MECHANISMS, EVOLUTION, AND PHENOTYPIC PLASTICITY OF HYPOXIA TOLERANCE AMONG BASS AND SUNFISH (FAMILY CENTRARCHIDAE)

By:

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TITLE: Mechanisms, evolution, and phenotypic plasticity of hypoxia tolerance among bass and sunfish (family Centrarchidae)

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ABSTRACT

Hypoxia events are increasing in severity, frequency, and duration in aquatic systems as pressure from pollution and global warming continues to accumulate. Many studies have examined hypoxia tolerance in fish, but few have determined the relationship that hypoxia tolerance has with other organismal traits, such as exercise performance or thermal tolerance. In this thesis, I used fish from the family Centrarchidae to examine the potential tradeoff between hypoxia tolerance and exercise performance, and to discover the physiological determinants of oxygen transport that underlie these traits. I found evidence for a tradeoff between hypoxia tolerance and exercise performance, with largemouth bass having the best exercise performance but the poorest hypoxia tolerance. Rock bass had the best tolerance of hypoxia but the worst exercise performance, while pumpkinseed sunfish and bluegill sunfish were intermediate in both traits. Hypoxia tolerance and exercise performance were each underlain by some distinct traits, with increased swimming muscle capillarity conveying high performance and the capacity for anaerobic metabolism in the heart granting augmented hypoxia tolerance. However, some common traits defined both the most hypoxia tolerant and the best swimming species, including total surface area of the gills and gluconeogenic capacity in the liver.

I also examined the effect that both temperature and acclimatization to the native environment had on hypoxia tolerance in sunfish. Increased temperature decreased hypoxia tolerance in all sunfish, and it had a greater effect on pumpkinseed sunfish than on the other species. Acclimatization to the native environment increased hypoxia tolerance compared to that in fish acclimated to the lab. This thesis advanced our
understanding of how organismal traits can interact with one another and showed evidence for a tradeoff between hypoxia tolerance and exercise performance. In addition, I found that both exercise performance and hypoxia tolerance are underlain by some common and some distinct physiological traits. My work will contribute to our understanding of the effects of environmental stressors on performance and fitness in aquatic organisms.
ACKNOWLEDGEMENTS

Heartfelt thanks to my supervisor, Dr. Graham Scott; it was the example you set in work ethic and commitment that guided me throughout this endeavour. Also thank you to the members of my committee, Dr. Grant McClelland and Dr. Chris Wood, for guidance and advice. To the rest of the members of the Scott lab, past and present: Brittney, Catie, Sajeni, Sherry, Paras, Danielle, Julie-Anne and Alex; this work is just as much yours as it is mine. Thank you for all of the support and good times. An extra special thank you to Alex Young, who performed the respirometry experiments on the sunfish at different temperatures. A great many thanks to all of those who showed me appreciation for the beautiful natural environment that we all rely on and whose preservation and knowledge of is the greatest incentive for any biologist. Last but not least, thank you to my family for supporting me as a student.
THESIS ORGANIZATION AND FORMAT

This thesis is organized in a sandwich format, as recommended and approved by the members of my supervisory committee, consisting of three main chapters. Chapter one is a general introduction outlining relevant background information and the objectives of my study. Chapter two consists of a manuscript that is being prepared for submission to a peer-reviewed scientific journal. Chapter three includes a general discussion of results collected not included in the manuscript as well as summary of the major findings of this thesis and suggestions for future steps.

Chapter 1: General Introduction

Chapter 2: Physiological tradeoffs underlie the evolution of hypoxia tolerance and exercise performance in bass and sunfish (Centrarchidae)

Authors: Kyle D. Crans, Hannah H.M. Chiu, and Graham R. Scott

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Comments: This study was conducted by KDC under the supervision of GRS. Some gill morphometric analyses were performed by HMMC.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ASR</td>
<td>Aquatic Surface Respiration</td>
</tr>
<tr>
<td>$P_{\text{crit}}$</td>
<td>Critical Oxygen Tension</td>
</tr>
<tr>
<td>$U_{\text{crit}}$</td>
<td>Critical Swimming Speed</td>
</tr>
<tr>
<td>COX</td>
<td>Cytochrome oxidase</td>
</tr>
<tr>
<td>HOAD</td>
<td>Hydroxyl-CoA dehydrogenase</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LOE</td>
<td>Loss of Equilibrium</td>
</tr>
<tr>
<td>$\text{MO}_{2\text{max}}$</td>
<td>Maximum rate of oxygen consumption</td>
</tr>
<tr>
<td>MCHC</td>
<td>Mean cell hemoglobin concentration</td>
</tr>
<tr>
<td>$\text{O}_2$</td>
<td>Oxygen</td>
</tr>
<tr>
<td>OLLT</td>
<td>Oxygen limitation of thermal tolerance</td>
</tr>
<tr>
<td>$\text{PO}_2$</td>
<td>Oxygen Tension</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PK</td>
<td>Pyruvate kinase</td>
</tr>
<tr>
<td>$\text{MO}_{2\text{rest}}$</td>
<td>Resting rate of oxygen consumption</td>
</tr>
<tr>
<td>BL</td>
<td>Standard body lengths</td>
</tr>
<tr>
<td>SDH</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>LOE$_{50}$</td>
<td>Tension at which half the subjects show loss of equilibrium</td>
</tr>
</tbody>
</table>
CHAPTER 1: GENERAL INTRODUCTION

Hypoxia in the Environment

Hypoxia, low dissolved oxygen (O₂), is a prevailing threat to aquatic ecosystems that has received much examination in the literature. It can be the result of both natural and anthropomorphic phenomenon, but in the last 50 years human influences on aquatic systems have increased the prevalence of hypoxia dramatically (Diaz, 2001). Natural causes of hypoxia include stratification, ice-cover, tidal action, diurnal fluctuations due to photosynthesis and when the respiration of aquatic organisms exceeds O₂ diffusion from the atmosphere ( Hecky, 2000; Magnuson et al., 1985; Martin, 1995; Miranda and Hodges, 2000). One of the main anthropogenic causes of hypoxia is eutrophication caused by nutrient loading (e.g., wastewater effluent, agricultural runoff, etc.) (Bricker et al., 1999), which has been linked to hypoxic “dead zones” in both Lake Erie in North America (Vanderploeg et al., 2009) as well as the Kattegat between Denmark and Sweden (Breitburg, 2002). The increases in temperature arising from climate change also decrease oxygen solubility in water and increase O₂ consumption in ectothermic organisms (Allen, 1955; Matear and Hirst, 2003). We can therefore expect to see an increase in the incidence of hypoxic events as pressure from eutrophication, global warming, and pollution continue to accumulate (Diaz, 2001).

Responses to Hypoxia

There are two broad classes of responses, behavioural and physiological, that fish can employ to minimize the effects of hypoxia. When possible, behavioural responses to hypoxia are employed first, and can include avoidance, aquatic surface respiration (ASR),
or changes in activity (Chapman and McKenzie, 2009). ASR is an attempt to ventilate the gills with the water in the oxygen-rich surface-air boundary (Kramer and McClure, 1982; McNeil and Closs, 2007), which helps the individual cope with hypoxia by increasing oxygen supply. Depending on the species, activity can increase or decrease in hypoxia. Active, fast swimming species (e.g., tuna) often increase their activity at the onset of hypoxia, presumably as part of an escape response that facilitates hypoxia avoidance (Dizon 1977). Slower more sluggish species (e.g., common sole) will often decrease their activity levels under hypoxic conditions, perhaps as an energy saving mechanism that reduces O₂ demands (Dalla Via et al. 1998).

If fish are unable to avoid hypoxia, many species cope by modifying their physiology. These physiological modifications can act primarily to improve oxygen uptake and transport, to increase energy supply from oxygen-independent pathways (anaerobic metabolism), and/or to reduce ATP demands via metabolic depression (Hochachka and Somero, 2002; Wu, 2002). For example, oxygen uptake can be increased by gill remodelling, leading to an enhancement of the functional area for gas exchange at their gills (Chapman et al. 2008; Sol lid et al., 2003). Interspecific differences in hypoxia tolerance are also positively related to variation in gill surface area (Crampton et al., 2008). The oxygen carrying capacity of the blood can increase during hypoxia acclimation due to erythropoiesis, increasing the number of erythrocytes in the circulation (Peterson, 1990). Across species of sculpins that differ in hypoxia tolerance, the more tolerant species have been shown to possess hemoglobin with a higher affinity for oxygen (Mandic et al., 2009). These and other mechanisms of increasing the ability to extract oxygen from the environment and transport it through the body are extremely beneficial
under hypoxia, but a severe lack of oxygen can make the use of anaerobic metabolism unavoidable.

The use of anaerobic metabolism in hypoxia may help organisms make up aerobic energy shortfalls. For example, fish can activate oxygen-independent pathways of metabolism, such as anaerobic glycolysis and CrP hydrolysis, and deactivate aerobic pathways, such as lipid oxidation, the TCA cycle, and the electron transport chain (Richards, 2009). The capacity for anaerobic glycolysis may also be augmented with hypoxia acclimation by increases in the expression and activity of glycolytic enzymes in several tissues (Martinez et al., 2006), particularly in the liver, because it contains the largest store of glycogen to act as a fuel for anaerobic glycolysis (Richards, 2009).

However, anaerobic glycolysis is a relatively inefficient form of energy production, yielding far fewer ATP for a given amount of carbohydrate fuel than oxidative phosphorylation, and can result in a metabolic acidosis.

The depression of metabolic rate is a mechanism to prolong survival in hypoxia by reducing the ATP demand of cells. Metabolic depression is common in hypoxia-tolerant organisms such as goldfish and crucian carp (van den Thillart and Waarde, 1985) and the painted turtle (Buck et al., 1993). The two most costly processes in the cell, protein synthesis and ion transport, which together can account for upwards of 80% of the cells energy budget, are some of the main targets for metabolic suppression (Boutilier, 2001). The energy requirements of ion transport by ATPases can be spared because membrane permeability is reduced (Hochachka, 1996). Some recent evidence suggests that AMP-activated protein kinase (AMPK) may be responsible for coordinating metabolic depression under hypoxia in some tissues (Jibb and Richards, 2008).
Measures of Hypoxia Tolerance

There are two common indices of resting hypoxia tolerance in the literature. Critical \( O_2 \) tension (\( P_{\text{crit}} \)) is the oxygen tension below which the resting \( O_2 \) consumption rate cannot be maintained (Beamish, 1976). A low \( P_{\text{crit}} \) indicates that resting metabolism can be maintained across a wide range of oxygen tensions, and is a typical feature of hypoxia-tolerant organisms (Mandic, 2009). At \( P_{\text{crit}} \), the scope for aerobic metabolism is essentially zero (Portner, 2010), so there should be no aerobic scope available for growth and reproduction. As oxygen tensions fall below the \( P_{\text{crit}} \), anaerobic metabolism becomes very important for fueling ATP demands, particularly in species that do not undergo metabolic depression (Portner, 2010).

