and at similar P_{w}O_{2} below the epaulette shark critical point for PO, the percentage depression from the normoxic resting value was always greater in the shovelnose rays (e.g., at \sim 3.1 kPa, PO in epaulette sharks is 30% lower than the resting level compared with 55% lower in shovelnose rays; at \sim 1.9 kPa, PO is decreased 40% in epaulette sharks and 75% in shovelnose rays) (Fig. 5.1C).

In both species, V_{SH} was similar under normoxic resting conditions and increased by 30-40% in response to progressive hypoxia exposure, with a plateau seen in shovelnose rays below \sim 3.3 kPa (Fig. 5.2A). The increase occurred at a significantly lower P_{w}O_{2} in epaulette sharks compared with shovelnose rays (Table 5.1). A decrease in P_{DA} (and calculated P_{VA} (data not shown)) of up to 45% was observed in both species during progressive hypoxia exposure (Fig. 5.2B). This decrease commenced at a significantly lower P_{w}O_{2} in epaulette sharks compared with shovelnose rays (Table 5.1). At P_{w}O_{2} below \sim 4.0 kPa, P_{DA} was higher.
in epaulette sharks, whereas at higher $P_wO_2$, $P_{DA}$ was similar in the two species (Fig. 5.2B). Systemic peripheral resistance was similar for both species and increased modestly during progressive hypoxia exposure in both species but reached statistical significance only for epaulette sharks (Fig. 5.2C). Critical $P_wO_2$ values for $R_{SYS}$ were not calculated because of the absence of major changes with decreasing $P_wO_2$.

The relationship between the critical $P_wO_2$’s of cardiovascular parameters and the critical $P_wO_2$ of $M_o\dot{O}_2$ ($P_{crit}$) measured in Chapter 4 were relatively similar between species. In each species the critical $P_wO_2$’s of $\dot{Q}$, $PO$, and $f_H$ were statistically similar to one another and to $P_{crit}$ (Table 5.1). In each species the critical $P_wO_2$ of $V_{SH}$ was significantly lower than that of $\dot{Q}$ only, except in epaulette sharks where it was also lower than the $P_{crit}$ (Table 5.1). In shovelnose rays the critical $P_wO_2$ of $P_{DA}$ was significantly lower than that of all other cardiovascular parameters as well as $P_{crit}$ whereas in epaulette sharks the same was true except that the critical $P_wO_2$’s for $V_{SH}$ and $f_H$ were not significantly different from $P_{DA}$ (Table 5.1).

During the first 30 min of normoxic recovery from progressive hypoxia exposure, $f_H$ in shovelnose rays was significantly elevated above the normoxic resting value. In contrast, $f_H$ in epaulette sharks was significantly lower than the resting value after 5 min of recovery, returning to resting values by 15 min of recovery (Fig. 5.1A). Cardiac output returned to resting values within 5 min of recovery in both species and remained unchanged for the full 60-min recovery period (Fig. 5.1B). Cardiac power output returned to resting values after 5 min of recovery in both species, but as recovery progressed $PO$ continued to increase to above resting values in epaulette sharks but not shovelnose rays (Fig. 5.1C). The same response was seen for $P_{DA}$ (and $P_{VA}$ (data not shown)) during recovery resulting in
significantly greater $P_{DA}$ during recovery in epaulette sharks compared with shovelnose rays (Fig. 5.2B). During recovery in both species, $V_{SH}$ and $R_{SYS}$ returned to resting values within 5 min and remained unchanged throughout the 60-min recovery period (Fig. 5.2A,C).

5.4.2 Experimental series II: Tissue metabolic status during relative hypoxia exposure

Cardiac [ATP] was similar in both species and was unaffected by 4 h of relative hypoxia exposure (Fig. 5.3A). Both species had similar normoxic levels of cardiac [CrP]. During the relative hypoxia exposure, heart [CrP] was unchanged in epaulette sharks but decreased significantly in the shovelnose rays (Fig. 5.3B). Cardiac [lactate] was similar in both species under normoxic conditions and increased significantly during relative hypoxia exposure in shovelnose rays but not in epaulette sharks (Fig. 5.4A). Cardiac [glucose] was similar in both species under normoxic conditions and was unaffected by relative hypoxia exposure (Table 5.2). Cardiac [glycogen] was significantly higher in epaulette sharks compared with shovelnose rays in normoxia and relative hypoxia exposure had no effect on cardiac [glycogen] in either species (Table 5.2). Cardiac [ADP$_{free}$] and [AMP$_{free}$] were unchanged by relative hypoxia exposure in epaulette sharks whereas levels of both increased significantly in shovelnose rays (Table 5.2).

Cardiac pH$_i$ was similar between species in normoxia (Fig. 5.5A). Shovelnose rays showed a significant decrease in cardiac pH$_i$ throughout the relative hypoxia exposure, whereas a significant decrease was not observed until 4 h in epaulette sharks. At 2 h of exposure, epaulette sharks had significantly higher cardiac pH$_i$ compared with shovelnose
rays, but not at 4 h (Fig. 5.5A). Blood pH remained unchanged during relative hypoxia exposure in epaulette sharks, but it decreased significantly in shovelnose rays (Fig. 5.5B).

White muscle [ATP] was unchanged in both species during relative hypoxia exposure but liver [ATP] decreased (Table 5.2) and did so more rapidly (within 2 h) in shovelnose rays compared with epaulette sharks (within 4 h). White muscle [CrP] was similar in both species in normoxia but was depleted more rapidly and by a greater amount in shovelnose rays during relative hypoxia exposure. Liver [CrP] was highly variable and unchanged in both species during relative hypoxia exposure (Table 5.2). Epaulette sharks had higher normoxic resting levels of liver [glycogen] compared with shovelnose rays (Table 5.2). Relative hypoxia exposure caused no change in white muscle [glycogen] whereas a significant decrease of liver [glycogen] occurred in shovelnose rays but not epaulette sharks (Table 5.2). White muscle [glucose] decreased after 4 h of relative hypoxia exposure in shovelnose rays but was unchanged in epaulette sharks. In liver, [glucose] was unaffected by relative hypoxia exposure (Table 5.2). [Lactate] in white muscle increased more rapidly and accumulated to a greater amount in shovelnose rays compared with epaulette sharks exposed to relative hypoxia (Fig. 5.4B). Lactate accumulation in liver, however, was similar for the two species (Fig. 5.4C).

Similar to heart and white muscle, plasma [lactate] increased significantly during relative hypoxia exposure in both species, but the magnitude of accumulation was greater in shovelnose rays compared with epaulette sharks (Fig. 5.4D). Plasma [glucose] was comparable between species and was unaffected by relative hypoxia exposure (Table 5.2). Plasma [β-HB] was higher in normoxia in epaulette sharks compared with shovelnose rays but levels were similar in both species during relative hypoxia exposure because plasma [β-
HB] in epaulette sharks decreased significantly whereas levels in shovelnose rays were unchanged (Table 5.2).

Compared with the normoxic group, the responses of metabolite levels in tissues of epaulette sharks exposed to 2 h of hypoxia at 1.0 kPa generally were similar to those exposed to 2.0 kPa (Table 5.2). However, the 2 h exposure at 1.0 kPa caused significant decreases in liver [ATP], white muscle [CrP] and cardiac pH\textsubscript{i} as well as significant increases in cardiac and white muscle [lactate], which at 2.0 kPa were only apparent after 4 h of exposure (Table 5.2; Fig. 5.4A,B; Fig. 5.5A). Qualitatively, a greater accumulation of plasma and liver [lactate] also was apparent in the epaulette sharks exposed to 1.0 kPa for 2 h compared with 2.0 kPa for 2 h (Fig. 5.4C,D). Finally, at 1.0 kPa but not at 2.0 kPa, 2 h of hypoxia exposure caused a significant decrease in cardiac [glycogen] and a trend (p=0.06) of decreased liver [glycogen] (Table 5.2).

5.5 DISCUSSION

The impressive hypoxia tolerance of the epaulette shark is associated with enhanced cardiovascular function and more stable cardiac energy status during environmental hypoxia exposure compared with the less hypoxia-tolerant shovelnose ray. Similar routine levels of cardiovascular function were maintained above P\textsubscript{crit} in the two species (=5.10±0.37 kPa, epaulette shark; 7.23±0.40 kPa, shovelnose ray; Chapter 4). Hypoxic cardiovascular responses occurred at or below P\textsubscript{crit} and always at a lower P\textsubscript{w}O\textsubscript{2} in epaulette sharks. Thus, routine cardiovascular function was maintained to lower P\textsubscript{w}O\textsubscript{2} in epaulette sharks compared with shovelnose rays. Also, epaulette sharks maintained greater levels of cardiovascular function, including higher PO, during environmental hypoxia exposure. Depression of
cardiac energy demand may be of secondary importance for hypoxia tolerance compared with the ability to maintain greater cardiovascular function during environmental hypoxia exposure.

The ability of epaulette sharks to maintain cardiac energy status and to minimize lactate accumulation and metabolic acidosis during hypoxia exposure also is superior to that of shovelnose rays. This is especially significant because the experimental exposure was carried out at a relative percentage of $P_{\text{crit}}$, therefore equalizing the between-species variation in arterial $O_2$ content ($C_aO_2$) (see Chapter 4). Thus, while $P_{\text{crit}}$ accurately reflects $O_2$ transport during hypoxia exposure (Chapter 4), it does not necessarily reflect tissue-level hypoxia tolerance, which may be affected by other factors such as tissue-specific MRD. The more stable cardiac energy status of the epaulette shark is not apparently related to greater depression of cardiac energy demand, but perhaps is due to greater $O_2$ delivery to the heart as a result of enhanced $\dot{Q}$ as well as extra-cardiac metabolic depression. Strategic $O_2$ delivery to the heart, as well as greater blood $O_2$ capacity (Chapter 4), may help explain the enhanced hypoxic cardiovascular function in the epaulette shark compared with the shovelnose ray.

### 5.5.1 Cardiovascular responses to progressive hypoxia and recovery

With the exception of $PO$, which was higher in shovelnose rays, normoxic levels of cardiovascular function were similar between epaulette sharks and shovelnose rays (Fig. 5.1A,B,C; Fig. 5.2A,B,C). The resting $f_{hi}$ and $P_{DA}$ of epaulette sharks match those measured previously in this species (Stensløkken et al., 2004). In epaulette sharks and shovelnose rays, resting $f_{hi}$, $V_{SH}$, $\dot{Q}$, $P_{DA}$, and $R_{SYS}$ were similar to those in other elasmobranchs with similar activity levels and accounting for differences in experimental temperature (Butler and Taylor,
1975; Lai et al., 1989; Lai et al., 1990; Sandblom et al., 2006; Satchell et al., 1970). To my knowledge, the only other in vivo measurement of elasmobranch PO is a mass-independent value for spotted catshark (Short et al., 1979), which is, in general, similar to the mass-specific values reported here for epaulette sharks and shovelnose rays. Elasmobranchs have higher routine PO values than typically seen in teleosts (0.5–3.0 mW/g) (Chapter 2; Stecyk and Farrell, 2006).

Progressive hypoxia exposure elicited a similar cardiovascular response in epaulette sharks and shovelnose rays, including bradycardia, increased $V_{SH}$, and decreased $\dot{Q}$, PO and $P_{DA}$ (Fig. 5.1A,B,C; Fig. 5.2A,B). Bradycardia induced by environmental hypoxia exposure is common in fishes and has been observed previously in elasmobranchs such as the epaulette shark, spiny dogfish (Squalus acanthias), and spotted catshark (Butler and Taylor, 1975; Sandblom et al., 2009; Stensløkken et al., 2004). Whereas spotted catsharks and spiny dogfish appear to achieve hypoxia-induced bradycardia via vagally-mediated cholenergic inhibition, this is not the case in epaulette sharks where the mechanism is unclear (Stensløkken et al., 2004); nothing is known about mechanisms of hypoxic bradycardia in shovelnose rays. Acidosis has a negative chronotropic effect in fishes but does not appear to be a primary cause of the bradycardia in epaulette sharks or shovelnose rays because, as shown in Chapter 4, blood pH decreased at a lower $P_{w}O_2$ compared with the commencement of bradycardia (cf. Fig. 4.5B in Chapter 4 and Fig. 5.1A in the present chapter). Bradycardia was initiated immediately below the $P_{w}O_2$ corresponding to a $P_aO_2$ just below the Hb-O$_2$ $P_{50}$ in each species (see Chapter 4) and direct hypoxemic effects on $f_H$ likely contributed to the bradycardia. Further studies are needed to elucidate the mechanisms of hypoxic bradycardia in elasmobranchs and especially the epaulette shark, including the suggested involvement of $\alpha$-adrenoceptors (Stensløkken et al., 2004).
Stroke volume increased in epaulette sharks and shovelnose rays during progressive hypoxia exposure but $\dot{Q}$ still decreased because of a large depression of $f_H$ (Fig. 5.1B; Fig. 5.2A). The large fall in $\dot{Q}$ with only a modest increase in $R_{SYS}$ resulted in the observed reduction in $P_{DA}$ in epaulette sharks and shovelnose rays, although the critical $P_wO_2$ for $P_{DA}$ was lower than that for $\dot{Q}$ (Table 5.1). Epaulette sharks show no change in gill resistance during hypoxia exposure so this is not likely to contribute to lowered $P_{DA}$ in this species (Stensløkken et al., 2004). The absence of a barostatic reflex to maintain arterial blood pressure is consistent with previous studies showing that the reflex is weak in response to hypotension in fishes and especially elasmobranchs (Olson and Farrell, 2006). Additionally, the barostatic reflex may be reset in the face of hypoxia-induced bradycardia in fishes (Stecyk and Farrell, 2006). Like the two species in the present study, an increase in $V_{SH}$ and a decrease in $P_{DA}$ were seen in spotted catsharks exposed to progressive hypoxia, but no decrease in $\dot{Q}$ was observed possibly because the level of hypoxia achieved was less severe ($P_wO_2=\sim 5$ kPa) (Butler and Taylor, 1975). In constrast, in spiny dogfish exposed to greater hypoxia ($P_wO_2=2.5$ kPa), Sandblom et al. (2009) observed decreases in $\dot{Q}$ and $V_{SH}$ but $P_{DA}$ was unchanged. These data suggest that the pattern of cardiovascular responses to environmental hypoxia, in particular $V_{SH}$ and $P_{DA}$, vary among elasmobranchs, as they do in teleosts.

