A NOVEL MECHANISM FOR ENHANCING TISSUE OXYGEN DELIVERY IN TELEOST FISHES

by

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ABSTRACT

Teleost fishes represent half of all extant vertebrates and possess a unique Root effect haemoglobin (Hb). The Root effect occurs when a reduction in pH greatly reduces Hb oxygen (O₂) affinity and carrying capacity, and is known to greatly enhance O₂ delivery to specialized structures (retia) at the eye and swimbladder. This phenomenon is thought to be a central component to the most successful adaptive radiation event in the evolutionary history of vertebrates. Here, I propose and demonstrate a role for the Root effect in general O₂ delivery. In Chapter 2, I demonstrated that in rainbow trout blood, which possesses a Root effect, the potential for enhanced O₂ delivery relative to that of an air-breathing vertebrate, such as a human, is an order of magnitude greater for a given arterial-venous blood pH change (ΔpHₐ₋ᵥ). However, large ΔpHₐ₋ᵥ are generally not thought possible away from retia. In Chapters 3 and 4, I proposed and validated a novel mechanism that induces a large ΔpHₐ₋ᵥ at the tissues permitting the Root effect to facilitate general O₂ delivery. During a generalized acidosis, teleosts secure gill O₂ uptake by protecting RBC pH via adrenergically-stimulated Na⁺/H⁺ exchange (βNHE). I proposed that short-circuiting βNHE at tissues with plasma-accessible carbonic anhydrase (CA) may create a large ΔpHₐ₋ᵥ that could greatly enhance O₂ delivery. In Chapter 3 this was validated in vitro in a closed system. I also validated this mechanism in vivo (Chapter 4), where rainbow trout were implanted with fiber-optic O₂-sensors to monitor red muscle (RM) partial pressure of O₂ (PO₂) in real-time. Resting RMPO increased by 65% following exposure to elevated environmental CO₂ and the associated mild acidosis. This was over 10-times what would be expected in an air-breathing vertebrate, such as a human. Furthermore, the ΔPO₂ was completely abolished in the presence of a CA inhibitor, C18. Thus, in my thesis, I have demonstrated that the Root effect may enhance general tissue O₂ delivery. Given that the Root effect evolved 150-270MY before eye and swimbladder retia, it may be that general O₂ delivery was the initial selection pressure in Root effect evolution.
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Figure 5.1.  A timeline illustrating the approximate first appearances of structures and systems related to Root effect Hbs in teleost fishes (MYA, million years ago).
LIST OF SYMBOLS AND ABBREVIATIONS

βNHE: β-adrenergically-activated sodium proton exchange

Δ: delta, change (e.g. ΔpH)

Φ: Bohr coefficient

A: absorbance

AD: adrenaline

AE: anion exchange

ANOVA: analysis of variance

atm: atmospheres of pressure, where 1 atm is 760 mm Hg or 101.33 kPa

ATP: adenosine triphosphate

C18: compound 18, carbonic anhydrase inhibitor

°C: degrees Celsius

C: content

CA: carbonic anhydrase

cAMP: adenylate cyclase and 3’, 5’ - cyclic monophosphate

Cl⁻: chloride

CO₂: carbon dioxide

DA: dorsal aorta

DHBA: 3,4-dihydroxybenzylamine hydrobromide

DMSO: dimethyl sulfoxide

EIPA: ethylisopropylamiloride

F3500: 4,4’-diisothiocyanostilbene-2,2’-disulfonic acid, carbonic anhydrase inhibitor

GPI: phosphatidylinositolglycan

H⁺: proton
Hb: haemoglobin
HCl: hydrochloric acid
Hct: haematocrit
HCO$_3^-$: bicarbonate
Hepes: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
ISO: isoproterenol
K$: potassium
K$_{cat}$: enzyme catalytic activity (s$^{-1}$)
K$_i$: enzyme inhibitor constant
kPa: kilopascals, where 1 kPa is 0.0099 atm or 7.50 mm Hg
LED: light emitting diodes
MCHC: mean cell haemoglobin concentration
mm Hg: millimeters of mercury, where 1 mm Hg is 0.1333 kPa or 0.0013 atm
MS-222: tricaine methanesulphonate (anaesthetic)
NA: noradrenaline
Na$: sodium
NaClO$_4$: sodium perchlorate
NaHCO$_3^-$: sodium bicarbonate
NaSO$_3$: sodium sulfite
NaSO$_4$: sodium sulfate
n$_H$: Hill coefficient
NHE: sodium proton exchange
NKA: Na$, K$, ATPase
O$_2$: oxygen
OEC: oxygen equilibrium curve
\( P_{50} \): partial pressure of oxygen where 50% of haemoglobin is saturated with oxygen

\( \text{pH}_a \): arterial blood pH

\( \Delta \text{pH}_{a-v} \): arterial-venous pH difference

\( \text{pH}_c \): extracellular, plasma pH

\( \text{pH}_i \): intracellular, red blood cell pH

\( \text{pH}_v \): venous blood pH

\( \text{PO}_2 \): partial pressure of \( \text{O}_2 \)

\( \text{PCO}_2 \): partial pressure of \( \text{CO}_2 \)

QAS: quaternary ammonium sulfanilamide, carbonic anhydrase inhibitor

\( \text{N}_2 \): nitrogen

RBC: red blood cell

RM: red muscle

RQ: respiratory quotient

S.E.M.: standard error around a mean value

\( \text{SO}_2 \): saturation of blood with oxygen

t: time

\( t_{1/2} \): the half-time of a reaction

Tris: tris(hydroxymethyl)aminomethane

\( \text{TO}_2 \): total oxygen content
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CO-AUTHORSHIP STATEMENT

Chapter One: General Introduction

Comments: This chapter was written by Jodie L. Rummer under the supervision of Dr. Colin J. Brauner who also supplied editorial advice.

Chapter Two: The influence of pH on haemoglobin-oxygen binding: A comprehensive investigation of the Bohr-Root effect system in rainbow trout, *Oncorhynchus mykiss*

Comments: All aspects of this study were conducted and written by Jodie L. Rummer under the supervision of Dr. Colin J. Brauner who also supplied expert advice and editorial suggestions.

Chapter Three: A mechanism at the level of the red blood cell for enhanced oxygen delivery in rainbow trout (*Oncorhynchus mykiss*): Short-circuiting βNHE in vitro

Comments: All aspects of this study were conducted and written by Jodie L. Rummer under the supervision of Dr. Colin J. Brauner who also supplied expert advice and editorial suggestions.

Chapter Four: A mechanism for enhanced oxygen delivery to muscle tissue in rainbow trout, *in vivo*

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Overview

Teleosts fishes represent over half of all extant vertebrates and possess Root effect haemoglobins (Hb). The Root effect is involved in the fine-tuning of buoyancy control, a complex physiological mechanism key to one of the most successful adaptive radiation events in vertebrate history. The Root effect occurs when a decrease in blood pH greatly reduces the affinity of Hb for oxygen (O$_2$). As with the Bohr effect found generally in most vertebrates, a decrease in pH elicits a right-shift in the O$_2$ equilibrium curve (OEC) and increases the driving force for O$_2$ delivery ($\Delta$PO$_2$) to tissues. Unique to the Root effect however, is that the carrying capacity of Hb for O$_2$ is also greatly reduced (Root, 1931; Root and Irving, 1943). The Root effect has long been understood to enhance O$_2$ delivery only to tissues where a substantial acidosis can be created, localized, and recycled, such as the choroid rete near the retina of the eye and the rete mirabile of the swimbladder (Alexander, 1966; Dafré and Wilhelm, 1989; Wittenberg and Wittenberg, 1974). A large arterial-venous blood pH difference ($\Delta$pH$_{a-v}$) is not thought possible in the general circulation, thus the potential for Root effect Hbs to enhance general tissue O$_2$ delivery has largely remained unexplored. The overall hypothesis of my thesis was that during a generalized acidosis in teleosts, through the exploitation of a novel mechanism that induces a large $\Delta$pH$_{a-v}$ at the tissues, general O$_2$ delivery can be enhanced via the combined Bohr-Root effect system.

In a fish possessing pH-sensitive Root Hbs, a generalized acidosis that would ensue during stressful conditions such as environmental hypercarbia, hypoxia or intense exercise would seemingly benefit O$_2$ delivery at the site of acid production. However, the low affinity and carrying capacity Hb would return to the gill unable to sufficiently bind and subsequently deliver O$_2$. Intracellular red blood cell (RBC) pH (pH$_i$) is regulated in many fish when catecholamines
released into the circulation activate sodium/proton (Na+/H+) exchange (βNHE) on the RBC membrane (Berenbrink and Bridges, 1994; Brauner et al., 1996; Nikinmaa and Huestis, 1984; Perry and Kinkead, 1989; Primmett et al., 1986; Val et al., 1998). The βNHE removes H+ from the RBC, which elevates pH_i. This increases Hb-O2 affinity, and thus O2 uptake at the respiratory surface is maintained (Berenbrink and Bridges, 1994). Carbonic anhydrase (CA) short-circuits the βNHE in vitro (Lessard et al., 1995). Therefore, it is not surprising that there is no plasma-accessible CA in the gills of teleost fishes (Lessard et al., 1995). However, there is evidence for plasma-accessible CA in other locations in vivo (Decker et al., 1996; Effros and Weissman, 1979; Geers and Gros, 2000; Gilmour et al., 1997; Henry et al., 1997; Henry and Swenson, 2000; Sender et al., 1994; Siffert and Gros, 1982; Yamamoto et al., 2003). In this thesis, the mechanism I propose to induce a large ΔpH_a-v during a generalized acidosis in rainbow trout involves short-circuiting of RBC pH_i regulation via βNHE by CA. The resulting large ΔpH_a-v would ultimately acidify the RBC and facilitate enhanced O2 delivery via the Bohr-Root effect, far in excess (an order of magnitude or more) of that thought possible by the Bohr effect alone.

The objectives of this thesis research were to 1) characterize the Bohr-Root effect system in rainbow trout to determine the potential for enhancing general O2 delivery associated with various blood pH changes relative to a system possessing a Bohr effect alone, such as that of a human, 2) characterize, in vitro, a mechanism whereby a large ΔpH_a-v could be created upon short-circuiting of the βNHE at the RBC by CA, thus permitting enhanced O2 delivery via the Bohr-Root effect system and 3) determine if the short-circuiting mechanism proposed in objective 2 is operational in vivo.
The remainder of the introduction will provide background information on Hb, the Bohr and Root effects, and how activation and short-circuiting of RBC βNHE may affect O₂ transport in teleost fishes, leading up to the specific objectives of each chapter in this thesis.

1.2 Background

With very few exceptions, aerobic metabolism and therefore O₂ is a prerequisite for vertebrate life. Oxygen uptake from the environment, transport through the circulatory system and ultimately delivery to metabolizing tissue, and the reverse for carbon dioxide (CO₂), defined together as the respiratory gas cascade, has been of interest to respiratory physiologists for centuries. This introduction will begin by focusing on one component of the respiratory cascade, the respiratory pigment, Hb.

1.2.1 Haemoglobin function in vertebrates

The respiratory pigment Hb is encapsulated in the RBCs and is universal for gas exchange in vertebrates, with very few exceptions. Under specific conditions, Hb binds O₂ at the respiratory surface, releases it to tissues in exchange for CO₂, and transports CO₂ back to the respiratory surface for removal. Due to its central role in both O₂ and CO₂ transport, Hb has become one of the most well studied proteins to date.

The Hb protein was discovered almost two centuries ago because of its role in respiration, and it is now known to be responsible for transporting 98-99% of O₂ in the blood. Hoppe-Seyler (1866) was the first to determine that Hb could reversibly bind O₂. Then, during the early 1900s, the reversible binding relationship between Hb and CO₂ was investigated by Bohr, Hasselbalch, and Krogh (1904), thus linking this molecule to the respiratory gas cascade. Several decades later, Perutz et al. (1960) examined the binding properties of these two
respiratory gases and Hb and determined that binding was largely determined by the structural and conformational changes incurred by the protein upon exposure to O\(_2\), CO\(_2\), and other ligands. While the aforementioned seminal studies linked O\(_2\) and CO\(_2\) transport to the Hb protein, Perutz (1960) linked the physiochemical structure of the Hb protein with respiratory function, forever changing the field of respiratory physiology, a discovery that also earned him the Nobel Prize for Chemistry in 1962.

When ligands bind to Hb, the process elicits conformational changes, which dictate how the molecule functions for O\(_2\) and CO\(_2\) exchange in vertebrates (Perutz et al., 1960). Perutz et al. (1960) determined human Hb to be a tetrameric protein consisting of 2 \(\alpha\) (141 amino acid) and 2 \(\beta\) (146 amino acid) subunits (globins), each containing a porphyrin ring with an iron (Fe\(^{2+}\))-containing haeme center, which is the site of O\(_2\) binding. Depending on the conformation of the Hb molecule, the O\(_2\) binding site could be concealed or exposed, thus directly affecting the ability of Hb to bind O\(_2\) (Monod et al., 1963; Perutz et al., 1960). When salt bridges form between the \(\alpha\)- and \(\beta\)- components of the Hb, O\(_2\) binding sites are obscured, creating a tense (T) conformational state. Non-covalent, electrostatic interactions lock the molecule into this deoxygenated conformation, thus preventing O\(_2\) binding (Perutz et al., 1960). The influence of ligands, such as organophosphates, ions such as Cl\(^-\), H\(^+\), and temperature can break salt bridges, shifting the structural conformation to a relaxed (R) state, revealing binding sites for oxygenation. Breaking salt bridges is energetically costly, especially for the number of salt bridges that must be broken to reveal the first O\(_2\) binding site. After the first O\(_2\) molecule binds, subsequent O\(_2\) binding at other haeme groups is easier because less salt bridges have to be broken to reveal binding sites. This is termed cooperativity, and ultimately results in an oxygenated, high affinity Hb (Bonaventura et al., 2004; Perutz, 1990; Perutz and Brunori, 1982; Perutz et al., 1980).
An O\textsubscript{2} equilibrium curve (OEC) represents O\textsubscript{2} binding to Hb (expressed as \% Hb-O\textsubscript{2} saturation), which depends on the partial pressure of O\textsubscript{2} (P\textsubscript{O}\textsubscript{2}) to which the system is equilibrated. The shape and position of the OEC (Figure 1.1) have been topics of intense investigation (Kobayashi et al., 1994). The OEC shape is dictated by the way in which individual Hb subunits interact upon binding. Cooperative binding is described by the Hill coefficient (n\textsubscript{H}) and calculated from the slope of the line when \[\frac{\log(\text{Hb-O}_2)}{1-(\text{Hb-O}_2)}\] is plotted against \log P\textsubscript{O}\textsubscript{2} (Hill, 1910; Lapennas and Reeves, 1983; Malte and Weber, 1987; Vorger, 1987). A sigmoidal OEC has a higher n\textsubscript{H} and therefore a higher degree of cooperative Hb subunit binding than a hyperbolic OEC. With a high cooperativity, substantial decreases in Hb-O\textsubscript{2} can occur with only very small decreases in P\textsubscript{O}\textsubscript{2} and can be functionally significant to an organism by enhancing O\textsubscript{2} unloading. High cooperatively results in a relatively constant driving force for tissue O\textsubscript{2} delivery, despite large variations in blood O\textsubscript{2} content (Lapennas and Reeves, 1983).

The position of the OEC relative to P\textsubscript{O}\textsubscript{2} is often used to characterize Hb-O\textsubscript{2} affinity. The position of the OEC is often characterized by one point, the P\textsubscript{O}_2 at which 50\% of Hb is oxygenated (P\textsubscript{50}), which differs markedly between and within organisms and therefore has important implications for gas exchange (Figure 1.1). For example, changes in environmental conditions, such as severe hypoxia, may alter the cellular environment of the Hb and decrease P\textsubscript{50} (left shift in the OEC), resulting in a higher affinity Hb that favours Hb-O\textsubscript{2} loading at the gills during times of low environmental O\textsubscript{2} (Jensen and Weber, 1982; Perry et al., 2004). Many factors are known to influence the shape, position, and magnitude of the shifts in the OEC, and this knowledge has contributed to a broader understanding of respiratory gas exchange in vertebrates.
1.2.2 The Bohr effect

Until Bohr, Hasselbalch, and Krogh’s first studies on canine blood in the early 1900s (Bohr et al., 1904), O₂ and CO₂ transport in the blood were generally regarded as two independent processes. Bohr et al. (1904) determined that, whereas CO₂ binding by the Hb was not affected by the presence of O₂, the extent of Hb-O₂ binding was reduced in the presence of CO₂. CO₂ decreased pH due to H⁺ formation from CO₂ hydration, and Hb was discovered to be sensitive to pH (Bohr et al., 1904). The key to Bohr’s early studies was that the effects of CO₂ on Hb-O₂ binding were investigated at low as well as high O₂ tensions (Bohr et al., 1904). Studies previous to 1904 only investigated O₂ binding at atmospheric O₂ tensions, where Bohr et al. found no effect of CO₂ on Hb-O₂ binding (1904). The fact that Hb-O₂ binding is influenced differently at high, compared to low atmospheric O₂ tensions was largely overlooked for 5 decades prior to the seminal Bohr study (Bohr et al., 1904). Many factors have since been determined to affect Hb-O₂ affinity, such as temperature, ions, and organophosphates (Clark et al., 2010; Clark et al., 2008; Eddy, 1973; Irving et al., 1941; Jensen, 2004; Nikinmaa, 1990; Nikinmaa, 1993b; Val, 2000; Val et al., 1991; Val et al., 1990; Val et al., 1995; Wells and Weber, 1989). Most relevant to this research are pH effects, for which the influence on Hb-O₂ affinity is now universally known as the Bohr effect (Bohr et al., 1904; Nikinmaa, 1997; Nikinmaa and Soivio, 1979).

1.2.3 The Bohr effect and the increase in the driving force for oxygen delivery (ΔPO₂)

The Bohr effect can be illustrated simply by the change in the position of the OEC due to an increase in H⁺ concentration and/or CO₂ (Figure 1.1). In the presence of an acidosis, such as in tissue capillaries due to metabolic CO₂ production, a low affinity Hb conformation is favored,
and the OEC shifts to the right (Figure 1.1). Thus, at a given blood Hb-O₂ saturation, blood PO₂ is increased (Figure 1.1), which is thought to enhance O₂ delivery to tissues. An increase in pH, which might occur at the respiratory surface due to CO₂ removal, shifts the OEC back to the left, where a lower PO₂ is required to saturate Hb with O₂ (Figure 1.1), and this is thought to enhance O₂ uptake. The Bohr effect is quantified by the Bohr coefficient (Φ), which describes the degree to which the OEC shifts for a given decrease in pH (Figure 1.1) (Bohr et al., 1904; Eddy, 1971; Malte and Weber, 1987; Nikinmaa, 1990; Nikinmaa and Soivio, 1979; Winslow et al., 1977). This is usually calculated for a single point on the OEC as the logarithm of the change in P₅₀ divided by the change in pH (Bohr et al., 1904) as indicated in the following equation:

\[ \Phi = \frac{\Delta \log P_{50}}{\Delta pH} \]

If the Φ is known, the magnitude of the right-shift in the OEC at a constant Hb-O₂ saturation (e.g. P₅₀) and a proposed ΔpHₐ₋ᵥ can be determined, and is referred to as ΔPO₂ (in mm Hg) throughout this thesis.

\[ \Delta PO₂ = 10^{[(\Phi * \Delta pH) + \log P_{50 (rest)}]} - P_{50 (rest)} \]

Intuitively, for a given ΔpHₐ₋ᵥ, a large Φ results in a greater ΔPO₂. However, the ΔPO₂ can only be calculated in this manner if the Φ is linear at a wide range of Hb-O₂ saturations (20-80%) within the OEC. This is generally the case in air-breathing vertebrates, making model development relatively straightforward. This is not the case for many teleost fishes, including the rainbow trout, where the Bohr effect is strongly non-linear over the OEC (Brauner and Jensen, 1999; Brauner and Randall, 1998; Brauner et al., 2001). Therefore modeling tissue O₂ delivery in a teleost may only be possible by directly interpolating ΔPO₂ from OECs (Chapter 2). Modeling tissue O₂ delivery for a teleost fish is also interesting due to their large Φ, relative to...
other vertebrates, and this is related to the presence of a unique, highly pH-sensitive Root effect Hb described below.

1.2.4 Evolution of Root effect haemoglobins

Teleost fishes, which represent over half of all extant vertebrate species, possess Hbs that are more pH-sensitive than Hbs in any other known vertebrate. Since Bohr’s seminal findings over one century ago, the magnitude of the pH-mediated, right-shift in the OEC and increase in $P_{50}$ has been a well-studied topic in vertebrate respiratory physiology (Brittain, 1987; Brittain, 2005; Dafré and Wilhelm, 1989; Eddy, 1971; Ingermann, 1982; Pelster and Decker, 2004; Pelster et al., 1998; Pelster and Weber, 1991; Root, 1931; Root and Irving, 1943; Scholander and Van Dam, 1954; Verde et al., 2007). The unique pH sensitivity in teleost fish Hbs is referred to as the Root effect, which is characterized by a reduction in the maximum carrying capacity of Hb for $O_2$ as well as a right-shift in the OEC. The Root effect has interesting implications for $O_2$ delivery and is the main focus of this dissertation.