The point at which fish undergo loss of equilibrium (LOE) in hypoxia is a useful indication of ‘ecological death’, shortly preceded by physiological death. There are different ways to measure this indicator of hypoxia tolerance, including the time to LOE at a specific oxygen tension, the oxygen tension at LOE during progressive hypoxia, and the oxygen tension at which half the subjects show LOE during progressive hypoxia (LOE\(_{50}\)). LOE indices are most useful in determining the tolerance of organisms to severe acute hypoxic events, and they are often well correlated with \( P_{\text{crit}} \) (Mandic et al., 2013).

Exercise Performance and Hypoxia

Fry (1947) introduced the idea of a metabolic scope for activity, the range of metabolism over which an animal’s energy requirements can be satisfied aerobically. The
metabolic scope of activity is usually measured as the difference (absolute or factorial) between resting (MO$_{2\text{rest}}$) and maximal (MO$_{2\text{max}}$) rates of oxygen consumption during exercise (Fry, 1947). Hypoxia usually inhibits MO$_{2\text{max}}$ at higher oxygen tensions than those that inhibit MO$_{2\text{rest}}$, such that hypoxia can decrease the scope for activity at oxygen tensions well above those that threaten survival (Claireaux and Lagardere, 1999). This effect of hypoxia is likely quite important, as a larger scope for activity allows an organism to devote more energy towards growth, foraging, and reproduction, and can thus improve their fitness (Clark et al., 2011). Therefore, measurements of MO$_{2\text{max}}$ can be relevant indicators of the effects of hypoxia on fitness.

Critical swimming speed ($U_{\text{crit}}$) is an indication of the maximum speed a fish can sustain for a prolonged period (Beamish, 1966). Sustainable swimming in fish has been suggested as a good indicator of fitness, as it reflects the range of speeds at which a fish can forage, evade predators, migrate, and interact with conspecifics without experiencing fatigue (Reidy et al., 2000). Burst swimming, by contrast, is usually only used in short periods of rapid movement for predator avoidance and prey capture (Reidy et al., 2000). Swimming speeds up to and including $U_{\text{crit}}$ are fueled largely through aerobic metabolism, and anaerobic metabolism (anaerobic glycolysis and creatine phosphate hydrolysis) contributes an increasing proportion of ATP supply at higher speeds (Brett, 1965; Richards et al., 2002). Since swimming at speeds below $U_{\text{crit}}$ is fuelled largely through aerobic metabolism, $U_{\text{crit}}$ is negatively affected by exposure to hypoxia (Dutil, 2007).

It has been noted that species exhibiting high performance, such as tuna, are often greatly affected by hypoxia, while sluggish species such as the crucian carp are extremely
tolerant to hypoxia, perhaps implying a tradeoff between exercise performance and hypoxia tolerance (Gooding et al., 1981; Johnston and Goldspink, 1973). Such a tradeoff has been demonstrated in cyprinid fishes, with fish from high flow habitats having good exercise performance and poor hypoxia tolerance, and closely related species from low flow habitats showing the opposite (Fu et al. 2014). This tradeoff has also been shown between different life stages of the same species, with a switch from free-swimming larvae to a sessile adult morph in coral reef fish coinciding with a loss of high exercise performance and development of hypoxia tolerance (Nilsson et al., 2007). The affinity of haemoglobin for oxygen has been suggested as the physiological basis for this apparent tradeoff, as although high O\textsubscript{2} affinity of haemoglobin increases hypoxia tolerance by improving oxygen uptake, it also limits O\textsubscript{2} unloading at tissues, which may degrade exercise performance (Burggren et al., 1991).

*The Effects of Temperature on Hypoxia Tolerance*

Temperature and hypoxia are likely to interact as stressors, as rising temperatures increase oxygen demand in fish while hypoxia limits oxygen supply in aquatic environments (McBryan et al. 2013). Indeed, increased temperatures have been shown to decrease hypoxia tolerance in killifish (Healy and Schulte, 2012) and coral reef fishes (Nilsson et al., 2010). The effects of temperature on metabolic rates are interesting as warmer temperatures increase both MO\textsubscript{2rest} as well as MO\textsubscript{2max} exponentially, up to an optimum (beyond which these traits decline), with a Q\textsubscript{10} value (fold increase in metabolic rate over 10 °C) of between 1.5 – 2 (White et al., 2006). However, rising temperatures cause a larger relative rise in MO\textsubscript{2rest} compared to MO\textsubscript{2max}, which shrinks aerobic scope.
(McBryan et al. 2013). As mentioned before, hypoxia tends to have a greater negative effect on MO$_{2\text{max}}$, also shrinking aerobic scope (Claireaux and Lagardere, 1999). Therefore when fish are exposed to hypoxia and thermal stress in concert, aerobic scope is greatly reduced by a combination of both stressors. This reduction in aerobic scope has grave consequences, as when aerobic scope collapses to zero, anaerobic metabolic processes must be used, putting a time-limitation on survival.

Thermal performance curves are a common conceptual framework for studying the effects of temperature on the physiology of ectotherms, and stipulate that there is an optimal temperature for physiological processes, and as temperatures increase or decrease from this optimum performance declines (Portner, 2010). The effects of other abiotic stressors, including hypoxia, have been shown to affect the shape of thermal performance curves, such as by shrinking the range at which optimum performance occurs (Portner, 2010). The interaction between hypoxia and temperature stress is often attributed to their effects on the same physiological traits, such as metabolic rate and tissue oxygenation levels (hypoxaemia) (Portner, 2010). Increased temperatures have also been shown to decrease the affinity of hemoglobin for oxygen, which could limit oxygen uptake, causing an increase in $P_{\text{crit}}$, hence a decrease in hypoxia tolerance (Jensen et al., 1993).

*Fish from the Family Centrarchidae*

Centrarchids (bass and sunfish) are a family of North American freshwater fishes, with 12 species found in Ontario (Holm, 2010). They inhabit a wide variety of habitats, and where they are found they are often the most abundant family of fishes in terms of biomass and diversity (Near and Koppelman, 2009). They encompass well-known and
studied species, including a number that are economically important, such as the largemouth bass (*Micropterus salmoides*), a popular sport fish (Chen et al., 2003). The centrarchid family is an interesting system for a comparative study due to the diversity of ecological niches and life histories amongst a group of closely related species (Aday et al., 2009; Near and Koppelman, 2009). They are particularly well suited for a study on hypoxia tolerance and exercise performance because some species regularly experience hypoxia in their natural environment and there are a wide variety of swimming styles and body shapes across the family (Kieffer and Cooke, 2009). There are also differences in thermal tolerance between centrarchid sunfish, with bluegill sunfish (*Lepomis macrochirus*) being tolerant of higher temperatures than pumpkinseed sunfish (*L. gibbosus*; a co-occurring congener) (Beitinger, 1977; Reuter and Herdendorf, 1975). They also tend to hybridize in areas where their ranges overlap (Dawley, 1987). We chose to conduct our study on four species of centrarchid fish that are found in Lake Opinicon in Ontario: largemouth bass, pumpkinseed sunfish, bluegill sunfish, and rock bass (*Ampholutes rupestris*).

**Objectives**

The aims of this thesis were two-fold. The first and primary aim was to determine if there is a tradeoff between hypoxia tolerance and exercise performance across centrarchid species and to identify physiological traits that may underlie this tradeoff. The second aim was to identify the effects of temperature and acclimatization to the natural environment on hypoxia tolerance and its underlying physiology in centrarchid sunfish species. In carrying out this investigation my objectives were to:
1) Determine if there are related patterns in the variation between resting hypoxia tolerance and swimming performance amongst centrarchid species.

2) Assess if the underlying determinants of oxygen transport capacity will help to explain these patterns.

3) Determine the effects of temperature on hypoxia tolerance in sunfish species.

4) Determine whether acclimatization to the native environment influences species differences in hypoxia tolerance, by comparing native-caught and lab-deacclimated individuals for each sunfish species.
CHAPTER 2: PHYSIOLOGICAL TRADEOFFS UNDERLIE THE EVOLUTION OF HYPOXIA TOLERANCE AND EXERCISE PERFORMANCE IN BASS AND SUNFISH (CENTRARCHIDAE)

Abstract

There is a frequently observed tradeoff between hypoxia tolerance and exercise performance in fish, even though both of these traits are often associated with a high $O_2$ transport capacity. We examined the physiological basis for this tradeoff in four species of bass and sunfish from the family Centrarchidae. Hypoxia tolerance was greatest in rock bass, intermediate in pumpkinseed and bluegill sunfishes, and lowest in largemouth bass, based on measurements of critical $O_2$ tension ($P_{crit}$) and the $O_2$ tension at loss of equilibrium. The least hypoxia-tolerant species had the highest critical swimming speed ($U_{crit}$) and maximal $O_2$ consumption rate during swimming, and suffered the greatest decrease in $U_{crit}$ during exercise in hypoxia, consistent with there being a tradeoff between hypoxia tolerance and exercise performance. Some traits appeared to contribute to both hypoxia tolerance and exercise performance, as reflected by the traits that were highest in both rock bass and largemouth bass, such as the gas-exchange surface area of the gills and the enzyme activities of lactate dehydrogenase and the gluconeogenic enzyme phosphoenolpyruvate carboxykinase in the liver. Some other traits were uniquely associated with hypoxia tolerance (pyruvate kinase and lactate dehydrogenase activities in the heart) or exercise performance (capillarity and fibre size of the axial swimming muscle). Therefore, the cumulative influence of a variety of respiratory and metabolic traits likely underlies the physiological tradeoffs associated with the evolution of hypoxia tolerance and exercise performance in fish.
Introduction

Hypoxia is a regular occurrence in many freshwater ecosystems. It can result from both natural and anthropogenic events, including eutrophication, prolonged ice cover, stratification of the water column, and when respiration exceeds the rate of diffusion from the atmosphere (Boesch et al., 2001; Bricker et al., 1999; Hecky, 2000; Magnuson et al., 1985; Miranda and Hodges, 2000; Vanderploeg et al., 2009). When severe, hypoxia can be the cause of fish and invertebrate kills, changes in trophic patterns, and loss of habitat (Mallin et al., 2006), and it is believed that the incidences and severity of aquatic hypoxia will increase in the future as global temperatures rise and as urbanization and pollution continue (Diaz, 2001).