Epaulette sharks and shovelnose rays showed a similar pattern of cardiovascular responses to progressive hypoxia and it remains unclear whether a specific suite of hypoxic cardiovascular responses correlates with hypoxia tolerance in fishes. There were, however, two key differences between the species: 1) all hypoxic cardiovascular responses in epaulette sharks occurred at a significantly lower $P_wO_2$ compared with shovelnose rays (Table 5.1); and
2) despite the presence of similar routine levels of cardiovascular function (i.e. above the critical $P_{wO_2}$ of each parameter; Table 5.1), the level of function of most parameters was greater at the same hypoxic $P_{wO_2}$ (below the critical $P_{wO_2}$) in the epaulette shark compared with the shovelnose ray (Fig. 5.1A,B,C; Fig. 5.2A,B). In other words, compared with the shovelnose ray, the more hypoxia-tolerant epaulette shark can maintain cardiovascular function at routine levels to lower $P_{wO_2}$ and once hypoxic responses commence the change from routine levels is smaller. This may benefit hypoxia tolerance by improving $O_2$ delivery and management of wastes.

The decreases in $f_H$, $\dot{Q}$, and $PO$ in epaulette sharks and shovelnose rays coincided with the decreases in whole-animal $\dot{M}_{O_2}$ seen during progressive hypoxia (see Chapter 4) and the critical $P_{wO_2}$ of these parameters were statistically similar within species (Table 5.1). A correlation between $P_{crit}$ and the critical $P_{wO_2}$ of heart rate appears to be a common phenomenon among fishes (Fig. 5.6), suggesting that $P_{crit}$ is a good predictor of the point at which hypoxic bradycardia is initiated. Likewise, co-occurrence of $P_{crit}$ and the critical $P_{wO_2}$ of $\dot{Q}$ and $PO$ also has been observed in fishes (Iversen et al., 2010; Chapter 2). These relationships are perhaps unsurprising considering the link between $\dot{M}_{O_2}$ and convective $O_2$ delivery (Webber et al., 1998). Other hypoxic cardiovascular responses (e.g. $V_{SH}$, $P_{DA}$) were not necessarily correlated with $P_{crit}$, although they always occurred at or below $P_{crit}$ (Table 5.1). Overall, these data suggest that in fishes exposed to environmental hypoxia, $P_{crit}$ indicates the lowest $P_{wO_2}$ at which routine cardiovascular function is maintained and this may explain in part why epaulette sharks maintain routine cardiovascular function to lower $P_{wO_2}$. However, $P_{crit}$ may not fully predict the capacity for hypoxic cardiovascular function at $P_{wO_2}$ below $P_{crit}$, because at the same relative percentages below $P_{crit}$ cardiovascular function
remained closer to routine levels in epaulette sharks compared with shovelnose rays (e.g. Fig. 5.1A,B,C; Fig. 5.2A,B). Because at relative percentages of $P_{crit}$ the $M_{\text{O}_2}$ and $C_{\text{aO}_2}$ are similar in the two species, this observation also suggests that the improved cardiovascular performance at $P_{w\text{O}_2}$ below $P_{crit}$ in epaulette sharks cannot be explained completely by its lower $P_{crit}$ and greater $C_{\text{aO}_2}$ (Chapter 4).

Cardiac energy demand as measured by $PO$ decreased as whole-animal $\dot{M}_{\text{O}_2}$ fell in both epaulette sharks and shovelnose rays (cf. Fig. 5.1C in present chapter and Fig. 4.1 in Chapter 4), likely due to the depression of $\dot{Q}$ associated with bradycardia. A similar result has been observed in tilapia (Chapter 2). Unlike tilapia, however, reductions in $P_{DA}$ and $P_{VA}$ probably also contribute to depressed $PO$ during hypoxia exposure in elasmobranchs, including epaulette sharks (Fig. 5.2B; Stensløkken et al., 2004). The results of the present study support previous findings showing that the depression of $PO$ is associated with hypoxia-induced whole-animal MRD in fishes and may, in some species (but apparently not in tilapia; see Chapter 3), contribute to hypoxia tolerance by matching cardiac energy demand to lowered energy supply (Farrell and Stecyk, 2007; Chapter 2). There was no evidence, however, that depression of $PO$ was associated with the greater hypoxia tolerance of the epaulette shark, because epaulette sharks maintained a higher $PO$ at all similar $P_{w\text{O}_2}$, even when expressed as a percentage of the normoxic resting level (Fig. 5.1C). Thus, $PO$ in epaulette sharks was maintained to lower $P_{w\text{O}_2}$ and at higher levels during environmental hypoxia compared with shovelnose rays, likely because of the similar maintenance of $f_{\text{Hb}}$, $\dot{Q}$, and $P_{DA}$. Only at ~0.1 kPa did epaulette sharks reach the level of $PO$ depression seen at the lowest $P_{w\text{O}_2}$ (~2.0 kPa) tolerated by shovelnose rays and at or below 0.1 kPa epaulette sharks (and presumably their hearts) remain responsive for at least 45 min (Renshaw et al., 2002).
While flow traces did not show marked cardiac arrhythmia in shovelnose rays or epaulette sharks, the plateau of $f_H$ and $\dot{Q}$ coupled with the continual decrease in $PO$ and $P_{DA}$ below a $P_{aO_2}$ of ~2.7 kPa in shovelnose rays (Fig. 5.1A,B,C; Fig. 5.2B) could be symptomatic of a failing heart, whereas no such pattern was evident for epaulette sharks.

Cardiovascular function returned to routine levels within 60 min of normoxic recovery following progressive hypoxia exposure in both epaulette sharks and shovelnose rays, with the exception of $PO$ in epaulette sharks, which steadily increased over time due to an increase in $P_{DA}$ (Fig. 5.1C; Fig. 5.2B). Shovelnose rays had elevated $f_H$ during the initial stage of recovery whereas epaulette sharks showed a non-elevated, gradual return to routine $f_H$ (Fig. 5.1A). Cardiac output showed qualitatively the same trends as $f_H$ (Fig. 5.1B). Further studies are needed to ascertain the role of these different cardiovascular responses for recovery from hypoxia exposure in epaulette sharks and shovelnose rays.

### 5.5.2 Metabolic status during relative hypoxia exposure

To investigate the effects of environmental hypoxia on tissue metabolic status and to test the hypothesis that $P_{crit}$ dictates tissue-level hypoxia tolerance, epaulette sharks and shovelnose rays were exposed for up to 4 h to a $P_{aO_2}$ representing 40% of respective $P_{crit}$, which resulted in a similar level of arterial hypoxemia in both species (see Fig. 4.2B in Chapter 4; interpolated $C_{aO_2}$ at 40% of respective $P_{crit}$ is ~0.6 vol % in both species). Contrary to my prediction of similar interspecific metabolic status under these conditions, I found greater decreases in pH (Fig. 5.5A,B), accumulation of lactate (Fig. 5.4A,B,D), and perturbation of tissue energy status (Fig. 5.3A,B; Table 5.2) in shovelnose rays compared with epaulette sharks. Perturbations of pH and energy status as well as high lactate load are
thought to contribute to hypoxic death and hypoxia-tolerant animals are able to avoid or at least postpone such perturbation (Nilsson and Östlund-Nilsson, 2008). The more stable metabolic state of the epaulette shark compared with the shovelnose ray at a similar level of arterial hypoxemia suggests that other factors work in concert with enhanced $O_2$ supply to explain the epaulette shark’s superior hypoxia tolerance, possibly including effective acid-base regulation, MRD, and strategic utilization of $O_2$ (see below).

In the heart, [ATP] was stable in both species but whereas [CrP] was stable in epaulette sharks, it fell in shovelnose rays, leading to increases in $[\text{ADP}_{\text{free}}]$ and $[\text{AMP}_{\text{free}}]$ (Fig. 5.3A,B; Table 5.2). The maintenance of cardiac energy status in epaulette sharks vs. perturbation in shovelnose rays is consistent with previous results for hypoxia-tolerant vs. sensitive fishes exposed to hypoxia (Dunn and Hochachka, 1986; Jorgensen and Mustafa, 1980; Chapter 2). Although [CrP] stabilized at a lower level in hypoxia-exposed shovelnose rays, this may not represent a stable functional state considering that the increase of inorganic phosphate associated with CrP depletion, rather than ATP depletion, is thought to be a major contributor to hypoxic heart failure in mammals and fishes (Arthur et al., 1992; Neubauer, 2007). Notably, the $P_{\text{w}O_2}$ of the relative hypoxia exposure of shovelnose rays was only marginally higher than the $P_{\text{w}O_2}$ where possible signs of cardiac failure were seen during the progressive hypoxia exposure (see above). The energy status measurements suggest that matching of cardiac energy supply and demand during hypoxia exposure is much less perturbed in epaulette sharks compared with shovelnose rays, even when hypoxemia is similar. Under the deeper hypoxia exposure ($P_{\text{w}O_2}=1.0 \text{ kPa}$), epaulette shark hearts still maintained stable energy status (Fig. 5.3A,B), suggesting that energy supply and demand remain well matched even under profound hypoxia which shovelnose rays cannot survive.
The ability of epaulette sharks to better match cardiac energy supply and demand is not due to a greater depression of energy demand (i.e., \( PO \)) compared with shovelnose rays because epaulette sharks actually maintain higher levels of \( PO \) during progressive hypoxia exposure, including when levels are compared at the species-specific \( P_{wO_2} \)'s used in the relative hypoxia exposure (Fig. 5.1C). Although \( PO \) in epaulette sharks may have decreased more over the duration of the relative hypoxia exposure compared with the shorter progressive hypoxia exposure, I consider this unlikely because the first sample time point (2 h) of the relative hypoxia exposure is comparable in duration to the progressive hypoxia exposure. Also, in other fish, levels of \( PO \) and other heart parameters measured at each \( P_{wO_2} \) during progressive hypoxia exposure appear to be a good indicator of the levels that are seen during prolonged exposure at the same, stable \( P_{wO_2} \) (e.g., tilapia, Chapter 2).

Strategic delivery of available \( O_2 \) to the heart during relative hypoxia exposure could explain, in part, how a stable cardiac energy status was maintained alongside greater cardiac function in epaulette sharks but not shovelnose rays, despite similar arterial hypoxemia. Consistent with this hypothesis, there was minimal lactate accumulation in the heart of epaulette sharks and greater lactate accumulation in heart of shovelnose rays during relative hypoxia exposure (Fig. 5.4A), even though cardiac energy demand was higher in the epaulette sharks (see above). Although not investigated in the present study, coronary perfusion of the myocardium could be more extensive in epaulette sharks, improving myocardial \( O_2 \) supply compared with shovelnose rays. In epaulette sharks, environmental hypoxia exposure causes changes in gill blood flow that may deliver blood directly to the heart (Stensløkken et al., 2004). Also, whole-animal MRD, and especially in non-essential tissues such as white muscle, could be greater in the epaulette sharks compared with
shovelnose rays, thus sparing $O_2$ for the heart. The lesser accumulation of lactate in white muscle and plasma in epaulette sharks (Fig. 5.4B,D) supports this hypothesis because, at similar arterial hypoxemia, lactate accumulation can be used as a rough proxy to compare energy demand between species. Liver [lactate] was similar between species (Fig. 5.4C), but in this case interpretation is complicated by the potential accumulation of lactate in liver for glycogen or glucose synthesis. A caveat is that the relative hypoxia exposure did not control for the higher $\dot{Q}$ seen during hypoxia exposure in epaulette sharks (Fig. 5.1B), which may facilitate tissue $O_2$ delivery in this species despite $C_\text{a}O_2$ being the same in both species. This may explain some of the species differences in lactate accumulation in non-cardiac tissues. Unfortunately, logistical constraints in the present study negated measurement of $O_2$ content in blood returning to the heart and use of the Fick method produced unreliable estimates of venous $O_2$ content, consistent with previous findings showing the inaccuracy of the Fick method during environmental hypoxia exposure in elasmobranchs (Metcalfe and Butler, 1982). Further studies are needed to directly assess the idea that strategic cardiac utilization of $O_2$ contributes to the superior hypoxia tolerance of epaulette sharks.