Because both Bohr and Root effects (teleost fishes only) elicit a right-shift in the OEC with decreasing pH (Figure 1.1) (Brauner and Val, 1996; Brauner et al., 2001; Bridges et al., 1983; Brittain, 1987; Brittain, 2005; Dafré and Wilhelm, 1989; Mylvaganam et al., 1996; Pelster and Decker, 2004; Pelster and Weber, 1991; Root, 1931), many investigators have suggested that the Root effect in teleost fishes is simply an exaggerated Bohr effect (Berenbrink et al., 2005; Bonaventura et al., 2004; Brittain, 1987; Mylvaganam et al., 1996; Perutz and Brunori, 1982; Vorger, 1985). However, molecular studies on Hb structure suggest different evolutionary and mechanistic origins. Structure-function analyses have revealed specific locations where amino acid substitutions have occurred supporting distinct molecular differences between the Bohr and Root effect combination in fish and the Bohr effect alone observed in other vertebrates.
It is known that $\text{H}^+$ binding at $\beta$-chain histidine residues stabilize the T-state, low $\text{O}_2$ affinity conformation of the protein, which is the molecular basis for the Bohr effect, but the Root effect results from an overstabilization of this conformation further impeding transition into the R-state, high $\text{O}_2$ affinity conformation (Berenbrink et al., 2005; Brittain, 2005). Various amino acid substitutions have been identified in Root effect Hbs, all of which differ distinctly from human Hb-A and thus are not thought to contribute to the mammalian Bohr effect. Furthermore, these substituted residues are thought to act synergistically. The hydrogen bonds between them anchor the positively charged cluster at the $\beta_1\beta_2$ interface, which decreases the $pK_a$ into the acidic range of what is known as the Root effect (Mylvaganam et al., 1996). Conserved in Root effect Hbs are three elements: the $\betaN$-terminus, an arginine for lysine at 21$\beta$, and histidine at 3$\beta$. Removal of any aspect can result in a decrease in the Root effect by 50%. It is also thought that a serine substituted for a cysteine residue at the $\beta93$ position allows salt bridges to form with the C-terminal histidine residue to overstabilize – with two new hydrogen bonds – the T-state conformation. However, additional substitutions have been identified, one of which may be essential, tryptophan at 3$\beta$. For the Root effect, $\text{H}^+$ bind not only at the histidine residue 147$\beta$ but also in the vicinity of this key amino acid, likely between two aspartate residues separating the $\alpha1$ and $\beta2$ subunits of the haemoglobin molecule, thus creating even stronger salt bridges further locking the molecule into the T-state conformation (Berenbrink et al., 2005; Brittain, 2005). Therefore, it follows that fish Hbs that exhibit a Root effect generally exhibit a large Bohr coefficient, as described above. The Bohr effect is thought to have evolved three times independently in vertebrates, but only in one instance (in fish) did it evolve in conjunction with the Root effect (Berenbrink, 2007; Berenbrink et al., 2005). Therefore although the molecular basis is slightly different, it could be that in fish, the Root effect is an extension of a
unique, non-mammalian Bohr effect (Berenbrink, 2007; Berenbrink et al., 2005). Furthermore, because it may be impossible to tease apart the Root effect from the traditionally understood Bohr effect within a given blood system, the teleost combined Bohr-Root effect mechanism is regarded as inseparable throughout this dissertation.

1.2.5 The role of the combined Bohr-Root effect in oxygen delivery to specialized structures

Root-effect Hbs found in teleost fishes enhance O$_2$ delivery by eliciting a state of incomplete Hb saturation, even at atmospheric PO$_2$ levels (Figure 1.1) as well as a right-shift in the OEC (and increase in P$_{50}$ value) (Pelster and Weber, 1991; Root, 1931; Root and Irving, 1943). The general understanding is that Root effect Hbs augment the capacity for O$_2$ delivery to specialized tissues such as the eye (Figure 1.2A) or the swimbladder (Figure 1.2B), tissues that are organized with a dense, counter-current capillary network (choroid rete or rete mirabile) and an acid producing gas gland (Brittain, 1987; Fänge, 1953; Ingermann, 1982; Pelster and Weber, 1991; Scholander and Van Dam, 1954). The rete mirabile and gas gland at the swimbladder serve to localize, magnify, and recycle the acidosis that drives O$_2$ from the Hb. The effect of this localized acidosis on Hb-O$_2$ elevates blood PO$_2$ to such an extent that O$_2$ can be secreted to inflate the swimbladder, apparently against enormous partial pressure gradients (in excess of 50 atm between the normal blood gas tension and that in the swimbladder) and at substantial depths (Berenbrink et al., 2005; D'Aoust, 1970; D'Aoust, 1973; Fänge, 1953; Harden-Jones and Marshall, 1953; Lapennas and Schmidt-Nielsen, 1977; Pelster and Weber, 1991; Scholander, 1954; Wittenberg and Haedrich, 1974; Wittenberg and Wittenberg, 1974). The Bohr-Root effect can play an important role in inflating the swimbladder which contributes to permitting precise buoyancy regulation in fish, possibly one of the most important factors responsible for the extensive adaptive radiation of teleosts (Alexander, 1966).
For fishes dependent on visually sensing prey and predators, the Bohr-Root effect in conjunction with the choroid rete and choroid gland, similar structures to those found at the swimbladder, also permits O\(_2\) delivery to the metabolically active, yet poorly vascularized retinal tissue of the eye, thus enhancing visual acuity (Figure 1.2A) (Berenbrink, 2000; Berenbrink et al., 2005; Dafré and Wilhelm, 1989; Herbert et al., 2002; Wittenberg and Haedrich, 1974; Wittenberg and Wittenberg, 1974). In rainbow trout, for example, retinal function relies on ocular PO\(_2\) that is at least three times the PO\(_2\) of arterial blood (Fonner et al., 1973). To date, this phenomenon that greatly facilitates O\(_2\) delivery and is unique to teleosts has been understood only to enhance O\(_2\) delivery to the aforementioned two tissues, where a substantial acidosis can be created, localized, and recycled. Interestingly, the retia near the retina of the eye (Figure 1.2A) is the first known use and location of this enhanced O\(_2\) delivery system, but the choroid retia appeared 150 million years after the Root effect Hbs were thought to have evolved in teleost fishes (Berenbrink, 2007; Berenbrink et al., 2005).

The common element in both structures that benefit from enhanced O\(_2\) delivery via the Bohr-Root effect is a localized, recycled acidosis, which is not thought to occur in general circulation. In this thesis, I hypothesize that a mechanism is in place in the general circulation whereby an acidosis can be localized and recycled at the level of the RBC, thus permitting the Bohr-Root effect to enhance general O\(_2\) delivery.

1.2.6 Why is exploitation of the Root effect thought to be restricted to specialized tissues?

One of the reasons that Root effect Hbs have not been considered important to general O\(_2\) delivery is because the \(\Delta pH_{a-v}\) at the tissues is thought to be negligible. The Root effect is generally associated with a large Bohr effect. However, a large Bohr effect does not necessarily result in enhanced tissue O\(_2\) delivery because as Hb-O\(_2\) saturation changes, so does the affinity
and therefore binding capacity of Hb for H\(^+\) and CO\(_2\). This phenomenon is called the Haldane effect and refers to the number of H\(^+\) that bind to Hb per mole of O\(_2\) released (Christiansen et al., 1914). The Haldane effect is quantified by the Haldane coefficient (H), which is calculated using the following equation:

\[
H = \frac{\Delta Hb-H^+}{\Delta Hb-O_2}
\]

At the tissues, metabolic CO\(_2\) diffuses down its partial pressure gradient to the plasma and into the RBC, where in some vertebrates (such as humans) it binds directly to the terminal amines on the Hb as carbamate, stabilizing the T state and reducing Hb-O\(_2\) affinity (Klocke, 1973; Klocke, 1988). In some vertebrates, such as teleost fish, terminal amine groups on both Hb subunits are acetylated, making them unavailable to bind CO\(_2\) directly (Galdames-Portus et al., 1979). Therefore in teleosts, when metabolic CO\(_2\) enters the RBC, because carbamate is not formed, a greater proportion of the total CO\(_2\) entering the RBC is hydrated to form H\(^+\) that reversibly bind to Hb (Jensen, 2004; Klocke, 1988; Nikinmaa, 1990). Inside the RBC, the CO\(_2\) hydration reaction is catalyzed by carbonic anhydrase (CA), and represented as:

\[
CO_2 + H_2O \leftrightarrow HCO_3^- + H^+
\]

As CO\(_2\) and H\(^+\) bind to Hb and induce the Bohr effect to facilitate O\(_2\) delivery to the tissues, the resulting reduction in Hb-O\(_2\) saturation increases Hb-H\(^+\) affinity. This increased Hb-H\(^+\) affinity reduces the magnitude of the pH change, and thus exploitation of the Bohr effect, but enhances CO\(_2\) removal from the tissues (Bohr et al., 1904; Brauner et al., 1996; Christiansen et al., 1914; Eddy, 1971; Eddy, 1974; Jensen, 1989; Klocke, 1973; Lapennas, 1983; Maren and Swenson, 1980; Vorger, 1985). The reverse processes occur at the respiratory surfaces, where Hb oxygenation releases H\(^+\) (Haldane effect). Inside the RBC, the H\(^+\) combine with HCO\(_3^-\) at a CA-catalyzed rate to form CO\(_2\) that diffuses out to the environment. The associated increase in pH increases Hb-O\(_2\) affinity (Bohr effect), thereby facilitating O\(_2\) loading. Thus, there is a clear
interaction between $O_2$ and $CO_2$ transport at the tissues and the respiratory surface (Brauner et al., 1996; Brauner and Randall, 1996; Brauner et al., 2001). In fact, the Bohr and Haldane effects tend to counteract one another to some degree. This is the reason that a large Bohr effect usually associated with a Root effect is not thought to enhance $O_2$ delivery and is further elaborated upon below.

1.2.7 Optimal Bohr coefficient for tissue oxygen delivery

The Bohr and Haldane effects are thermodynamically-linked functions with reciprocal effects (Wyman, 1979). As described earlier, the Bohr effect describes changes in Hb-$O_2$ affinity due to changes in pH or $H^+$ concentration (Bohr et al., 1904); whereas the Haldane effect describes the change in Hb-$H^+$ binding in relation to changes in Hb-$O_2$ saturation (Christiansen et al., 1914). The Bohr and Haldane effects have, for this reason, been referred to as mirror images of the same phenomenon (Brauner, 1995; Brauner et al., 1996; Brauner and Randall, 1996), a linkage recognized in the classic equation:

$$(\log PO_2/pH)(Hb-O_2) = (H^+/(Hb-O_2))pH$$

by Wyman (1979), which concludes that the Bohr and Haldane coefficients are numerically equivalent. While it is beyond the scope of this introduction to explore this in more detail, the important point is that if the blood of an animal has a large Bohr coefficient, it will also have a large Haldane coefficient. Consequently, there has been considerable interest in determining whether there exists an optimal $\Phi$ for $O_2$ delivery (Lapennas, 1983).

Lapennas calculated that an optimal $\Phi$ for $O_2$ delivery was 50% of the respiratory quotient (RQ) (Lapennas, 1983). Under steady state conditions, vertebrate tissues produce about the same amount of $CO_2$ as $O_2$ that is consumed, a ratio called the RQ:
RQ = (moles of CO₂ eliminated) / (moles of O₂ consumed)

Assuming most animals have an RQ of between 0.7 and 1.0, the Φ would range from -0.35 to -0.5 (Bohr coefficients are negative because a decrease in pH right-shifts the OEC) (Figure 1.3) (Lapennas, 1983). As Φ values increased (as is observed with a Root effect Hb) or decreased from that value, the influence of the Bohr effect on O₂ delivery would be reduced (Figure 1.3) (Lapennas, 1983; Wyman, 1979). The optimal Φ (-0.35 to -0.5), as calculated by Lapennas, is very similar to Bohr coefficients determined for air breathing vertebrates (Lapennas, 1983).

Consequently, Lapennas concluded that the Bohr coefficients in air-breathing vertebrates are optimized for O₂ delivery (Lapennas, 1983). A large Φ that is equivalent to RQ (i.e. -1.0), which is often observed in teleosts possessing a combined Bohr-Root effect, would result in no benefit to O₂ delivery (Figure 1.3). This is because all H⁺ coming from CO₂ produced at the tissues (RQ = 1, and assume 1 mole of H⁺ per mole of O₂ consumed) would be bound by the Haldane effect (Φ and H of 1: 1 mole of H⁺ bind to Hb per 1 mole of O₂ released to the tissues), and there would be no resulting ΔpHₐ₋ᵥ during blood transit. Thus, although the pH sensitivity of this Hb is high, if there is no pH change, there is no benefit to tissue O₂ delivery. A Φ that is greater than RQ (which is often the case in Root effect Hbs) might result in a reverse ΔpHₐ₋ᵥ, actually hindering O₂ delivery (Figure 1.3) (Lapennas, 1983). Thus according to Lapennas’ analyses (1983), one might expect that the combined Bohr-Root effect may actually be detrimental to general tissue O₂ delivery.

Lapennas’ analyses (1983) assume steady-state conditions and that equilibrium conditions exist within the blood, which may not ever be achieved within the blood of fish. During non-steady state conditions such as environmental hypoxia or exercise, the ΔpHₐ₋ᵥ may be substantial, and a large Φ (>0.5 × RQ), as associated with the presence of a combined Bohr-Root effect in teleosts, may greatly facilitate O₂ delivery to the tissues. However, this would be
limited to a one-time phenomenon at the tissues. The adrenergic activation of red blood cell 
\( \text{Na}^+/\text{H}^+ \) exchange (\( \beta \text{NHE} \)) described below is an example of a mechanism whereby a large blood 
\( \Delta p\text{H}_{\text{a-v}} \) could occur at the tissues, thus permitting a Bohr-Root effect to be exploited for general 
\( \text{O}_2 \) delivery continuously during the period of activation. This idea is also explored extensively 
in Chapters 3 and 4 of this thesis.

1.2.8 Adrenergic red blood cell pH regulation during stress

In this thesis, I propose a novel mechanism whereby a large \( \Delta p\text{H}_{\text{a-v}} \) at the level of the 
RBC may occur under stressful conditions to exploit the highly pH-sensitive Bohr-Root effect 
for general \( \text{O}_2 \) delivery in teleosts. Root effect Hbs are so sensitive that even very small changes 
in blood pH can induce a very fast “Root off” effect in the blood of many fishes (Nikinmaa,
1990), with half-times in eels calculated at 44.8 ms (Pelster et al., 1992). Therefore, away from a 
retæ, where the acidosis is not localized to a specific tissue and rather results in a general blood 
acidosis, the combined Bohr-Root effect may only enhance \( \text{O}_2 \) delivery during one pass through 
the circulatory system (Figure 1.4). Following this, the resulting reduced affinity and \( \text{O}_2 \) 
carrying capacity of the blood would limit \( \text{O}_2 \) uptake at the gill, ultimately compromising 
subsequent \( \text{O}_2 \) transport and delivery (Figure 1.4).

During a generalized acidosis, such as hypoxia or stress, many teleosts release 
catecholamines, typically adrenaline (AD) and noradrenaline (NA), which stimulate \( \beta \)–
adrenergic activation of sodium (\( \text{Na}^+ \)), proton (\( \text{H}^+ \)) exchange (\( \beta \text{NHE} \)) on the RBC membrane 
(Figure 1.5) (Borgese et al., 1987; Jensen, 2004; Malapert et al., 1997; Nikinmaa and Huestis,
1984; Perry and Gilmour, 1996; Perry et al., 2004; Primmett et al., 1986; Salama and Nikinmaa,
1988). The purpose of the \( \beta \text{NHE} \) in this context is to protect RBC intracellular pH (pH\text{i}) and 
safeguard \( \text{O}_2 \) uptake at the gills (Baroin et al., 1984; Berenbrink and Bridges, 1994; Nandi, 1961;
Nikinmaa, 2003; Nikinmaa and Huestis, 1984; Nilsson et al., 1976). Activation of βNHE transports H⁺ (produced via the carbonic anhydrase (CA) catalyzed CO₂ hydration) out of the cell in exchange for Na⁺ (Figure 1.5). In conjunction with the H⁺, HCO₃⁻ is rapidly produced inside the cell via the CA-catalyzed reaction and is removed via anion exchange (AE) for Cl⁻, but at a slower rate (Figure 1.5) (Nikinmaa, 1990). This combination results in an intracellular alkalosis and an increase in Hb-O₂ affinity (Figure 1.5) (Baroin et al., 1984; Cossins and Richardson, 1985; Heming et al., 1987; Nikinmaa, 1982; Nikinmaa, 1983; Nikinmaa and Huestis, 1984; Nikinmaa and Jensen, 1986). The H⁺ removed from the RBC acidify the plasma, resulting in a decrease in extracellular pH (pHₑ) and at an uncatalyzed rate, H⁺ will combine with HCO₃⁻ to form CO₂, resulting in a slow plasma alkalinization after the initial pH decrease (Geers and Gros, 2000; Lessard et al., 1995) (Figure 1.5). Subsequent elevation of intracellular Na⁺ and Cl⁻ results in RBC swelling through the movement of osmotically-obliged water and activation of Na⁺, K⁺, ATPase (NKA) (Figure 1.5) (Borgese et al., 1987; Guizouarn et al., 1993a; Guizouarn et al., 1993; Nikinmaa et al., 1990). This β-adrenergic response at the RBC is one of the best-characterized non-mammalian adrenergic responses to date (Motais et al., 1992; Nikinmaa, 1982; Nikinmaa and Jensen, 1992; Nikinmaa and Salama, 1988; Nikinmaa et al., 1990) and is thought to have evolved in teleosts to safeguard O₂ uptake at the respiratory surfaces during a generalized acidosis in the presence of extremely pH-sensitive Bohr-Root shift Hbs (Borgese et al., 1987; Jensen, 2004; Malapert et al., 1997; Nikinmaa et al., 1984; Perry and Kinkead, 1989; Primmett et al., 1986; Salama and Nikinmaa, 1988).
1.2.9 Selectively short-circuiting red blood cell pH regulation during stress to enhance oxygen delivery

The presence of plasma-accessible CA within the circulatory system could potentially short-circuit RBC βNHE, transfer a blood acidosis into the RBC, and permit enhanced tissue O₂ delivery via the Bohr-Root effect (Figure 1.6), which is the central theme of this thesis. Short-circuiting the βNHE to acidify the RBC has been shown to occur in vitro with very high CA concentrations (Lessard et al., 1995; Motais et al., 1989; Nikinmaa, 1990; Wood and Munger, 1994). Teleost fish lack plasma-accessible CA at the gills so that O₂ uptake at the gills is secured during a generalized acidosis when RBC βNHE has been activated. Furthermore, it is unlikely that fish possess free CA in general circulation, as it is a small protein (29kDa) that would be filtered by the glomerulus and lost in the urine (Henry and Swenson, 2000). Several CA isoforms are membrane-bound via a phosphatidylinositolglycan (GPI) anchor and potentially plasma-accessible in select locations away from the gill, such as muscle endothelia (Decker et al., 1996; Effros and Weissman, 1979; Geers and Gros, 2000; Gilmour et al., 1997; Henry et al., 1997; Henry and Swenson, 2000; Sender et al., 1994; Siffert and Gros, 1982; Wang et al., 1998; Yamamoto et al., 2003). Effros and Weissman (1979) suggest that small amounts of plasma-accessible CA exist but appear localized in specific areas, particularly the endothelium, where they may be membrane-bound but oriented toward the extracellular fluid (Wang et al., 1998). Fish red muscle (RM) is of particular interest regarding the potential for plasma-accessible CA, as it is a key site for O₂ demand and the primary location of CO₂ (and therefore acid) production during exercise. Selectively located CA on extracellular surfaces of capillary membranes could catalyze the H⁺ removed via the βNHE and the HCO₃⁻ removed via the anion exchange to form CO₂, which could back-diffuse into and acidify the RBC, elevating PO₂ (Figure 1.6). Thus, short-circuiting RBC βNHE in acidified blood could create a marked increase in ΔpHₐᵥ during
capillary transit (Figure 1.6), which, in conjunction with a large $\Phi$ ($\geq 1.0$) and combined Bohr-Root effect, may greatly enhance $O_2$ delivery to those particular tissues during a stressful situation. This mechanism outlined above forms the basis for the experiments comprising Chapters 3 and 4 of this thesis, where the mechanism was validated and extensively characterized in vitro (Chapter 3) and through the use of isoform-specific CA inhibitors, also characterized in vivo (Chapter 4).

1.3 This Thesis

The combined Bohr-Root effect is a fascinating adaptation, a phenomenon that influences the respiratory system of over half of all extant vertebrate species, but whether it can facilitate general $O_2$ delivery to areas other than the swimbladder and eye has remained unclear for decades. During my Ph.D. thesis research, I proposed and validated a mechanism whereby CA short-circuits RBC $\beta$NHE (Figure 1.6), which elicits a $\Delta pH_{a-v}$, thus permitting enhanced $O_2$ delivery via the Bohr-Root effect system. I validated this mechanism through both in vitro and in vivo experiments, while utilizing traditional methods in comparative physiology. In order to test the hypothesis that the combined Bohr-Root effect unique to teleost fishes facilitates general $O_2$ delivery, I performed experiments addressing three main questions:

1. To what degree do blood pH changes in a teleost possessing a unique Bohr-Root effect system increase the potential for enhancing $O_2$ delivery? How does this compare with that observed in a vertebrate possessing a Bohr effect alone?