Hypoxia tolerance in fish is associated with a variety of physiological traits, arising via plasticity (e.g., acclimatization or developmental plasticity) or evolutionary specialization (e.g., genetic adaptation). These can include adjustments in traits that improve oxygen uptake from the water and oxygen transport to tissues, increase anaerobic ATP production, or depress ATP demands in hypoxia (Boutilier, 2001; Bickler and Buck, 2007; Hochachka et al., 1996; Richards, 2009; Wu, 2002). For example, many tolerant species have an increased capacity for O$_2$ transport in hypoxia compared to intolerant species, whose underlying mechanisms can include a high functional area for oxygen uptake at the gills and/or a high haemoglobin-O$_2$ affinity (Chapman et al. 2008; Mandic et al., 2009; Nilsson, 2007). Many tolerant species also tend to live a relatively sedentary lifestyle and reduce their activity in hypoxia (Chapman and McKenzie, 2009; Johnston and Goldspink, 1973; Zhang et al. 2010). On the other hand, many active
species (e.g., tuna, rainbow trout) have similarly high O\textsubscript{2} transport capacities to support high rates of aerobic metabolism, but are very sensitive to hypoxia (Bernal, 2001; Bushnell et al., 1990; Davis, 1975; Gooding et al., 1981; Matey et al., 2011). Therefore, increasing O\textsubscript{2} transport capacity can improve both hypoxia tolerance and exercise performance, traits that are seemingly at odds with one another.

There have been relatively few studies examining the mechanistic basis for tradeoffs between hypoxia tolerance and exercise performance in fish. It has been found that cyprinid fish from habitats with high water flows tend to have a higher capacity for sustained swimming but a lesser hypoxia tolerance than those from slow flowing habitats, suggesting that there is a mechanistic tradeoff between hypoxia tolerance and performance among closely related species, even after phylogenetic relationships are taken into account (Fu et al., 2014). An increase in hypoxia tolerance coincident with a decrease in sustained swimming performance has also been observed during development in coral reef damselfish (Nilsson et al., 2007). It has been suggested that the apparent tradeoff between hypoxia tolerance and performance arises due to the oxygen-binding properties of haemoglobin, such that a high haemoglobin-O\textsubscript{2} affinity improves hypoxia tolerance by increasing branchial O\textsubscript{2} uptake and a low affinity improves exercise performance by facilitating O\textsubscript{2} unloading at the tissues (Burggren et al., 1991). However, the physiological basis for this tradeoff has not been examined among closely related species from across the O\textsubscript{2} cascade.

Centrarchidae (bass and sunfish) is a family of 4 genera and 32 species of North American freshwater fish (Near et al., 2003; Page and Burr, 2011) that exhibit appreciable variation in hypoxia tolerance and exercise performance. For example,
pumpkinseed sunfish (*Lepomis gibbosus*) can tolerate deep hypoxia for longer periods than its congener, the bluegill sunfish (*L. macrochirus*) (Farwell et al., 2007; Mathers et al., 2014). Largemouth bass (*Micropterus salmoides*) also appear to be more tolerant of hypoxia than smallmouth bass (*M. dolomieu*), as largemouth maintain higher blood O$_2$ content in hypoxia and can maintain blood pH at levels of hypoxia that induce a metabolic acidosis in smallmouth (Furimsky et al., 2003). Largemouth and smallmouth bass are generally good swimmers, with higher sustained swimming performance than both bluegill and pumpkinseed sunfish (Brett and Sutherland, 1965; Dahlberg et al., 1967; Kelsch, 1996).

The objectives of this study were to examine (i) the nature of the potential tradeoff between hypoxia tolerance and swimming performance among a group of centrarchid species (largemouth bass, bluegill sunfish, pumpkinseed sunfish, and rock bass) inhabiting a similar environment (Lake Opinicon, Canada), and (ii) the underlying physiological determinants of this tradeoff from across the oxygen transport pathway. We studied fish that were well acclimated to common conditions in the lab to help determine the inherent evolved differences between species. We found that some respiratory traits differed between species in association with both hypoxia tolerance and exercise performance, while several other respiratory and metabolic traits that might contribute to an observed tradeoff were associated with only one of these organismal traits.
Materials and Methods

Study animals

Fish were collected from Lake Opinicon, Ontario, Canada (44.559°N, -76.328°W) by angling or seining in August and October 2012. We caught an overlapping size range of largemouth bass (*Micropterus salmoides*; body mass ranged from 36.8 to 85.1 g, mean ± s.e.m. of 54.5 ± 7.5 g), rock bass (*Ambloplites rupestris*; 36.6 to 93.8 g, 62.7 ± 6.2 g), bluegill sunfish (*Lepomis macrochirus*; 49.1 to 117.3 g, 80.0 ± 5.3 g), and pumpkinseed sunfish (*Lepomis gibbosus*; 53.1 to 128.9 g, 87.8 ± 4.3 g). Fish were then transported to McMaster University and housed in 500 l flow-through tanks at a 12:12 h light:dark photoperiod. Tanks were supplied with dechlorinated, aerated City of Hamilton tap water at 12-15°C. Fish were fed a mix of commercially purchased squid or beef organs (heart, liver, kidney) four to five times weekly (~3% body mass daily). Fish were housed for at least a month under these conditions before any experimentation took place. Passive integrated transponder tags (PIT) (Biomark, Boise, Idaho, USA) were implanted into the body cavity of each fish to allow ongoing identification of individuals. All animal procedures followed guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board (Animal Utilization Protocol 12-01-02).

Resting respirometry and hypoxia tolerance measurements

Resting O\(_2\) consumption rate (MO\(_2\)) was measured by stop-flow respirometry during stepwise reductions in PO\(_2\) in fish that were fasted for ~24 h. Fish were held in respirometry chambers (volume of 2 l) for at least 10 h before measurements began.
During this period, the chambers (which were situated in a darkened glass buffer tank) were continuously flushed with normoxic water (flushing circuit). The chambers were also connected to a recirculating circuit in which the PO$_2$ was measured continuously using a fibre-optic O$_2$ sensor (PreSens, Regensburg, Germany). Both circuits were driven by pumps controlled by AutoResp software (Loligo systems, Tjele, Denmark). Temperature in the buffer tank was continuously maintained at 15°C using a stainless steel cooling coil through which chilled water was pumped by a circulating refrigerated water bath.

MO$_2$ measurements began in normoxia and proceeded for progressive step-wise hypoxia, in which the PO$_2$ was reduced by 10% air saturation every 20 min from 100% to 20%. This was accomplished by regulating the PO$_2$ in the buffer tank in a feedback loop using a galvanic oxygen sensor that automatically controlled the bubbling of the water with nitrogen gas using a solenoid valve (Loligo Systems). Each 20 min step contained two successive flush and measurement periods. During the ~5 min flush periods, both the flush and recirculating pumps were active and the chamber and buffer tank were equilibrated. During the 5 min measurement periods, the flush pump was turned off so the chamber was isolated from the buffer tank, and the MO$_2$ was measured as the change in O$_2$ concentration over time. The 20 min step at 20% air saturation was followed by another step at 15% saturation, and then the chamber was sealed from the buffer tank and the fish was allowed to consume the O$_2$ in the chamber until it lost equilibrium (at which point the PO$_2$ at LOE was noted). The critical PO$_2$ ($P_{crit}$) – the inflection point of the relationship between MO$_2$ and PO$_2$ when fish transition from oxyregulating to oxyconforming – was determined using Regress software (Yeager and Ultsch, 1989).
Swimming respirometry

Fish metabolic rates and swimming performance were measured in fish that were fasted for ~24 h in a 5L swim-tunnel respirometer, submerged in a ~60 l buffer tank (Loligo Systems, Tjele, Denmark). Each individual fish was swum at water PO\textsubscript{2} of 20 kPa, 9 kPa, and 6 kPa. The order of each swim trial was randomized, and fish were allowed to recover for at least 2 weeks between trials. Temperature (15°C) and PO\textsubscript{2} in the buffer tank were maintained as described for the resting respirometry experiments. PO\textsubscript{2} in the swim tunnel was measured continuously using a fibre-optic O\textsubscript{2} sensor (PreSens, Regensburg, Germany). Fish were weighed and measured for standard body length before each swim trial and were allowed 30-60 min to habituate to the swim tunnel. The swimming trials then started at a velocity of 0.33 standard body lengths (BL) s\textsuperscript{-1}. Swimming velocity was increased by 0.33 BL s\textsuperscript{-1} every 10 min until the fish exhausted (tunnel water speeds were calibrated with an inline flow meter, HFA, Höntzsch GmbH, Waiblingen, Germany). Exhaustion was defined as when the fish could no longer maintain their position in the water flow and were forced into the screen at the back of the chamber for >30 s, despite repeated attempts to motivate the fish to resume swimming (e.g., tapping the chamber, shining a light, etc.). Two successive flush-measure cycles (90 s flush, 210 s measure) were used to measure MO\textsubscript{2} at each speed. Critical swimming speed (U\textsubscript{crit}) was calculated from the following equation (Brett, 1965):

\[ U_{\text{crit}} = u_{i} + u_{ii} \left( t_{ii} / t_{i} \right) \]
$u_i$ is the highest velocity step that the fish completed, $t_i$ is the duration of a full velocity step (10 min), $u_{ii}$ is the increment of increase in velocity between steps (0.33 BL s$^{-1}$), and $t_{ii}$ is the duration the fish swam at last velocity step. $MO_2$ max was recorded as the maximum $MO_2$ value that was measured during the course of the trial.

**Sampling**

Fish were euthanized with an overdose of buffered MS-222 (1 g l$^{-1}$) and sampled within 30 min of reaching LOE. A transverse cut was made through the trunk at the anterior base of the anal fin. Blood was collected into capillary tubes and centrifuged for 5 min at 14,000 rpm to measure haematocrit, and an additional 6 µl blood sample was taken to measure haemoglobin content (using Drabkin’s Reagent following manufacturer instructions; Sigma-Aldrich, Oakville, ON, Canada). A second transverse cut was then made ~2-3 mm anterior to the first section, and this resulting block of muscle was frozen in liquid N$_2$ for later use in enzyme assays (see below). A third transverse cut was made ~2-3 mm posterior to the first cut, an image was taken to measure the total transverse area of the musculature, and one lateral hemisphere of the steak was cut into dorsal, midline, and ventral blocks of tissue for histology. These three blocks were then covered in embedding medium (Shandon Cryomatrix, Fisher Scientific, Ottawa, Canada), frozen on cork in liquid N$_2$-cooled isopentane, and stored at -80°C until sectioning was performed. The entire heart was weighed, and the heart, brain, and liver were frozen in liquid N$_2$ for later use in enzyme assays. The entire gill basket was removed from each fish, fixed for ~24 h at 4°C (in 2% paraformaldehyde and 2% glutaraldehyde in phosphate-buffered
saline, PBS, at pH 7.8), and then stored in PBS until morphometric analyses were performed.