There was no increase in plasma [glucose] during relative hypoxia exposure in either species, similar to the results of the progressive hypoxia exposure (Chapter 4) and similar to previous studies on epaulette sharks and other elasmobranchs exposed to hypoxia (Routley et al., 2002; Speers-Roesch and Treberg, 2010). However, glycogen was mobilized in the liver (Table 5.2), suggesting that glucose flux increases. Plasma [$\beta$-HB] decreased during relative hypoxia exposure in the epaulette sharks (Table 5.2). The role of ketone bodies during hypoxia exposure in epaulette sharks warrants attention considering the protective effects of ketone bodies seen in mammalian ischaemia and because of the parallels with the hypoxic
decreases of plasma free fatty acids seen in some hypoxia-tolerant teleosts, which could be related to MRD (Speers-Roesch and Treberg, 2010; Chapter 2).

5.5.3 Conclusions and perspectives

The results of the present study and the accompanying study (Chapter 4) show that in comparison with the relatively hypoxia-sensitive shovelnose ray, the hypoxia-tolerant epaulette shark possesses greater blood $O_2$ transport, greater cardiovascular function, and superior maintenance of pH and cardiac energy status during environmental hypoxia exposure. These attributes probably contribute to the exceptional hypoxia tolerance of the epaulette shark and generally may be hallmarks of hypoxia tolerance in fishes. The enhanced hypoxic cardiorespiratory performance of the epaulette shark also highlights the importance for hypoxia tolerance of maintenance of superior $O_2$ supply including cardiovascular function in order to optimize aerobic energy production during hypoxia exposure.

A regulated depression of $PO_2$ in fishes is thought to be an important component of cardiac and consequently whole animal hypoxia tolerance (Farrell and Stecyk, 2007; but see Chapter 3). I hypothesized that the hypoxia-tolerant epaulette shark would show greater hypoxia-induced depression of $PO_2$ compared with the hypoxia-sensitive shovelnose rays. In fact, the epaulette shark’s greater hypoxic cardiovascular function was associated with a smaller reduction of $PO_2$ compared with the shovelnose ray (Fig. 5.1C). Although this finding does not refute the importance of depression of $PO_2$ for hypoxia tolerance, it does suggest that rather than outright cardiac depression, the maintenance of higher levels of cardiac function and therefore energy demand may be an equally important strategy for hypoxia tolerance in fishes because of the benefits for $O_2$ supply and management of fuel and waste. Interestingly,
unlike shovelnose rays, epaulette sharks appear to be able to avoid perturbation of cardiac energy status during hypoxia exposure (Fig. 5.3A,B) despite maintaining a higher cardiac energy demand. Considering that this relative hypoxia exposure equalized $C_{a}O_2$ between species, these results and those for tissue [lactate] imply that during hypoxia exposure, $O_2$ delivery to the heart in epaulette sharks is superior to that of shovelnose rays, possibly in part due to $O_2$ sparing related to metabolic depression in non-essential tissues. Further studies are needed to test this hypothesis directly. Overall, epaulette sharks, unlike shovelnose rays, appear to be able to coordinate depression of cardiac energy demand (i.e. decreases in $PO$) with improvements in cardiac energy supply (e.g. $O_2$ supply) in order to achieve stable cardiac energy status, enhanced cardiac function, and thus improved hypoxia tolerance.

Finally, the present study provides insight into the use of $P_{crit}$ as a measure of hypoxia tolerance in fishes. Previous work has shown that $P_{crit}$ is an excellent indicator of the ability of a fish to take up and transport $O_2$ at low $P_{v}O_2$ (Mandic et al., 2009; Chapter 4). Here, I also provide support for the idea that $P_{crit}$ provides an indication of the ability of a fish to maintain routine cardiovascular function to low $P_{v}O_2$. However, data from the relative hypoxia exposure show that $P_{crit}$ does not necessarily determine the metabolic status and hypoxia tolerance of tissues, where metabolic depression also may play a major role. Overall, the results of the present study suggest that $P_{crit}$ is an important measure of respiratory hypoxia tolerance and that improved hypoxic $O_2$ supply associated with a low $P_{crit}$ is only one, albeit important, component of hypoxia tolerance in fishes.

5.6 ACKNOWLEDGEMENTS

See Section 4.6.
Table 5.1. Critical $P_{w}O_2$ of cardiovascular parameters and whole animal $O_2$ consumption rate in epaulette sharks and shovelnose rays exposed to progressive decreases in water $P_{w}O_2$.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Critical $P_{w}O_2$ (kPa)</th>
<th>Epaulette shark</th>
<th>Shovelnose ray</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\dot{Q}$</td>
<td>5.12±0.95$^a$</td>
<td>8.86±1.13$^{\Psi,y}$</td>
<td></td>
</tr>
<tr>
<td>$\dot{M}_{O_2}$</td>
<td>5.10±0.37$^a$</td>
<td>7.23±0.40$^{\Psi,y}$</td>
<td></td>
</tr>
<tr>
<td>$PO$</td>
<td>4.80±0.48$^{ab}$</td>
<td>7.03±0.59$^{\Psi,y}$</td>
<td></td>
</tr>
<tr>
<td>$f_H$</td>
<td>3.94±0.61$^{abc}$</td>
<td>7.66±0.59$^{\Psi,y}$</td>
<td></td>
</tr>
<tr>
<td>$V_{SH}$</td>
<td>3.22±0.58$^{bc}$</td>
<td>6.99±0.59$^{\Psi,x}$</td>
<td></td>
</tr>
<tr>
<td>$P_{DA}$</td>
<td>2.61±0.51$^c$</td>
<td>4.78±0.68$^{\Psi,x}$</td>
<td></td>
</tr>
</tbody>
</table>

See Materials and methods for information on time course and starting and ending $P_{w}O_2$ of the exposures. For comparison, the critical $P_{w}O_2$ of oxygen consumption rate ($P_{cro}$) is included from Chapter 4. Parameters are listed in descending order of the values for epaulette sharks. Values are means±s.e.m. ($n=5$). $P_{w}O_2$, water PO$_2$; $\dot{Q}$, cardiac output; $\dot{M}_{O_2}$, whole animal O$_2$ consumption rate; $PO$, cardiac power output; $f_H$, heart rate; $V_{SH}$, stroke volume; $P_{DA}$, dorsal aortic blood pressure. $\Psi$ denotes significant difference between shovelnose and epaulette values. Values with different letters within species are significantly different (two-way ANOVA with Holm-Sidak tests, $p<0.05$). The critical $P_{w}O_2$ for ventral aortic blood pressure ($P_{VA}$) is the same as for $P_{DA}$ and the critical $P_{w}O_2$ for systemic resistance ($R_{SYS}$) was not calculated (see text).
Table 5.2. Levels of selected metabolites of energy metabolism in heart, liver, white muscle, and plasma of epaulette sharks and shovelnose rays exposed either to normoxia or to 2 h and 4 h of hypoxia representing 40% of each species’ respective P_{crit} (P_{W,O_2} = 2.0 and 3.1 kPa, respectively) and in epaulette sharks exposed to 20% of P_{crit} (P_{W,O_2} = 1.0 kPa).

<table>
<thead>
<tr>
<th></th>
<th>Epaulette shark</th>
<th></th>
<th>Shovelnose ray</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normoxia</td>
<td>2h at 2.0 kPa</td>
<td>4h at 2.0 kPa</td>
<td>2h at 3.1 kPa</td>
</tr>
<tr>
<td><strong>Heart</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[ADP\text{free}]</td>
<td>19.3±3.8\text{a}</td>
<td>25.2±4.5\text{a}</td>
<td>15.4±6.0\text{a}</td>
<td>22.1±6.3\text{a}</td>
</tr>
<tr>
<td>[AMP\text{free}]</td>
<td>0.22±0.07\text{b}</td>
<td>0.25±0.08\text{b}</td>
<td>0.12±0.07\text{b}</td>
<td>0.29±0.16\text{b}</td>
</tr>
<tr>
<td>[Glycogen]</td>
<td>80.4±4.4\text{a}</td>
<td>77.6±14.4\text{a}</td>
<td>67.7±10.4\text{a}</td>
<td>43.4±10.6\text{a}</td>
</tr>
<tr>
<td>[Glucose]</td>
<td>1.09±0.19\text{a}</td>
<td>1.27±0.46\text{a}</td>
<td>1.42±0.56\text{a}</td>
<td>1.13±0.48\text{a}</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[ATP]</td>
<td>1.41±0.14\text{a}</td>
<td>1.10±0.25\text{a}</td>
<td>0.44±0.07\text{b}</td>
<td>0.37±0.06\text{b}</td>
</tr>
<tr>
<td>[CrP]</td>
<td>0.94±0.75\text{a}</td>
<td>1.00±0.93\text{a}</td>
<td>0.78±0.43\text{a}</td>
<td>2.25±1.28\text{b}</td>
</tr>
<tr>
<td>[Glycogen]</td>
<td>75.0±26.5\text{a}</td>
<td>66.4±23.3\text{a}</td>
<td>24.4±16.3\text{a}</td>
<td>15.7±7.2\text{b}</td>
</tr>
<tr>
<td>[Glucose]</td>
<td>2.92±0.24\text{a}</td>
<td>3.33±0.75\text{a}</td>
<td>2.81±0.86\text{a}</td>
<td>4.21±1.37\text{a}</td>
</tr>
<tr>
<td><strong>White muscle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[ATP]</td>
<td>5.91±0.84\text{a}</td>
<td>6.59±0.82\text{a}</td>
<td>5.54±1.13\text{a}</td>
<td>7.40±0.41\text{a}</td>
</tr>
<tr>
<td>[CrP]</td>
<td>41.7±2.7\text{a}</td>
<td>38.1±1.5\text{a}</td>
<td>32.7±3.1\text{a}</td>
<td>25.7±4.1\text{a}</td>
</tr>
<tr>
<td>[Glycogen]</td>
<td>22.8±3.2\text{a}</td>
<td>23.4±5.1\text{a}</td>
<td>16.1±3.5\text{a}</td>
<td>22.0±2.4\text{a}</td>
</tr>
<tr>
<td>[Glucose]</td>
<td>0.20±0.08\text{a}</td>
<td>0.19±0.07\text{a}</td>
<td>0.30±0.13\text{a}</td>
<td>0.37±0.07\text{a}</td>
</tr>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Glucose]</td>
<td>1.87±0.12\text{a}</td>
<td>1.86±0.64\text{a}</td>
<td>2.23±0.67\text{a}</td>
<td>1.47±0.49\text{a}</td>
</tr>
<tr>
<td>[β-HB]</td>
<td>0.97±0.13\text{a}</td>
<td>0.52±0.05\text{a}</td>
<td>0.34±0.07\text{a}</td>
<td>0.57±0.10\text{a}</td>
</tr>
</tbody>
</table>