   In Chapter 2, I performed experiments to determine the degree to which the combined Bohr-Root effect system could increase the driving force for $O_2$ delivery for a range of pH changes in the blood. This type of determination has been and is routinely conducted for Bohr
effect-alone systems (e.g. humans) (Roughton and Severinghaus, 1973) but has not been previously conducted in an organism with a non-linear, combined Bohr-Root effect system. The potential benefit to O₂ delivery was quantified by determining the magnitude of the right-shift in the OEC, reported as ΔPO₂ (mm Hg) at constant Hb-O₂ saturations, for a given ΔpH. I predicted that the ΔPO₂ in a teleost would far exceed that of a Bohr effect-alone system. Complete OECs were generated for rainbow trout blood over a broad physiological pH range that might be encountered in vivo. The ΔPO₂ for each simulated ΔpHᵢ₋ᵥ was quantified and compared to a theoretical model developed for an air-breathing vertebrate possessing a Bohr effect alone (e.g. human). A supplemental objective was to investigate the effects of different blood sampling and preparation techniques and analytical protocols (tonometry and a new commercially available system (P wee 50)) for generating OECs. Validating simpler, more efficient protocols, where a smaller blood volume can be used, and/or potentially stored overnight opens opportunities to study other teleost species that could not have been otherwise investigated due to, for example, small size or protected status, but may be key to gaining further insight into the evolutionary basis for this complex biological system involving Root effect Hbs.

2. Does short-circuiting of red blood cell βNHE with carbonic anhydrase in vitro in a closed system elevate PO₂, and could this have implications toward enhancing O₂ delivery in vivo?

In Chapter 3, I performed experiments to test the hypothesis that during an acidosis, adrenergic RBC pHᵢ regulation via the βNHE can be short-circuited in the presence of plasma-accessible CA. This short-circuiting mechanism would be expected to reduce Hb-O₂ affinity, elevate PO₂, and increase the driving force for O₂ delivery (ΔPO₂). As a proof of principle, an in vitro closed-system was created to test this mechanism in rainbow trout blood pre-equilibrated to
pre-defined Hb-O\textsubscript{2} saturations. This blood was sequentially subjected to an acidosis (of different magnitudes), followed by β-adrenergic stimulation, and then CA addition. Throughout this sequence, changes in both pH and PO\textsubscript{2} in this closed-system were monitored in real-time using fiber-optic sensors to assess both the magnitude and the time course of the response. The ultimate goal was to gain insight into whether βNHE short-circuiting could be operational \textit{in vivo} and estimate the degree to which it might influence O\textsubscript{2} delivery.

3. Is carbonic anhydrase-mediated red blood cell βNHE short-circuiting operational \textit{in vivo} in rainbow trout, such that elevations in PO\textsubscript{2} can be observed at the tissue level during a generalized blood acidosis and abolished when plasma-accessible CA is inhibited?

In Chapter 4, I performed experiments to test the hypothesis that during a generalized acidosis, plasma-accessible CA short-circuits the adrenergically-activated RBC βNHE to generate the large ΔpH\textsubscript{a-v} that may enhance red muscle O\textsubscript{2} delivery via the combined Bohr-Root effect. The resulting increase in the O\textsubscript{2} partial pressure gradient (ΔPO\textsubscript{2}) is presumed to increase the driving force for O\textsubscript{2} delivery. To determine this, arterial PO\textsubscript{2} (P\textsubscript{a}O\textsubscript{2}) and RMPO\textsubscript{2} were monitored in real-time in resting rainbow trout, prior to and following the development of a mild acidosis associated with CO\textsubscript{2} exposure, when catecholamines may be released into circulation. To address the role of plasma-accessible CA to tissue oxygenation, a new, potent, membrane-impermeant CA inhibitor (compound-18, C18) was characterized and used to selectively inhibit plasma-accessible CA. According to the hypothesis, in the absence of the inhibitor, there would be an increase in RMPO\textsubscript{2} associated with RBC βNHE short-circuiting of acidified blood, but this ΔPO\textsubscript{2} would be abolished in the presence of C18. A secondary objective in this chapter was to test the effectiveness of C18 in comparison to CA inhibitors that have been commonly used in rainbow trout. Ultimately, the aim of Chapter 4 was to validate, \textit{in vivo}, the mechanisms that
were effectively demonstrated to increase the $\Delta$PO$_2$ in rainbow trout blood \textit{in vitro} in Chapter 3 and draw conclusions as to the potential for enhanced O$_2$ delivery to the red muscle.

Chapter 5 of this thesis is a general discussion, where the ideas generated from the aforementioned chapters are summarized and conclusions drawn, all placed within the broader context of respiratory physiology and the evolution of this complex biological system involving the Root effect Hb in teleost fishes, as well as O$_2$ delivery in vertebrates in general.
1.4 Figures

**Figure 1.1** A theoretical oxygen equilibrium curve (OEC) depicting the relationship between haemoglobin-oxygen saturation (%Hb-O₂) and blood partial pressure for O₂ (PO₂) at a routine pH value (black line), and at a reduced pH, resulting in either a Bohr effect (blue dashed line) right-shift, or a combined Bohr-Root effect (red dashed line) rightward and downward shift. Thin black drop lines depict P₅₀ values, the PO₂ at which 50% of Hb is saturated with O₂, for each of the three OECs.
Figure 1.2 A schematic illustrating the arrangement of the dense counter-current capillary network, the choroid rete, and acid-producing choroid gland at the teleost fish eye (Panel A) and the dense counter-current capillary network, rete mirabile, and acid-producing gas gland of a physoclistous swimbladder in a teleost fish (Panel B).
Figure 1.3 A graph depicting the optimal Bohr coefficient (Φ) for O₂ delivery, as per Lapennas’ steady state analyses (Lapennas, 1983). The y-axis represents the qualitative (numbers suppressed) change in P₅₀ (ΔLogP₅₀) predicted to occur during arterial-venous blood transit over a range of Bohr-Haldane coefficients (x-axis) at two respiratory quotients (RQ; 0.7 or 1.0). The ΔLog P₅₀ represents the potential for enhanced oxygen delivery associated with calculated arterial-venous pH changes in the blood at 50% Hb-O₂ saturation. The point at which each curve is the highest represents the optimal Bohr coefficient (A) and (B) for each RQ, respectively.
Figure 1.4 A schematic illustrating general circulation in a teleost fish, where Hb is oxygenated (Hb-O₂) at the gill and with a high affinity for O₂, travels through the arterial system to the respiring tissues, where CO₂ is released, and the protons (H⁺) acidify the red blood cell, thus releasing O₂ from the Hb. The O₂ is delivered to the tissue, and H⁺ bind to Hb (Hb-H⁺). Blood travels through the venous system through the heart to return to the gill where CO₂ is excreted. During a general acidosis, the blood could return to the gill, with a low affinity Hb and a limited ability to bind O₂ due to the combined Bohr-Root effect.
During a stressful situation, catecholamine release in fish activates the sodium and proton (Na\(^+\)/H\(^+\)) exchanger (\(\beta\)NHE) on the RBC membrane through a G-protein activated cascade including adenylate cyclase and 3’, 5’- cyclic monophosphate (cAMP). The \(\beta\)NHE removes H\(^+\) (produced via the carbonic anhydrase (CA) catalyzed CO\(_2\) hydration) from the cell, for Na\(^+\). In conjunction with the H\(^+\), bicarbonate (HCO\(_3^-\)) is rapidly produced inside the cell via the CA-catalyzed reaction and is removed via anion exchange (AE) for Cl\(^-\). This results in an intracellular alkalosis and an increase in Hb-O\(_2\) affinity. The H\(^+\) removed from the RBC acidify the plasma, resulting in a decrease in extracellular pH (pH\(_e\)), and at an uncatalyzed rate, H\(^+\) will combine with HCO\(_3^-\) to form CO\(_2\), resulting in a slow plasma alkalinization after the initial pH decrease. Subsequent elevation of intracellular Na\(^+\) and Cl\(^-\) results in RBC swelling through the movement of osmotically-obliged water and activation of Na\(^+\), K\(^+\), ATPase (NKA).
Figure 1.6 A schematic illustrating the activation and short-circuiting of the βNHE upon exposure to carbonic anhydrase (CA) that is bound (anchored) to endothelia cells and plasma-accessible. The βNHE is short-circuited when the plasma-accessible CA catalyzes the H⁺ removed via the βNHE, along with bicarbonate (HCO₃⁻) removed via anion exchange (AE), to form CO₂ that back-diffuses into the RBC. The RBC is acidified when CO₂ is hydrated with CA inside the RBC to form H⁺ that displaces O₂ from the Hb enhancing O₂ delivery.
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CHAPTER 2: THE INFLUENCE OF pH ON HAEMOGLOBIN-OXYGEN BINDING: A COMPREHENSIVE INVESTIGATION OF THE BOHR-ROOT EFFECT SYSTEM IN RAINBOW TROUT, Oncorhynchus mykiss

2.1 Introduction

The affinity of Hb for O₂ is reduced when blood pH decreases, a phenomenon termed the Bohr effect. For over a century, the Bohr effect has been studied to understand the degree to which CO₂ released from the tissues may increase the partial pressure gradient for O₂ (PO₂) and thus facilitate O₂ delivery (Bohr et al., 1904; Nikinmaa, 1997; Nikinmaa and Soivio, 1979). At the tissues, the potential benefit for O₂ delivery associated with the Bohr effect at the P₅₀ of blood (PO₂ at which Hb is 50% saturated) can be calculated as the product of the arterial-venous pH difference (ΔpHₐ-ᵥ) and the Bohr coefficient (Φ = Δlog P₅₀/ΔpH), reported as a ΔPO₂ (Bohr et al., 1904; Brauner et al., 2001; Jensen, 1989; Lapennas, 1983; Nikinmaa, 1993; Nikinmaa, 1997; Nikinmaa, 2001; Nikinmaa and Soivio, 1979). By assuming a constant Bohr coefficient over the oxygen equilibrium curve (OEC) and knowing the resting P₅₀ value, a ΔPO₂ can be calculated as a function of ΔpHₐ-ᵥ. By definition, this can only be calculated at P₅₀ to indicate the potential benefit for O₂ delivery associated with the Bohr effect. For example, a human with a Bohr coefficient of -0.35 and ΔpHₐ-ᵥ of 0.035, which is probably the largest change that can occur in vivo (Rang et al., 2002), will therefore exhibit a ΔPO₂ of less than 1 mm Hg. Tissue PO₂ values in mammals have been measured at 40 mm Hg (Behnke et al., 2001; Hutter et al., 1999; Jung et al., 1999; Suttner et al., 2002), and so a ΔPO₂ of 1 mm Hg would represent a modest benefit

¹A version of this chapter has been prepared to submit for publication. Rummer, J.L. and Brauner, C.J. The influence of pH on haemoglobin-oxygen binding: A comprehensive investigation of the Bohr-Root effect system in rainbow trout, Oncorhynchus mykiss.
(~2.5%) to the driving force for O$_2$ delivery. In this way, any vertebrate Hb that exhibits a Bohr shift can benefit in terms of O$_2$ delivery by the release of acid equivalents in the tissue capillaries.

Among vertebrates, teleost fish possess unique Hbs, whereby an acidosis not only greatly decreases the affinity of Hb for O$_2$ but also reduces the blood O$_2$ carrying capacity, a phenomenon termed the Root effect (Pelster and Weber, 1991; Root, 1931; Root and Irving, 1943). The Root effect is generally discussed in terms of its role in enhancing the capacity for O$_2$ delivery to the eye and swimbladder of fishes. These tissues are equipped with retia, dense capillary networks that can serve to localize and magnify an acidosis, which in conjunction with the Root effect greatly elevates arterial PO$_2$. In the eye, the high arterial PO$_2$ serves to overcome great diffusion distances to oxygenate the metabolically active, yet poorly vascularized retinal tissue (Dafré and Wilhelm, 1989; Nikinmaa and Huestis, 1984; Wittenberg and Wittenberg, 1974). In the swimbladder, the high arterial PO$_2$ serves to fill the swimbladder apparently against large pressure gradients (>50 atm) associated with depth (Alexander, 1966).

The Root effect has long been referred to as an exaggerated Bohr effect (Brittain, 1987). The Bohr effect evolved three times independently in vertebrates but in only one of these instances in conjunction with what is now known as the Root effect in fishes (Berenbrink, 2007; Berenbrink et al., 2005). Structure-function analyses have revealed distinct molecular differences between the Root effect in fishes and the Bohr effect observed in mammals, indicating that the Root effect is not simply an exaggerated Bohr effect as once thought (Berenbrink, 2007; Berenbrink et al., 2005). Instead, the Root effect may represent an extension of a unique, non-mammalian Bohr effect. Regardless, within a given Hb system, the Bohr and Root mechanisms are functionally inseparable and are referred to as a Bohr-Root effect system in this study. The decrease in O$_2$ carrying capacity produced by the combined Bohr-Root effect also decreases Hb affinity, as reflected in the increase in P$_{50}$. This change in P$_{50}$ for a given pH
change in teleost blood that possesses a combined Bohr-Root effect is much greater than what occurs in air-breathing vertebrates with a Bohr effect alone. Consequently, it is not surprising that many researchers have considered the potential for this system to enhance O₂ delivery to tissues in general, beyond the swimbladder and eye (Brauner and Randall, 1998; McKenzie et al., 2004; Nikinmaa, 1997).

Enhanced O₂ delivery via the combined Bohr-Root effect is therefore qualitatively intuitive, but this hypothesis lacks quantitative support for two main reasons. First, teleosts, unlike most air-breathing organisms, exhibit a non-linear Bohr effect, a disproportionate binding and release of oxylabile protons (H⁺) over the OEC with the greatest Bohr shift existing between 60 and 100% Hb-O₂ saturation (Brauner et al., 1996; Brauner et al., 2001; Jensen, 1986). This violates the assumption used to calculate ΔPO₂ using only P₅₀. Second, it is well understood that Root effect Hbs, depending on the pH change, may not reach anywhere near 100% saturation, even at O₂ tensions that are orders of magnitude greater than atmospheric PO₂. Therefore ΔPO₂ does not apply near full saturation. Since ΔPO₂ cannot simply be calculated as described above because the effects are not uniform across the OEC, ΔPO₂ must be interpolated directly from OECs generated at relevant pH and PO₂ values. Indeed, to assess the benefit to O₂ delivery associated with tissue release of acid equivalents in teleosts possessing this unique Bohr-Root effect system, complete OECs generated for intact red blood cells (RBCs) are required over a range of pH values encountered in vivo. Surprisingly, such a data set does not currently exist.

I chose to investigate the Bohr-Root effect system in rainbow trout, *Oncorhynchus mykiss*, a species that is one of the most comprehensively investigated teleosts to date, which would allow me to make comparisons to the various data sets that exist from previous studies. My objectives were as follows: i) generate complete OECs over a predetermined pH range and calculate ΔPO₂ that would be associated with the right-shift in the OEC for each pH change over the entire range of Hb-O₂ saturations for rainbow trout blood, ii) compare this with the ΔPO₂
calculated from models developed for a vertebrate possessing a Bohr effect alone, iii) model the degree to which the Bohr-Root effect system could enhance O$_2$ delivery potential, relative to the Bohr effect alone, for a given expected ΔpH$_{a-v}$ over the entire range of the OEC, and iv) validate three independent protocols for generating OECs. By comparing the non-linear Bohr-Root effect that exists in a teleost with the linear Bohr effect, a framework can be built and hypotheses generated revolving around the idea that this unique Bohr-Root effect system – which evolved approximately 400 million years ago in teleosts, and 150 million years before evidence of the choroid rete at the eye (Berenbrink et al., 2005), the first known structure to benefit from the mechanism – may have first evolved to enhance general O$_2$ delivery and later for the specialized structures (retia) of the eye and swimbladder.

2.2 Materials and Methods

2.2.1 Series1: The influence of pH on the oxygen equilibrium curves of rinsed red blood cells

2.2.1.1 Experimental animals and holding conditions

Rainbow trout, (O. mykiss, 300-600 g wet body mass), were obtained from Spring Valley Trout Farm (Langley, British Columbia, Canada). Fish were maintained in the Department of Zoology at the University of British Columbia in 4,000 l tanks supplied with flow-through Vancouver dechlorinated municipal tap water (average 12°C) under a natural photoperiod. Fish were fed every other day to satiation using commercial trout pellets (Skretting, Orient 4-0). Food was withheld 24 h prior to experimentation. All protocols comply with the guidelines approved by the Canadian Council on Animal Care, protocol # A07-0080.
2.2.1.2 Caudal puncture sampling protocol

Fish were quickly removed from holding tanks and placed into a 20 l bucket of clean, well-aerated water containing benzocaine (0.2 mM final concentration, p-aminobenzoate, Sigma-Aldrich cat. no. E1501; St. Louis, MO, USA), to anaesthetize fish. Blood was drawn from the caudal vein and collected in heparinized syringes, and RBCs were rinsed twice and resuspended in ice-cold Cortland's saline (Wolf, 1963) according to Caldwell et al. (2006). Haematocrit (Hct) of the rinsed RBCs was measured in duplicate by centrifuging 60 µl whole blood in heparinized micro-capillary tubes for 3 min at 12,000 rpm. The Hct was standardized to 25% by removing either saline or RBCs, and blood was stored at 4°C overnight until experiments commenced, ensuring that any catecholamines present within the sample had degraded (Bourne and Cossins, 1982; Randall and Perry, 1992; Tetens et al., 1988).

2.2.1.3 Oxygen equilibrium curves derived from tonometry using rinsed red blood cells

The Hct of rinsed RBCs stored at 4°C overnight was readjusted to 25% as needed. Then 2.5 ml was added to each of four Eschweiler tonometers, which were incubated at 12°C, and equilibrated for one hour with a humidified gas mixture to one of the following CO₂ proportions: 0.25, 0.5, 1, 2, or 4% balanced with air (21% O₂). To generate an OEC at each of the above CO₂ proportions (n=8), each tonometer was subjected to a step-wise decrease in O₂ proportions (21, 20, 19, 13, 9, 6.5, 4, 2.5, 1.5, or 0%) by increasing the proportion of N₂ using a DIGAMIX Wösthoff gas-mixing pump (DIGAMIX 275 6KM 422 Wösthoff, Bochum, Germany). Following a 20-min incubation period at each O₂ tension, a 50 µl aliquot of rinsed RBCs was withdrawn into a pre-gassed Eppendorf™ tube or Hamilton™ syringe for measurement of total O₂ content (TO₂), haemoglobin concentration ([Hb]), Hct, extracellular pH (pHₑ), and intracellular pH (pHᵢ). TO₂ was measured according to Tucker (1967), [Hb] (mM per tetramer)
was measured after adding rinsed RBCs to Drabkin’s solution (Sigma-Aldrich cat. no. D5941; St. Louis, MO, USA) and measuring absorbance at 540 nm and applying a millimolar extinction coefficient of 11. The freeze-thaw technique (Zeidler and Kim, 1977) was used to measure pH, where both pH_e and pH_i were measured using a BMS 3 Mk2 Blood Microsystem in conjunction with a PHM 84 meter (Radiometer, Copenhagen). All assays were performed in triplicate.

2.2.1.4 Calculations and statistical analyses

Data are presented as mean ± S.E.M. Mean corpuscular haemoglobin concentration (MCHC) was calculated as Hb/(Hct/100). Haemoglobin saturation (SO_2) was calculated as a percentage by dividing TO_2 (after subtracting physically dissolved O_2 according to Boutilier et al. (1984) and dividing this by the theoretical maximum carrying capacity of the rinsed RBCs based upon the tetrameric Hb concentration obtained spectrophotometrically according to Tucker (1967). The SO_2 values were plotted as a function of incubation PO_2 (mm Hg) for each CO_2 proportion (0.25, 0.5, 1.0, 2.0, and 4.0%), and an Exponential Rise to Maximum, Simple Exponent curve was fit to the data using the Dynamic Fit Wizard function in SigmaPlot for Windows 10.0.1.25 (Systat Software Inc., 2006) to generate the OECs. The P_{50} and n_H were interpolated from log [SO_2/(1 – SO_2)] vs. log PO_2 plots (20-80% saturation). Bohr coefficients (Φ) were calculated as ΔlogP_{50}/ΔpH_e for pH values corresponding to each CO_2 incubation condition relative to 0.25% CO_2. Data were compared statistically between CO_2 treatments, and statistical differences were detected via ANOVA and, when necessary, a post-hoc Holm-Sidak multiple comparisons test. All statistical analyses were conducted using SigmaStat for Windows 3.5.0.54 (Systat Software, Inc., 2006), using α < 0.05 to determine statistical significance.
2.2.2 Series 2: Modeling oxygen delivery potential in a combined Bohr-Root effect system (rainbow trout) and a system with a Bohr effect alone (human).

**2.2.2.1 Human model with a Bohr effect alone.**

Oxygen delivery potential ($\Delta P_{O_2}$) was calculated by rearranging the equation for the Bohr coefficient, $\Phi = \Delta \log P_{50}/\Delta p\text{H}$, using a range of pH changes that might occur in the circulation. A $\Delta p\text{H}$ of 0.1, 0.2, 0.55 and 1.0 pH units were chosen to be consistent with those values determined for rainbow trout below. Values published for human blood, starting/resting $P_{50}$ of 27 mm Hg at pH = 7.4, as per (Wells and Weber, 1989), were used for the human model, the air-breathing vertebrate for which the most information is available. The Bohr coefficient used was $\Phi = -0.35$, according to the optimal Bohr coefficient, as determined by (Lapennas, 1983), and was assumed constant at all pH values between 20 and 80% SO$_2$. It was assumed that Hb always reached 100% saturation at atmospheric O$_2$ tensions. Therefore, the $\Delta P_{O_2}$ at 0 and 100% SO$_2$ will always equal zero.