*Gill morphometrics*

Gill morphometrics were quantified on the left arches. Total gill surface area was measured using standard methods (Hughes, 1984). The four arches were dissected and digital pictures of all filaments were taken using a stereomicroscope to count filament number and measure the length of all filaments lengths. The density of lamellae on the filaments (i.e., the average number of lamellae per filament length) was measured along the length of every tenth filament on the first gill arch. Five transverse sections through each tenth filament was used to quantify lamellar area. All measurements were made using ImageJ software (Rasband, 2014). Total gill surface area (TGSA) was calculated by the equation TGSA = 2 L n B, where L is the total filament length (product of filament number and average filament length), n is lamellar density, B is the average bilateral surface area of the lamellae (mm²), all of which is doubled to account for there being two sides to a fish (Hughes, 1984). TGSA was expressed relative to body mass.

*Muscle histology*

Muscle blocks were sectioned 10 µm thick in a cryostat (Model: CM 1850, Leica Microsystems, Wetzlar, Germany) at -20°C, mounted on Superfrost Plus slides (Fisher Scientific), and air-dried. We stained freshly sectioned tissue for succinate dehydrogenase (SDH) activity to identify oxidative fibres, and the remaining slides were stored at -80°C until stained to identify capillaries. SDH activity was stained by incubating slides for one
hour at room temperature in a working buffer of 41.7 mM Na₂HPO₄, 8.3 mM NaH₂PO₄, 80 mM sodium succinate, 0.1% NBT at pH 7.6. Capillaries were identified by staining for ATPase activity in a neutral Pb²⁺-containing medium (Rosenblatt et al., 1987). Slides were first fixed for 5 min at 4°C in a PBS solution containing 4% paraformaldehyde and 68 mmol l⁻¹ CaCl₂ (pH 7.6). Slides were then stained in a solution of 1 mmol l⁻¹ ATP, 3.8 mmol l⁻¹ Pb(NO₃)₂, 6.5 mmol l⁻¹ CaCl₂, 300 mg gelatin, 100 mmol l⁻¹ tris (pH 7.2) for 1 h at 37°C, and were mounted with Aquamount (Fisher Scientific). Stained tissue was imaged with a Nikon Eclipse E800 light microscope (Melville, USA).

The total transverse area and total number of oxidative fibres was measured from SDH-positive staining. The total area was calculated as twice (to account for there being two sides of the fish) the sum of all transverse areas of oxidative muscle in the three blocks taken for each fish. The areal density of oxidative fibres (%) was determined by dividing the total transverse area of oxidative fibres by the total transverse area of the musculature that was determined from the image taken during sampling. Capillary density and the number of capillaries per muscle fibre were quantified in areas that were clearly oxidative (red) muscle and glycolytic (white) muscle. The average transverse area of each fibre type was calculated as the total area imaged for each type divided by the number of fibres within that area. All measurements were made in ImageJ.

**Enzyme assays**

Tissue was homogenized on ice in 20 volumes of homogenization buffer (20 mM hepes, 1 mM EDTA, and 0.1% Triton X-100) at pH 7.0. Preliminary assays were carried out to determine the substrate concentrations needed to elicit maximal enzyme activity
Cytochrome c oxidase activity was measured on freshly homogenized samples in 50 mmol l\(^{-1}\) tris containing 100 µmol l\(^{-1}\) of fully reduced cytochrome c and 0.5% tween-20 (pH 8.0). The remaining assays were performed after one or more freeze-thaw cycles, the number of which was consistent for each enzyme. Phosphoenolpyruvate carboxykinase (PEPCK) activity was assayed in the liver under the following conditions (concentrations in mmol l\(^{-1}\)): 1.1 phosphoenolpyruvate (PEP), 0.15 NADH, 0.5 dGDP, 20 NaHCO\(_3\), 1 MnCl\(_2\), 50 imidazole, and excess coupling enzyme (20 U ml\(^{-1}\) malate dehydrogenase). PEPCK activity was not detected in the muscle, brain, or heart tissue. The remaining enzyme assays were assayed in all tissues (except that hydroxyacyl coA dehydrogenase was not assayed in brain) under the following conditions (concentrations in mmol l\(^{-1}\)): Citrate synthase (CS), 0.5 oxaloacetate, 0.3 acetyl-coA, 0.1 DTNB, 50 tris, pH 8.0; hydroxyacyl coA dehydrogenase (HOAD), 0.05 acetoacetyl-coA, 0.3 NADH, 50 imidazole, pH 7.2; lactate dehydrogenase (LDH), 1 pyruvate 0.15 NADH, 50 hepes, pH 7.4; pyruvate kinase (PK), 5 PEP, 0.15 NADH, 5 ADP, 100 KCl, 10 MgCl\(_2\), 0.010 fructose-1,6-bisphosphate, 50 mops, and excess coupling enzyme (10 U/ml LDH), pH 7.4.

All enzyme assays were run in triplicate in a 96-well microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA), with temperature controlled at 25°C, by measuring the rate of change in absorbance at 550 nm (COX), 412 nm (CS), or 340 nm (HOAD, LDH, PEPCK, and PK). Activities were determined by subtracting the background reaction rate without a key substrate (COX, fully reduced cytochrome c; CS, oxaloacetate; HOAD, acetoacetyl-CoA; LDH, pyruvate; PEPCK, dGDP; PK, phosphoenolpyruvate) from the rates measured in the presence of all
substrates. We used extinction coefficients ($\varepsilon$) of 28.5 and 13.6 optical density (mmol l$^{-1}$) cm$^{-1}$ for COX and CS assays, respectively, and determined $\varepsilon$ empirically for HOAD, LDH, PEPCK, and PK by constructing standard curves of absorbance versus NADH in the buffers appropriate for each assay.

Statistics
Data are reported as means ± s.e.m. One-way ANOVA followed by Bonferroni multiple comparisons post-tests were used to compare between species. A significance level of $P<0.05$ was used throughout.

Results
Hypoxia tolerance

There was distinct variation in hypoxia tolerance across species, as evidenced by differences in critical oxygen tension ($P_{\text{crit}}$) – the oxygen tension ($P_O$) at which fish transition from oxyregulating to oxyconforming – and the $P_O$ at loss of equilibrium (LOE). Rock bass had the lowest $P_{\text{crit}}$ (~2.3 kPa) of any of the species and the lowest $P_O$ at LOE (~0.7 kPa) (Fig. 2-1A,B). $P_{\text{crit}}$ was similar in the remaining species at ~4 kPa (Fig. 2-1A). Largemouth bass had the highest $P_O$ at LOE (~1.6 kPa), and the sunfish were somewhat intermediate (~0.8 kPa in pumpkinseed and ~1.0 kPa in bluegill) (Fig. 2-1B). These results suggest that rock bass are the most hypoxia tolerant of the species examined, followed by pumpkinseed and bluegill, and that largemouth bass are the least tolerant. However, there were no significant differences in resting $O_2$ consumption rate
between species (rock bass, 59.7 ± 6.2; pumpkinseed, 81.6 ± 5.2; bluegill, 79.4 ± 4.4; largemouth bass, 86.1 ± 12.2; all in units mg O$_2$ kg$^{-1}$ body mass h$^{-1}$).

Swimming performance

Swimming performance in normoxia varied across centrarchid species in a similar manner to the variation in hypoxia tolerance. Largemouth bass had a much higher critical swimming speed ($U_{\text{crit}} \sim 2.7$ body lengths s$^{-1}$) than rock bass and the sunfish species (~2 body lengths s$^{-1}$) (Fig. 2-1C). There was a strong correlation between $U_{\text{crit}}$ and maximal O$_2$ consumption rate (MO$_2$ max) across all swimming experiments, but the variation in MO$_2$ max between species in normoxia was lower in magnitude than the variation in $U_{\text{crit}}$ (Fig. 2-1D). This may have arisen because there is also variation in body shape between species – largemouth bass are much more streamlined with a substantially higher fineness ratio (body length:body depth of 3.47 ± 0.10, compared to 2.33 ± 0.02 in rock bass, 1.98 ± 0.02 in pumpkinseed, and 2.00 ± 0.01 in bluegill; P<0.001) – which could alter the relationship between $U_{\text{crit}}$ and MO$_2$ max. Nevertheless, our data suggest that largemouth bass have the greatest capacity for performing sustainable exercise in normoxia.

Hypoxia had a substantial effect on swimming performance, particularly in largemouth bass and rock bass (Fig. 2-1D). In these species, $U_{\text{crit}}$ measured in mild hypoxia (water PO$_2$ of 9 kPa) was only ~60% and ~67% of what it was in normoxia, respectively, and $U_{\text{crit}}$ was only ~50% of normoxic values in these species in moderate hypoxia (6 kPa). The reduction in $U_{\text{crit}}$ imposed by hypoxia was less in the sunfish species, as $U_{\text{crit}}$ measured in mild hypoxia (≥90% of normoxic values) and moderate
hypoxia (~80% of normoxic values) were a much higher proportion of $U_{\text{crit}}$ measured in normoxia.

**Gill morphology**

There were substantial differences in gill morphology between species, with the most hypoxia tolerant species and the best swimming species having the greatest surface areas for branchial gas exchange. Largemouth bass and rock bass had 20-40% larger total gill surface areas (TGSA) on average than both of the sunfish species, a difference that was significant in rock bass and neared significance in largemouth bass (Fig. 2-2). Most of this variation in TGSA could be accounted for by the largemouth bass and rock bass having a greater number of gill filaments, which more than compensated for the shorter average filament length and smaller lamellae of largemouth bass (Table 2-1). Although pumpkinseed possessed lamellae with a relatively large surface area, TGSA was comparable to bluegill due to the offsetting effects of the other morphometric traits on TGSA (Table 2-1).

**Phenotype of the swimming muscle**

Capillarity of the oxidative (red) fibre region of the axial swimming muscle was highest in the best swimming species, largemouth bass (Fig. 2-3A). Largemouth bass had both the highest density of capillaries (~1300 capillaries mm$^{-2}$; 1.5- to 2.3-fold higher than the other species) and the highest capillary to fibre ratio (~1.7; 1.3- to 2.1-fold higher than the other species) in the oxidative muscle (Fig. 2-3A). Largemouth bass also had a 1.5- to 1.8-fold higher capillary density in the glycolytic muscle, but capillary to
fibre ratio was relatively low in largemouth bass compared to the other species (Fig. 2-3B). This distinction between indices of capillarity may have arisen because largemouth bass also had the smallest glycolytic muscle fibres (Table 2-2).