Values are means±s.e.m. (n=5-8 for epaulette sharks; n=4-7 for shovelnose rays). All data are expressed as μmol · g ww\text{-1} except glycogen (μmol glucosyl units · g ww\text{-1}), ADP\text{free} and AMP\text{free} (nmol · g ww\text{-1}), and plasma glucose and β-HB (mM). β-HB, β-hydroxybutyrate; CrP, creatine phosphate. Within each species, values with the same letter are not significantly different from one another (two-way ANOVA with Holm-Sidak post-hoc test, p<0.05). \text{\textsuperscript{y}} indicates that the value for shovelnose ray is significantly different from the value for epaulette shark in normoxia at 2.0 kPa (two-way ANOVA with Holm-Sidak post-hoc test, p<0.05). \text{\textsuperscript{*}} indicates the epaulette shark value for 2h at 1.0 kPa is significantly different from the epaulette shark normoxia value (Student’s t-test, p<0.05).
Figure 5.1 (overleaf). Heart rate ($f_H$) (A), cardiac output ($\dot{Q}$) (B), and cardiac power output ($PO$) (C) of epaulette sharks (closed circles) and shovelnose rays (open circles) exposed to progressive decreases in water $PO_2$ ($P_{wO_2}$) over 135±8 min and 71±6 min, respectively, followed by up to 60 min of recovery in normoxic water. Data are means±s.e.m. ($n=5$ for both species except epaulette sharks in recovery, where $n=4$ for $f_H$ and $\dot{Q}$ and $n=3$ for $PO$). Recovery values are offset at each time point for clarity. * denotes value is significantly different from the first, normoxic resting value at ≥15.3 kPa within species. ** denotes where the two species values bracketed by a horizontal { (at statistically similar $P_{wO_2}$) are significantly different between species; in the absence of ** the two bracketed species values are not statistically different from each other. For recovery, ** denotes a significant difference between epaulette sharks and shovelnose rays at each time point.
Figure 5.2. Stroke volume ($V_{SH}$) (A), dorsal aortic blood pressure ($P_{DA}$) (B), and systemic resistance ($R_{SYS}$) (C) of epaulette sharks (closed circles) and shovelnose rays (open circles) exposed to progressive decreases in water PO$_2$ ($P_{wO_2}$) over 135±8 min and 71±6 min, respectively, followed by up to 60 min of recovery in normoxic water. Data are means±s.e.m. ($n=5$ for both species except epaulette sharks in recovery, where $n=4$ for $V_{SH}$ and $n=3$ for $P_{DA}$ and $R_{SYS}$). Recovery values are offset at each time point for clarity. See Figure 5.1 caption for details on symbols denoting statistical comparisons.
Figure 5.3. Cardiac [ATP] (A) and [CrP] (B) in epaulette sharks (black closed circles) and shovelnose rays (open circles) exposed either to normoxia or to 2 h and 4 h of hypoxia representing 40% of each species’ respective critical \( P_O_2 \) of whole animal \( O_2 \) consumption rate (\( P_{crit} \)) (water \( P_O_2 = 2.0 \) and 3.1 kPa, respectively) and in epaulette sharks exposed to 20% of \( P_{crit} \) (water \( P_O_2 = 1.0 \) kPa) (gray closed circles). Data are means±s.e.m. (\( n = 5-7 \) for epaulette sharks and \( n = 4-7 \) for shovelnose rays). Values are offset at each time point for clarity. Values with different lowercase letters are significantly different from each other within species; between species differences at each time point are denoted by \( \psi \). For epaulette sharks, a significant difference between the 1.0 kPa group and the normoxic group is denoted by different uppercase letters.
Figure 5.4. [Lactate] in heart (A), white muscle (B), liver (C), and mixed arterial-venous plasma (D) in epaulette sharks (black closed circles) and shovelnose rays (open circles) exposed either to normoxia or to 2 h and 4 h of hypoxia representing 40% of each species’ respective $P_{\text{crit}}$ (water $P_{O_2} = 2.0$ and 3.1 kPa, respectively) and in epaulette sharks exposed to 20% of $P_{\text{crit}}$ (water $P_{O_2} = 1.0$ kPa) (gray closed circles). Data are means±s.e.m. ($n=5$-7 for epaulette sharks and $n=4$-7 for shovelnose rays). Values are offset at each time point for clarity. See Figure 5.3 caption for details on symbols denoting statistical comparisons.
Figure 5.5. Cardiac intracellular pH (A) and blood (mixed arterial-venous) pH (B) in epaulette sharks (black closed circles) and shovelnose rays (open circles) exposed either to normoxia or to 2 h and 4 h of hypoxia representing 40% of each species’ respective P\textsubscript{crit} (water PO\textsubscript{2} = 2.0 and 3.1 kPa, respectively) and in epaulette sharks exposed to 20% of P\textsubscript{crit} (water PO\textsubscript{2} = 1.0 kPa) (gray closed circles). Data are means±s.e.m. (pH\textsubscript{i}, n=4 for epaulette sharks and n=4-7 for shovelnose rays; blood pH, n=4-6 for epaulette sharks and n=3-6 for shovelnose rays). Values are offset at each time point for clarity. See Figure 5.3 caption for details on symbols denoting statistical comparisons.
Figure 5.6. The relationship between critical PO$_2$ (P$_w$O$_2$) of oxygen consumption rate (P$_{crit}$) and critical P$_w$O$_2$ of heart rate (i.e., P$_w$O$_2$ at initiation of hypoxia-induced bradycardia) in fishes. Legend: (1) *Hoplias malabaricus*, 25°C, Rantin et al., 1993; (2) *Oreochromis* hybrid, 22°C, Speers-Roesch et al., 2010; (3) *Piaractus mesopotamicus*, 25°C, Rantin et al., 1998; (4) *Leiopotherapon unicolor*, 25°C, Gehrke and Fielder, 1988; (5) *Hoplias lacerdae*, 25°C, Rantin et al., 1993; (6) *Hemiscyllium ocellatum*, 28°C, present study and Speers-Roesch et al. (submitted); (7) *Aptychotrema rostrata*, 28°C, present study and Speers-Roesch et al. (submitted); (8) *Gadus morhua*, 10°C, McKenzie et al., 2009; (9) *Gadus morhua*, 10°C, Petersen and Gamperl, 2011; (10) *Scyliorhinus canicula*, 12°C, Butler and Taylor, 1975 (values estimated from graphical data); (11) *Anguilla anguilla*, 20°C, Iversen et al., 2010. The linear regression is significant (p=0.001).
CHAPTER 6: GENERAL DISCUSSION

Cardiac failure occurs in most vertebrates including humans following even short periods of hypoxia exposure due to an inability to match cardiac energy demand to the limited cardiac energy supply. In contrast, hypoxia-tolerant ectothermic vertebrates show the remarkable ability to maintain cardiac energy balance and stable cardiac function during prolonged exposure to severe hypoxia. The major objectives of my thesis were to investigate how this cardiac hypoxia tolerance is achieved and how it is related to whole-animal hypoxia tolerance. My work shows that maintenance of cardiac energy balance is a key component of cardiac hypoxia tolerance and suggests that enhanced $O_2$ supply, greater MGP, and depression of $PO$ all play important roles in cardiac hypoxia tolerance, although their relative importance is species- and situation-specific (e.g., the high MGP of the tilapia heart may be useful primarily for short-term bouts of increased hypoxic performance). On the other hand, hypoxia-induced modulation of cardiac aerobic energy production pathways including provision of aerobic fuels (i.e., fatty acids) appears to be relatively unimportant for cardiac hypoxia tolerance. I have also made novel findings showing that hypoxic cardiac performance and in particular $PO$ is closely associated with changes in whole-animal $\dot{M}_O_2$, and possibly metabolic rate, suggesting that modulation of whole-animal demand for blood flow (e.g., by MRD) can influence cardiac hypoxia tolerance. Finally, I have shown for the first time that a lower $P_{crit}$, which is a widely used metric of hypoxia tolerance, is associated with greater hypoxic blood $O_2$ transport, which has consequences for cardiac and whole-animal hypoxia tolerance. In this chapter, I summarize my salient findings in a broad context and suggest future research directions. I also present a model based on my work and other
studies that describes the variation in hypoxic cardiac performance and cardiac hypoxia
tolerance in ectothermic vertebrates.

Before continuing, I must acknowledge the limitations imposed by 1) my choice of
study species and 2) my use of a two-species comparative model. Because of the
evolutionary distance and substantial physiological differences between teleosts (tilapia) and
elasmobranchs (epaulette shark and shovelnose ray), comparisons of mechanisms of cardiac
and whole-animal hypoxia tolerance in these species must be made cautiously. Nevertheless,
the hypoxic cardiac responses I observed show a convergence (particularly with regard to \(PO\)
depression) and ultimately I think my conclusions have been strengthened by the use of
divergent groups of fishes that allow cardiac hypoxia tolerance to be considered broadly
among ectothermic vertebrates. On the second point, two-species comparisons of hypoxia
tolerance (or any other physiological state) should be viewed cautiously because
physiological mechanisms that appear to be beneficial for hypoxia tolerance may have
evolved for other reasons or may represent random differences between phylogenetically-
disparate species (Garland and Adolph, 1994). Ideally, phylogenetically-corrected multiple
species comparisons should be carried out (e.g., Mandic et al., 2009), although logistical
constraints often preclude such analyses. For example, obtaining a wide variety of cichlids or
elasmobranchs that vary in their hypoxia tolerance is difficult and the extent of \textit{in vivo}
measurements of cardiorespiratory performance carried out in the present thesis presents a
significant time constraint if applied to a wide range of species. In any case, the epaulette
shark and shovelnose ray show a clear difference in their hypoxia tolerance that is consistent
with the degree of environmental hypoxia in their habitats (see section 4.2) and I have been
careful only to infer that the responses observed are consistent with hypoxia tolerance rather than inferring that they represent an adaptation underlying hypoxia tolerance.

6.1 HYPOXIC MODULATION OF CARDIAC AEROBIC ENERGY SUPPLY IS MODEST IN FISHES (CHAPTER 2)

Control of aerobic energy supply pathways, especially at the level of the mitochondria, is thought to underlie the initiation of decreased ATP turnover in at least some animal models of MRD (Bishop et al., 2002; Staples and Buck, 2009) and also may help avoid impaired substrate oxidation and accumulation of associated deleterious by-products (e.g., ROS, fatty acid intermediates) (Aragonés et al., 2008; Chen et al., 2007; Corr et al., 1984; van der Vusse et al., 1992; Wheaton and Chandel, 2011). *In vivo* profound hypoxia exposure in tilapia, however, caused few responses in aerobic ATP supply pathways in the heart (Chapter 2). There were no changes in components of the fatty acid oxidation pathway and only a slow, modest decrease in the activity of PDHα, an enzyme that has been implicated in hypoxic control of substrate oxidation and ATP turnover in mammalian and fish skeletal muscle (Aragonés et al., 2008; Richards et al., 2007; Richards et al., 2008; Semenza, 2007). Thus, my findings provide little support for the hypothesis that downregulation of cellular aerobic ATP supply contributes to decreased ATP turnover or hypoxia tolerance in the fish heart during hypoxia exposure.

Perhaps this is not surprising considering that the ATP turnover of the heart is determined largely by its mechanical work, which is reflected by *PO*. *PO* is greatly depressed in hypoxic tilapia *via* a vagally-mediated bradycardia (Chapter 2). Thus, metabolic depression in the heart is likely driven mostly by modulation of energy demand associated...
with contractile activity rather than by energy supply. Indeed, the decrease in PDH$_a$ activity may be explained in part by depressed $PO$, considering that PDH$_a$ activity is highly responsive to the level of mechanical work in fish skeletal muscle (Richards et al., 2002b). Nevertheless, PDH$_a$ activity decreased at a considerably slower rate than $PO$ after the onset of hypoxia exposure and decreases in PDH$_a$ activity could also be affected by regulation through the PDK pathway, which can be mediated by HIF-1$\alpha$ (Semenza, 2007), although my supporting evidence (acute increases in mRNA levels of PDK-2 and PPAR$\alpha$) is only suggestive and does not directly implicate HIF-1$\alpha$. It remains possible that decreases in cardiac PDH$_a$ in tilapia are involved in depression of non-contractile ATP turnover, which appeared to occur in the profoundly hypoxic in situ tilapia heart (Chapter 3). This hypotheses is lent credence by the observation that decreases in substrate oxidation can cause depression of energy demand processes such as protein synthesis in mammalian neonatal cardiomyocytes (Casey et al., 2002).

Finally, decreased PDH$_a$ activity could be involved in a coordinated slowing of substrate oxidation and mitigation of ROS production under hypoxia, which has been shown to improve hypoxia tolerance in mammalian cells and tissues (Aragonés et al., 2008; Semenza, 2007; Wheaton and Chandel, 2011). As yet there is no clear evidence for such a role for PDH$_a$ in hypoxia-tolerant fish. However, in one of the first studies on mitochondrial function in fishes exposed to severe hypoxia, the hypoxia-tolerant epaulette shark showed significantly less ROS production in permeabilized heart fibres compared with the hypoxia-sensitive shovelnose ray (Hickey et al., 2012). Mitochondrial function (including PDH$_a$ activity) and its relationship with whole organ function and MRD during hypoxia exposure in hypoxia-tolerant animals is a field of inquiry in its infancy and further work is needed.
6.2 DEPRESSION OF PLASMA [NON-ESTERIFIED FATTY ACIDS] IS NOT IMPORTANT FOR CARDIAC HYPOXIA TOLERANCE (CHAPTERS 2 AND 3)

Although there were only modest responses of cellular aerobic energy supply pathways to hypoxia exposure in tilapia heart, there was a profound decrease in fatty acid provision to heart and other tissues as indicated by an ~80% decrease in the level of plasma non-esterified fatty acids (NEFA) (Chapter 2), which are important for fatty acid oxidation in peripheral tissues (Henderson and Tocher, 1987). As discussed in section 1.4.2.2, this hypoxic response is common to several species of hypoxia-tolerant fishes. I hypothesized that this depression of plasma [NEFA] contributes to cardiac hypoxia tolerance by obviating lipotoxic perturbation of cardiac function during hypoxia exposure when fatty acid oxidation is limited, but found no evidence to support this hypotheses based on my studies of the in situ tilapia heart (Chapter 3).