**2.2.2.2 Rainbow trout model with a combined Bohr-Root effect**

Rainbow trout blood $\Delta P_{O_2}$ values were obtained by direct interpolation from the Bohr-Root effect system OECs generated in Series 1. The SO$_2$ values from 0 to 100% for a $\Delta p\text{H}_e$ of 0.1 (by comparing the 0.25 and 0.5% CO$_2$ OECs), $\Delta p\text{H}_e$ of 0.2 (by comparing the 0.25 and 1% OECs), $\Delta p\text{H}_e$ of 0.5 (by comparing the 0.25 and 2% CO$_2$ OECs), and $\Delta p\text{H}_e$ of 1.0 (by comparing 0.25 and 4% CO$_2$ OECs) were used. For comparative purposes, the same four pH shifts ($\Delta p\text{H}$) simulated in rainbow trout blood were used for the human blood calculations.
2.2.3 Series 3: Comparison of tonometry and $P_{\text{wee}}$ system methods

2.2.3.1 Experimental animals and sampling protocol

All fish used in this series were obtained and maintained as outlined in Series 2. Fish were anaesthetized as described above, placed on a surgical table, gills intubated and continuously irrigated with water containing anaesthetic (0.2 mM final concentration, p-aminobenzoate), and an indwelling cannula (PE50) was surgically implanted into the dorsal aorta (DA) according to Soivio et al. (1980). Following surgery, fish were gently force-ventilated to recover from anaesthetic. When involuntary ventilation commenced, fish were left to recover in a Perspex box supplied with aerated 12°C clean water for 48 h prior to sampling. Cannulae were flushed twice daily with heparinized (10 IU ml$^{-1}$ ammonium heparin, Sigma-Aldrich cat. no. H0878; St. Louis, MO, USA) Cortland’s saline (Wolf, 1963). Prior to experimentation, blood was withdrawn through the DA cannula into a heparinized syringe, but at the first sign of struggling, no further blood was removed to ensure resting plasma catecholamine levels.

2.2.3.2 Haemoglobin-oxygen saturation in whole blood using tonometry

As described above for the rinsed RBCs described in Series 1, whole blood drawn from the DA of 2-3 fish was pooled, adjusted to 25% haematocrit, and divided equally between four tonometers. The samples were incubated and analyzed at 13% $O_2$ (96 mm Hg) at each $CO_2$ (0.25, 0.5, 1, 2, and 4%, n=4 for each), balance $N_2$. The $SO_2$ values generated on whole blood were compared with those values obtained at the same $O_2$ in Series 1 to validate using rinsed RBCs to generate OECs for this and future studies.
2.2.3.3 Oxygen equilibrium curves using a microdiffusion chamber and spectrophotometer (P\textsubscript{wee}-50 system)

A modified version of the microdiffusion chamber and spectrophotometer (P\textsubscript{wee}-50, La Trobe University, Bundoora, Australia, abbreviated P\textsubscript{wee}) described by Clark et al. (2008) and utilizing the protocol from Clark et al. (2010) was used as the third independent method for determining the relationship between pH and Hb-O\textsubscript{2} saturation. Briefly, a 1 \textmu l sample of whole blood, drawn from the DA of cannulated fish was deposited between two 6-\textmu m Teflon membranes secured with a neoprene O-ring on a stainless steel holding apparatus. The apparatus was placed into an airtight chamber in a temperature-controlled room so that analyses could be performed at 12°C, consistent with the tonometry experiments. In the apparatus, the blood sample was positioned directly above the light-emitting diodes (LEDs), which switched between 435 nm (approximate peak absorption for deoxy-Hb) and 390 nm (the approximate isosbestic point between oxy- and deoxy-Hb). Humidified gas mixtures were introduced into the sample chamber at a flow rate of 50 ml min\textsuperscript{-1} using a Corning 192 Precision Gas Mixer (Corning Medical and Scientific, Medfield, Massachusetts, USA). Each sample took approximately 10 min to equilibrate at each gas tension, and the difference in absorbance at the two wavelengths were used to calculate SO\textsubscript{2} at each O\textsubscript{2} tension. Three OECs were constructed using the P\textsubscript{wee} at each of the five CO\textsubscript{2} tensions (generated from 0.25, 0.5, 1, 2, and 4% CO\textsubscript{2} gas mixtures, n=3 for each), using stepwise increments in O\textsubscript{2}, balance N\textsubscript{2}. The pH\textsubscript{e} values (required for calculation of the Bohr coefficient) were measured after incubating blood samples in tonometers at each experimental CO\textsubscript{2} tension, balance air, as conducted in Series 1. To account for the Root effect, 100% SO\textsubscript{2} was defined as the peak absorbance value obtained when the blood sample was exposed to a CO\textsubscript{2}-free gas mixture (21% O\textsubscript{2} and balance N\textsubscript{2}).
2.2.3.4 Calculations and statistical analyses

Data obtained from tonometry of whole blood are presented as mean ±S.E.M. The SO$_2$ values were calculated as described above and were plotted as a function of incubation PO$_2$ (mm Hg) for each proportion of CO$_2$ (0.25, 0.5, 1.0, 2.0, and 4.0%). For data derived from the P$_{wee}$ system, curves were fit to raw data and 95% confidence intervals were plotted with best-fit regression curves. The SO$_2$ values calculated at ~96 mm Hg for each CO$_2$ tension were compared statistically between all three methods – tonometry of rinsed RBCs (Series 1), tonometry of whole blood (Series 3), and P$_{wee}$ system using whole blood (Series 3) – with ANOVA and when necessary, a post-hoc Holm-Sidak multiple comparisons test. The P$_{50}$ and n$_H$ were interpolated from P$_{wee}$ data, and the Bohr coefficients (Φ) were calculated as described in Series 1. Data were compared statistically between CO$_2$ treatments and between techniques, and statistical differences were detected via ANOVA and, when necessary, a post-hoc Holm-Sidak multiple comparisons test. All statistical analyses were conducted using SigmaStat for Windows 3.5.0.54 (Systat Software, Inc., 2006), using α < 0.05 to determine statistical significance.

2.3 Results

2.3.1 Series 1: The influence of pH on the oxygen equilibrium curves of rinsed red blood cells

Rinsed RBCs incubated under progressively higher CO$_2$ tensions (generated from 0.25 to 4% gas mixtures) significantly reduced both pH$_i$ and pH$_e$ at each CO$_2$ tension (P<0.001) (Table 2.1). Changes in pH$_i$ were significantly and linearly correlated with changes in pH$_e$ (pH$_i$ = 2.747 + (0.574 * pH$_e$), R$^2$ = 0.930), similar to previous determinations with whole blood *in vitro* (pH$_i$ = 2.708 + (0.595 * pH$_e$), R$^2$ = 0.950) (Boutilier et al., 1986). The standardized Hct of 25% prior to each CO$_2$ incubation remained stable throughout each experiment (Table 2.1), except the
higher CO₂ incubation conditions (1, 2, and 4%) statistically increased Hct compared with 0.25 and 0.5% CO₂. Mean Hb-O₂ saturation at each incubation PO₂ significantly decreased with each increase in CO₂ tension, resulting in the characteristic rightward (Bohr effect) and downward (Root effect) shifts in the OEC (Figure 2.1). The exceptions were points generated from samples incubated at 0.25, 0.5, and 1% CO₂ for a PO₂ of 156 to 158 mm Hg, which were not statistically different from each other. This pattern was evident again when those samples were incubated at a PO₂ of 47 to 49 mm Hg. However, at air-saturated oxygen tensions (~160 mm Hg), the Root effect alone resulted in a reduction in Hb-O₂ saturation from 95 and 96% at 0.25 and 0.5% CO₂, to 89% at 1%, 75% at 2% and 47% at 4% CO₂ (Figure 2.1).

The P₅₀ values calculated from Hill plots for samples incubated at each CO₂ tension were approximately 25, 38, 41, 75, and >160 mm Hg (generated from 0.25 to 0.5, 1, 2, and 4% CO₂ mixtures, respectively), each significantly different from one another (<0.001) except for 0.5 and 1% CO₂ samples (P=0.05) (Table 2.1, Figure 2.2A). Statistical analyses did not include the >160 mm Hg P₅₀ from samples, as determination of a nominal value was beyond the scope of this study. Low cooperativity in Hb-O₂ binding was exhibited, as indicated by low nₜ for each OEC (1.34, 1.48, 1.37, 1.03, and 0.55 at 0.25, 0.5, 1, 2, and 4% CO₂, respectively). The nₜ using 2 and 4% CO₂ were the only significantly different values (Table 2.1, Figure 2.2A). The calculated Bohr coefficients were -0.91, -0.67, -0.87, and -0.77 based upon the change in P₅₀ values from 25 mm Hg. (Table 2.1).

2.3.2 Series 2: Modeling oxygen delivery potential in a combined Bohr-Root effect system (rainbow trout) and a system with a Bohr effect alone (human).

For human blood and using a starting P₅₀ of 27 mm Hg and Bohr coefficient of Φ = -0.35, the ΔPO₂ values determined for ΔpH values of -0.2, -0.3, -0.55, and -1.0 were 4.0, 6.3, 12.8, and
28.5 mm Hg, respectively. The $\Delta$PO$_2$ for each $\Delta$pH, was assumed constant between 20 and 80% Hb-O$_2$ saturation and are plotted in Figure 2.3A.

For rainbow trout blood, the $\Delta$PO$_2$ values directly interpolated from the OECs for $\Delta$pH values of -0.2, -0.3, -0.55, and -1.0 are shown in Figure 2.3B. These values range from 14.6 to 295.1 at 50% Hb-O$_2$ saturation (Figure 2.3B).

The $\Delta$PO$_2$ for human and rainbow trout determined at a similar $\Delta$pH value are shown in Figure 2.3C. The $\Delta$PO$_2$ for each $\Delta$pH was at least 2.5-times greater in rainbow trout blood (rinsed RBCs) than human blood at 40% Hb-O$_2$ and higher. For Hb-O$_2$ saturations of 80% and higher, and at $\Delta$pH close to that expected in vivo, the $\Delta$PO2 for rainbow trout was, at minimum, 8-fold that of the human $\Delta$PO$_2$ (Figure 2.3C). At larger $\Delta$pH values and Hb-O$_2$ saturations, the $\Delta$PO$_2$ for rainbow trout is orders of magnitude greater than that of the human (Figure 2.3C).

### 2.3.3 Series 3: Comparison of tonometry and P$_{wee}$ system methods

Tonometry of whole blood at a PO$_2$ of ~96 mm Hg, yielded SO$_2$ values of 93.5 ±2.9, 85.5 ±1.0, 66.5 ±3.9, 49.0 ±5.7, and 40.0 ±4.8% at 0.5, 1, 2, and 4% CO$_2$, respectively (Figure 2.4). Except for the SO$_2$ for blood incubated at 1% CO$_2$, these values were not significantly different than SO$_2$ values at the same PO$_2$s determined using tonometry of rinsed RBCs (Series 1, Figure 2.1) and fell within the 95% confidence intervals for whole blood measurements using the P$_{wee}$ system (Figure 2.4).

Five OECs were generated using the P$_{wee}$ system for whole blood. At air-saturated oxygen tensions (~160 mm Hg), the Root effect reduced Hb-O$_2$ saturation from 104% to 97, 87, 66, and 49% as the CO$_2$ in the gas mixture increased from 0.25% to 0.5, 1, 2, and 4% (Figure 2.4). The $P_{50}$ values calculated from Hill plots for samples incubated at each CO$_2$ tension differed significantly from one another ($P$<0.001) except for those determined at gas mixtures
containing 0.5 and 1% CO₂ (P=0.395; Table 2.1). Hill coefficients (n_H) were low, with the only significantly distinct value being for the gas mixture containing 0.25% CO₂ OEC (Table 2.1, Figure 2.2). Bohr coefficients calculated for each P₅₀ and pH shifts from 0.25% CO₂ are displayed in Table 2.1.

OECs generated using tonometry of rinsed RBCs (Series 1) always fell within the 95% confidence intervals surrounding the OECs generated using the P_wec microdiffusion chamber, with one exception. Samples incubated at 2% CO₂, for tonometry of rinsed RBCs just exceeded the 95% confidence intervals above a PO₂ of about 120 mm Hg (Figure 2.4). Similarly, the SO₂ values determined at a PO₂ of 96 mm Hg for the three methods were not significantly different, despite the qualitatively low value obtained for whole blood tonometry at 2% CO₂ (Figure 2.4). Furthermore, P₅₀ values, n_H values, and P₅₀ Bohr coefficients did not differ significantly among methods. For both methods, the lowest Bohr coefficient was observed when calculated between the 0.25 and 1% CO₂ OECs. In general, data obtained to quantify Hb-O₂ relationships were consistent between the three methods utilized in this study (Figure 2.4).

### 2.4 Discussion

The present study is the most extensive analysis of the O₂ delivery potential associated with a pH change (ΔPO₂) of the blood in any teleost with a combined Bohr-Root effect system. This potential is also compared with a system with a Bohr effect alone (human blood). The ΔPO₂ in rainbow trout blood at 40% Hb-O₂ saturation was at least 2.5-fold greater than that of a system with a Bohr effect alone with the same -0.2 pH unit change (Figure 2.3). At Hb-O₂ saturations greater than 50%, the difference was even more pronounced with the Bohr-Root effect system exceeding that of a Bohr effect alone system by 8-fold (Figure 2.3). This difference has not previously been quantified, despite qualitative predictions (Brauner and Weber, 1998; Brauner
and Randall, 1998; Brauner et al., 2001). The validity of my quantitative estimates is greatly enhanced by the fact that SO₂ values and OECs, and therefore a full Hb-O₂ binding profile could be generated in rainbow trout using three independent sampling protocols (rinsed RBCs or whole blood drawn from an indwelling dorsal aortic cannula), or analytical protocols (tonometry or the \( P_{\text{wee}} \) microdiffusion technique) (Figure 2.4). All three independent methods yielded similar results in terms of the general shapes of the OECs, and calculated Bohr and Hill coefficients and \( P_{50} \) values. Sampling protocols for whole blood usually involve cannulation and recovery chambers, and often more fish than those where RBCs can be rinsed and resuspended, making the latter a more efficient and simpler protocol overall. The \( P_{\text{wee}} \) system required the smallest volume of blood of any technique and can now be used to account for the Root effect with appropriate modifications (Clark et al., 2010; Clark et al., 2008), which has previously not been possible with most gas diffusion chambers and has lead to some of the discrepancies in the literature. Ultimately, these validations mean that future studies can be reliably performed on small fish where blood volume is limiting and do not have to be restricted to fish implanted with blood collecting cannulae.

2.4.1 Past information on haemoglobin-oxygen relationships in rainbow trout

Rainbow trout is arguably one of the most universally investigated teleost species with respect to O₂ transport and respiratory physiology. The absence of a comprehensive data set sufficient to model O₂ delivery potential with a change in pH (Table 2.2) is therefore surprising. A review of the available literature reveals that reported O₂ transport-related variables in rainbow trout blood are highly variable. For example, great variability in Bohr coefficients are observed from \(-0.15\) to \(-1.97\) (Figure 2.5) and \( P_{50} \) values for control or resting animals as low as 11 but as high as 40 mm Hg (Ingermann, 1982; Irving et al., 1941; Nikinmaa, 1983; Tetens and
Christensen, 1987; Vorger, 1985). This variability is likely due to the range of animal holding conditions, sampling techniques, blood preparation and protocols (Table 2.2; Figure 2.5) but it is unknown how these differences may affect the values obtained (Eddy, 1971; Eddy, 1974; Eddy, 1977; Eddy et al., 1977; Eddy and Morgan, 1969; Holk and Lykkeboe, 1995; Ingermann, 1982; Irving et al., 1941; Malte and Weber, 1985; Nikinmaa, 1983; Tetens and Christensen, 1987; Tetens and Lykkeboe, 1981; Vorger, 1985; Weber et al., 1976). Clearly, the complete data set to investigate ΔPO$_2$ on a Bohr-Root effect system presented here represents an important advance.

2.4.2 Modeling oxygen delivery potential in a combined Bohr-Root effect system (rainbow trout) and a system with a Bohr effect alone (human)

Christiansen and colleagues calculated in vivo in human blood, a maximal P$_{50}$ shift (ΔPO$_2$) of 3 mm Hg that was associated with the Bohr effect (Christiansen et al., 1914). Therefore, the predicted benefit to O$_2$ delivery associated with the Bohr effect alone is quite modest, and this has been known for some time. In modeling ΔPO$_2$ associated with a pH change in human blood, a Bohr coefficient of –0.35 was assumed, a value deemed optimal for O$_2$ delivery (Lapennas, 1983). It was also assumed that the Bohr coefficient was constant between 20 and 80% SO$_2$. A P$_{50}$ value of 27 mm Hg was chosen, which is midway between resting values of 24 and 29 mm Hg, which have previously been determined at a pH of 7.4 (Roughton and Severinghaus, 1973; Vorger, 1985; Wells and Weber, 1989; Winslow, 2005; Winslow et al., 1977). The ΔpH values chosen were consistent with those determined in Series 1 for rainbow trout OECs to allow direct comparison between the two systems. If anything, this would underestimate the difference between the two systems because pH is a log scale, and starting pH values are lower in humans (7.4) than in rainbow trout (8.0). Thus, a similar ΔpH would represent a greater addition of H$^+$ to the system in the former, which has a lower H$^+$ sensitivity.
The measured arterial to venous pH differences ($\Delta pH_{a-v}$) in humans are quite small, 0.03-0.04 pH units (Adrogue et al., 1989; Kelly et al., 2001; Middleton et al., 2006), relative to even the lowest $\Delta pH$ value of 0.2 units used in this study, which between 20 and 80% saturation, results in a $\Delta PO_2$ less than 5 mm Hg. A $\Delta pH$ of 0.03-0.04 would result in a $\Delta PO_2$ of less than 1 mm Hg, which is consistent with previous findings.

The potential for $O_2$ delivery associated with a blood $\Delta pH_{a-v}$ in a system with a teleost combined Bohr-Root effect differs from that of air-breathing vertebrates with a Bohr effect. First, the Root effect results in incomplete Hb-$O_2$ saturation at low pH, despite atmospheric PO$_2$ (Pelster and Weber, 1991; Root, 1931; Root and Irving, 1943). This was evident in the OECs generated for rainbow trout, as larger pH changes resulted in curves that approached upper asymptotic maximum Hb-$O_2$ saturations that were distinctly lower than 100%, which translated to infinite $\Delta PO_2$ values upon interpolation (Figures 2.1 and 2.3). Second, Root effect Hbs typically exhibit large Bohr coefficients (Brauner and Weber, 1998; Brauner and Randall, 1998; Brauner et al., 2001). As a result, the $\Delta PO_2$ in rainbow trout blood greatly exceeds that of human blood at all comparable $\Delta pH$ values (Figure 2.3). A $-0.2 \Delta pH_{a-v}$ difference in human blood is unlikely, but a difference of this magnitude is common in rainbow trout during exercise, based on arterial and venous blood pH measurements (Brauner et al., 2000; Kiceniuk and Jones, 1977; Nikinmaa and Vihersaari, 1993). Even at a $\Delta pH_{a-v}$ of -0.035, the $\Delta PO_2$ in rainbow trout blood still exceeds that of the human blood by more than 3-fold (data not shown). Third, Root effect Hbs exhibit a non-linear Bohr coefficient (Brauner and Jensen, 1999; Brauner and Randall, 1998; Brauner et al., 2001). The importance of the non-linear Bohr effect is revealed here because the difference in $\Delta PO_2$ for a given pH change between the Bohr and Bohr-Root effect is almost non-existent below the $P_{50}$ (Figure 2.3). Brauner and colleagues determined that in rainbow trout, during resting conditions, venous Hb-$O_2$ likely never falls below 50% (Brauner and Jensen,
A Φ calculated over the region of the OEC used under resting conditions then may be equivalent to the RQ (~1.0), as it would only encompass changes occurring in the upper reaches of the OEC. According to Lapennas’ analyses (1983), and as outlined in Chapter 1, this would convey enhanced CO₂ transport, as all metabolically produced CO₂ would bind to Hb as H⁺. During non-steady state conditions, such as during exposure to environmental hypoxia or when white muscle H⁺ production occurs during burst activity, teleosts may have a greater likelihood of achieving large ΔpH_{a-v} at the tissues. During these conditions, a greater portion of the OEC may be utilized and the resulting Φ calculated would be lower, perhaps more closely resembling 50% RQ, which Lapennas suggested was optimal for O₂ delivery (Brauner and Randall, 1998; Brauner et al., 2001; Lapennas, 1983). It is clear that the ΔO₂ associated with the ΔpH_{a-v} in the blood of a system that possesses a Bohr-Root effect, such as rainbow trout, is far greater than that for a Bohr effect alone, such as a human.