Although there were large differences in capillarity between species, there was no variation in the abundance of oxidative fibres in the axial musculature, or in the maximal activity of oxidative enzymes. The transverse area of oxidative fibres was similar between species, in regards to both total absolute area scaled to body mass (~0.0012-0.0015 cm² g⁻¹) or areal density (~4-5% of the transverse area of the musculature) (Fig. 2-3C). There were also no differences in average oxidative fibre size (Table 2-2) or in the total number of oxidative fibres in the axial musculature (Fig. 2-3D). Consistent with these findings, there were no differences in the maximal activities in the muscle for citrate synthase (CS; which catalyzes the first step of the citric acid cycle) or cytochrome oxidase (COX; complex IV and the terminal O₂ acceptor in the electron transport chain) (Fig. 2-4A) when assayed in samples that contained the entirety of the red and white muscle in one lateral hemisphere.

There was variation between species in the activity of enzymes involved in carbohydrate and lipid catabolism in the muscle (Fig. 2-4A). The activity of the glycolytic enzyme pyruvate kinase (PK) was highest in rock bass and lowest in largemouth bass, in possible association with the variation in hypoxia tolerance. However, the substantial variation in both lactate dehydrogenase (LDH) and the β-oxidation enzyme hydroxyacyl-coA dehydrogenase (HOAD) did not occur in parallel to the variation in hypoxia tolerance or exercise performance.
**Characteristics of the heart and blood**

There was substantial variation in heart mass across species, with largemouth bass – the best swimming species examined – having the largest heart. Curiously, bluegill had very small hearts compared to the other species, contributing only ~0.1% to total body mass (Table 2-3). As a result, largemouth bass had hearts that were almost twice as large as those of bluegill. In contrast, there were no differences between species in blood hemoglobin content, hematocrit, or mean cell hemoglobin concentration (MCHC) (Table 3).

Variation in the biochemical capacity for glycolysis and anaerobic metabolism in the heart appeared to parallel the variation in hypoxia tolerance (Fig. 2-4B). Rock bass had 1.5- to 1.9-fold higher maximal activity of PK in the heart than all other species, and had 1.5- to 1.7-fold higher LDH activity than bluegill sunfish and largemouth bass. Neither oxidative capacity nor the capacity for β-oxidation in the heart appeared to be clearly related to the variation in hypoxia tolerance or swimming performance, because COX activity was invariant across all species, CS activity was highest in the sunfish species, and HOAD only differed in pumpkinseed sunfish (Fig. 2-4B).

**Enzyme activities in the liver and brain**

There was appreciable variation in enzyme activities in the liver that was associated with hypoxia tolerance and/or exercise performance. The activity of both LDH and phosphoenolpyruvate carboxykinase (PEPCK; an enzyme involved in gluconeogenesis) were elevated in both rock bass (the most hypoxia tolerant species) and largemouth bass (the best swimmer) relative to the sunfish species (Fig. 2-4C). Liver PK...
activity was also uniquely elevated by 2- to 3-fold in largemouth bass (Fig. 2-4C).
Pumpkinseed sunfish had the highest activities of COX and HOAD in the liver, variation that was not related to the variation in hypoxia tolerance or exercise performance. In contrast, there were no significant differences in enzyme activity in the brain between species (Fig. 2-4D).
Fig. 2-1. Hypoxia tolerance and swimming performance vary across species from the family Centrarchidae. (A) Critical oxygen tension ($P_{crit}$) was lowest in rock bass (n=8) and was similar in pumpkinseed sunfish (n=17), bluegill sunfish (n=13), and largemouth bass (n=7). (B) Oxygen tension ($PO_2$) at loss of equilibrium (LOE) was highest in largemouth bass. (C) Critical swimming speed ($U_{crit}$) measured in normoxia was highest in largemouth bass. The units (body lengths s$^{-1}$) refer to standard lengths. Different letters indicate a significant difference between species (P<0.05). (D) Hypoxia reduced $U_{crit}$ and maximal O$_2$ consumption rate ($MO_2$ max) in all species (data for water $PO_2$ of ~20 kPa, 9 kPa, and 6 kPa proceed from right to left for each species), and there was a strong linear correlation ($U_{crit} = 0.00806 MO_2$ max + 0.415, $R^2 = 0.800$, P<0.0001) between $U_{crit}$ and $MO_2$ max.
Fig. 2-2. Gill surface area is greater in rock bass than in bluegill sunfish or pumpkinseed sunfish. Total gill surface area was expressed relative to body mass. Different letters indicate a significant difference between species (P<0.05) (n as follows: rock bass, 8; pumpkinseed sunfish, 16; bluegill sunfish, 11; largemouth bass, 5).
<table>
<thead>
<tr>
<th></th>
<th>Rock bass</th>
<th>Pumpkinseed sunfish</th>
<th>Bluegill sunfish</th>
<th>Largemouth bass</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Filament Number</strong></td>
<td>1649 ± 36&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1255 ± 27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1277 ± 19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2194 ± 201&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Average Filament Length (mm)</strong></td>
<td>3.47 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.57 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.67 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.73 ± 0.19&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Lamellar Density (mm&lt;sup&gt;-1&lt;/sup&gt;)</strong></td>
<td>23.9 ± 0.3</td>
<td>25.2 ± 0.7</td>
<td>27.2 ± 1.3</td>
<td>27.5 ± 0.5</td>
</tr>
<tr>
<td><strong>Lamellar Bilateral Surface Area (mm&lt;sup&gt;2&lt;/sup&gt;)</strong></td>
<td>0.242 ±</td>
<td>0.339 ±</td>
<td>0.247 ±</td>
<td>0.183 ±</td>
</tr>
<tr>
<td></td>
<td>0.020&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.008&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.011&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.004&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are means ± s.e.m. (n as in Fig. 2).

Different letters indicate a significant difference between species (P<0.05).
Fig. 2-3. Capillarity of the swimming muscle was higher in largemouth bass than in other centrarchids. Capillarity, expressed as capillary density and capillary to fibre ratio, was assessed in both the oxidative (A) and glycolytic (B) regions of the axial swimming muscle. In contrast, there were no significant differences between species in the area of oxidative fibres in the muscle (C), expressed in either absolute units or as a percentage of the transverse area of the entire musculature, or in the total number of oxidative fibres. Different letters indicate a significant difference between species (P<0.05) (n as follows: rock bass, 7; pumpkinseed sunfish, 12; bluegill sunfish, 9; largemouth bass, 5).
Table 2-2. Muscle fibre sizes

<table>
<thead>
<tr>
<th></th>
<th>Oxidative fibres ($\mu$m$^2$)</th>
<th>Glycolytic fibres ($\mu$m$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rock Bass</td>
<td>1455 ± 103</td>
<td>4696 ± 408$^a$</td>
</tr>
<tr>
<td>Pumpkinseed sunfish</td>
<td>1473 ± 91</td>
<td>5419 ± 358$^a$</td>
</tr>
<tr>
<td>Bluegill sunfish</td>
<td>1674 ± 65</td>
<td>4805 ± 200$^a$</td>
</tr>
<tr>
<td>Largemouth bass</td>
<td>1357 ± 150</td>
<td>3035 ± 277$^b$</td>
</tr>
</tbody>
</table>

The transverse area of oxidative (red) and glycolytic (white) muscle fibres are shown as means ± s.e.m. (n as in Fig. 3). Different letters indicate a significant difference between species (P<0.05).
Fig. 2-4. Maximal activities of hydroxyacyl-CoA dehydrogenase (HOAD), citrate synthase (CS), cytochrome oxidase (COX), lactate dehydrogenase (LDH), phosphoenolpyruvate carboxykinase (PEPCK), and pyruvate kinase (PK) in the muscle (A), heart (B), liver (C), and brain (D) of centrarchid fish species. Different letters indicate a significant difference between species (P<0.05) (n as follows: rock bass, 8; pumpkinseed sunfish, 16; bluegill sunfish, 12; largemouth bass, 5).
Table 2-3. Haematology and heart size

<table>
<thead>
<tr>
<th></th>
<th>Rock bass</th>
<th>Pumpkinseed sunfish</th>
<th>Bluegill sunfish</th>
<th>Largemouth bass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart mass (% body mass)</td>
<td>0.147 ± 0.006&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.148±0.008&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.104 ± 0.010&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.187 ± 0.024&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Blood [haemoglobin] (g dl&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>6.47 ± 0.56</td>
<td>5.81±0.69</td>
<td>6.01±0.79</td>
<td>5.1±0.7</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>31.0 ± 1.9</td>
<td>30.3±0.9</td>
<td>28.0±1.3</td>
<td>28.3±1.7</td>
</tr>
<tr>
<td>MCHC (g dl&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>20.7 ± 1.2</td>
<td>19.4±2.2</td>
<td>20.7±1.2</td>
<td>17.8±1.5</td>
</tr>
</tbody>
</table>

MCHC, mean cell haemoglobin concentration.

Data are means ± s.e.m. (n as in Fig. 1).

Different letters indicate a significant difference between species (P<0.05).
Discussion

The mechanistic basis for the frequently observed tradeoff between hypoxia tolerance and exercise performance is paradoxical when considering that both traits rely on a high capacity for O$_2$ transport. Our current findings in fish from the family Centrarchidae, along with recent observations in other taxa (Fu et al., 2014), suggest that this tradeoff can exist among closely related species. Here, we identify a variety of respiratory and metabolic traits from across the O$_2$ cascade that are associated with hypoxia tolerance and exercise performance. Our data suggest that some of the determinants of hypoxia tolerance and exercise performance are shared in common, including a large surface area for gas exchange at the gills and a high gluconeogenic capacity in the liver. A variety of other physiological traits distinguish hypoxia tolerance and exercise performance and may therefore contribute to a tradeoff between them.