An alternative hypothesis that requires testing is that hypoxic depression of plasma [NEFA] in fishes decreases fatty acid provision to tissues and contributes to MRD by decreasing tissue fatty acid oxidation and thus tissue $\dot{M}_{\text{O}_2}$. In this way, depression of plasma [NEFA] may represent energy supply control of MRD. Another hypothesis is that decreasing fatty acid provision to tissues represents preferential fuel selection for carbohydrate (glucose and lactate) oxidation during hypoxia exposure, when there may still be low levels of oxidative phosphorylation occurring. Indeed, my work on blood $\text{O}_2$ transport and its effect on cardiac function in elasmobranchs (Chapters 4 and 5) demonstrates the importance of tissue $\text{O}_2$ supply even during severe hypoxia (see section 6.3). Carbohydrate oxidation should be favoured over fatty acid oxidation because it is more efficient per mol $\text{O}_2$ and it would
mitigate accumulation of lactate, as suggested for hypoxic rainbow trout (Omlin and Weber, 2010). These two hypotheses, which are not mutually exclusive, could be tested in whole-animal *in vivo* experiments in tilapia. For example, arterial infusion with a synthetic lipid emulsion (e.g., Intralipid) could be used to artificially increase plasma [NEFA] during progressive and prolonged hypoxia exposure while \( \dot{M}_{O_2} \), plasma lactate, and hypoxia survival time was monitored. Combining this protocol with radioactive measurements of NEFA and glucose turnover rate would allow for simultaneous measurement of flux through these pathways (Haman et al., 1997) and provide insight into how fuel selection is affected by modulation of plasma [NEFA]. Finally, cardiovascular function could be monitored to confirm my findings of a lack of an effect of fatty acid provision on hypoxic performance of the *in situ* heart.

6.3 **ENHANCED O\(_2\) SUPPLY IS AN IMPORTANT COMPONENT OF HYPOXIA TOLERANCE AND CAN BE PREDICTED BY P\(_{crit}\) (CHAPTERS 4 AND 5)**

My comparative work on the hypoxia-tolerant epaulette shark and the hypoxia-sensitive shovelnose ray affirms the important role of O\(_2\) supply for hypoxia tolerance in fishes and provides novel information supporting the use of P\(_{crit}\) as a measure of hypoxia tolerance. I found that a lower P\(_{crit}\) is associated with greater \( \dot{M}_{O_2} \) at \( P_{wO_2} \) below P\(_{crit}\) and provided the first evidence that a lower P\(_{crit}\) is associated with greater blood O\(_2\) transport at a given low \( P_{wO_2} \), which was previously only assumed. This appears to be explained by the presence of a lower *in vivo* Hb-O\(_2\) P\(_{50}\) in the species with the lower P\(_{crit}\) (the epaulette shark). Another novel result of my work was that P\(_{crit}\) was predictive of arterial O\(_2\) content (C\(_aO_2\)) and Hb-O\(_2\) saturation and explained the interspecies differences in these parameters. These
findings provide strong support for the notion that Hb-O\textsubscript{2} P\textsubscript{50} is a major determinant of P\textsubscript{crit} (Mandic et al., 2009) and allow for a synthesis of the long-standing hypotheses that P\textsubscript{crit} (Fry, 1947; Hughes 1973; Mandic et al., 2009) and Hb-O\textsubscript{2} P\textsubscript{50} (Nikinmaa, 2001; Powers, 1980) each are important underlying factors of hypoxia tolerance in fishes. As a point of comparison, in tilapia the P\textsubscript{crit} was lower than in the elasmobranchs and the \textit{ex vivo} whole blood Hb-O\textsubscript{2} P\textsubscript{50} was also lower by a roughly proportional amount (Chapter 2), which is consistent with a link between P\textsubscript{crit} and Hb-O\textsubscript{2} P\textsubscript{50}. Simultaneous measurements of P\textsubscript{crit}, C\textsubscript{a}O\textsubscript{2}, and Hb-O\textsubscript{2} P\textsubscript{50} during progressive hypoxia, as described in Chapter 4, should be made in multiple species with varied P\textsubscript{crit} from other fish taxa to confirm the results I have obtained for the two elasmobranch species.

The increased blood O\textsubscript{2} transport at low P\textsubscript{s}O\textsubscript{2} associated with a lower P\textsubscript{crit} and Hb-O\textsubscript{2} P\textsubscript{50} presumably enhances energy supply and decreases the accumulation of deleterious anaerobic end-products, thus improving hypoxia tolerance. It is likely that enhanced O\textsubscript{2} supply to tissues, in part, underlies the superior hypoxia tolerance of the epaulette shark, including the greater levels of hypoxic cardiovascular function seen in this species compared with the comparatively hypoxia-sensitive shovelnose ray (see Chapter 5 and section 6.4.2). However, the results of the relative hypoxia exposure (Chapter 5), which controlled for the variation in arterial O\textsubscript{2} content between species, showed that epaulette sharks were still better able to maintain a stable cardiac high-energy phosphate pool and to minimize metabolic acidosis and lactate accumulation in tissues. These results suggest that tissue hypoxia tolerance in fishes is not solely determined by the ability to take up and transport O\textsubscript{2} but also is determined by tissue-specific metabolic responses, possibly including the ability to undergo a deep MRD especially in non-essential tissues (as I have argued for the epaulette
shark [see section 5.5.2]). It is reasonable to hypothesize that MRD becomes increasingly important with progressively greater hypoxia, with the end point being anoxia where enhanced O₂ supply is not useful and MRD is the only way to ensure a matching of energy supply and demand while minimizing reliance on anaerobic glycolysis. Though logistically challenging, the relationship between O₂ uptake, MRD (measured via direct calorimetry), and hypoxia tolerance at different levels of absolute and relative PₗO₂ should be examined comparatively in fishes. Similarly, I predict that in comparisons of species that have a limited ability for MRD (e.g. zebrafish, Stangl and Wegener, 1996), tissue hypoxia tolerance will be largely determined by Pₗ and the capacity for hypoxic blood O₂ transport.

My results suggest that Pₗ can be used to design relative hypoxia exposures that control for interspecific variation in O₂ uptake and transport. In fact, the relative hypoxia exposure described in Chapter 5 is one of the first experiments to examine comparative metabolic responses of fishes under such conditions. Such exposures represent a potentially powerful tool to examine the relative importance of O₂ availability for hypoxia tolerance compared with other mechanisms such as MRD. They also can be used to standardize hypoxia exposures between species such that similar hypoxemia results, using the relatively easily measured Pₗ as the basis. Similarly, my results lend support to the notion of using Pₗ in setting management guidelines for thresholds for environmental hypoxia, which are typically set at somewhat arbitrary levels (Farrell and Richards, 2009). However, it is important that the relationship I have observed between Pₗ and blood O₂ transport be validated in other species before being applied widely in this way. At the very least, knowledge of Pₗ for individual species allows for the selection of appropriate experimental hypoxia exposures for the level of desired hypoxemic stress (e.g., Gracey et al., 2001). This
is an important consideration because, traditionally, the level of hypoxia exposure has often been somewhat arbitrarily selected, resulting in the question of whether hypoxemic stress was actually achieved (e.g., Driedzic et al., 1985; Johnston and Bernard, 1982).

The results of Chapter 4 provide equivocal support for the hypothesis that hypoxic stimulation of anaerobic glycolysis occurs at $P_{\text{crit}}$ because epaulette sharks showed plasma lactate accumulation at $P_{\text{crit}}$ whereas in shovelnose rays lactate accumulation occurred at $P_{wO_2}$ below $P_{\text{crit}}$. This discrepancy is consistent with the related literature for other vertebrates, where accumulation of plasma lactate has been observed to occur at or below $P_{\text{crit}}$ in fishes and above $P_{\text{crit}}$ in amphibians (McKenzie et al., 2000; Nonnotte et al., 1993; Pörtner and Grieshaber, 1993; Pörtner et al., 1991a; Routley et al., 2002; Ultsch et al. 2004). This variation may, in part, be due to species-specific differences in activity level, MRD, and lactate handling (Omlin and Weber, 2010; Tattersall and Ultsch, 2008; Ultsch et al. 2004). In addition, the method that has been used to address this hypothesis (serial sampling of plasma during progressive hypoxia while simultaneously measuring $\dot{M}_{O_2}$) may not be able to account for temporal variation of lactate dynamics during hypoxia exposure nor be sensitive enough to accurately detect the temporal correlation between stimulation of anaerobic glycolysis and $P_{\text{crit}}$. An instructive experiment would be to expose a small species of fish to multiple discrete levels of $P_{wO_2}$ above and below $P_{\text{crit}}$ and monitor whole-body lactate content over a time course. A similar exposure with measurements of lactate turnover rate would also be useful. Ideally, NMR spectroscopy could be used to obtain integrated data on lactate and other biochemical profiles in real-time. In the absence of such experiments, at present we can only conclude that lactate accumulation does not occur above $P_{\text{crit}}$ in the species of fish examined to date.
6.4 MECHANISMS AND PATTERNS OF INTEGRATED CARDIAC HYPOXIA TOLERANCE IN ECTOTHERMIC VERTEBRATES

The findings of my thesis provide novel insight into the intrinsic and extrinsic factors that affect hypoxic cardiac performance and the ability to achieve cardiac energy balance in ectothermic vertebrates during environmental hypoxia exposure. Prior to my thesis, there were relatively few measurements of \textit{in vivo} PO in hypoxia-exposed ectothermic vertebrates and thus my work has added greatly to our knowledge of the hypoxic responses of cardiac energy demand. In all three study species, a decrease in cardiac energy demand occurred \textit{via} a bradycardia and a consequent fall in $\dot{Q}$ and $PO$, which is consistent with the second pattern of decreasing $\dot{Q}$ during hypoxia exposure in fishes suggested by Gamperl and Driedzic (2009) (see section 1.5.1). However, the extent of this decrease in $\dot{Q}$ and $PO$ and its importance for maintaining cardiac energy balance and sustained hypoxic cardiac performance appears to be affected by species-specific differences in MGP and O$_2$ supply. Furthermore, the decreases in cardiac parameters closely paralleled decreases in $M_{O_2}$, suggesting that whole-animal demand for blood flow, which can be modulated \textit{via} whole-animal MRD, exerts a powerful extrinsic influence over hypoxic cardiac performance. In this section, I discuss first how my work on tilapia (Chapter 2 and 3; also see Lague et al., 2012) has led to a revision of the limits of MGP in the ectothermic vertebrate heart and the strategies by which hypoxia-tolerant species ensure cardiac energy balance during profound hypoxia and anoxia exposure when MGP is the primary determinant of cardiac energy supply. Then, I discuss my work on elasmobranchs (Chapter 4 and 5) that indicates that enhanced O$_2$ supply also is an important component of cardiac hypoxia tolerance during
environmental hypoxia, allowing greater levels of hypoxic cardiac performance while minimizing reliance on anaerobic energy production. Following a consideration of the relationship between $M_{O_2}$ and cardiac performance during hypoxia exposure, I integrate all of my findings to propose a model describing the different patterns of cardiovascular responses to hypoxia exposure in fishes and other ectothermic vertebrates and their relationship to cardiac and whole-animal hypoxia tolerance.

6.4.1 The limits of MGP in the ectothermic vertebrate heart and strategies of anaerobic cardiac energy balance (Chapters 2 and 3)

The MGP is thought to be of central importance in ensuring cardiac energy balance during severe hypoxemia or anoxia when $O_2$ supply to the heart is most constrained and anaerobic energy production is heavily relied upon; in hypoxia-tolerant ectothermic vertebrates, a level of $PO$ can be achieved under these conditions that is sustainable by the MGP (Farrell and Stecyk, 2007). My results (Chapter 2 and 3) and those of Lague et al. (2012) on in vivo and in situ performance of the tilapia heart support this concept but force an upward revision of the limit of MGP in the ectothermic vertebrate heart. Furthermore, my findings (Chapter 3) and those of Lague et al. (2012) indicate that hypoxic depression of routine $PO$ does not necessarily occur to maintain hypoxic cardiac energy balance, contrary to previous suggestions (Farrell and Stecyk, 2007; Chapter 2).