Most reports of blood pHₐ and pHᵥ values represent equilibrium values which do not likely exist in capillaries in vivo, suggesting previous literature may be underestimating ΔpH_{a-v}, which has large implications for O₂ delivery. Samples are typically drawn from the animal into a collecting vessel, and then analyzed outside of the animal over a short period of time while minimizing air exposure and temperature change. Given that uncatalyzed hydration and dehydration rates for CO₂ are within 1 to 2 min, it is likely that pH has come to equilibrium by the time a value is recorded. If the in vivo capillary ΔpH_{a-v} at the tissues are much larger than those reported based upon equilibrium values, the implications for the Bohr-Root effect to enhance O₂ delivery may be substantial. While the in vivo ΔpH_{a-v} is very difficult to measure, insight into ΔPO₂ at the tissues may be provided through the use of implantable O₂ optodes. McKenzie and colleagues (2004) measured, for the first time, PO₂ in the red muscle (RM) prior
to, during, and following both sustained and exhaustive exercise. The RMPO$_2$ values were higher than expected for a mammalian system (Behnke et al., 2001; Hutter et al., 1999; Jung et al., 1999; McKenzie et al., 2004; Suttner et al., 2002) with similar starting arterial PO$_2$ ($P_{a}O_2$) values, and values that were higher than those for their own and previously reported mixed venous blood PO$_2$ values (Farrell and Clutterham, 2003; Holeton and Randall, 1967; Hutter et al., 1999; McKenzie et al., 2004). Thus, it appears that RMPO$_2$ is maintained during, and following recovery from exercise, despite the corresponding decreases in arterial supply, and it was suggested that this may be associated with the presence of the Root effect (McKenzie et al., 2004). A $\Delta$H of 0.2 is a $\Delta$H$_{a-v}$ that has been measured during sustained swimming in *O. mykiss* but is likely an underestimate due to disequilibrium states. Based on calculations from this study and using this $\Delta$H, the $\Delta$PO$_2$ to the RM could be between 20 and 30 mm Hg, which is consistent with the *in vivo* values reported by (McKenzie et al., 2004). Although it is thought that the only locations where the full extent of the combined Bohr-Root effect facilitates enhanced O$_2$ delivery are where the acidosis can be magnified, localized, and recycled, like at the retia. A mechanism may be in place in the RM vasculature to capitalize on this physiological phenomenon that clearly requires further investigation, and was the focus of Chapters 3 and 4.

2.4.4 Conclusions

Three independent sampling and analytical protocols resulted in a high level of consistency between methods to determine OECs. Quantitative results confirmed theoretical predictions that the combined Bohr-Root effect in rainbow trout has the potential to convey an enormous benefit to O$_2$ delivery when compared with human blood having only a Bohr effect for a given pH change. Teleost fish clearly evolved an extraordinary O$_2$ delivery mechanism using the Bohr-Root effect that allows O$_2$ delivery to the eye and swimbladder, which may be one of
the most important factors responsible for their extensive adaptive radiation. Here I provide empirical evidence to suggest that the Bohr-Root effect system can also facilitate general $O_2$ delivery, consistent with recent *in vivo* studies by McKenzie et al. (2004). If this proves to be the case, it may be that Root effect Hbs, which evolved prior to the appearance of the anatomical structures (*retia*) typically associated with exceptional $O_2$ delivery systems at the eye and swimbladder, were initially selected for enhancing general $O_2$ delivery.
2.5 Chapter Summary

1. The dramatic increase in the $\Delta$PO$_2$ for a range of pH values associated with a combined Bohr-Root effect was quantified over the entire O$_2$ equilibrium curve for the first time in *Oncorhynchus mykiss*.

2. The $\Delta$PO$_2$ for the combined Bohr-Root effect in *O. mykiss* was at least 2.5 fold greater than the $\Delta$PO$_2$ for a system with a Bohr effect alone (human blood) at 40% Hb-O$_2$ saturation (and greater) with a pH change of -0.2.

3. The $\Delta$PO$_2$ interpolated at 80% Hb-O$_2$ saturations for *O. mykiss* were a minimum of 8-fold greater than that of human blood, a phenomenon consistent with the existence of a non-linear Bohr effect evident in most teleosts.

4. For pH differences greater than 0.2, the $\Delta$PO$_2$ for the combined Bohr-Root effect system was orders of magnitude greater than that of the Bohr effect alone.

5. Three independent combinations of blood sampling (cannulation and caudal puncture) and preparation techniques (whole blood and rinsed RBCs) and analytical protocols (tonometry and a new commercially available system ($P_{\text{wee}}$ 50)) were validated and found to generate statistically identical OECs and Hb-O$_2$ saturations.
### Table 2.1 Effects of carbon dioxide (% CO$_2$) on haematology, pH and oxygen transport-related variables for rinsed red blood cells and whole blood of rainbow trout, *Oncorhynchus mykiss*.

<table>
<thead>
<tr>
<th>CO$_2$ (%)</th>
<th>0.25</th>
<th>0.50</th>
<th>1</th>
<th>2</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCO$_2$ (mm Hg)</td>
<td>1.9</td>
<td>3.8</td>
<td>7.6</td>
<td>15.2</td>
<td>30.4</td>
</tr>
<tr>
<td>[Hb] (mM)</td>
<td>$0.9 \pm 0.0^{abc}$</td>
<td>$0.9 \pm 0.0^{abc}$</td>
<td>$1.0 \pm 0.0^{b}$</td>
<td>$1.0 \pm 0.0^{b}$</td>
<td>$0.8 \pm 0.1^{c}$</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>$23.2 \pm 0.0^{abcd}$</td>
<td>$22.5 \pm 0.3^{b}$</td>
<td>$25.3 \pm 0.8^{c}$</td>
<td>$26.1 \pm 0.4^{c}$</td>
<td>$24.5 \pm 0.2^{cd}$</td>
</tr>
<tr>
<td>MCHC</td>
<td>$3.7 \pm 0.2^{ab}$</td>
<td>$4.2 \pm 0.1^{a}$</td>
<td>$4.1 \pm 0.1^{a}$</td>
<td>$4.0 \pm 0.1^{a}$</td>
<td>$3.1 \pm 0.4^{b}$</td>
</tr>
<tr>
<td>pH</td>
<td>$7.40 \pm 0.00^{a}$</td>
<td>$7.24 \pm 0.05^{b}$</td>
<td>$7.12 \pm 0.03^{c}$</td>
<td>$6.96 \pm 0.05^{d}$</td>
<td>$6.79 \pm 0.02^{e}$</td>
</tr>
<tr>
<td>pH$_e$</td>
<td>$8.01 \pm 0.00^{a}$</td>
<td>$7.81 \pm 0.04^{b}$</td>
<td>$7.69 \pm 0.02^{c}$</td>
<td>$7.46 \pm 0.03^{d}$</td>
<td>$6.96 \pm 0.01^{e}$</td>
</tr>
<tr>
<td>P$_{50}$ (mm Hg)</td>
<td>Tonometry</td>
<td>$24.8 \pm 0.3^{a}$</td>
<td>$37.7 \pm 0.3^{b}$</td>
<td>$40.6 \pm 2.2^{o}$</td>
<td>$75.0 \pm 4.8^{c}$</td>
</tr>
<tr>
<td></td>
<td>P$_{wee}$</td>
<td>$25.2 \pm 2.0^{o}$</td>
<td>$35.7 \pm 1.4^{b}$</td>
<td>$37.6 \pm 1.2^{b}$</td>
<td>$73.8 \pm 1.3^{c}$</td>
</tr>
<tr>
<td>n$_H$</td>
<td>Tonometry</td>
<td>$1.3 \pm 0.1^{a}$</td>
<td>$1.5 \pm 0.0^{a}$</td>
<td>$1.4 \pm 0.0^{b}$</td>
<td>$1.0 \pm 0.0^{b}$</td>
</tr>
<tr>
<td></td>
<td>P$_{wee}$</td>
<td>$2.1 \pm 0.2^{o}$</td>
<td>$1.5 \pm 0.1^{b}$</td>
<td>$1.3 \pm 0.1^{b}$</td>
<td>$0.9 \pm 0.1^{c}$</td>
</tr>
<tr>
<td>Φ</td>
<td>Tonometry</td>
<td>$-0.91 \pm 0.18$</td>
<td>$-0.67 \pm 0.04$</td>
<td>$-0.87 \pm 0.06$</td>
<td>$-0.77 \pm 0.05$</td>
</tr>
<tr>
<td></td>
<td>P$_{wee}$</td>
<td>$-0.76 \pm 0.06^{a}$</td>
<td>$-0.54 \pm 0.02^{b}$</td>
<td>$-0.85 \pm 0.02^{o}$</td>
<td>$-0.75 \pm 0.01^{e}$</td>
</tr>
</tbody>
</table>

Note: Haemoglobin concentration [Hb], haematocrit (Hct), mean corpuscular haemoglobin concentration (MCHC), intracellular pH (pH$_i$), extracellular or plasma pH (pH$_e$), the Hill coefficient (n$_H$) = (Δlog(S/(1−S)))/(ΔlogPO$_2$), the Bohr coefficient (Φ) = (ΔlogP$_{50}$/ΔpH$_e$). All data were collected according to Series 1 protocols on rinsed RBCs using tonometry (labeled as “Tonometry” when necessary). For P$_{50}$, n$_H$, and Φ, data from Series 3 on whole blood using P$_{wee}$ methodology are also displayed for comparison and labeled “P$_{wee}$.” The n$_H$ value derived from tonometry data is the average of eight replicate slopes from lines generated using four SO$_2$ points taken between 20 and 80% saturation on each OEC. The n$_H$ values calculated from P$_{wee}$ data are done so based on three OEC plots, but still the only difference between methods was in the first n$_H$ value 0.25% CO$_2$ (P<0.001). Statistical significance for comparisons within a CO$_2$ treatment group but between tonometry and P$_{wee}$ sampling techniques is indicated with an asterisk. All analyses were interpreted using α < 0.05 to determine statistical significance.
Table 2.2 Oxygen transport-related variables (Bohr coefficients, $P_{50}$, $pH_e$, $pH_i$ and Hill coefficients) from a selection of data published on rainbow trout, *Oncorhynchus mykiss*, from previous studies.

<table>
<thead>
<tr>
<th>Bohr</th>
<th>$P_{50}$</th>
<th>$pH_e$</th>
<th>$pH_i$</th>
<th>Temperature ($°C$)</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>15.0</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td></td>
<td>Irving et al., 1941</td>
</tr>
<tr>
<td>-</td>
<td>13.0</td>
<td>-</td>
<td>-</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>13.0</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>19.0</td>
<td>-</td>
<td>-</td>
<td>15</td>
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<td>-</td>
<td>22.0</td>
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<td>-</td>
<td>30.0</td>
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<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>30.0</td>
<td>-</td>
<td>-</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>38.0</td>
<td>-</td>
<td>-</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>30.0</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>36.0</td>
<td>-</td>
<td>-</td>
<td>5</td>
<td></td>
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<tr>
<td>-</td>
<td>30.0</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td></td>
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</tr>
<tr>
<td>-</td>
<td>34.0</td>
<td>-</td>
<td>-</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>35.0</td>
<td>-</td>
<td>-</td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>40.0</td>
<td>-</td>
<td>-</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>40.0</td>
<td>-</td>
<td>-</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>52.0</td>
<td>-</td>
<td>-</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-0.79</td>
<td>15.0</td>
<td>7.63</td>
<td>-</td>
<td></td>
<td>Blood was drawn via cardiac puncture, immediately incubated for 20 min, and analyzed in a Haldane apparatus. The pH was not measured, and so Bohr coefficients cannot be calculated from these data. The shift in the OEC is represented by $P_{50}$ values calculated at each temperature at either PCO$_2$ of 1 or 10 mm Hg.</td>
<td>Eddy and Morgan, 1969</td>
</tr>
<tr>
<td>-0.40</td>
<td>61.5</td>
<td>6.85</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-0.40</td>
<td>15.0</td>
<td>7.63</td>
<td>-</td>
<td>13-19</td>
<td>Blood was drawn via cardiac puncture, and tonometry protocols were used with O$_2$ content determined via microsyringe technique of (Roughton and Scholander, 1944; Scholander and van Dam, 1955) with modifications of (Grant, 1947; Grigg, 1967). This was one of the first in vitro characterizations on rainbow trout blood.</td>
<td>Irving et al., 1941</td>
</tr>
<tr>
<td>-0.96</td>
<td>27.0</td>
<td>6.99</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-0.56</td>
<td>35.7</td>
<td>6.87</td>
<td>-</td>
<td></td>
<td>The Root effect onset was determined at pH$_e$ = 7.6, and it was determined that normal metabolic function of rainbow trout RBCs deteriorates above 20°C.</td>
<td>Eddy et al., 1971; 1977</td>
</tr>
<tr>
<td>-0.59</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-0.52</td>
<td>-</td>
<td>7.85 - 7.32</td>
<td>-</td>
<td>15</td>
<td>Blood was obtained via caudal puncture.</td>
<td>Weber et al., 1976</td>
</tr>
<tr>
<td>-0.48</td>
<td>-</td>
<td>6.50 - 8.25</td>
<td>-</td>
<td>15</td>
<td>Stripped hemolysates were used.</td>
<td></td>
</tr>
<tr>
<td>-0.60</td>
<td>-</td>
<td>6.75 - 8.25</td>
<td>-</td>
<td>5</td>
<td>Blood was collected by caudal puncture and acclimated to normoxia and then switched to anoxia.</td>
<td>Tetens and Lykkeboe, 1981</td>
</tr>
<tr>
<td>-0.49</td>
<td>25.0</td>
<td>7.80 - 7.90</td>
<td>-</td>
<td>20</td>
<td>Blood was collected by caudal puncture and acclimated to normoxia and then switched to anoxia.</td>
<td></td>
</tr>
<tr>
<td>-0.50</td>
<td>20.0</td>
<td>-</td>
<td>-</td>
<td>2.05</td>
<td>Fish were acclimated to hypoxia, blood samples were drawn via caudal puncture.</td>
<td></td>
</tr>
<tr>
<td>-0.52</td>
<td>22.0</td>
<td>-</td>
<td>-</td>
<td>2.15</td>
<td>Fish were acclimated to hypoxia, blood samples were drawn via caudal puncture.</td>
<td></td>
</tr>
<tr>
<td>-0.49</td>
<td>24.1</td>
<td>7.80</td>
<td>-</td>
<td>2.30</td>
<td>Fish were acclimated to $PO_2 = 150$ mmHg, then switched to 80, then 50 mmHg; blood samples were drawn via caudal puncture.</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>21.7</td>
<td>-</td>
<td>-</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>16.8</td>
<td>-</td>
<td>-</td>
<td></td>
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</tr>
</tbody>
</table>
Table 2.2 (continued) Oxygen transport-related variables (Bohr coefficients, $P_{50}$, pH$_e$, pH$_i$ and Hill coefficients) from a selection of data published on rainbow trout, *Oncorhynchus mykiss*, from previous studies.

<table>
<thead>
<tr>
<th>Bohr</th>
<th>$P_{50}$</th>
<th>pH$_e$</th>
<th>pH$_i$</th>
<th>Hill</th>
<th>Temperature ($^\circ$C)</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>-0.78</td>
<td>20.0</td>
<td>7.40</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>An interpolated OEC was depicted based on information used for the review, and we calculated a Bohr coefficient based on those data.</td>
<td>Ingermann, 1982</td>
</tr>
<tr>
<td></td>
<td>120.0</td>
<td>6.40</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td></td>
</tr>
<tr>
<td>-2.02</td>
<td>40.0</td>
<td>7.73</td>
<td>7.25</td>
<td>-</td>
<td>-</td>
<td>Blood was collected via cardiac puncture and then rinsed and resuspended in pH 7.8 buffer. We calculated Bohr coefficients from their reported $P_{50}$ and pH$_i$ values.</td>
<td>Nikinmaa, 1983</td>
</tr>
<tr>
<td>-1.95</td>
<td>46.0</td>
<td>7.70</td>
<td>7.21</td>
<td>-</td>
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<tr>
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<td>7.49</td>
<td>6.98</td>
<td>-</td>
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<tr>
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<td>6.89</td>
<td>-</td>
<td>12-13</td>
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</tr>
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<td></td>
<td>30.1</td>
<td>7.65</td>
<td>-</td>
<td>2.00</td>
<td>10</td>
<td>Isolated RBCs were suspended in a TrisHCl buffer and analyzed spectrophotometrically using a dual wavelength protocol.</td>
<td>Vorger, 1985</td>
</tr>
<tr>
<td>-0.82</td>
<td>22.2</td>
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<td>-</td>
<td>2.09</td>
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<td>2.00</td>
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<td>-</td>
<td>1.75</td>
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<td></td>
<td>26.7</td>
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<td>1.98</td>
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<td>2.18</td>
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<td>17.6</td>
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<td>-</td>
<td>2.25</td>
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<td></td>
<td>15.4</td>
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<td>2.30</td>
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<td></td>
<td>13.6</td>
<td>8.60</td>
<td>-</td>
<td>2.34</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-0.36</td>
<td>19.0</td>
<td>-</td>
<td>$\Delta$H = 0.23</td>
<td>-</td>
<td>-</td>
<td>Mathematical modeling was performed based on values from past literature.</td>
<td>Malte and Weber, 1985; 1987</td>
</tr>
<tr>
<td></td>
<td>23.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>-1.41</td>
<td>23.0</td>
<td>7.87</td>
<td>-</td>
<td>-</td>
<td>15</td>
<td>An in vivo, &quot;apparent Bohr&quot; was calculated, but data were collected upon analyzing blood drawn from the DA and using Tucker methodology.</td>
<td>Tetens and Chrisensen, 1987</td>
</tr>
<tr>
<td></td>
<td>18.0</td>
<td>7.93</td>
<td>-</td>
<td>-</td>
<td>15</td>
<td></td>
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</tr>
<tr>
<td>-0.50</td>
<td>21.4*</td>
<td>7.67</td>
<td>7.51</td>
<td>1.60</td>
<td>15</td>
<td>Blood was collected via caudal puncture, incubated in tonometers at $PCO_2 = 0.5%$, $Ar = 8%$. Bohr was calculated using $\Delta$H$<em>i = -0.73$. *values were calculated for $P</em>{50}$.</td>
<td>Holf and Lykkeboe, 1995</td>
</tr>
<tr>
<td>-0.50</td>
<td>14.8*</td>
<td>7.90</td>
<td>7.76</td>
<td>2.20</td>
<td>15</td>
<td></td>
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</tr>
</tbody>
</table>

Note: Data included in this table were either derived directly from data reported in published manuscripts, extrapolated from graphs, or calculated from other data that were available.
2.7 Figures

Figure 2.1 Oxygen equilibrium curves (OECs) for rainbow trout, *Oncorhynchus mykiss*, rinsed RBCs generated at 0.25, 0.5, 1.0, 2.0, and 4.0% CO\(_2\) with stepwise decreases in PO\(_2\) (mm Hg) from 160 to 0 mm Hg (balance N\(_2\)). The ΔpH\(_e\) relative to the 0.25% CO\(_2\) curve (black, pH\(_e\) = 8.01) is also noted for each treatment. Data are means ±S.E.M. Brackets surround statistically homogenous measurements among CO\(_2\) treatments within a given O\(_2\) incubation condition. Dotted lines extend from 50% Hb-O\(_2\) saturation to indicate the P\(_{50}\) for each CO\(_2\) treatment.
Figure 2.2 Hill plots for rainbow trout rinsed RBCs generated using tonometry (dashed lines) and whole blood using P\textsubscript{wee} methodology (solid lines) Hill coefficients, n\textsubscript{H} and P\textsubscript{50} values derived from this plot are reported in Table 2.1. Data points represent mean ± S.E.M.
Figure 2.3 The potential for oxygen delivery (ΔPO₂) over the entire oxygen equilibrium curve associated with a range of pH changes (ΔpH represented as different colours) for a haemoglobin system with A) a Bohr effect alone (human, dashed lines), B) a combined Bohr-Root effect (rainbow trout, solid lines) and C) both systems presented together to permit comparison. In A, Φ was assumed to be –0.35 and linear over the OEC, and the starting P₅₀ was 27 mm Hg. It was also assumed that saturation always reached 100% at the highest PO₂ (no Root effect). Curves for the different ΔpH values were calculated as described in the materials and methods (2.2). In B, curves were interpolated directly from Figure 2.1 to account for the presence of a Root effect at different pH values and the non-linear Bohr effect over the OEC.
Figure 2.4 Oxygen equilibrium curves generated on whole blood using $P_{\text{wee}}$ methodology (Series 3c; coloured line with 95% confidence intervals indicated by dashed lines) and tonometry of rinsed RBCs (solid black curve) at gas mixtures of a) 0.25%, b) 0.5%, c) 1%, d) 2%, and e) 4% CO$_2$. The OECs generated using tonometry of RBCs (solid black line) is the best-fit curve from Figure 2.1 (Series 1) determined at the respective CO$_2$ tension. Thin black (dotted) drop lines indicate the $P_{50}$ for each curve, which are also reported in Table 2.1. Panel f) represents all data combined including Hb-O$_2$ saturation values for whole blood tonometry (Series 3b) where samples were incubated at 96 mm Hg PO$_2$ at each CO$_2$ tension (means ±S.E.M.) to permit direct comparison among the three techniques.
Figure 2.5 Bohr coefficients ($\Phi$) and the pH$_e$ range over which they were calculated are plotted for the present study and those previously published for rainbow trout. If the $\Phi$ was not explicitly reported in the paper, it was extrapolated from the reported data. ($\Phi$) = ($\Delta \log P_{50}/\Delta \text{pH}_e$). All values reported in this figure were determined *in vitro*, but for different preparations, including whole blood, RBC suspensions, isolated stripped Hb suspensions, using different conditions to generate the decrease in pH (hypoxia, CO$_2$, HCl, or buffer suspensions) or from data sets built from an array of studies.
2.8 References


CHAPTER 3: A MECHANISM AT THE LEVEL OF THE RED BLOOD CELL FOR ENHANCED OXYGEN DELIVERY IN RAINBOW TROUT (Oncorhynchus mykiss): SHORT-CIRCUITING βNHE IN VITRO

3.1 Introduction

The Bohr effect represents the decrease in Hb-O₂ affinity with a reduction in pH (Bohr et al., 1904). The resulting right-shift in the O₂ equilibrium curve (OEC) is thought to play an important role in enhancing O₂ delivery to the tissues (Bohr et al., 1904; Brauner et al., 2001; Jensen, 1989; Jensen, 2001; Jensen, 2004; Lapennas, 1983; Nikinmaa, 1993; Nikinmaa, 1997; Nikinmaa and Soivio, 1979). For teleost fish haemoglobins (Hbs), a reduction in pH also reduces the O₂ carrying capacity of blood, a phenomenon referred to as the Root effect (Root, 1931; Root and Irving, 1943). Chapter 2 compared, quantitatively, the potential for O₂ release from Hb as a function of blood pH for the human Bohr effect and the rainbow trout, Oncorhynchus mykiss combined Bohr-Root effect systems. The combined Bohr-Root effect system potentially increases the driving force for O₂ delivery to a tissue (ΔPO₂) 2.5- to 8-times that of a Bohr effect-alone system for a pH decrease of 0.2 pH units, depending upon the starting Hb-O₂ saturation (Chapter 2).