*Physiological basis for hypoxia tolerance*

Rock bass appeared to have the greatest hypoxia tolerance among the species examined, with a lower $P_{\text{crit}}$ than all others and a PO$_2$ at LOE that was less than largemouth bass and similar to (or slightly less than on average) that of the sunfish. The $P_{\text{crit}}$ of this species is low compared to most other fish (Mandic et al., 2009; Nilsson and Östlund-Nilsson, 2008; Scott et al., 2008), suggesting that rock bass possess an enhanced capacity to extract and transport oxygen and to sustain routine metabolism across a wide range of water PO$_2$ (Richards, 2009). $P_{\text{crit}}$ is often, but not always related to the ability to resist losing equilibrium in hypoxia (Speers-Roesch et al., 2013), likely because they are underlain by different subordinate physiological traits, which perhaps accounts for the
slight discrepancy in the interspecific variation exhibited for \( P_{\text{crit}} \) and \( \text{PO}_2 \) at LOE here. In fact, the determinants of the \( \text{PO}_2 \) at LOE measured here may differ from those that dictate the time to LOE at a constant \( \text{PO}_2 \). For example, pumpkinseed sunfish are able to avoid LOE in severe hypoxia for much longer than bluegill sunfish, even though \( P_{\text{crit}} \) and the \( \text{PO}_2 \) at LOE are similar between species (Fig. 2-1) (Farwell et al., 2007; Mathers et al., 2014). Interestingly, it has been suggested that the northern range of centrarchid species is limited by hypoxia tolerance, due to there being more frequent potential for winterkill events from ice cover in the north, and among the species studied rock bass have the northernmost range limit and largemouth bass have the southernmost (Bailey and Smith, 1981; Near et al., 2003).

The biochemical capacity of the heart for glycolysis and anaerobic metabolism, as reflected by the activities of PK and LDH, appeared to vary in parallel with the variation in hypoxia tolerance. The spongy myocardial tissue may be especially \( \text{O}_2 \) limited during hypoxia, because it is supplied with \( \text{O}_2 \) from the venous blood that has already perfused other upstream tissues in the peripheral circulation (Farrell and Clutterham, 2002; Marvin and Heath, 1968), so it may require the use of anaerobic metabolism to maintain ATP supply. Hypoxia acclimation may also increase the activity of these enzymes in the heart in some hypoxia-tolerant species (Chippari-Gomes et al., 2005; Martinez et al., 2006). The increase in LDH activity in particular may also increase the capacity of the heart to oxidize the lactate produced by other tissues in hypoxia. The activity of PK in the muscle also varied between species in relation to the observed differences in hypoxia tolerance, even though there were no differences in fibre-type composition. There were no interspecific differences in enzyme activity in the brain underlying the variation in
hypoxia tolerance, consistent with recent observations in some fish taxa (Crocker et al., 2013) but inconsistent with some others (Mandic et al., 2013).

**Physiological basis of exercise performance**

Largemouth bass had the greatest swimming performance in normoxia among all species examined, consistent with previous findings (Brett and Sutherland, 1965; Dahlberg et al., 1967; Kelsch, 1996). However, this difference was extremely dependent on water \( \text{PO}_2 \), because \( U_{\text{crit}} \) was much more sensitive to hypoxia in largemouth bass than in the sunfish (Fig. 2-1D). The limiting effect of hypoxia on swimming performance has been documented in many other species (Dutil et al., 2007; Lefrancois et al., 2005), so the relative insensitivity of sunfish to hypoxia is somewhat surprising and may help allow these benthic species to remain active if their environment becomes mildly hypoxic.

Largemouth bass, the best swimming performers in normoxia, were clearly distinguished from other species by having the highest capillarity in the swimming muscle. High performing species, such as lamnid sharks and tunas, also have high capillarity in the red muscle (Bernal et al., 2001), and there is good support for a strong influence of muscle capillarity on aerobic capacity (Scott and Milsom, 2006; Wagner, 1996). Furthermore, swim training increases the capillarity of red muscle (Sanger, 1992), all of which emphasize the importance to swimming performance of the heightened capillarity in the red muscle of largemouth bass. However, capillarity and oxidative capacity are usually tightly linked (Hepple, 2000), and our observation that these traits did not co-vary between species (Figs. 2-3 and 2-4) is uncommon. The importance of the high capillarity in the glycolytic muscle of largemouth bass is less clear, but it could
contribute to swimming performance at very high speeds when the white muscle is first recruited (Rome et al., 1984; Tudorache et al., 2007). The increased capillarity of the glycolytic muscle in largemouth bass may also facilitate the clearance of lactate and metabolic acid during recovery from exhaustive exercise (Egginton, 2011).

Some of the traits that appeared to be related to exercise performance were also associated with hypoxia tolerance, as reflected by those traits that were highest in both rock bass and largemouth bass. Total gill surface area was one such trait (Fig. 2-2), consistent with the crucial role of the gills for oxygen uptake, as well as previous observations that gill surface area increases in response to hypoxia exposure (Chapman et al., 1999; Sollid et al., 2003) and is larger in fish with greater hypoxia tolerance (Mandic et al., 2009). Larger gills are also characteristic of the high performance tunas (Brill, 1994; Muir and Kendall, 1968).

Both exercise performance and hypoxia tolerance also appeared to be associated with an elevated capacity for gluconeogenesis in the liver, reflected by a higher PEPCK activity in both rock and largemouth bass (Fig. 2-4C). The concurrent elevation of LDH activity in these species could further suggest that there is a high capacity for lactate as a fuel source for gluconeogenesis in these species. If so, a high gluconeogenic capacity in the liver could facilitate lactate clearance from the blood during or in recovery from exercise or hypoxic exposure. Consistent with an important role of gluconeogenesis in hypoxia, acclimation to hypoxia increases the activities of enzymes involved in this process (malate dehydrogenase and fructose-1,6-bisphosphatase) in the liver of killifish (Martinez et al., 2006).
Physiological basis for tradeoffs between hypoxia tolerance and performance

The apparent tradeoff between hypoxia tolerance and exercise performance that we observed across centrarchid species is consistent with previous findings in other fish taxa (Fu et al., 2014; Nilsson, 2007). The best swimming performer in normoxia, largemouth bass, also had the lowest hypoxia tolerance and the sharpest decline in performance when swimming in moderate hypoxia (Fig. 2-1). The most hypoxia-tolerant species, rock bass, was also the poorest performer in normoxia. The cumulative influence of the variety of respiratory and metabolic traits that distinguish exercise performance and hypoxia tolerance, such as muscle capillarity and the capacity for anaerobic energy production in the heart, likely contributes to this tradeoff.

The nature of oxygen supply to the heart in particular could partly underlie a tradeoff between hypoxia tolerance and exercise performance. The spongy myocardium of the heart receives its oxygen supply from the venous blood in the heart lumen (Farrell, 2007), so cardiac performance can become impaired if the heart becomes deprived of oxygen during exercise (when muscle extracts appreciable O\(_2\) from the venous blood) or exposure to hypoxia (which can lead to hypoxaemia). Cardiac impairment could be especially severe in hypoxia if (i) venous O\(_2\) is depleted by the combined influence of arterial hypoxaemia and high O\(_2\) extraction from the blood, and (ii) the heart does not possess a high anaerobic capacity to help maintain ATP supply during O\(_2\) deprivation. This could be the case in largemouth bass, whose hearts have lower anaerobic potential than other centrarchids, if the high capillarity of their swimming muscle (the most abundant tissue in the body) is also reflective of high O\(_2\) extraction by the muscle in hypoxia. During exercise in normoxia, the arterial oxygen content remains high so the
swimming muscle is less likely to deplete the venous O₂ supply to the heart. Therefore, this unique combination of traits associated with exercise performance in largemouth bass could potentiate O₂ limitation to the heart during hypoxia, and reduce their hypoxia tolerance.

As discussed earlier, a tradeoff between hypoxia tolerance and exercise performance could also arise from the conflicting influence of haemoglobin-O₂ affinity on branchial O₂ uptake and peripheral O₂ unloading (Burggren et al., 1991). The evolution of hypoxia tolerance is often associated with a low P₅₀ – the PO₂ at which haemoglobin is 50% saturated – presumably because it improves branchial O₂ uptake in hypoxia (Mandic et al., 2009; Weber and Fago, 2004). However, a low P₅₀ may reduce O₂ unloading at respiring tissues because it reduces mean capillary PO₂ and thus the driving force for diffusion. Exercise performance should therefore be increased by a lower haemoglobin-O₂ affinity as long as the arterial blood can still be fully saturated with oxygen in normoxia. As a result, the evolution of haemoglobin-O₂ affinity to facilitate hypoxia tolerance or exercise performance can come at the expense of having haemoglobin that is less well suited to the other trait. Future work should examine whether the P₅₀ of haemoglobin varied across centrarchid species and contributes to the apparent tradeoff between hypoxia tolerance and exercise performance.
CHAPTER 3: GENERAL DISCUSSION AND SUMMARY

Physiological Tradeoffs Between Hypoxia Tolerance and Exercise Performance

In this study, I have provided evidence for a tradeoff between hypoxia tolerance and exercise performance among fish species within the family Centrarchidae. In addition, I have identified traits that underlie both hypoxia tolerance and exercise performance. The species that had the highest hypoxia tolerance, rock bass, were the poorest performers (Fig. 2-1). Additionally, largemouth bass, which had the highest performance, were the least hypoxia tolerant of the four species (Fig. 2-1). Finding evidence for a tradeoff between hypoxia tolerance and exercise performance, similar to that seen in Fu et al. (2014) and Nilsson et al. (2007), suggests that in the evolution of a species, a choice may have to be made between these two organismal traits. Tolerance of hypoxia and high exercise performance may represent two distinct fitness optima.

My thesis identified the respiratory and metabolic traits that underlie hypoxia tolerance and exercise performance in centrarchids. Two traits, larger gill surface area and increased PEPCK activity, appeared to benefit both hypoxia tolerance and performance (Fig. 2-2,2-4). However, the high capillarity of the swimming muscle in largemouth bass, without any concurrent change in oxidative capacity, appeared to increase exercise performance alone (Fig. 2-3). Increased hypoxia tolerance in the rock bass appeared to be associated with an increased capacity for anaerobic metabolism in their heart (Fig. 2-4). It therefore appears that the tradeoff between hypoxia tolerance and exercise performance is linked to the particular physiological variables that are associated with just one of these traits.
The Effects of Temperature on Hypoxia Tolerance

Hypoxia tolerance can be strongly influenced by environmental temperature (McBryan et al., 2013). One of the objectives of my thesis was to evaluate this influence in centrarchid fishes, for which I collected preliminary data on pumpkinseed sunfish, bluegill sunfish, and their naturally occurring first generation hybrids (which do not themselves reproduce). I evaluated hypoxia tolerance in fish that were acclimated to common conditions in the lab (T=12-15 °C) using respirometry (using the same methods for measuring resting MO$_2$, P$_{crit}$, and the PO$_2$ at LOE in progressive hypoxia as those in Chapter 2) and time to LOE tests (assessed at a constant water O$_2$ content of 1.0 mg O$_2$/l, as described in the Supplemental Materials and Methods in the Appendix) across a range of experimental temperatures (15, 25, and 28 °C).