The MGP of the tilapia heart at 22°C is approximately 148 to 172 nmol ATP s$^{-1}$ g$^{-1}$, which sustains a $PO_{\text{max}}$ of approximately 2.7 to 3.3 mW g$^{-1}$ (Chapter 3; Lague et al., 2012). This level of MGP greatly exceeds the previous estimate for the ectothermic vertebrate heart ($\sim$70 nmol ATP s$^{-1}$ g$^{-1}$ at 15°C, achieving a $PO$ of $\sim$0.7 mW g$^{-1}$) (Farrell and Stecyk, 2007),
which because of a paucity of available data was based primarily on results from rainbow trout, hagfishes and freshwater turtles only (see Fig. 9 in Lague et al., 2012). Even when the potential effect of temperature is considered (assuming a $Q_{10}$ for anaerobic glycolysis of 2.1; Overgaard et al., 2004), the original estimate is only 118 nmol ATP s$^{-1}$ g$^{-1}$ and 1.2 mW g$^{-1}$ at 22°C, which remains much lower than the cardiac MGP of tilapia. This previous underestimate may be explained by the fact that hagfishes have a low in vivo routine $PO$, rainbow trout are relatively hypoxia-sensitive, and freshwater turtles undergo a profound whole-animal and cardiac metabolic depression when experiencing hypoxia; in all cases, the result is that the MGP is comparatively low. Tilapia, on the other hand, have higher routine $PO$ than hagfishes, are hypoxia-tolerant compared with rainbow trout, and in contrast with turtles undergo a less profound hypoxic metabolic depression marked by greater levels of activity. Indeed, it is possible that the comparatively great cardiac MGP of tilapia evolved to permit an appreciable scope for anaerobic cardiac performance, which would be beneficial for short-term predator avoidance in the commonly hypoxic environments that tilapia inhabit (Chapman et al., 2002; Lague et al., 2012). It seems likely that examination of the cardiac MGP of other hypoxia-tolerant teleosts that maintain greater levels of activity during hypoxia exposure will provide new insight into the limits of MGP and hypoxic cardiac performance in the ectothermic vertebrate heart. For example, the MGP of the crucian carp is similar to that of the tilapia if differences in temperature are accounted for (see Lague et al., 2012).

The impressive MGP of the tilapia heart allowed it to operate in situ at routine $PO$ for at least 60 min of profound hypoxia exposure (Chapter 3; Lague et al., 2012). On the other hand, $PO$ is rapidly depressed by up to ~50% during in vivo exposure to profound hypoxia (Chapter 2). Thus, contrary to my previous hypothesis (Chapter 2), depression of $PO$
probably does not occur primarily to balance cardiac energy demand with energy supply during hypoxia exposure, because the MGP can easily sustain routine $PO$. Instead, as discussed in section 3.5.4, depression of $PO$ in tilapia may have the benefit of slowing fuel use and minimizing acidosis at a time when circulatory demands are decreased as a result of whole-animal MRD. Although the heart (ventricle) represents a relatively small percentage of total body mass (approximately 0.037% in tilapia; B. Speers-Roesch, unpublished results), it has a high mass-specific energy demand. In tilapia exposed to a $P_o$ of 1 kPa, venous $PO_2$ is approximately 0.5 kPa (B. Speers-Roesch, unpublished results) and at this $PO_2$ and a routine $PO$, cardiac lactate efflux rate of the heart is ~2800 nmol min$^{-1}$ g ventricle$^{-1}$ (Lague et al., 2012). A 500 g tilapia with a 185 mg ventricle under these conditions would produce 0.249 mmol lactate over an 8 hour exposure. Assuming an equal distribution of this lactate in the body (which is probably unlikely), this results in a minimum lactate load of 0.5 umol/g wet tissue, which is not insubstantial (e.g., it is 10% of the peak in cardiac lactate content; Fig. 2.6B). Equally important, glycogen levels in tilapia heart and liver are about 0.24 and 0.92 mmol glucosyl units g$^{-1}$, respectively (Wright et al., 1998); production of 0.249 mmol lactate represents about 50% of the heart stores or 14% of liver stores (assuming 2 lactate produced from one glucosyl unit). Overall, these calculations demonstrate that depression of $PO$ is of quantitative significance for minimizing fuel use and waste production.

Additionally, minimizing cardiac anaerobic energy production by depressing $PO$ could reduce the degree of localized intracellular acidosis; there are significant depressive effects of acidosis on cardiac performance in tilapia (Lague et al., 2012).

These results lead us to slightly revise Farrell and Stecyk’s (2007) proposal of the two strategies by which cardiac energy balance is achieved during profound hypoxia or anoxia.
exposure in hypoxia-tolerant ectothermic vertebrates. In both strategies, the \( PO \) achieved is sustainable by the MGP and this appears to be a crucial component of cardiac hypoxia tolerance in hypoxia-tolerant ectothermic vertebrates. In the first strategy (Fig. 6.1 panel I), observed in hagfishes, crucian carp, and tilapia, routine \( PO \) can be sustained by the MGP but there are two patterns of \textit{in vivo} responses of \( PO \) during hypoxia exposure: 1) maintenance of routine \( PO \) in species with a very low routine \( PO \) (e.g., 0.1 to 0.2 mW g\(^{-1}\) for hagfishes at 10-18°C; Axelsson et al., 1990; Cox et al., 2010; Forster, 1991) or with unique waste management strategies (e.g., crucian carp; Vornanen et al., 2009) (\( PO \) level “B” in Fig. 6.1 panel I), and 2) downregulation of routine \( PO \) in species with a higher routine \( PO \) (e.g. \(~1.3\) mW g\(^{-1}\) for tilapia at 22°C; Chapter 2) for reasons independent of the requirement for balancing cardiac energy supply and demand, possibly including minimizing fuel use and waste production (\( PO \) level from “A” to “B” in Fig. 6.1 panel I). Consequently, within species that exhibit the first strategy, the level of MGP varies greatly and the level may depend upon the activity level of the animal and the selective pressures associated with hypoxic cardiac performance in their natural environment. The second strategy is the same as that originally proposed by Farrell and Stecyk (2007), where routine \( PO \) is above the level sustainable by the MGP and downregulation of \( PO \) is required to match energy supply and demand during profound hypoxia or anoxia exposure (\( PO \) level from “A” to “B” in Fig. 6.1 panel II). This strategy has only been conclusively demonstrated in freshwater turtles (Arthur et al., 1997; Hicks and Farrell, 2000; Stecyk et al., 2004a), which as discussed above have a comparatively low MGP. Based on indirect evidence Farrell and Stecyk (2007) suggested that the common carp may also show this strategy, but the work on tilapia in the present thesis and in Lague et al. (2012) indicates that the MGP in hypoxia-tolerant fishes may be
more impressive than previously anticipated, suggesting that common carp could in fact display the second pattern of the first strategy. Further studies are required to identify which of these strategies (and which of the two patterns of the first strategy) is most universal among hypoxia-tolerant ectothermic vertebrates.

6.4.2 Superior \( {\text{O}}_2 \) supply is associated with enhanced hypoxic cardiac performance and greater hypoxia tolerance (Chapters 4 and 5)

Despite the importance of MGP for maintaining cardiac energy balance in hypoxia-tolerant animals when \( {\text{O}}_2 \) availability is extremely limited during profound hypoxia or anoxia, my work on the epaulette shark and the shovelnose ray affirms that \( {\text{O}}_2 \) supply also plays an important role in cardiac hypoxia tolerance and the ability to sustain cardiac performance during environmental hypoxia. Essentially, cardiovascular performance is less sensitive to decreases in \( P_{wO_2} \) in the more hypoxia-tolerant epaulette shark, with improved hypoxic maintenance of routine performance and less hypoxic depression of \( PO \) all while showing better maintenance of cardiac energy balance and less accumulation of anaerobic end-products. This appears to be largely explained by an enhanced blood \( {\text{O}}_2 \) content via a lower \( \text{Hb-}{\text{O}}_2 P_{50} \) as well as strategic cardiac \( {\text{O}}_2 \) delivery via \( {\text{O}}_2 \) sparing related to metabolic depression in non-essential tissues. Although \( {\text{O}}_2 \) sparing for essential tissues via preferential blood delivery has been suggested to be an important response in hypoxia-tolerant animals (Gracey et al., 2001; Stecyk and Farrell, 2006; Stenslokken et al., 2004), my data suggest that MRD in non-essential tissues also may play an important role in \( {\text{O}}_2 \) sparing. However, my evidence is indirect and further studies are necessary to thoroughly test the notion of \( {\text{O}}_2 \) sparing, including measurement of venous blood \( {\text{O}}_2 \) content in the sinus venosus to confirm
the prediction that it is indeed higher in the epaulette shark compared with the shovelnose ray. Such measurements could be made in other hypoxia-tolerant and -sensitive species to ascertain if O₂ sparing for the heart is a common extrinsic mechanism to improve hypoxic cardiac performance. Based on available studies in fishes showing fewer hypoxic transcriptional responses in heart compared with other tissues (Gracey et al., 2001) as well as hypoxia-induced increases in cardiac myoglobin (Roesner et al., 2006), aerobic enzymes (Ngan and Wang, 2009), and lactate transporters involved in lactate uptake (presumably for oxidation) (Ngan and Wang, 2009), optimization of cardiac oxidative metabolism may be a priority in hypoxia-exposed fishes.

As discussed in section 6.4.1, depression of PO has been focused on as a key element of cardiac hypoxia tolerance. However, my data show that the maintenance of greater levels of cardiac performance including PO (via enhanced and strategic cardiac O₂ supply) may be an equally important strategy for hypoxia tolerance in fishes, possibly because of the benefits for peripheral O₂ delivery (especially to the brain) and management of fuel and waste. In fact, like the similarly hypoxia-tolerant epaulette shark, the tilapia also appears to show less depression of routine PO during hypoxia exposure compared with the more hypoxia-sensitive shovelnose ray (Fig. 6.2). The tilapia had a Hb-O₂ P₅₀ even lower than that of the epaulette shark so it is possible that improved O₂ supply may also benefit hypoxic cardiac performance in this species. Nevertheless, even in hypoxia-tolerant species with greater levels of cardiac performance, hypoxic decreases of PO occur and may be important for maintaining cardiac energy balance and/or minimizing reliance on anaerobic energy production in the face of declining blood O₂ content (see section 6.4.4).
6.4.3 Hypoxic responses of whole-animal \( \dot{M}_{O_2} \) and cardiac performance are closely associated but this association is species-specific among fishes (Chapters 2 and 5)

Cardiac performance, and in particular \( \dot{Q} \), is matched to the whole-animal metabolic demand for blood flow, including the requirement for \( O_2 \) delivery to tissues (Webber et al., 1998). Consequently, hypoxic modulation of whole-animal demand for blood flow, for example via MRD, is thought to influence hypoxic cardiac performance (Jackson, 2000). For example, an MRD of up to 90% occurs in anoxic freshwater turtles and \( \dot{Q} \) (as well as \( PO \)) is decreased by a similar amount (Hicks and Farrell, 2000; Jackson, 2000). My findings are consistent with this concept because in all the study species there was a close association between hypoxic decreases in cardiac performance (in particular, \( f_{Hi} \), \( \dot{Q} \), and \( PO \)) and \( P_{crit} \) as well as subsequent declines in \( \dot{M}_{O_2} \) (Fig. 6.3). Furthermore, in tilapia I observed a \( \sim 50\% \) decrease in \( f_{Hi} \), \( \dot{Q} \), and \( PO \) in tilapia exposed to profound hypoxia (\( P_{wO_2} = 1 \) kPa) (Chapter 2), which matches the \( \sim 50\% \) decrease in whole-animal heat production (i.e., MRD) observed in tilapia exposed to the same \( P_{wO_2} \) (van Ginneken et al., 1997). A close relationship between MRD and bradycardia also has been observed in goldfish (van Ginneken et al., 2004). Interestingly, the \( \dot{M}_{O_2} \) of tilapia at a \( P_{wO_2} \) of 1 kPa is decreased by a greater amount (70-80\%) (van Ginneken et al., 1997; Chapter 2), suggesting that during profound hypoxia when \( O_2 \) uptake and transport to tissues is limited, cardiac performance remains matched to metabolic demand rather than \( \dot{M}_{O_2} \) \textit{per se}, at least in tilapia. Nonetheless, \( \dot{M}_{O_2} \) remains a useful approximation of whole-animal metabolic rate, especially at less severe levels of hypoxia (van Ginneken et al., 1997; van Ginneken et al., 2004). Overall, my data suggest that hypoxic cardiac performance is influenced by decreases in whole-animal metabolic rate in
my study species, although the observed hypoxic cardiac responses may additionally have intrinsic benefits for cardiac hypoxia tolerance because decreased $\dot{M}_{O_2}$ below $P_{\text{crit}}$ is associated with increasing hypoxemia (Chapter 4) that can affect cardiac function (see section 6.4.4).

An association between the initiation of hypoxic bradycardia and $P_{\text{crit}}$ appears to be common to all fishes that have been studied (summarized in Figure 5.6), with further decreases in $\dot{M}_{O_2}$ below $P_{\text{crit}}$ being associated with further decreases in $f_H$. However, although $\dot{M}_{O_2}$ decreases towards zero with progressive decreases in $P_{wO_2}$ below $P_{\text{crit}}$, the magnitude of bradycardia may be limited by the minimum pacemaker rhythm or by whole-animal demand for blood flow, as suggested by the plateau in $f_H$ seen in shovelnose ray at the lowest $P_{wO_2}$ (Fig. 5.1A) and by the aforementioned matching of cardiac performance to metabolic rate rather than $\dot{M}_{O_2}$ in tilapia.