The Bohr-Root O₂ delivery mechanism unique to teleosts is used to great advantage for filling a swimbladder against apparently large pressure gradients (>50 atm) associated with depth (Alexander, 1966) and for oxygenating the metabolically active, yet avascular retinal tissue of the eye (Dafré and Wilhelm, 1989; Wittenberg and Haedrich, 1974; Wittenberg and Wittenberg, 1974). To harness this potential requires localizing and recycling the

¹A version of this chapter has been prepared to submit for publication. Rummer, J.L. and Brauner, C.J. A mechanism for enhanced oxygen delivery at the level of the red blood cell in rainbow trout (Oncorhynchus mykiss): Short-circuiting βNHE in vitro.
acidosis with a unique vascular architecture at these specialized structures, specifically the *rete mirabile* at the swimbladder and the choroid *rete* at the eye. Chapter 2 demonstrated that a substantial $\Delta P_{O_2}$ could be elicited in rainbow trout Hb by a $\Delta pH_{a-v}$ (-0.2 pH units) typical of stress (e.g. acute hypercapnia or hypoxia) or after burst or exhaustive exercise (Brauner et al., 2000; Kiceniuk and Jones, 1977; Milligan and Wood, 1987; Nikinmaa and Vihersaari, 1993; Perry and Gilmour, 1996; Primett et al., 1986). Despite this potential, no research has addressed whether this mechanism unique to teleosts may be important in optimizing $O_2$ delivery to tissues other than the swimbladder and eye, such as muscle, during a generalized acidosis.

The Bohr-Root effect may enhance general tissue $O_2$ delivery, provided a large $\Delta pH_{a-v}$ occurs.

In this research, I proposed and validated, *in vitro*, in rainbow trout, a novel mechanism whereby this large $\Delta pH_{a-v}$ may be possible.

Most teleost fish that exhibit a pronounced Bohr-Root effect have some ability to regulate red blood cell (RBC) pH adrenergically to maintain $O_2$ loading at the gills. I hypothesized that $pH_i$ regulation may be short-circuited at the tissues to enhance $O_2$ delivery potential via expression of the Bohr-Root effect. Catecholamines (e.g. adrenaline and noradrenaline) are released into the general circulation and bind to $\beta$-adrenergic receptors on the RBC membrane that, via adenylate cyclase and 3’, 5’ - cyclic monophosphate (cAMP) activate a $\beta$-adrenergic sodium/proton ($Na^+/H^+$) exchange ($\beta$NHE) (Salama and Nikinmaa, 1990). The carbonic anhydrase (CA)-catalyzed hydration of $CO_2$ inside the RBC produces $H^+$ that are removed in exchange for $Na^+$ via $\beta$NHE, and $HCO_3^-$ that is removed via anion exchange (AE) for $Cl^-$ at a slower rate, which in combination result in an intracellular alkalosis and increase in Hb-$O_2$ affinity. The $H^+$ removed from the RBC acidify the plasma, resulting in a decrease in extracellular pH ($pH_e$), and at an uncatalyzed rate, $H^+$ will combine with $HCO_3^-$ to form $CO_2$, resulting in a slow plasma alkalinization after the initial pH decrease (Figure 3.1) (Geers and
Gros, 2000; Lessard et al., 1995). Adrenergic RBC βNHE is thought to have evolved to safeguard O₂ uptake at the respiratory surfaces during a generalized acidosis in the presence of Bohr-Root shift Hbs (Borgese et al., 1987; Jensen, 2004; Malapert et al., 1997; Nikinmaa et al., 1984; Perry and Kinkead, 1989; Primmett et al., 1986; Salama and Nikinmaa, 1988).

Carbonic anhydrase (CA) is prevalent in the RBC where it is responsible for the hydration/dehydration of CO₂. CA is one of the fastest enzymes known, with an average k₉₅ of 10⁶ s⁻¹ and half time (t₁/₂) of 5x10⁻³ to 10⁻³ s, 6,000-times faster than the uncatalyzed reaction (Cardenas et al., 1998; Geers and Gros, 2000; Henry and Swenson, 2000). Although plasma-accessible CA is not present in the teleost gill, membrane-bound and plasma-accessible CA (e.g. CA IV-like isoforms) may exist in select locations such as bound to muscle endothelia (Decker et al., 1996; Effros and Weissman, 1979; Geers and Gros, 2000; Gilmour et al., 1997; Henry et al., 1997; Henry and Swenson, 2000; Sender et al., 1994; Siffert and Gros, 1982; Yamamoto et al., 2003). Fish are thought to possess plasma-accessible CA isoforms similar to mammalian CA IV, but their location and function are undetermined (Gilmour and Perry, 2009). I propose that if CA is available to the plasma in tissue capillaries, H⁺ removed from the RBC via βNHE could combine with plasma HCO₃⁻ to reform CO₂, which would and back-diffuse into the RBC, and create a larger RBC ΔpHₐ-ᵥ at the tissues than would otherwise occur (Figure 3.2). The large acidosis transferred to the RBC would elevate PO₂ via the combined Bohr-Root effect, facilitating tissue O₂ delivery. Furthermore, provided the rate of RBC βNHE short-circuiting in the tissue and subsequent pHᵢ recovery during transit to the gill was sufficiently rapid, a generalized acidosis could provide protons that could repeatedly be used to increase the intracellular ΔpHₐ-ᵥ at the tissues with every pass through the circulation elevating PO₂ at the tissues at a time when O₂ delivery is especially needed.

Rainbow trout blood was pre-equilibrated at pre-defined Hb-O₂ saturations, and then, in a closed system, acidified, β-adrenergically-stimulated, and then exposed to CA. Throughout this
sequence, changes in both pH and PO\textsubscript{2} were monitored in real-time to assess both the magnitude and time course of the response to gain insight into whether βNHE short-circuiting could be operational \textit{in vivo} and estimate the degree to which it might influence O\textsubscript{2} delivery. I hypothesized that in this \textit{in vitro}, closed-system, and in the presence of an acidosis, plasma-accessible CA short-circuits the adrenergically-stimulated RBC βNHE, thus creating a decrease in RBC pH that elevates the driving force for O\textsubscript{2} delivery, ΔPO\textsubscript{2}, due to the combined Bohr-Root effect.

### 3.2 Materials and Methods

#### 3.2.1 Animals and rearing conditions

Rainbow trout \textit{Oncorhynchus mykiss} (300-600 g wet body mass) were obtained from Spring Valley Trout Farm (Langley, British Columbia, Canada). Fish were maintained in 4,000 l flow-through tanks supplied with Vancouver dechlorinated municipal tap water under a natural photoperiod. Fish were fed every other day to satiation using commercial trout pellets (Skretting, Orient 4-0). All procedures complied with the guidelines approved by the Canadian Council on Animal Care (UBC protocol # A07-0080).

#### 3.2.2 Sampling protocol

Fish were quickly anaesthetized in a 20 l bucket of clean, well-aerated water containing benzocaine solution (0.2 mM final concentration, p-aminobenzoate). Fish were then placed on a surgery table, and their gills were intubated and continuously irrigated with water containing a more dilute anaesthetic (0.02 mM p-aminobenzoate). An indwelling cannula (PE50) was surgically implanted into the dorsal aorta according to Soivio et al. (1975). Following surgery,
fish were placed in a Perspex box supplied with aerated 12°C clean water and gently force-ventilated until they regained equilibrium. Fish were left to recover for at least 24h prior to sampling, during which time cannulae were flushed twice with heparinized (10 IU ml$^{-1}$ lithium heparin, Sigma-Aldrich cat. no. H0878; St. Louis, MO, USA) Cortland’s saline (Wolf, 1963). Prior to experimentation, blood was removed from the cannulae into a heparinized syringe, but at the first sign of struggling, no further blood was removed to ensure negligible plasma catecholamine levels. Blood was pooled from 2-3 fish and haematocrit (Hct) was measured in duplicate by centrifuging 60 µl of whole blood in heparinized micro-capillary tubes for 3 min at 12,000 rpm. Prior to experimentation, the pooled blood sample was standardized to a Hct of 25% by removing either plasma or RBCs. Approximately 2.5 ml aliquots were added to four Eschweiler tonometers. Tonometers containing blood were equilibrated for one hour at 12°C (LAUDA Brinkman™ Model S-1 recirculating chilling unit) with a humidified gas mixture, varying in O$_2$ proportions regulated by a gas-mixing pump (DIGAMIX 275 6KM 422 Wösthoff, Bochum, Germany; PCO$_2$ = 0.5%, balance N$_2$). The aim was to incubate blood at O$_2$ tensions between 20 and 80% Hb-O$_2$ saturation, the linear region of the OEC in rainbow trout. Nominal values of 30, 50, 65 and 75% Hb-O$_2$ saturation were targeted and the required incubation PO$_2$ was determined from the rainbow trout OECs generated in Chapter 2 at 12°C and 0.5% CO$_2$. Following incubation of the tonometers at respective gas proportions, a sub-sample of blood (600 µl) was removed so that haemoglobin concentration ([Hb]), haematocrit (Hct), extracellular pH (pH$_e$), and intracellular pH (pH$_i$) could be measured. The remaining blood was then loaded into the closed-system for experimentation, as described below.
3.2.3 Closed-system preparation

Following blood tonometry, a 2 ml aliquot of blood was transferred using a pre-gassed Hamilton™ syringe and slowly ejected into a pre-gassed 2 ml glass vial until overflow, at which time the vial was sealed with a septum (Figure 3.3). A pre-calibrated fiber optic implantable O₂ sensor and fiber optic implantable pH sensor (PreSens, Loligo Systems ApS, Denmark) (tip diameters 50-140 µm), presoaked in heparinized Cortland’s saline, were inserted through the septum to monitor blood PO₂ and pH in real-time in the closed-system (Figure 3.3). The vial was thermostatted at 12°C, equipped with a small stir bar and positioned on a stir plate set at 400 revolutions min⁻¹ to ensure adequate mixing throughout the experiment. Oxygen and pH signals were amplified using an Oxy-4 micro four-channel oxygen meter and signal amplifier (Loligo Systems ApS, cat #OX11700) and a pH-1 micro single-channel meter (Loligo Systems ApS, cat #PH10450), respectively. Data were collected in real-time throughout the duration of each experiment at a sampling rate of 1 s⁻¹, and integrated with the manufacturer’s software packages for PC Windows. All data were saved as text files and analyzed using Acqknowledge® Data Acquisition Software (Version 3.7.3, BIOPAC Systems, Inc.) (Figure 3.3).

3.2.4 Series 1: Closed-system in vitro characterization of red blood cell β-adrenergic stimulation during an acidosis in the presence of carbonic anhydrase

Blood PO₂ and pH in the closed-system stabilized within 5 min and values were recorded for an additional 5 min. Blood was then acidified following injection of 20 µl of 100, 150, or 200 mM HCl prepared in Cortland’s saline via a 50 µl Hamilton™ syringe achieving a final concentration of 1, 1.5, or 2 mM, respectively, resulting in a nominal 0.15, 0.30 or 0.50 pH unit reduction in blood pH (see Table 3.2 for actual pH values). Blood PO₂ and pH stabilized to a new value within 2-3 min of acidification. Then, 5 min following addition of HCl, 20 µl of the
β-adrenergic agonist isoproterenol (ISO) (Sigma-Aldrich cat. no. I5627; St. Louis, MO, USA) was added. Isoproterenol was prepared fresh in Cortland’s saline immediately prior to use and injected to obtain a final concentration of 1 mM which is known to elicit a maximum response in rainbow trout blood (Figure 3.4) (Tetens et al., 1988). Blood pH and PO$_2$ were monitored for an additional 5 min before CA (from bovine erythrocytes, E.C. 4.2.1.1, Sigma-Aldrich cat. no. C3934; St. Louis, MO, USA) was injected into the system for a final concentration of $10^{-3}$ mM, which is similar to mammalian RBC concentrations and a concentration previously shown to short-circuit the β-NHE response in rainbow trout blood in vitro (Table 3.1) (Henry et al., 1997; Nikinmaa et al., 1990). This entire experimental treatment will be referred to as HCl+ISO+CA (Figure 3.4).

3.2.5 Series 2: Closed-system, in vitro characterization during the absence of red blood cell β-adrenergic stimulation during an acidosis in the presence of carbonic anhydrase

Preparations were identical to those described for Series 1, as were data collection and analyses. Only one level of acidification (100 mM HCl) was used in Series 2, one that generated a ΔpH$_e$ of -0.15. Blood PO$_2$ and pH stabilized to a new value within 2-3 min, and 5 min following addition of HCl, CA was injected into the system for a final concentration of $10^{-3}$ mM which is the same concentration used in Series 1 experiments. This entire experimental treatment will be referred to as HCl+CA (Figure 3.5).

3.2.5 Series 3: Closed-system, in vitro characterization while inhibiting red blood cell Na$^+$, H$^+$ exchange during an acidosis in the presence of carbonic anhydrase

Ethylisopropylamiloride (EIPA) (Sigma-Aldrich cat. no. A3085; St. Louis, MO, USA), is a potent inhibitor of Na$^+$/H$^+$ exchange, specifically the NHE1 isoform (Kristensen et al., 2007).
It was used to validate the effects of ISO and the role of an adrenergically-mediated NHE in the model proposed. The protocol followed that as described above with only one incubation condition (0.5% CO₂, 65% air, balance N₂) except that 5 min following addition of HCl, EIPA was injected into the system for a final concentration of 0.1 mM. Blood PO₂ and pH stabilized to a new value within 2-3 min, and 5 min following addition of EIPA, CA was injected into the system for a final concentration of 10⁻³ mM which is the same concentration used in Series 1 and 2 experiments. This entire experimental treatment will be referred to as HCl+EIPA+CA (Figure 3.6).

3.2.6 Controls and blood analysis

Sham injections of Cortland’s saline were also introduced at every interval in a separate and final control experiment to account for potential injection effects (data not shown, as no effects were observed). At 30 min in all experiments, blood was removed for further analysis. Haematocrit was determined in duplicate after centrifuging two heparinized Hct tubes, each filled with 40 µl of blood, at 12,000 rpm for 3 min. Haemoglobin was measured in duplicate using the cyanomethaemoglobin method and an extinction coefficient of 11 mM cm⁻¹ at 540 nm. The remaining aliquot of blood was centrifuged at 6,000 rpm for 3 min, plasma removed and discarded, and RBCs were immediately frozen in liquid nitrogen and later stored at -80°C until later analysis. Intracellular pH (pHᵢ) was measured in duplicate using a thermostatted BMS 3 Mk2 Blood Microsystem (Radiometer, Copenhagen) in conjunction with a Radiometer PHM73 acid-base analyzer after samples were prepared using the freeze/thaw method of Zeidler and Kim (Zeidler and Kim, 1977).
3.2.7 Data analyses

Representative traces were chosen for both the HCl+ISO+CA (Figure 3.4) and the HCl+CA (Figure 3.5) experiments. Otherwise, data are presented as mean ±S.E.M. For every level of acidification at every starting Hb-O₂ saturation used and in each experiment, sample size was n=6. (Table 3.2). For all responses, time to half-maximal response (t₁/₂) was calculated by using a double reciprocal plot. Data were compared statistically within acidification treatments and to baseline values, and when necessary, statistical differences were detected via one-way analysis of variance (ANOVA). All data met the assumptions of normality (Kolmogorov-Smirnov test) and homogeneity of variance. When a significant difference was identified, a post-hoc Holm-Sidak multiple comparisons test was applied to compare means. All statistical analyses were performed using SigmaStat 3.5 (Systat Software, San Jose, California) statistics software using α < 0.05 to determine statistical significance.

3.3 Results

3.3.1 Series 1: Red blood cell β-adrenergic stimulation during an acidosis in the presence of carbonic anhydrase (HCl+ISO+CA)

The mean starting Hct, pHₑ, and pHᵢ immediately following tonometry was 25.0 ±0.1%, 7.93 ±0.02, and 7.40 ±0.00, respectively. Within each acidification group, experiments began with four statistically distinct Hb-O₂ saturations (P<0.001), nominally 34, 54, 63, and 68% for the lowest level of acidification, 32, 59, 66, and 78% for the middle level of acidification, and 30, 53, 63, and 75% for the highest level of acidification (Table 3.2). The addition of HCl significantly reduced blood pHₑ by 0.15, 0.33, and 0.49 units, all of which differed significantly from one another (Table 3.2). Upon HCl addition, there was a rapid (t₁/₂ = 40.9 ±2.1 s, pooled for all Hb-O₂ saturations and acidification levels) and significant increase in PO₂ (ΔPO₂) of between
55 and 87 mm Hg, depending on the starting Hb-O₂ saturation and degree of acidification (P<0.001) (Table 3.2). Within a given acidification group, ΔPO₂ did not differ significantly among the four different starting Hb-O₂ saturations and consequently values were pooled. There were no significant differences in ΔPO₂ among the three acidification groups (P=0.271) (Table 3.2). However, ΔPO₂ values were all significantly different from 0 (P<0.001). For reference, data for this experimental series are presented in tabular format in Table 3.2, and a representative trace from a single trial is depicted in Figure 3.4.

Adrenergic stimulation significantly decreased PO₂ in all acidification groups and at all Hb-O₂ saturations except for in the lowest two starting Hb-O₂ saturations in the ΔpHₑ = -0.49 acidification group (P>0.05) (Table 3.2). Qualitatively, pHₑ decreased, but the change was not significant. Compared to the acidosis response, the ISO response was twice as slow (t₁/₂ = 102.1 ±8.1 s, pooled for all Hb-O₂ saturations and acidification levels, P<0.001) (Table 3.2; Figure 3.4).

CA addition significantly increased PO₂ in every acidification group and at every starting Hb-O₂ saturation (P<0.001), except within the ΔpHₑ = -0.15 group, in the subgroup where starting Hb-O₂ saturation was 33.7% (P=0.104) (Table 3.2). Qualitative increases in pHₑ were evident on most traces (Figure 3.4). Although, changes in pHₑ were not significant within or between groups. Overall, the CA-mediated response was 2 to 5-times faster than the HCl and ISO-mediated responses respectively (t₁/₂ = 21.8 ±2.2 s, pooled for all Hb-O₂ saturations and acidification levels, p=0.003 and p<0.001 compared to HCl and ISO, respectively) (Table 3.2; Figure 3.4).

In Series 1, pHᵢ was measured only at the start and end of each experiment and was always significantly higher at the beginning of the experiment (P<0.001) with one Hb-O₂ saturation level in the ΔpHₑ = -0.49 acidification group being the exception (P=0.127) (Table 3.2). Differences in final pHᵢ between Hb-O₂ saturation levels within each acidification group
were only observed in the $\Delta p\text{H}_e = -0.33$ and -0.49 acidification groups (Table 3.2). Final Hct measured as the proxy for RBC $\beta$-adrenergic stimulation, significantly increased relative to the initial value in all acidification groups at all starting Hb-O$_2$ saturations ($P<0.001$), resulting in up to a 70% RBC volume increase (Table 3.2). A significant correlation existed between the starting Hb-O$_2$ saturation and the negative $\Delta$PO$_2$ following the addition of ISO to previously acidified blood ($R^2 = 0.706$, $P<0.001$). The correlation was also evident with the positive $\Delta$PO$_2$ following CA addition ($R^2 = 0.289$, $P<0.05$). When the decrease in $\Delta$PO$_2$ due to ISO was pronounced, the increase in $\Delta$PO$_2$ due to CA was pronounced ($R^2 = 0.355$, $P<0.05$). This relationship was evident within and among each acidification group (Table 3.2). Consistent with these responses, in previously acidified blood a significant relationship could be detected between the degree of RBC swelling and the ISO-induced decrease in $\Delta$PO$_2$ ($R^2 = 0.394$, $P<0.03$).

3.3.2 Series 2: The absence of red blood cell $\beta$-adrenergic stimulation during an acidosis in the presence of carbonic anhydrase (HCl+CA).