There was a noticeable negative effect of increased temperature on hypoxia tolerance in all of the sunfish species, but pumpkinseed sunfish showed a greater sensitivity to temperature than either bluegill or hybrid sunfish. There was a significant effect of temperature on resting MO$_2$, P$_{crit}$, the PO$_2$ at LOE in progressive hypoxia, and the time to LOE at 1.0 mg O$_2$/l in all species (P < 0.001) (Fig. 3-1B-D). Although pumpkinseed showed the greatest hypoxia tolerance, as reflected by time to LOE, they showed a greater decrease in their tolerance to hypoxia relative to the other species as temperature rose (Fig. 3-1B,D). Pumpkinseed also possessed a higher P$_{crit}$ at 28 °C than bluegill and hybrids. Taken together, these results suggest that the hypoxia tolerance of sunfish is negatively correlated with temperature, and that there is a stronger interaction between hypoxia and warming temperature in pumpkinseed sunfish.
The greater hypoxia tolerance of pumpkinseed sunfish, as shown by a longer time to LOE than bluegill and hybrids, is associated with differences in the capacity for anaerobic metabolism in the heart. Pumpkinseed sunfish have a significantly higher LDH activity in the heart than bluegill, which may serve to increase cardiac energy production under hypoxic conditions as described in Chapter 2 (Table 3-2). This is especially important as the heart may be especially O₂ limited during hypoxia, due to the tendency of the venous blood, from which it draws its oxygen supply, to become hypoxic (Farrell and Clutterham, 2002). LDH activity is greater in the muscle of pumpkinseed sunfish as well, similar to the results seen by Davies et al. (2012) (Table 3-2). This may increase hypoxia tolerance, because they may allow the heart and muscle to supplement limitations in aerobic energy production by anaerobic means.

Although rising temperature reduced hypoxia tolerance in all sunfish species, it was surprising that pumpkinseed sunfish – the species with the highest hypoxia tolerance – seemed to be more strongly affected by high temperatures than either bluegill or hybrids. The highest temperatures to which these fish were exposed experimentally were very close to preferred temperatures reported in the literature (27.7°C for pumpkinseed and 31.2°C for bluegill), which are only slightly lower than their critical thermal maxima (~30-36 °C), but are likely higher than the temperatures to which they are exposed most often in the wild (Becker and Genoway, 1979; Beitinger, 1997; Reutter and Herdendorf, 1975; Jobling, 1981). Although there was a strong effect of temperature on the resting metabolic rate of all sunfish species, this effect did not differ between species, arguing that temperature effects on resting metabolic rate was not the primary cause of the noted interspecific differences in hypoxia tolerance across temperatures. A more likely
explanation is an interaction between hypoxia and temperature as might be expected by the oxygen limitation of thermal tolerance (OLTT) hypothesis (Portner and Knust, 2007). Hypoxia has been shown to shrink the thermal tolerance window of species by bringing upper and lower critical temperatures closer together and by magnifying the reduction in aerobic scope with rising temperature (Portner, 2010). It is possible that bluegill sunfish, and to a lesser extent bluegill-pumpkinseed hybrids, may be less effected by hypoxia at higher temperatures because their thermal optimum range extends to warmer temperatures in normoxia, such that aerobic scope is higher, hypoxia is less likely to collapse aerobic scope to zero, and $P_{crit}$ is lower (Fig. 3-1).

*The Effects of Acclimatization to the Native Environment on Hypoxia Tolerance*

I also evaluated hypoxia tolerance in wild-caught fish in the field (at an experimental temperature of 25 °C) using respirometry and time to LOE, and I compared these data to those for fish that were well acclimated to the lab to assess the potential importance of acclimatization to the native environment to variation in hypoxia tolerance in the wild.

There was a significant effect of removing the influence of acclimatization to the native environment (‘lab deacclimation’) on hypoxia tolerance and resting $MO_2$ in sunfish, but there were no clear patterns of variation indicating that this influence differed between species. There was a strong effect of acclimatization on hypoxia tolerance, as wild-caught sunfish had lower $P_{crit}$, $PO_2$ at LOE in progressive hypoxia, and resting $MO_2$ compared to those that were acclimated to laboratory conditions ($P < 0.001$) (Fig. 3-2A-C). In contrast, there was no effect of acclimatization status on the time to LOE at 1.0 mg
Overall, these results indicate that wild-caught fish that are acclimatized to their native environment are more hypoxia tolerant than fish acclimated to the lab. The underlying physiological basis of this observation has not yet been well explored, but wild fish did have significantly higher haematocrit than lab fish (Fig. 3-3).

The noticeable effect of acclimatization to the native environment on hypoxia tolerance and physiology of sunfish could have arisen because wild populations may regularly experience hypoxic events. Hypoxia tolerance is plastic and has been shown to improve with hypoxia acclimation in zebrafish and goldfish (Fu et al., 2011; Rees et al., 2001). There appeared to be interspecific differences in the effect of lab deacclimation on $P_{\text{crit}}$, with bluegill having the lowest $P_{\text{crit}}$ in the wild but not in the lab (Fig. 3-2B). This may indicate that bluegill sunfish are exposed to longer or more severe bouts of hypoxia then pumpkinseed and hybrids in the wild. Alternatively, the results may indicate that there are differences in each species’ capacity for plasticity in response to environmental hypoxia. Nevertheless, this largely similar overall affect of acclimatization to the native environment on hypoxia tolerance across the species is paralleled by wild-caught fish having smaller brains than lab fish (Fig. 3-3), possibly as a means to curtail oxygen demands. This observation is congruent with that of brain size in mormyrid fish, where species living in hypoxic swamps have smaller brains than those dwelling in open-water normoxic habitats (Chapman and Hulen, 2001).
Fig. 3-1. Hypoxia tolerance in sunfish is negatively affected by increased temperature. (A) Resting metabolic rate (MO$_{2\text{rest}}$) is similar between pumpkinseed sunfish (n=8), bluegill sunfish (n=8) and hybrid sunfish (n=8), across temperatures. Temperature has a strong effect on MO$_{2\text{rest}}$ (P<0.001), but neither the species effect nor the interaction between temperature and species was significant. (B) Critical oxygen tension (P$_{\text{crit}}$) is highest in pumpkinseed sunfish at 28°C. There was a significant effect of species, temperature and their interaction on P$_{\text{crit}}$ (P<0.05). (C) The oxygen tension (PO$_2$) at loss of equilibrium (LOE) is raised in sunfish when exposed to higher temperatures. Temperature has a definitive effect on PO$_2$ at LOE (P<0.0001), but there is no difference in species effect or the interaction between species and temperature. (D) Pumpkinseed sunfish have a longer time to LOE across all experimental temperatures. The species, temperature and interaction effects on time to LOE were are significant (P<0.0001). A * indicates a significant difference within an experimental temperature between pumpkinseed sunfish and hybrid and bluegill sunfish (P<0.05). A † indicates a significant difference between only pumpkinseed sunfish and hybrid sunfish within a temperature group (P<0.05). Symbols are offset for hybrid sunfish (to the right) and bluegill sunfish (to the left), for clarity.
Fig. 3-2. Lab deacclimation decreases hypoxia tolerance in sunfish in comparison to wild-caught populations. (A) Resting metabolic rate (MO$_{2\text{rest}}$) is elevated in both wild-caught and lab-deacclimated hybrid sunfish. There is a strong effect of both temperature and species on MO$_{2\text{rest}}$ (P<0.05), but the interaction between them is not significant. (B) Critical oxygen tension (P$_{\text{crit}}$) is lower in wild-caught bluegill sunfish than either pumpkinseed or hybrid sunfish. There is a strong effect of both temperature and species on P$_{\text{crit}}$ (P<0.05), but the interaction between them is not significant. (C) Lab-deacclimated sunfish have higher oxygen tensions (PO$_2$) at loss of equilibrium (LOE) than wild-caught sunfish. There is a strong effect of acclimation on PO$_2$ at LOE (P<0.05). However, there is no effect of species or the interaction between species and acclimation. (D) Pumpkinseed sunfish have a longer time to LOE in both wild-caught and lab deacclimated populations. There is a significant species effect on time to LOE (P<0.05), but no effect of acclimation or the interaction between acclimation and species. A * indicates a significant difference within an experimental temperature between pumpkinseed sunfish vs. hybrid and bluegill sunfish (P<0.05). A † indicates a significant difference between only bluegill sunfish and hybrid sunfish within a temperature group (P<0.05) (n as follows: wild-caught: pumpkinseed sunfish, 14; bluegill sunfish, 11; hybrid sunfish, 15; lab-deacclimated: pumpkinseed sunfish, 8; bluegill sunfish, 8; hybrid sunfish, 8). Symbols are offset for hybrid sunfish (to the right) and bluegill sunfish (to the left), for clarity.
**Fig. 3-3. Lab deacclimation has an effect on the physiology of sunfish.** (A) Hematocrit is lower in sunfish that are deacclimated to lab conditions. There is a significant effect of acclimation on hematocrit (P<0.0001), but there is no effect of species or the interaction between species and temperature on hematocrit. (B) Relative heart mass is higher in pumpkinseed and hybrid sunfish deacclimated to lab conditions. There is a strong effect on relative heart size in sunfish from acclimation, species and the interaction between them (P<0.05). (C) Relative liver weight is higher in wild-caught pumpkinseed sunfish. There is a definitive species effect on relative liver weight (P<0.05). However there is no effect of acclimation or the interaction between acclimation and species. (D) Lab deacclimation increases relative brain mass in sunfish species relative to wild caught populations. There is a significant effect of acclimation on relative brain weight (P<0.001), but there is no effect of species or the interaction between species and temperature on relative brain weight. A * indicates a significant difference within an experimental temperature between pumpkinseed and hybrid sunfish vs. bluegill sunfish (P<0.05). A † indicates a significant difference between pumpkinseed sunfish vs. hybrid and bluegill sunfish within a temperature group (P<0.05) (n are the same as in Fig. 3-2). Symbols are offset for hybrid sunfish (to the right) and bluegill sunfish (to the left), for clarity.
Table 3-1. Maximum metabolic rate, swimming performance, gill morphology and muscle histology of sunfish