Much less information is available on the relationship between $P_{\text{crit}}$ and $\dot{Q}$ or $PO$, but it appears to be more species-specific than that between $P_{\text{crit}}$ and bradycardia. I observed that decreases in $\dot{M}_{O_2}$ below $P_{\text{crit}}$ are closely associated with decreases in $\dot{Q}$ (due to bradycardia) in tilapia, epaulette sharks, and shovelnose ray, which is similar to findings for Anguilla spp. eels (Chan, 1986; Iversen et al., 2010). My studies also provide the first direct evidence that decreases in $PO$ accompany decreases in $\dot{M}_{O_2}$ below $P_{\text{crit}}$, at least in fishes that allow $\dot{Q}$ to decrease (Gamperl and Driedzic, 2009). On the other hand, studies on Atlantic cod show that this species attempts to defend $\dot{Q}$ (and probably $PO$, based on measurements of $\dot{Q}$ and $P_{\text{DA}}$) below $P_{\text{crit}}$ even while $\dot{M}_{O_2}$ falls (Petersen and Gamperl, 2011). These species-specific
differences may be related to differences in hypoxia tolerance and whole-animal metabolic demand for blood flow during hypoxia exposure (see section 6.4.4).

6.4.4 Integration of MGP, O₂ supply, and whole-animal demand for blood flow: a model for cardiac hypoxia tolerance in fishes and other ectothermic vertebrates

The above discussion demonstrates that both intrinsic (e.g., MGP, PO₂ depression, cardiac O₂ utilization) and extrinsic factors (e.g., declines in ṀO₂ and metabolic rate and their influences on O₂ availability and the demand for blood flow) appear to impact hypoxic cardiac performance and cardiac hypoxia tolerance in ectothermic vertebrates. In this section, I present a model for cardiac hypoxia tolerance that integrates these intrinsic and extrinsic factors and builds upon Gamperl and Driedzic’s (2009) scheme of three patterns of hypoxic responses of ˙Q in fishes. I propose that the cardiovascular responses in fishes exposed to environmental hypoxia can be classified as hypoxia-sensitive, hypoxia-tolerant, or anoxia-tolerant (including type “A” that downregulate cardiac performance and type “B” that maintain cardiac performance) (Fig. 6.4).

In the hypoxia-sensitive response, which corresponds to pattern 1 of Gamperl and Driedzic’s (2009) scheme and is observed in rainbow trout (Gamperl et al., 1994; Holeton and Randall, 1967b; Wood and Shelton, 1980) and Atlantic cod (Petersen and Gamperl, 2011), bradycardia occurs at P_{crit} and f_{H} and ṀO₂ decrease together as P_{w}O₂ falls. This bradycardia is initially compensated for by increased V^{SH}_{SH} such that ˙Q is maintained at or even increased above resting normoxic levels, perhaps in an attempt to continue to meet the whole-animal demand for blood flow (including O₂ demand), which may be little changed because hypoxia-sensitive species may have a comparatively limited ability for hypoxic
MRD. Because $\dot{Q}$ is maintained and is accompanied by increased blood pressure, the bradycardia apparently does not occur in these species to decrease $PO$ nor does it appear to be a response to extrinsic decreases in $\dot{M}_{O_2}$; rather, it is possible that bradycardia in the hypoxia-sensitive response occurs primarily to improve myocardial $O_2$ delivery during the severe hypoxemia occurring below $P_{crit}$ (Farrell, 2007; see section 1.5.1). If $P_aO_2$ continues to fall, however, eventually $\dot{Q}$ also falls and this may be associated with a large drop in blood pressure and $f_H$ (Gamperl et al., 1994; Petersen and Gamperl, 2011). I suggest that, at this point, cardiac failure is imminent and results from a mismatch between $PO$ and cardiac $O_2$ supply; MGP in these species may be limited (certainly it is in trout; Arthur et al., 1992) and cannot sustain the operating $PO$. Accumulation of lactate and metabolic acidosis may hasten the cardiac failure (Overgaard et al., 2004). This hypothesis is supported by the observation of cardiac failure in hypoxia-exposed rainbow trout (Gamperl et al., 1994) where blood $PO_2$ was at a level ($P_aO_2 = \sim 2.7$ kPa; $P_vO_2 = \sim 1.3$ kPa) that is associated with cardiac arrhythmia in exercised coronary-ligated trout (Steffensen and Farrell, 1998) as well as failure of routine $PO$ in situ ($\leq 2.7$-3.4 kPa; Farrell et al., 1989; Hanson et al., 2006; Overgaard et al., 2004). Also, the in vivo hypoxic cardiac failure in cod occurs at a venous $PO_2$ of $\sim 2$ kPa (Petersen and Gamperl, 2011), which approaches the $PO_2$ at which routine $PO$ cannot be sustained in an in situ preparation that operated at a lower $PO$ (considering the hypoxic increases of $\dot{Q}$ and blood pressure that occur in vivo) and under non-acidotic conditions (Petersen and Gamperl, 2010). Importantly, in cod, the hypoxic cardiac failure has clearly been shown to be closely associated with hypoxic loss of equilibrium (Petersen and Gamperl, 2011). Overall, in the hypoxia-sensitive response, the heart attempts to maintain blood flow to sustain the relatively unchanged whole-animal metabolic demand but it fails because of a limited cardiac
O₂ supply and MGP, leading to a circulatory collapse that is associated with whole-animal loss of equilibrium.

The hypoxia-tolerant response (Fig. 6.4) corresponds to pattern 2 of Gamperl and Driedzic’s (2009) scheme. It is exemplified by the shovelnose ray (Chapter 5) but may be characteristic of many fish species, including Anguilla eels (Chan, 1986; Iversen et al., 2010; Peyraud-Waitzenegger and Soulier, 1989), lingcod (Farrell, 1982), sea bass (Axelsson et al., 2002), short-horn sculpin (MacCormack and Driedzic, 2004), and smallmouth bass (Furimsky et al., 2003). However, in the available studies the experimental hypoxia exposures have been limited in their severity so it may be that some of these species (e.g. eel, which have anoxia-tolerant myocardium (Bailey et al., 1990; Hartmund and Gesser, 1992)) actually demonstrate the anoxia-tolerant response (see below). On the other hand, I propose that the species suggested to correspond to pattern 3 of Gamperl and Driedzic’s (2009) scheme are in fact representatives of the hypoxia-tolerant response, as discussed in section 1.5.1.

In the hypoxia-tolerant response, similar to the hypoxia-sensitive response, there is a bradycardia at P_{crit} and a subsequent decline in f_{H} with decreasing M_{O₂} that may benefit myocardial O₂ delivery (Farrell, 2007). However, unlike the hypoxia-sensitive response, the bradycardia also drives a decrease in Ṕ that may or may not be partially compensated for by an increase in V_{SH} (e.g., an increase in shovelnose ray (Chapter 5) vs. no increase in short-horn sculpin (MacCormack and Driedzic, 2004)). I suggest that the depression of Ṕ is important for decreasing PO, as demonstrated in shovelnose rays, because this allows cardiac energy demand to be matched to the decreasing energy supply resulting from hypoxemia below P_{crit}, thus ensuring continued cardiac function for as long as fuel stores last and
metabolic acidosis can be tolerated. Importantly, the depression of $PO$ may be facilitated by whole-animal MRD, which decreases circulatory demands. However, the magnitude of MRD that can be achieved may be limited indicating that there is a minimum level of $PO$ required to sustain the minimum systemic requirement for internal convection. Alternatively, the minimum pacemaker rhythm or the requirement to maintain blood pressure above the critical closing pressure of capillaries (Panerai, 2003) could constrain the magnitude of $PO$ depression that is possible. In either case, if $P_{wO_2}$ continues to decrease, eventually a point will be reached where the minimum $PO$ can no longer be sustained by available $O_2$ or by the MGP, which may not be large. Thus, cardiac energy demand exceeds energy supply and cardiac failure occurs. My data on shovelnose rays appears to support this line of reasoning.

At the most severe level of progressive hypoxia exposure, which was just above the $P_{wO_2}$ at which shovelnose rays rapidly lost equilibrium, there was a plateau of $f_H$ and $\dot{Q}$ coupled with a continued decrease in $PO$ and $P_{DA}$ that could be symptomatic of a failing heart (Fig. 5.1 and Fig. 5.2). Furthermore, exposure at this level of hypoxia was also associated with evidence of perturbation of cardiac energy balance as indicated by cardiac $[CrP]$ (Fig. 5.3). Further support for this hypothesis could be obtained by determining the $PO_2$ thresholds for sustainable in vivo and in situ cardiac performance at different levels of $PO$ and linking these findings with measurements of cardiac energy balance (e.g. ATP and CrP).

An organism showing the hypoxia-tolerant response can be less tolerant or more tolerant of environmental hypoxia depending on the $P_{wO_2}$ at which cardiac failure occurs (Fig. 6.4). Maintenance of cardiac function to a lower $P_{wO_2}$ (i.e., an increase in cardiac hypoxia tolerance) can be achieved in several ways. Because the hypoxia-tolerant response is initiated at $P_{crit}$, species with lower $P_{crit}$ can therefore maintain routine cardiac performance to
a lower $P_{wO_2}$. Furthermore, my work shows that a lower $P_{crit}$ (and a lower Hb-O$_2$ $P_{50}$) is associated with greater blood O$_2$ transport at $P_{wO_2}$ below $P_{crit}$ (Chapter 4) and the enhanced O$_2$ supply can improve cardiac performance, as suggested for the epaulette shark (Chapter 5). The ability to depress $PO$ to a greater extent (e.g., by achieving a greater whole-animal MRD) may also be important to ensure cardiac energy balance and minimize reliance on anaerobic energy production, especially as $P_{wO_2}$ approaches anoxia. Finally, a greater MGP can improve anaerobic energy production, facilitating maintenance of cardiac energy balance but with the cost of inefficiency and waste production. In fact, given the downsides of high rates of anaerobic energy production, full exploitation of a greater MGP may ultimately only be useful for short-term bouts of greater hypoxic cardiac performance, such as predator evasion (as argued for tilapia; Chapter 3).

As described in section 6.4.1, the ability to achieve a $PO$ that is sustainable by the MGP is a central strategy of cardiac hypoxia tolerance among the most hypoxia- and anoxia-tolerant ectothermic vertebrates, including crucian carp, hagfishes, tilapia, and freshwater turtles. These organisms demonstrate the anoxia-tolerant response, of which there are species that downregulate cardiac function (type “A”) and those that maintain it during hypoxia exposure below $P_{crit}$ (type “B”) (Fig. 6.4). Type “A” species include tilapia (Chapter 2) and freshwater turtles (Hicks and Farrell, 2000; Stecyk et al., 2004a). The anoxia-tolerant epaulette shark (Renshaw et al., 2002; Routley et al., 2002) may also be a type “A” species, considering that stable cardiac energy status and cardiac function were present even under profound hypoxia exposure (Chapter 5). Similarly, the common carp can maintain a stable level of depressed cardiac function during profound hypoxia (Stecyk and Farrell, 2006). In type “A” species, the hypoxic cardiac responses are similar to those seen in the hypoxia-
tolerant response except that the responses occur at a lower $P_{w}O_2$ as a result of a lower $P_{crit}$, cardiac performance is greater at any given $P_{w}O_2$ below $P_{crit}$ due to enhanced $O_2$ supply, and, most importantly, a level of hypoxic $PO$ can be achieved that is sustainable by the MGP, allowing continued cardiac function in the absence of $O_2$ (but this cardiac hypoxia tolerance is tempered by acidosis and fuel depletion). The downregulation of $PO$, which is facilitated by a large MRD (e.g., Jackson, 1968; van Ginneken et al., 1997), may be needed to balance energy supply and demand (e.g., in turtles) or simply to minimize reliance on anaerobic energy production (e.g., in tilapia where routine $PO$ can be sustained by MGP) (see section 6.4.1).

In type “B” species showing an anoxia-tolerant response, including crucian carp and hagfishes, a bradycardia occurs (presumably at $P_{crit}$ and probably with the same benefits as in the other responses) but $\dot{Q}$ and $PO$ are little changed from routine levels even during anoxia exposure (Axelsson et al., 1990; Cox et al., 2010; Stecyk et al., 2004b) (Fig. 6.4). This is possible because routine $PO$, which is low, is sustainable by the MGP (see section 6.4.1 for further discussion). Essentially, these species represent the true representatives of the third pattern mentioned by Gamperl and Driedzic (2009).

In vivo and in situ studies of the hypoxic cardiac performance of a greater variety of hypoxia-sensitive, hypoxia-tolerant, and anoxia-tolerant species of ectothermic vertebrates are necessary to test my proposed model for cardiac hypoxia tolerance. In future studies, progressive hypoxia exposure should be paired with prolonged exposure at different levels of $P_{w}O_2$ (similar to my work on tilapia; Chapter 2), to better ascertain both the sensitivity of cardiac performance to changes in $P_{w}O_2$ as well as the stability at given levels of hypoxia.
6.5 FINAL THOUGHTS: IS THE HEART THE SLAVE OR THE MASTER?