When adrenergic stimulation was omitted from the sequence, starting Hb-O$_2$ saturations were nominally 47, 59, 65, and 74% (Table 3.2). Immediately following tonometry, Hct, pH$_e$, and pH$_i$ were not significantly different from values measured in Series 1 and consequently Series 1 and 2 starting values were pooled. Following HCl addition, pH$_e$ was significantly reduced by 0.15 units, consistent with the lowest level of acidification in Series 1 (Figure 3.5). Upon acidification, PO$_2$ increased significantly ($P<0.001$) by an average of 49 mm Hg (Table 3.2). The time to half-maximal acidosis response was $33.0 \pm 4.1$ s (pooled for all starting Hb-O$_2$ saturations) and not significantly different than the $t_{1/2}$ for the same level of acidification in Series 1 experiments (Student’s t-test, $t=1.372$, df=6, $P=0.219$) (Table 3.2; Figure 3.5). CA
addition increased PO2 by an average of 0.3 to 5.9 mm Hg, depending on starting Hb-O2 saturation (Table 3.2; Figure 3.5). The time to half-maximal CA-mediated response was 21.9 ±3.6 s (pooled for all starting Hb-O2 saturations) and not significantly different than the pooled t1/2 for the same level of acidification in Series 1 experiments (Student’s t-test, t=-0.577, df=6, P=0.585) (Table 3.2). At the end of the 30-min monitoring period, pHi had decreased to 7.10 or lower, and although blood from this experiment was not adrenergically-stimulated with ISO, Hct had increased significantly over starting values (P<0.001), but to a significantly lesser degree (16% opposed to 70% increase) relative to Series 1 (P<0.001) (Table 3.2). For reference, a representative trace is presented in Figure 3.5 and mean values are listed in Table 3.2.

3.3.3 Series 3: Inhibiting red blood cell Na+, H+ exchange during an acidosis in the presence of carbonic anhydrase (HCl+EIPA+CA).

For experiments conducted with EIPA, starting Hct, PO2, pHc, and pHi were 25.2 ±0.1%, 92.8 ±2.3 mm Hg, 7.83 ±0.03, and 7.20 ±0.03 respectively, and Hb-O2 saturation was 75.8 ±1.9%. Acidification significantly increased blood PO2 by 73 mm Hg, reaching a value of 165.4 ±17.9 while pHc significantly decreased by 0.22 ±0.03 units (Figure 3.6). Addition of EIPA did not significantly affect either blood PO2 or pHc. Following CA addition, there were no significant changes in either PO2 or pHc (Figure 3.6). At the end of the 30-min experimental and recording period, Hct was unchanged from starting values (P>0.05). Blood PO2 continued to fall over the duration of the experiment, reaching 133.8 ±8.1 mm Hg at 30 min, but remained significantly elevated over initial values (P<0.01). Blood pHc stabilized over the last 15 min of the recording period, but was still significantly lower than initial values (P<0.001), as was pHi (7.09 ±0.03; P=0.016).
3.4 Discussion

*In vitro* results are consistent with my hypothesis that, adrenergic RBC pH regulation during an acidosis via βNHE can be short-circuited by plasma-accessible CA. In doing so, Hb-O₂ affinity is reduced and ΔPO₂ is elevated in this closed-system. The increase in ΔPO₂ with CA was in excess of 30 mm Hg in some treatments, and occurred twice as rapidly relative to acidification without CA. This response also occurred to a lesser degree in the absence of adrenergic stimulation (Figure 3.5), but the response was abolished in the presence of EIPA when the NHE is directly inhibited (Figure 3.6). Thus, the addition of plasma-accessible CA to acidified blood, in the presence or absence of adrenergic stimulation, appears to increase ΔPO₂ through NHE short-circuiting. If, this mechanism is operational *in vivo* (supported in Chapter 4), short-circuiting RBC NHE in conjunction with a highly pH-sensitive combined Bohr-Root effect, system could markedly enhance tissue O₂ delivery over that which would occur in vertebrates possessing a Bohr effect alone (Chapter 2). This may shed further insight into the evolution of Root effect Hbs, which evolved prior to RBC βNHE and specialized *retia* at the eye and swimbladder.

3.4.1 Justification of parameters chosen

The specific *in vitro* treatments were chosen to mimic *in vivo* conditions where possible (i.e. initial Hb-O₂ saturations and acidification levels). Excess levels of ISO and CA ensured maximal effects in demonstrating proof-of-principle. Starting Hb-O₂ saturations (between 30 and 78%) encompassed the region of the OEC most commonly used in rainbow trout *in vivo*, and acknowledges the non-linear Bohr effect that exists in rainbow trout (Brauner et al., 1996; Brauner et al., 2001). The levels of initial acidification (0.15, 0.3 and 0.5 unit decreases in pHₐ) corresponded to *in vivo* changes in pHₐ documented in rainbow trout following exposure to
hypoxia or strenuous exercise (Brauner et al., 2000; Kiceniuk and Jones, 1977; Milligan and Wood, 1987; Nikinmaa and Vihearsaari, 1993). An acid-base disturbance of this magnitude in vivo also rapidly elevates plasma catecholamine levels (both adrenaline and noradrenaline) from resting levels that are usually less than $2 \times 10^{-5}$ mM (Motais et al., 1989; Tetens et al., 1988) to as high as $8.5 \times 10^{-5}$ mM (Butler et al., 1986; Milligan and Wood, 1987). ISO is a more potent β-adrenergic agonist, and concentrations known to generate a maximal βNHE response at the RBC (Tetens et al., 1988) were used (Table 3.1) to demonstrate proof-of-principle.

Two factors were considered when choosing CA concentrations higher than what might be expected in muscle: the importance of matching $\text{H}^+$ production rates between the RBC and plasma, and overwhelming endogenous CA inhibitors potentially present in the plasma (Dimberg, 1994). The final CA concentrations used in this study exceeded, by 20-times, those found in rabbit white muscle (likely CA IV, $K_{cat} \approx 1.1 \times 10^{-6}$ s$^{-1}$, similar to CAII) (Hilvo et al., 2008) (Table 3.1), a similar membrane-bound isoform to what may be available to rainbow trout muscle in vivo (Effros and Weissman, 1979; Wang et al., 1998). However, the concentrations used were slightly lower than those determined for mammalian RBCs ($5 \times 10^{-3}$ mM) (Henry et al., 1997), but still in line with what Nikinmaa, who used bovine erythrocyte CA, found to short-circuit β-adrenergically-stimulated rainbow trout RBCs incubated in tonometers (Nikinmaa et al., 1990) (Table 3.1). The isoform used was from bovine erythrocytes, likely mammalian CA II, which is not expected to be affected by plasma inhibitors, which are not only thought to be species-specific but also particular to the RBC isoform (Henry et al., 1997; Peters et al., 2000).

3.4.2 The $\Delta P_{O_2}$ associated with red blood cell βNHE short-circuiting

The $\Delta P_{O_2}$ measured in this closed-system was used as “proof of principle” for βNHE short-circuiting in this study. Insight was also gained relative to the time course over which
short-circuiting and subsequent adrenergic pHi recovery occurs. The optode response time for O2 is much faster than for pH (pH optodes ≥30 s; PO2 optodes <1 s). Thus, the ΔPO2 was a very sensitive, indirect measurement of changes in RBC pHi, which could not be measured in real-time. Therefore, regardless of the level of pHi detection, which was limited by optode response time, even the subtlest changes at the level of Hb-O2 could be identified, via PO2.

The magnitude of the CA-mediated ΔPO2 response following in vitro acidification by -0.33 or -0.49 units was very similar to the ΔPO2 values calculated by direct interpolation between OECs generated at pH values that differed by a similar amount (Chapter 2) (Figure 3.7). This suggests that nearly the entire acid load initially added to the closed-system may have been available for βNHE short-circuiting in this in vitro set-up. If this system is operational in vivo, (see below for a detailed discussion), there would be further acidification from CO2 produced from the tissues that could even further increase ΔPO2 as discussed in Chapter 2. Tissues would also continuously consume O2, potentially reducing the magnitude of ΔPO2. The Bohr effect is thought to be important in enhancing tissue O2 delivery, but the anticipated ΔPO2 in humans with a ΔpHa-v of 0.15 is estimated as 2-3 mm Hg (Chapter 2) (Behnke et al., 2001; Hutter et al., 1999; Jung et al., 1999; Rang et al., 2002; Suttner et al., 2002). In contrast, the ΔPO2 associated βNHE short-circuiting in the in vitro set-up employed in this study can be up to 25 mm Hg (Table 3.2). Whether this system can operate in vivo is discussed below.

3.4.3 Potential for short-circuiting of red blood cell βNHE to be operational in vivo

In order for this system to operate in vivo, there are many requirements that must be satisfied. Minimally, CA must be plasma-accessible. Also, the rate at which βNHE is short-circuited in acidified blood must be less than the time required for blood transit from the gills to the tissues. Furthermore, the rate of βNHE to recover pHi and secure O2 uptake at the gills must
be faster than that required for transit from the tissues to the gills. A resting fish has a cardiac output of 26.6 ml min\(^{-1}\) kg\(^{-1}\) (Brauner et al., 2000; Thorarensen et al., 1996), which, for a 1 kg fish with 5% blood volume indicates that blood transit time through the entire circulatory system is in the order of 2 min. The t\(_{1/2}\) for O\(_2\) release from Hb is very rapid, <10 ms (Roughton, 1964), but the protons from the initial HCl extracellular acidification enter the RBC as CO\(_2\) at the uncatalyzed rate of formation in the plasma, as protons do not typically cross plasma membranes (Pelster and Niederstatter, 1997).

The intracellular acidification in the first step of this in vitro model, indirectly indicated by \(\Delta P_{\text{O}_2}\) following acid addition, may be rate limited by the uncatalyzed (t\(_{1/2}\) >90 s) dehydration of HCO\(_3^-\) and H\(^+\) to form CO\(_2\) in the plasma that can freely enter the RBC. There were immediate effects of the acidification, but the t\(_{1/2}\) (28-44 s) was orders of magnitude slower than values published in the literature. For example, at the swimbladder of the eel, Pelster and colleagues reported a “Root off” t\(_{1/2}\) of 44.8 ms (Pelster et al., 1992), almost 1,000-times faster than this report. However, Pelster’s studies also suggest that CA is available to the plasma in the vicinity of the acid-producing gas gland (Pelster, 1995; Pelster and Niederstatter, 1997; Pelster and Scheid, 1992), which would greatly facilitate a fast “Root off” effect. My in vitro system was set to record every second and report every other data point. Also, the response time for the pH optodes is not matched with the faster (yet still not as fast as 1 ms) responding PO\(_2\) optodes. The t\(_{1/2}\) for the increase in PO\(_2\) was faster for the CA-mediated response, however, which may be a closer match to what Pelster et al. observed in the eel where CA was available (Pelster et al., 1992).
3.4.4 The role of general red blood cell Na\(^+\), H\(^+\) exchange

In Series 2 it was determined that a CA-mediated increase in PO\(_2\) could occur in the absence of red blood cell adrenergic stimulation (Figure 3.5). Yet, some isoform of NHE was involved, as evidenced in Series 3 experiments where blocking NHE eliminated all CA-mediated responses (Figure 3.6). While it is known that rainbow trout possess a highly sensitive βNHE (Borgese et al., 1987; Motais et al., 1987; Nikinmaa and Jensen, 1986; Nikinmaa et al., 1990; Nikinmaa, 1983; Nikinmaa and Huestis, 1984; Nikinmaa and Tufis, 1989), it may be that other NHEs are also present on the RBC, acting as “housekeeping” H\(^+\) exchangers that can be activated independent of adrenergic stimulation (Claiborne et al., 1999). Indeed, nearly all eukaryotes possess an isoform of NHE to regulate cell pH and volume (Claiborne et al., 1999; Deigweiher et al., 2008; Wakabayashi et al., 1997; Yun et al., 1995), and it has been suggested that at least one derived teleost species of the five groups that have secondarily lost the βNHE, maintains a general RBC NHE for those purposes (Rummer et al., 2010). These data support this hypothesis, and raise questions regarding what may be activating NHE isoforms in the absence of catecholamines on the RBC. The conditions under which catecholamines are released and βNHE is activated may be limited to extremely stressful scenarios in vivo such as when arterial PO\(_2\) falls below 20 mm Hg or 45-60% Hb-O\(_2\) saturation or when water PO\(_2\) falls below 60 mm Hg (Perry and Gilmour, 1996; Perry and Thomas, 1991). If the system also functions via short-circuiting of a general NHE with pH disturbances of as little as −0.15, for example, O\(_2\) delivery could be enhanced in select locations where CA is plasma-accessible under much less stressful conditions that may occur more frequently.

If NHE can be initiated quickly and the full response prolonged over several minutes, it may mean that pH\(_i\) has ample time to recover from an acidosis that is perpetuated at the muscle tissue when CA short circuits the protective βNHE system, at least by the time the blood returns
back to the gill, which could take up to 1 min. The CA-mediated response observed in this study, with $t_{1/2}$ ranging 10-35 s depending on starting Hb-O$_2$ saturation and the level of initial acidification, and occurred almost twice as fast as the HCl-induced PO$_2$ increases, where $t_{1/2}$ ranged 29-46 s, and almost 5-times faster than the $\beta$NHE (Table 3.2). If the PO$_2$ increase, *in vivo*, is as fast as previous studies suggest (45 ms) (Pelster et al., 1992), then it may be expected that the CA-mediated $\beta$NHE short-circuiting that elevates PO$_2$ also happens very fast. The transit time of a RBC through the capillaries is 1-3 s (Bhargava et al., 1992; Honig et al., 1977; Randall, 1982; Tetens and Lykkeboe, 1981), which is also ample time for CA to short-circuit the H$^+$ extrusion mechanism on the RBC. After this phenomenon permits enhanced O$_2$ delivery at the tissues, blood leaves the site of plasma-accessible CA, and $\beta$NHE is no longer short-circuited. Therefore blood returns to the respiratory surface with pH$_i$ and therefore Hb-O$_2$ binding protected once again by $\beta$NHE. Even if only a fraction of the response observed *in vitro* could be realized *in vivo*, this would significantly affect O$_2$ transport in comparison to systems possessing a Bohr effect alone, as illuminated in Chapter 2. Most importantly, however, the short-circuiting system appears to be operational *in vivo* as indicated in the following Chapter 4.

### 3.4.5 Conclusions

The main conclusion of this study is that *in vitro*, NHE on the RBC membrane can be short-circuited during an acidosis in the presence of plasma-accessible CA, effectively decreasing Hb-O$_2$ saturation and elevating the PO$_2$ of the blood. This finding has implications for the Bohr-Root effect to enhance O$_2$ delivery *in vivo*. An acidosis as small as $\Delta$pH = 0.15 can be recycled via the CA-mediated NHE short-circuiting mechanism in the presence as well as absence of catecholamines, indicating that the mechanism may not be restricted to circumstances where a severe acidosis is created and/or where catecholamines are released into and remain in
circulation for an extended period of time. The next step is to apply this *in vitro* model at the whole organism level, as was done in Chapter 4.
3.5 Chapter Summary

1. Adrenergic RBC pH\textsubscript{i} regulation via \(\beta\)NHE in the presence of an acidosis can be short-circuited following the addition of plasma-accessible CA \textit{in vitro}, in a closed-system. This resulted in a reduction in Hb-O\textsubscript{2} affinity and elevation in \(\Delta\text{PO}_2\) in excess of 30 mm Hg, depending on starting Hb-O\textsubscript{2} saturation and level of initial acidification, and occurred rapidly with a \(t_{1/2}\) of as fast as 7 s.

2. Interestingly, the addition of CA to acidified blood in the absence of adrenergic stimulation resulted in an increase in \(\Delta\text{PO}_2\), but was abolished when general NHE was inhibited.

3. If, this mechanism is operational \textit{in vivo}, during an acidosis, short-circuiting RBC NHE in conjunction with a highly pH-sensitive combined Bohr-Root effect system could markedly enhance tissue O\textsubscript{2} delivery over that which would occur in vertebrates possessing a Bohr effect alone, a finding that may shed further insight into the evolution of the Root effect Hbs, which evolved prior to RBC \(\beta\)NHE and specialized \textit{retia} at the eye and swimbladder.
### 3.6 Tables

Table 3.1 Concentrations of carbonic anhydrase (CA) and catecholamines such as noradrenaline (NA) and adrenaline (AD) and adrenergic agonists such as isoproterenol (ISO) that have been used in previous studies (including this one, Chapter 4) and determined in various tissues or levels of stress in rainbow trout.

<table>
<thead>
<tr>
<th>[CA]</th>
<th>[CA] justification</th>
<th>[catecholamine] or [l-agonist] justicification</th>
<th>[ISO] justification</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x 10⁻⁵ mM</td>
<td>Mammalian white skeletal muscle (Henry et al., 1997)</td>
<td>1.2 x 10⁻⁵ mM</td>
<td>Resting rainbow trout plasma (Tetens et al., 1988)</td>
</tr>
<tr>
<td>10⁻⁵ mM</td>
<td>Promotes rapid change in pH₄, tonometry experiments, rainbow trout (Motaïs et al., 1989)</td>
<td>5.3 x 10⁻⁷ mM</td>
<td>Resting rainbow trout plasma, overnight recovery from dorsal aorta cannulation surgery (Chapter 4)</td>
</tr>
<tr>
<td>1.5 x 10⁻⁴ mM</td>
<td>Rainbow trout red blood cells (Chapter 4)</td>
<td>1.7 x 10⁻⁵ mM</td>
<td>Rainbow trout blood in vitro (Motaïs et al., 1989)</td>
</tr>
<tr>
<td>2 x 10⁻⁷ mM</td>
<td>Stopped flow experiments with spiny dogfish, Squalus acanthias (Perry et al., 1999)</td>
<td>2 x 10⁻⁵ mM</td>
<td>Acute hypoxia, 60 min. exposure in rainbow trout (Tetens et al., 1988)</td>
</tr>
<tr>
<td>6.7 x 10⁻³ mM</td>
<td>Final concentration, bovine CALL injected into rainbow trout (Wood and Munger, 1994)</td>
<td>8.5 x 10⁻⁸ mM</td>
<td>After repeated burst swimming in rainbow trout (Butler et al., 1986)</td>
</tr>
<tr>
<td>5 x 10⁻⁸ mM</td>
<td>Mammalian red blood cell levels (Henry et al., 1997)</td>
<td>3 x 10⁻⁵ mM to 3.5 x 10⁻⁴ mM</td>
<td>Elicits half-maximum β-adrenergic pH₄ regulation in rainbow trout (Nikinmaa, 1992)</td>
</tr>
<tr>
<td>0.01 mM</td>
<td>Elicits a marked (&gt;1 pH unit) pH₄ recovery in β-adrenergically stimulated rainbow trout blood in vitro (Nikinmaa et al., 1990)</td>
<td>5 x 10⁻⁴ mM</td>
<td>in vitro studies on rainbow trout and eel (Anguilla anguilla) (Borgese et al., 1987; Romero et al., 1996)</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>A. anguilla blood in vitro (Hyde and Perry, 1990)</td>
<td>10⁻⁴ mM</td>
<td></td>
</tr>
<tr>
<td>10⁻³ mM</td>
<td>Elicits half-maximum β-adrenergic pH₄ regulation in rainbow trout (Nikinmaa 1982)</td>
<td>0.01 mM</td>
<td>Following injection into A. anguilla circulatory system (Hyde and Perry, 1990)</td>
</tr>
<tr>
<td></td>
<td>Following injection into rainbow trout circulatory system (Nikinmaa et al., 1990)</td>
<td>0.1 mM</td>
<td>Following injection into rainbow trout circulatory system (Nikinmaa et al., 1990)</td>
</tr>
<tr>
<td></td>
<td>Elicits maximum (saturated) response, rainbow trout blood in vitro (Caldwell et al., 2006)</td>
<td></td>
<td>Elicits maximum (saturated) response, rainbow trout blood in vitro (Tetens et al., 1988)</td>
</tr>
</tbody>
</table>
Table 3.2 The effect of HCl, isoproterenol (ISO), and carbonic anhydrase (CA) on ΔPO₂ (mm Hg) and pH, and associated half times (t₁/₂) in rainbow trout blood *in vitro* in a closed-system.