<table>
<thead>
<tr>
<th></th>
<th>Pumpkinseed sunfish</th>
<th>Hybrid sunfish</th>
<th>Bluegill sunfish</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maximum Rate of Oxygen Uptake</strong> (mg O\textsubscript{2} kg\textsuperscript{-1} body mass hr\textsuperscript{-1} at 15\textdegree C and at:**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 kPa</td>
<td>235±8</td>
<td>233±12</td>
<td>223±8</td>
</tr>
<tr>
<td>9 kPa</td>
<td>178±6</td>
<td>174±12</td>
<td>163±6</td>
</tr>
<tr>
<td>6 kPa</td>
<td>122±6</td>
<td>122±10</td>
<td>136±6</td>
</tr>
<tr>
<td><strong>Critical Swimming Speed</strong> (body lengths s\textsuperscript{-1} at 15\textdegree C and at:**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 kPa</td>
<td>2.08±0.09</td>
<td>2.37±0.2</td>
<td>2.149±0.09</td>
</tr>
<tr>
<td>9 kPa</td>
<td>1.84±0.07</td>
<td>1.73±0.14</td>
<td>2±0.1</td>
</tr>
<tr>
<td>6 kPa</td>
<td>1.71±0.08</td>
<td>1.57±0.13</td>
<td>1.6±0.11</td>
</tr>
<tr>
<td><strong>Total Gill Surface Area</strong> (cm\textsuperscript{2} g\textsuperscript{-1})</td>
<td>4.34±0.19</td>
<td>4.49±0.24</td>
<td>4.11±0.21</td>
</tr>
<tr>
<td><strong>Lamellar Bilateral Surface Area</strong> (mm\textsuperscript{2})</td>
<td>0.34±0.02\textsuperscript{a}</td>
<td>0.32±0.02\textsuperscript{a}</td>
<td>0.25±0.01\textsuperscript{b}</td>
</tr>
<tr>
<td><strong>Oxidative Fibre Areal Density</strong> (%)</td>
<td>4.86±0.19</td>
<td>5.66±0.67</td>
<td>4.77±0.37</td>
</tr>
<tr>
<td><strong>Capillary Density of Oxidative Muscle Fibres</strong> (mm\textsuperscript{2})</td>
<td>895±64\textsuperscript{a}</td>
<td>823±145\textsuperscript{ab}</td>
<td>622±39\textsuperscript{b}</td>
</tr>
<tr>
<td><strong>Capillary Density of Glycolytic Muscle Fibres</strong> (mm\textsuperscript{2})</td>
<td>193±12</td>
<td>229±16</td>
<td>211±9</td>
</tr>
</tbody>
</table>

Data are means ± s.e.m (n as follows: pumpkinseed sunfish, 16; hybrid sunfish, 8; bluegill sunfish, 11). Different letters indicate a significant difference between species (P<0.05).
Table 3-2. Enzyme activity in the liver, heart, brain and muscle of sunfish

<table>
<thead>
<tr>
<th>Enzyme Activity (µmol g tissue⁻¹ min⁻¹)</th>
<th>Pumpkinseed sunfish</th>
<th>Hybrid sunfish</th>
<th>Bluegill sunfish</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEPCK Activity</td>
<td>Liver</td>
<td>4.21±0.28</td>
<td>5.07±0.45</td>
</tr>
<tr>
<td></td>
<td>Heart</td>
<td>4.12±0.22</td>
<td>3.43±0.26</td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>1.78±0.09</td>
<td>1.36±0.38</td>
</tr>
<tr>
<td>HOAD Activity</td>
<td>Liver</td>
<td>4.54±0.32</td>
<td>3.03±0.4</td>
</tr>
<tr>
<td></td>
<td>Heart</td>
<td>4.54±0.32</td>
<td>3.03±0.4</td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>1.78±0.09</td>
<td>1.36±0.38</td>
</tr>
<tr>
<td>CS Activity</td>
<td>Liver</td>
<td>2.03±0.14</td>
<td>2.66±0.47</td>
</tr>
<tr>
<td></td>
<td>Heart</td>
<td>15.25±0.87</td>
<td>12.49±1.27</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>9.86±0.52</td>
<td>10.19±0.56</td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>2.16±0.12</td>
<td>2.68±0.57</td>
</tr>
<tr>
<td>COX Activity</td>
<td>Liver</td>
<td>53.63±3.75</td>
<td>35.93±4.33</td>
</tr>
<tr>
<td></td>
<td>Heart</td>
<td>39.17±2.48</td>
<td>29.23±4.25</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>32.69±3.24</td>
<td>27.18±4.46</td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>10.53±0.75</td>
<td>11.45±2.07</td>
</tr>
<tr>
<td>PK Activity</td>
<td>Liver</td>
<td>16.99±1.59</td>
<td>33.87±10.73</td>
</tr>
<tr>
<td></td>
<td>Heart</td>
<td>235.8±18.4</td>
<td>211.3±23.28</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>161±9.32</td>
<td>195.7±17.35</td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>420.3±24.81</td>
<td>436.3±39.53</td>
</tr>
<tr>
<td>LDH Activity</td>
<td>Liver</td>
<td>29.02±0.95</td>
<td>35.37±6</td>
</tr>
<tr>
<td></td>
<td>Heart</td>
<td>498.7±26.65</td>
<td>368.8±27</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>224.8±9.13</td>
<td>218.5±13.12</td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>778.9±36.61</td>
<td>704.8±53.4</td>
</tr>
</tbody>
</table>

Data are means ± s.e.m (n as follows: rock bass, 8; pumpkinseed sunfish, 16; bluegill sunfish, 12; largemouth bass, 5). Hydroxyacyl-CoA dehydrogenase (HOAD), citrate synthase (CS), cytochrome oxidase (COX), lactate dehydrogenase (LDH), phosphoenolpyruvate carboxykinase (PEPCK), and pyruvate kinase (PK).

Different letters indicate a significant difference between species (P<0.05).
Conclusions

These results illustrate the variation in hypoxia tolerance in centrarchid fishes and demonstrate the tradeoff that exists between hypoxia tolerance and exercise performance. Hypoxia tolerance is plastic, as reflected by the greater overall tolerance of wild-caught fish compared to fish that are acclimated to the lab, and it is affected by environmental temperature. I have pinpointed some of the physiological determinants of exercise performance and hypoxia tolerance in centrarchids, as well as traits that contribute to both or that may underlie the tradeoff between these traits. Hypoxia tolerance, temperature tolerance, and exercise capacity are some of the most important traits influencing the life history, evolution, and behaviour of fish. Hypoxia and warm temperatures are becoming more prevalent in the aquatic environment due to pollution and climate change. Understanding the physiological basis of hypoxia tolerance and thermal tolerance, as well as how they interact with other organismal traits, such as exercise performance, is an important step in understanding how species will react and change to meet the environmental challenges they face in an uncertain future.

Future Directions

This study has produced evidence for a tradeoff between hypoxia tolerance and exercise performance in centrarchid species and has illuminated the underlying physiological basis of this tradeoff. However, further investigation into the physiology of these fish to improve the understanding of this tradeoff is warranted and interesting. Another avenue for research would be to take a closer look at cardiac physiology in these fish, by measuring cardiac performance (heart rate, stroke volume, cardiac output) and
blood oxygen tensions (venous and arterial) during swimming and at rest in both hypoxia and normoxia (Gamperl and Driedzic, 2009; Speers-Roesch et al. 2010). It may also be possible to manipulate hypoxic conditions at the heart by doing isolated heart experiments and measuring cardiac performance under a range of oxygen tensions. Taking in vivo measurements of oxygen tension in the venous and arterial blood, perhaps just before and just after it passes through the capillary beds supplying the axial swimming muscle and directly at the heart would be extremely useful in determining if the heart is the “weak link” that limits hypoxia tolerance or contributes to the tradeoff between hypoxia tolerance and exercise performance (Farrell and Clutterham, 2002).

Another important avenue to pursue would be to measure the $P_{50}$ of haemoglobin in these species, as this has been hypothesized as a candidate physiological trait underlying a tradeoff between hypoxia tolerance and performance (Burggren et al., 1991).

We also showed evidence of variation in the interactive effects of temperature on hypoxia tolerance among species of sunfish, and that acclimatization to the native environment has an influence on hypoxia tolerance in these species. These measurements should be complemented by additional work to understand the underlying physiological bases for the observations. This could include measurements of gill morphology or the activity of metabolic enzymes across the same range of temperatures as our hypoxia tolerance experiments.
Supplemental Materials and Methods

DNA Extraction and Genotyping

Genotyping was conducted in order to identify hybrids from purebred pumpkinseeds by following a similar procedure to that of Near et al. (2004). Firstly, small clips of the caudal fin on fish displaying the pumpkinseed phenotype were taken, and DNA was extracted via a REDEnd-N-Amp Tissue PCR Kit (Sigma-Aldrich). The nuclear S7 fragment was amplified through PCR using the following primers taken from Davies et al., 2012 (Table 4-1). The PCR reaction (25 µl) included 1 µl of each primer, 5 µl of template, 10 µl of master mix (Sigma) and 8 µl of RNA/DNA free water with 30 cycles of denaturation (30 sec at 94 °C) annealing (30 sec at 59 °C) and elongation (30 sec at 72 °C). Amplified products were electrophoresed on a 3% agarose gel at 60 volts. For pumpkinseed a single band is observed at 385 bp and in hybrids a double band is seen at 363 and 385 bp. After identification, fish identified as purebreds and hybrids were separated.

Time to Loss of Equilibrium

The time to loss of equilibrium at 1.0 mg O₂/L was measured in both the lab and the field. Fish were placed into either 2L respirometry chambers, or 3L Tupperware containers equipped with a flow through pump and immersed in either a 60 gallon tank in the lab or a 20 gallon tank in the field. Experiments were run at 15, 25 or 28 °C in dechlorinated Hamilton tap water in the lab, or at 25 °C in lake water in the field (Queens University Biological Station on Lake Opinicon). After fish were placed into the
chambers they were left for a minimum of 8 hours on well-aerated flow through conditions at the experimental temperature. When the experiments were commenced nitrogen gas was bubbled into the tanks to reduce the oxygen tension to 1.0 mg O$_2$/l within 15 min. Fish were then monitored continuously for loss of equilibrium and the tension at which they could no longer recover equilibrium was noted. Fish were then removed to a well-oxygenated recovery tank.

<table>
<thead>
<tr>
<th>Gene Identification</th>
<th>5’ – Forward – 3’</th>
<th>5’ – Reverse – 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>S7 - Intron</td>
<td>TGTAACGGGGAGCAGTTAGC</td>
<td>ACAGCCGATGTTAGGAAACAG</td>
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</tbody>
</table>
Chapter 5: References


the nations estuaries. NOAA, National Ocean Service, Special Projects Office and the National Centers for Costal Ocean Science.