In the General Introduction I posed the question of whether the hypoxic depression of cardiac performance in hypoxia-tolerant fishes and other ectothermic vertebrates primarily serves the extrinsic requirements of the whole animal for blood flow (i.e., the heart is the “slave”) or whether it primarily serves the intrinsic requirements of the heart itself to ensure continued cardiac metabolic energy balance and function (i.e., the heart is the “master”). Based on my findings and my proposed model of cardiac hypoxia tolerance, I suggest this question represents a false dichotomy and that both factors have an important impact on hypoxic cardiac performance and cardiac hypoxia tolerance. In general, the systemic requirement for blood flow does appear to exert a powerful influence over the level of hypoxic cardiac performance. This finding does not, however, exclude the possibility that hypoxic cardiac responses such as depression of \( PO \) are intrinsically important. For example, in anoxia-exposed freshwater turtles, downregulation of \( PO \) matches the profound MRD but this downregulation is also required to balance cardiac energy supply and demand because routine \( PO \) cannot be sustained by the MGP (Farrell and Stecyk, 2007). On the other hand, tilapia initially appear to support the argument that the heart is merely the slave, because hypoxic depression of \( PO \) is not required to sustain cardiac energy balance yet occurs anyways in concert with MRD (see section 6.4.3). However, acidosis of a magnitude seen during \textit{in vivo} hypoxia exposure has a large effect on the routine performance of the \textit{in situ} tilapia heart and during long-term exposure could make routine \( PO \) unsustainable (Lague et al., 2012). Bradycardia also appears to be of intrinsic benefit to the heart, possibly for one of the four major reasons proposed by Farrell (2007), because it occurs even in hypoxia-sensitive species that defend \( \dot{Q} \) and \( PO \) (see section 6.4.4). Indeed, abolishment of hypoxic
bradycardia via vagotomy in Atlantic cod was associated with a greater likelihood of cardiac failure and loss of whole-animal equilibrium at $P_{wO_2}$ below $P_{crit}$ (McKenzie et al., 2009). Similarly, a greater incidence of cardiac arrhythmia occurred when bradycardia was abolished in atropinized European eels during hypoxia exposure (Iversen et al., 2010). Iversen et al.’s (2010) study is particularly instructive because, unlike untreated eels, the atropinized eels showed no decrease in $\dot{Q}$ during progressive hypoxia exposure. This suggests that, assuming minimal effects of atropine on blood pressure (Stecyk and Farrell, 2006), $PO$ also would not have decreased. Thus, the cardiac dysfunction observed could have resulted from a mismatch between cardiac energy supply and demand or from perturbing effects of localized intracellular acidosis resulting from higher $PO$ (e.g., Overgaard et al., 2004). Further studies on atropinized and vagotomized fishes during hypoxia exposure are needed to examine the relationship between $PO$, cardiac hypoxia tolerance, and whole-animal hypoxia tolerance.

I also propose that the ability of the heart to perform at the level of $PO$ that is set by the minimum whole animal metabolic demand during hypoxia exposure ultimately is a “master” determinant of hypoxic survival and may be, along with the brain, a weak link in hypoxia tolerance. For example, in hypoxia-sensitive species, such as Atlantic cod and rainbow trout, cardiac failure appears to coincide with loss of whole-animal equilibrium (Gamperl et al., 1994; Petersen and Gamperl, 2011). Also, in the shovelnose ray, $PO$ depression occurs below $P_{crit}$ but if $P_{wO_2}$ falls below $\sim 2.7$ kPa evidence of cardiac failure is observed (including perturbed cardiac energy balance) and loss of equilibrium follows rapidly (Chapter 5). In more hypoxia- or anoxia-tolerant species, such as tilapia, the heart may be less of a weak link because cardiac energy balance can be maintained under anoxia
(Chapter 3; Lague et al., 2012), but, ultimately, progressive metabolic acidosis may lead to cardiac dysfunction including severe bradycardia, with lethal consequences for the animal. Interestingly, failure of the hypoxia-sensitive rainbow trout heart following 20-30 min of in situ severe hypoxia or anoxia exposure does not appear to be associated with myocardial necrosis (Faust et al., 2004; Overgaard et al., 2004), which contrasts with the typical situation in mammals (Jennings and Reimer, 1991) (although it is worth noting that <20 min of coronary occlusion in dogs does not result in necrosis (Bolli and Marbán, 1999)). Nevertheless, even if hypoxic cardiac failure in fishes is not associated with myocardial necrosis (i.e., it may be reversible in the short-term), the resulting impairment of peripheral blood flow, especially to the brain, can be fatal.

A final hypothesis that warrants discussion is that the decreases in whole-animal \( \dot{M}_{O_2} \) and metabolic rate below \( P_{\text{crit}} \) are caused by tissue hypoperfusion resulting from the depression of \( \dot{Q} \) (Coulson, 1986; van Ginneken et al., 2004). Hypoperfusion causes oxyconformation of tissue \( \dot{M}_{O_2} \) below a critical \( O_2 \) delivery rate in a wide variety of animals and tissues (Boutilier, 2001b; Forgan and Forster, 2012; Schlichtig et al., 1992). This phenomenon is best studied in skeletal muscle, which receives the vast proportion of \( \dot{Q} \) in fishes (Schultz et al., 1999). The oxyconformation of tissue \( \dot{M}_{O_2} \) during hypoperfusion occurs because tissue \( \dot{M}_{O_2} \) decreases instantaneously with a decrease in the \( P_{O_2} \) gradient between the perfusate and the tissue (Boutilier, 2001b; Forgan and Forster, 2010). The underlying mechanisms of oxyconformation remain unclear, but appear to involve intracellular sensing of decreasing \( P_{O_2} \) and consequent downregulation of energy demand pathways, rather than a diffusion limitation (Forgan and Forster, 2010; Forgan and Forster,
Based on these findings, it seems reasonable that depression of $\dot{Q}$ might result in a hypoperfusion of skeletal muscle that could contribute to the decline in whole animal $\dot{M}_{O_2}$ as well as MRD below $P_{\text{crit}}$. To date, however, the evidence for this hypothesis is weak. A strong correlation between $f_{\text{H}}$ and metabolic heat production in hypoxia-exposed goldfish was taken by van Ginneken et al. (2004) as supporting evidence for the hypothesis, but $\dot{Q}$ was not measured. The close associations between $\dot{M}_{O_2}$ and $\dot{Q}$ below $P_{\text{crit}}$ observed in the three study species in the present thesis are likewise correlative and may simply reflect matching of $\dot{Q}$ to metabolic demand (Fig. 6.3). Janssen et al. (2010) showed that in whole anaesthetized snapper ($\textit{Pagrus auratus}$) exposed to progressive hypoxia, perfusion of white muscle decreased prior to local tissue hypoxemia and suggested that upstream modulation of blood delivery (rather than local responses to tissue hypoxemia) was the explanation. However, the decrease in perfusion preceded the observed hypoxic bradycardia ($\dot{Q}$ was not measured), so it is unclear whether changes in $\dot{Q}$ or autonomically-regulated vasoconstriction underlie the hypoperfusion.

Finally, Iversen et al.’s (2010) study on eels appears to directly contradict the proposed hypothesis because abolishment of hypoxic decreases in $f_{\text{H}}$ and $\dot{Q}$ via treatment with atropine had no effect on the response of whole-animal $\dot{M}_{O_2}$ below $P_{\text{crit}}$. Even if depression of $\dot{Q}$ is not the primary cause of decreases in $\dot{M}_{O_2}$ below $P_{\text{crit}}$, hypoperfusion of non-essential tissues such as white muscle or the gut via other mechanisms (e.g., $\alpha$-adrenergic vasoconstriction (Stecyk and Farrell, 2006)) may still be an important mechanism by which $\dot{M}_{O_2}$ is decreased below $P_{\text{crit}}$ (Boutilier, 2001b; Forgan and Forster, 2012; Janssen et al., 2010). The close association between $P_{\text{crit}}$ and increases in peripheral resistance in tilapia (Chapter 2) is
consistent with this notion, although no similar clear relationship was observed in the elasmobranchs (Chapter 5). Further mechanistic studies, including pharmacological manipulation, are needed to better understand the relationship between $\dot{Q}$, peripheral blood flow, and whole-animal and tissue $\dot{M}_\text{O}_2$ during hypoxia exposure in ectothermic vertebrates.

There is much work yet to be done on the mechanisms and patterns of cardiac hypoxia tolerance and performance and its relationship to whole-animal hypoxia tolerance in ectothermic vertebrates. The present thesis highlights the importance of considering the contribution of extrinsic as well as intrinsic factors in future studies given the integrated role of the heart in whole-animal metabolism. Finally, the evolution of the mechanisms of cardiac hypoxia tolerance is a topic that has been relatively understudied but which presents a ripe field for inquiry.
Figure 6.1. Schematic diagrams, revised from Farrell and Stecyk (2007), illustrating how cardiac metabolic energy balance and sustained cardiac function can be ensured in hypoxia-tolerant ectothermic vertebrates during profound hypoxia or anoxia exposure when O₂ supply to the heart is greatly constrained and anaerobic energy production is heavily relied upon. In both panels, the dashed box labeled “aerobic” indicates the maximum PO sustained under normoxic conditions. Panel I represents species showing strategy 1 where routine PO can be sustained by the MGP but there are two patterns of in vivo responses of PO during hypoxia exposure: #1) maintenance of a low routine PO at level B that is sustainable by a low MGP ("MGP 1", which is similar to the original estimate of Farrell and Stecyk (2007) for the ectothermic vertebrate heart), or #2) downregulation of PO from routine level A to a hypoxic depressed level B despite the presence of an MGP that can sustain routine PO ("MGP 2", which represents the new limit for the MGP in the ectothermic vertebrate heart based on studies on tilapia [Chapter 3; Lague et al., 2012]). The estimated limits of MGP 1 and MGP 2 are indicated but for clarity are not shown to scale. Panel II represents species showing strategy 2 where routine PO is above the level sustainable by the MGP (which is similar to the original estimate of Farrell and Stecyk (2007)) and hypoxic downregulation of PO is required to match energy supply and demand (PO level from “A” to “B”). See section 6.4.1 for further details.
Figure 6.2. Cardiac power output (PO) as a % of the normoxic routine value in tilapia, epaulette sharks, and shovelnose rays exposed to progressive decreases in water PO$_2$ (P$_w$O$_2$). At P$_w$O$_2$ below P$_{crit}$, PO is depressed in all species but is depressed less in the more hypoxia-tolerant tilapia and epaulette shark (see section 6.4.2).
Figure 6.3. Relationships between whole animal $\dot{M}_{O_2}$ and heart rate (A), cardiac output ($\dot{Q}$) (B), and cardiac power output ($PO$) (C) in tilapia, epaulette sharks, and shovel nose rays, expressed as a percentage of the routine normoxic value. In all species, there was a significant correlation between each cardiovascular parameter and $\dot{M}_{O_2}$ (linear regression, $p<0.0001$).
Figure 6.4. (overleaf). A model for cardiac hypoxia tolerance in fishes and other ectothermic vertebrates. Cardiovascular responses in fishes exposed to environmental hypoxia can be classified as hypoxia-sensitive, hypoxia-tolerant, or anoxia-tolerant (including type “A” that downregulate cardiac performance and type “B” that maintain cardiac performance). Panel A shows responses of heart rate and panel B shows the responses of $\dot{Q}$ and $PO$. In all three responses, bradycardia occurs below $P_{\text{crit}}$ and this may be beneficial for myocardial $O_2$ delivery (Panel A). In the hypoxia-sensitive response, $\dot{Q}$ and $PO$ are defended as bradycardia develops, but eventually cardiac failure occurs as a result of a mismatch between $PO$ and cardiac $O_2$ supply and the absence of a sufficiently large MGP to sustain the $PO$ that is required to meet whole animal demand for blood flow (Panel A). In the hypoxia-sensitive response, $\dot{Q}$ and $PO$ are downregulated via bradycardia, allowing cardiac energy demand to be matched to the decreasing energy supply resulting from hypoxemia below $P_{\text{crit}}$, thus ensuring continued cardiac function for as long as fuel stores last and metabolic acidosis can be tolerated. If $P_{wO_2}$ continues to decrease, eventually a point will be reached where the minimum $PO$ can no longer be sustained by available $O_2$ or by the MGP, which may not be large, and cardiac failure occurs. The $P_{wO_2}$ at which cardiac failure occurs in the hypoxia-tolerant response determines whether an organism is less tolerant or more tolerant of environmental hypoxia and this point can be modified by a number of factors (see section 6.4.4). The type “A” anoxia-tolerant response is similar to the hypoxia-tolerant response, except that cardiac performance is enhanced during environmental hypoxia and a level of hypoxic $PO$ can be achieved that is sustainable by the MGP, allowing continued cardiac function in the absence of $O_2$ (but again, limited by fuel stores and tolerance of acidosis). In the type “B” anoxia-tolerant response, $PO$ is sustainable by the MGP and remains unchanged even during anoxia exposure. See section 6.4.4 for further details.
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