<table>
<thead>
<tr>
<th>Starting Hb-O₂ saturation (mm Hg)</th>
<th>Starting PO₂ (mm Hg)</th>
<th>HCl-induced ΔPO₂ (mm Hg)</th>
<th>pH disturbance from 7.93 ± 0.02</th>
<th>ISO-induced ΔPO₂ (mm Hg)</th>
<th>pH disturbance from 7.93 ± 0.02</th>
<th>CA-induced ΔPO₂ (mm Hg)</th>
<th>pH disturbance from 7.93 ± 0.02</th>
<th>Final pH</th>
<th>Final pH from 7.40 ± 0.00</th>
<th>Final Hct from 25.02 ± 0.12 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>53.7 ± 0.4a</td>
<td>26.5 ± 0.5a</td>
<td>44.0 ± 4.6a</td>
<td>-8.0 ± 1.9a</td>
<td>128.5 ± 2.6a</td>
<td>1.3 ± 0.1a</td>
<td>10.0 ± 0.0a</td>
<td>7.22 ± 0.04a</td>
<td>6.96 ± 0.10</td>
<td>42.66 ± 0.74a</td>
<td></td>
</tr>
<tr>
<td>54.1 ± 1.3a</td>
<td>55.4 ± 1.0a</td>
<td>41.0 ± 3.5a</td>
<td>-10.1 ± 1.4a</td>
<td>112.0 ± 2.9a</td>
<td>5.5 ± 1.1a</td>
<td>28.0 ± 0.7a</td>
<td>7.49 ± 0.8a</td>
<td>n/a</td>
<td>37.75 ± 1.04a</td>
<td></td>
</tr>
<tr>
<td>63.4 ± 0.7c</td>
<td>69.3 ± 1.c</td>
<td>39.7 ± 3.3</td>
<td>-14.3 ± 2.6a</td>
<td>80.7 ± 12.3a</td>
<td>13.0 ± 4.9c</td>
<td>23.0 ± 4.92a</td>
<td>7.43 ± 0.09c</td>
<td>7.14 ± 0.08a</td>
<td>35.13 ± 0.99a</td>
<td></td>
</tr>
<tr>
<td>67.8 ± 0.8c</td>
<td>77.3 ± 1.6c</td>
<td>33.7 ± 1.8a</td>
<td>-33.3 ± 1.4a</td>
<td>74.7 ± 5.6c</td>
<td>4.5 ± 1.0a</td>
<td>16.0 ± 0.0c</td>
<td>7.09 ± 0.00c</td>
<td>6.99 ± 0.01a</td>
<td>32.75 ± 2.20a</td>
<td></td>
</tr>
<tr>
<td>46.7 ± 0.0</td>
<td>45.3 ± 0.0</td>
<td>32.7 ± 12.4</td>
<td>ΔPH = -0.15 ± 0.04</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>0.02 ± 0.00</td>
<td>2.89 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>59.0 ± 1.6a</td>
<td>62.5 ± 1.2a</td>
<td>3.6 ± 1.2a</td>
<td>22.0 ± 4.3a</td>
<td>14.7 ± 4.9a</td>
<td>5.1 ± 2.2a</td>
<td>30.0 ± 0.7a</td>
<td>7.67 ± 0.08</td>
<td>7.06 ± 0.05</td>
<td>27.11 ± 0.37</td>
<td></td>
</tr>
<tr>
<td>65.2 ± 1.7a</td>
<td>72.5 ± 1.2a</td>
<td>5.1 ± 1.7a</td>
<td>14.7 ± 4.9a</td>
<td>5.1 ± 2.2a</td>
<td>30.0 ± 0.7a</td>
<td>7.67 ± 0.08</td>
<td>7.06 ± 0.05</td>
<td>27.11 ± 0.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>73.7 ± 1.3a</td>
<td>88.5 ± 2.0a</td>
<td>5.1 ± 1.7a</td>
<td>14.7 ± 4.9a</td>
<td>5.1 ± 2.2a</td>
<td>30.0 ± 0.7a</td>
<td>7.67 ± 0.08</td>
<td>7.06 ± 0.05</td>
<td>27.11 ± 0.37</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Data are presented for Series 1 and Series 2 (shaded region), and categorized by starting Hb-O₂ saturation (first column) and the magnitude of the initial pH disturbance (fifth column) of -0.15 (Series 1 and 2), -0.33 (Series 1), or -0.49 (Series 1) pH units. When no significant differences were observed for a variable within a pH disturbance group, data were pooled for the four starting Hb-O₂ saturations and a single value reported. For values presented for each Hb-O₂ saturation within a pH disturbance, capital letters that differ indicate significant differences. An asterisk indicates a significant difference from 0. All data are presented as means ±S.E.M.
Figure 3.1 A schematic illustrating the cascade associated with red blood cell (RBC) adrenergic stimulation at the level of the tissue. (CA, carbonic anhydrase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; βNHE, β-adrenergically-activated sodium proton exchanger; AE, anion exchange; NKA, Na\(^{+}\), K\(^{+}\), -ATPase; Hb, haemoglobin). Modified from (Bidani and Crandall, 1988; Cardenas et al., 1998; Heming, 1984).
Figure 3.2 A schematic illustrating the proposed changes in extracellular pH (pH_e), intracellular pH (pH_i), and the partial pressure of oxygen (PO_2) associated with initial contact with plasma-accessible CA (in red) and the proposed RBC βNHE short-circuiting at the tissues. (CA, carbonic anhydrase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; βNHE, β-adrenergically-activated sodium proton exchanger; AE, anion exchange; NKA, Na^+K^+-ATPase; Hb, haemoglobin). See description in Figure 3.1 for further details.
Figure 3.3 A schematic (not to scale) illustrating *in vitro* closed-system set-up. Compressed gas (A), mixed and regulated by a Wösthoff gas-mixing pump (B) was humidified and pumped into oscillating Eschweiler tonometers (1 of 4 in original set-up) (C) incubated at 12°C via water bath (D). Blood was drawn from the tonometer into a pre-gassed Hamilton syringe (E) and ejected into a 2 ml glass vial (F) through a gas-impermeable septum until overflow. The glass vial was secured in a glass water jacket (G) thermostatted at 12°C using a water bath (D) and affixed atop an electric stirring plate (H) set to rotate a mini-stir bar inside the glass vial at a rate of 400 revolutions • min⁻¹. Two fiber optic sensors (I) were guided through the septum via glass Pasteur pipettes and were connected to respective pH (J) and PO₂ (K) microx amplifiers and data acquisition systems, which were integrated to a desktop PC (L) where data were collected and analyzed.
Figure 3.4 Representative trace documenting real-time changes (in min) in blood PO₂ (red) and pHₑ (blue) in the *in vitro* closed-system over the 30-min duration of the experiment for Series 1 (HCl+ISO+CA). Dashed vertical lines represent the time at which the blood was exposed to the respective treatment indicated on the x-axis: HCl: hydrochloric acid, ISO: isoproterenol, and CA: carbonic anhydrase. Mean values ±S.E.M. for all variables measured or calculated in Series 1 are reported in Table 3.2.
Figure 3.5 Representative trace documenting real-time changes (in min) in blood PO\textsubscript{2} (red) and pH\textsubscript{e} (blue) in the \textit{in vitro} closed-system over the 30-min duration of the experiment for Series 2 (HCl+CA). Dashed vertical lines represent the time at which the blood was exposed to the respective treatment indicated on the x-axis: HCl: hydrochloric acid or CA: carbonic anhydrase. Mean values ±S.E.M. for all variables measured or calculated in Series 2 are reported in Table 3.2.
Figure 3.6 Representative trace documenting real-time changes in blood $PO_2$ (red) and $pH_e$ (blue) in the *in vitro* closed-system over the 30-min duration of the experiment from Series 3 (HCl+EIPA+CA). Dashed vertical lines represent the time at which the blood was exposed to the respective treatment indicated on the x-axis: HCl: hydrochloric acid, EIPA: ethylisopropylamiloride, and CA: carbonic anhydrase. Means ±S.E.M. are plotted on this trace, as only one starting Hb-O$_2$ saturation and one level of acidification were used for this experiment. Letters that differ within a variable, indicate a significant difference.
Figure 3.7 The ΔPO₂ (mm Hg) following addition of carbonic anhydrase to the *in vitro* closed-system, representing the potential benefit to oxygen delivery that could result from short-circuiting of βNHE at different starting Hb-O₂ saturations. Symbols are means ±S.E.M. from Series 1 and Series 2 (reported in Table 3.2) fitted with a second order polynomial regression. Green triangles and solid line (Series 1 experiments, $R^2 = 0.9902$) represents an initial acidification of $-0.49 \Delta pH_e$ prior to ISO and CA addition. Blue triangles and solid line (Series 1 experiments, $R^2 = 0.9994$) represents an initial acidification of $-0.33 \Delta pH_e$. White (Series 1, $R^2 = 0.9999$) and black (Series 2, $R^2 = 0.9782$) circles and associated black lines represent ΔPO₂ represents an initial acidification of $-0.15 \Delta pH_e$ units. All dashed lines represent the potential ΔPO₂ for the combined Bohr-Root effect system in rainbow trout blood for a ΔpHₑ of 0.5 (green) and 0.3 (blue) pH units as described in Chapter 2.
3.8 References


CHAPTER 4: A MECHANISM FOR ENHANCED OXYGEN DELIVERY TO MUSCLE TISSUE IN RAINBOW TROUT, *IN VIVO*¹

4.1 Introduction

The oxygen (O₂) transport system of teleost fish differs from that of other vertebrates. In teleosts, a decrease in pH greatly reduces the O₂ carrying capacity of blood (Root effect) as well as the affinity of Hb for O₂ (similar to higher vertebrates possessing a Bohr effect alone) even at high oxygen partial pressures (PO₂) (Berenbrink et al., 2005; Bohr et al., 1904; Brittain, 1987; Jensen, 2004; Nikinmaa, 2004; Pelster and Weber, 1991; Root, 1931; Root and Irving, 1943; Walsh and Milligan, 1993). This combined Bohr-Root effect manifests as a pronounced rightward and downward shift in the O₂ equilibrium curve (OEC). In Chapter 2, I determined that for a given pH change, the potential benefit to tissue O₂ delivery of a combined Bohr-Root effect system exceeds that of a Bohr effect alone and by up to an order of magnitude.

The combined Bohr-Root system is known to enhance O₂ delivery at only two specialized structures, the swimbladder and eye, where a localized acidosis drives O₂ from the blood to those tissues, resulting in PO₂ values that far-exceed that of blood in the dorsal aorta. For example, swimbladder PO₂ has been measured in excess of 50 atm or ~38,000 mm Hg (Alexander, 1966). Moreover, by restricting the acidosis to these tissues, through the use of retia, there is no negative effect on gill O₂ uptake that would otherwise occur if the acidosis extended into the general circulation (Alexander, 1966; Dafré and Wilhelm, 1989; Wittenberg and Haedrich, 1974; Wittenberg and Wittenberg, 1974). As proposed here, the Bohr-Root system may influence O₂ delivery in other tissues in the presence of a significant ΔpHₐᵥ. Under most

¹A version of this chapter has been prepared to submit for publication. J. L. Rummer, D. J. McKenzie, A. Innocenti, C. T. Supuran, and C. J. Brauner A mechanism for enhanced oxygen delivery to muscle tissue in rainbow trout.
conditions, the ΔpH_{a-v} in fish is not large, but the following mechanism may permit a large and localized acidosis at the level of the red blood cell that could greatly facilitate O_2 delivery.

During a general acidosis, teleosts protect RBC intracellular pH (pH_i) by activating βNHE (Borgese et al., 1987; Jensen, 2004; Malapert et al., 1997; Nikinmaa et al., 1984; Perry and Kinkead, 1989; Primmett et al., 1986; Salama and Nikinmaa, 1988), which prevents any negative effects that would ensue if O_2 loading at the gills was compromised. However, it is now known that a plasma-accessible CA can selectively short-circuit the βNHE in vitro (Chapter 3) because as H^+ and HCO_3^- is removed from the RBC by βNHE, CA in the plasma rapidly reforms CO_2, which rapidly back-diffuses into the RBC, decreasing Hb-O_2 affinity, and greatly elevated PO_2 in a closed-system. In fact, virtually the entire acid load added to the blood prior to βNHE activation in vitro appears to contribute to the short-circuiting mechanism, as large increases in PO_2 were observed with initial pH reductions (ΔpH) as small as -0.15 units. Since red muscle endothelia may possess plasma-accessible CA isoforms (Effros and Weissman, 1979; Geers and Gros, 1984; Geers and Gros, 2000; Henry and Swenson, 2000; Wang et al., 1998), βNHE short-circuiting could operate in vivo, which would greatly enhance general O_2 delivery in an animal with a Bohr-Root system.

I investigated this possibility in rainbow trout, where highly vascularized, oxidative slow-twitch red muscle (RM) tissue is anatomically separated from underlying fast-twitch, white muscle fibers. This makes it possible to directly monitor RM tissue O_2 levels, which is key to addressing whether this mechanism is operating in vivo. In rainbow trout, RMPO_2 has been monitored in real-time in only one other study. McKenzie and colleagues (2004) observed that upon exposure to hypoxia, muscle O_2 tensions decreased, but not nearly to the extent of the decreases observed in arterial blood O_2 levels (P_aO_2). Furthermore, RMPO_2 remained higher
than venous PO$_2$ (P$_O$2). They attributed both unusual responses in part to the Root effect, consistent with the hypothesis proposed here.

If RBC βNHE short-circuiting is operational in vivo and enhances O$_2$ delivery to the tissues, the addition of a membrane-impermeable CA inhibitor into the general circulation should abolish this response. For the first time in a teleost, a new, potent CA-inhibitor, compound-18 (C18) was used (Figure 4.1) for this purpose. Pyridinium benzenesulfonamide (C18) was first characterized in mammalian systems (Supuran et al., 1998) for its limited ability to cross plasma membranes due to its cationic nature. Therefore, C18 injected into the blood will only inhibit plasma-accessible CA isoforms, including CA IV isolated from bovine lung microsomes (Scozzafava et al., 1999; Supuran, 2008), and not those within the tissues, including the RBC. The objectives of this study were as follows: i) characterize a new isoform-specific, relatively membrane-impermeant C18 for the first time in a teleost and compare its efficacy with benzolamide and acetazolamide, which have been commonly used in rainbow trout and ii) determine whether βNHE short-circuiting may be operational within the red muscle by exposing rainbow trout to hypercarbia to induce a generalized blood acidosis in the absence and presence of C18 and observing changes in RMPO$_2$. I predict that exposure to hypercarbia will result in an elevation in RMPO$_2$ that will be abolished following C18 injection into the general circulation. The ultimate aim of this study was to test the hypothesis that CA-mediated RBC βNHE short-circuiting is operational in vivo in rainbow trout, a mechanism that creates a localized acidosis at the level of the RBC which elevates RMPO$_2$ through exploitation of the combined Bohr-Root effect.
4.2 Materials and Methods

4.2.1 Experimental animals, holding conditions

Rainbow trout, *(O. mykiss*, mean wet body mass 1,178 g), obtained from Spring Valley Trout Farm (Langley, British Columbia, Canada), were maintained in outdoor aquatic facilities in the Department of Zoology at the University of British Columbia until used for experiments. Fish were held in 8,000 l tanks supplied with flow-through Vancouver dechlorinated municipal tap water (average 12°C) under a natural photoperiod. Fish were fed every two days to satiation using commercial trout pellets (Skretting, Orient 4-0), but food was withheld 24 h prior to experimentation. All protocols complied with the guidelines approved by the Canadian Council on Animal Care, protocol # A07-0080.

4.2.2 Series 1: Characterizing C18 for use in rainbow trout

4.2.2.1 Comparing C18 to known teleost carbonic anhydrase inhibitors

A modified version of the electrometric delta pH CA assay (Gervais and Tufts, 1998; Henry, 1991; Maren, 1960; Maren and Couto, 1979; Sundram et al., 1986) was used to compare C18 with known teleost CA sulfonamide inhibitors (acetazolamide and benzolamide). The reaction buffer used was 6 ml 10 mM Tris Buffer (Tris(hydroxymethyl) amino-methane Sigma-Aldrich cat. no. 252859; St. Louis, MO, USA), pH 7.4 and thermostatted to 4°C. The pH was monitored continuously with a combination electrode (Beckman 511080) and meter (Beckman Φ 340 pH/Temperature meter) connected to Acqknowledge® Data Acquisition Software (Version 3.7.3, BIOPAC Systems, Inc.). Then, 100 μl of CO₂-saturated deionized (Millipore Direct-Q® #ZRQSOP030) water, also at 4°C, was added via a gas-tight Hamilton™ syringe. The slope of
the decrease in pH over time was monitored for 30 s and was used as the standard uncatalyzed rate of the CO$_2$ hydration reaction. This procedure was repeated with CA (from bovine erythrocytes, E.C. 4.2.1.1, Sigma-Aldrich cat. no. C3934; St. Louis, MO, USA), using a final concentration 10 µM. From Chapter 3, this concentration was known to short-circuit the βNHE response in rainbow trout RBCs *in vitro*. CA was dissolved in Tris buffer prior to the addition of CO$_2$-saturated water (this treatment would be the standard catalyzed rate). The assay was also repeated with three inhibitors (C18, benzolamide, and acetazolamide) in the presence of CA, and % CA inhibition was calculated. The C18 concentration used corresponded to 200 µM (Hilvo et al., 2008; Hisar et al., 2005), but five other concentrations (500, 100, 10, 1, 0.1 µM, data not shown) were tested as well to determine the most effective concentration to use prior to *in vivo* experimentation below. The final concentrations of benzolamide and acetazolamide used were 94 µM (Gilmour and Perry, 2004) and 100 mM (98% inhibition in rainbow trout for 6 h (Perry et al., 1999) respectively, both sufficient to inhibit a multitude of CA isoforms (Geers and Gros, 1984). Each trial was replicated 7- to 10-times, and slopes (over 30 s), as well as individual points were compared between uncatalyzed, catalyzed, C18-inhibited, benzolamide-inhibited, and acetazolamide-inhibited reactions via ANOVA and, when applicable, a post-hoc Holm-Sidak multiple comparisons test. All analyses were interpreted using $\alpha < 0.05$ to determine statistical significance.

4.2.2.2 Temporal exposure of rainbow trout red blood cell to C18

Fish were quickly removed from holding tanks and anaesthetized in a 50 l container filled with clean, well-aerated water containing benzocaine (0.2 mM final concentration, p-aminobenzoate). Blood was drawn from the caudal artery/vein into heparinized syringes, and RBCs were rinsed twice with and resuspended in ice-cold Cortland's saline (Wolf, 1963)
according to Caldwell et al. (2006). Haematocrit (Hct) was measured in duplicate by centrifuging 60 µl of the rinsed RBCs in heparinized micro-capillary tubes for 3 min at 12,000 rpm. The Hct was standardized to 25% by removing either saline or RBCs, and blood was stored at 4°C overnight until experiments commenced, ensuring that any catecholamines released during sampling had degraded (Bourne and Cossins, 1982; Randall and Perry, 1992; Tetens et al., 1988). The following morning, Hct of rinsed RBCs was readjusted to 25%, if necessary, and then divided in 6 ml aliquots in four Eschweiler tonometers, incubated at 12°C, and equilibrated for 1 h with a humidified gas mixture of 0.5% CO₂ balanced with air (21% O₂) using a DIGAMIX Wösthoff gas-mixing pump (DIGAMIX 275 6KM 422 Wösthoff, Bochum, Germany).

Following a 60-min incubation period, control samples were taken (time zero). A 1-ml aliquot of rinsed RBCs was withdrawn into a pre-gassed Eppendorf™ tube, centrifuged at 6,000 rpm for 3 min, and the saline was discarded. The remaining aliquot of RBCs was rinsed and resuspended 3 times with ice-cold Cortland’s saline, and when the last volume of saline was removed, the remaining RBC pellet was immediately frozen in liquid nitrogen for later analysis of CA activity. To determine the degree to which C18 permeated the RBCs, a bolus of stock C18 prepared in Cortland’s saline and DMSO (20% by volume, as would be prepared for in vivo use) was added to remaining rinsed RBCs in each tonometer (final concentration of 200 µM, as determined in 1a), and a 1-ml sample was withdrawn after 30, 60, 150, 210, and 260 min and prepared as described above. These samples were analyzed to determine the degree to which RBC CA activity was inhibited, presumably by C18 that had entered the RBC. All samples were stored at -80°C until later analysis.
4.2.2.3 Analyzing red blood cell for C18 activity

Frozen, packed RBCs were thawed on ice, washed twice with NaCl (0.9%), subjected to three freeze–thaw cycles using dry ice followed by the addition of five volumes of ice-cold distilled water, and then sonicated (Vibra Cell - VCX 130, #2.7775.82 Bioclass, Pistoia, Italy). The pH of the lysed RBC homogenate was adjusted to 8.7 using solid TRIS HCl (TRIS hydrochloride, Tris (hydroxymethyl) aminomethane hydrochloride, Sigma-Aldrich cat. no. T3253; St. Louis, MO, USA). The CA from the lysed RBCs was purified according to the methods of Bülbül and colleagues with activated CH Sepharose 4B gel (Amersham, Biosciences) and were used to determine the presence of and activity of the inhibitor (Bülbül et al., 2003). The ligand (4-(2aminoethyl)-benzensulfonamide) was dissolved in coupling buffer (100 mM NaHCO₃ pH = 8, containing 500 mM NaCl), gel packed and poured into a prepared activated CH Sepharose 4B-4-(2aminoethyl)-benzensulfonamide affinity column (1.36 cm x 30 cm), following the standard protocol supplied with the resin, eluted (500 mM sodium perchlorate in 100 mM sodium acetate pH 5.0), and column effluents were extensively dialysed into a solution of 10 mM Hepes (pH 7.5), 10 mM Tris HCl, 100 mM Na₂SO₄ and 1 mM ZnCl₂. Protein concentrations from column effluents were measured spectrophotometrically at 280 nm.

An SX.18MV-R Applied Photophysics (Oxford, UK) stopped-flow instrument was used to assay the CA catalytic activity of the samples by following the CA-catalyzed CO₂ hydration reaction either alone or in the presence of inhibitors as reported by Khalifah (Khalifah, 1971). A dual-reservoir reaction chamber was used, containing water (25°C), saturated with CO₂, as the substrate, and CA with or without inhibitors, prepared with 10 mM Hepes, pH 7.4 (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N -(2-ethanesulfonic acid), Sigma-Aldrich cat. no. H3375; St. Louis, MO, USA) as buffer, 100 mM Na₂SO₄ or NaClO₄ (for maintaining constant ionic strength; these anions are not inhibitory over the concentrations used here). Phenol red (0.2 mM) was used as the pH indicator in the CA
assay. Upon mixing of the two solutions, $H^+$ are formed, the indicator turns from red to yellow, and absorbance is measured at 557 nm, which is the maximum absorbance of the pH indicator. The change in absorbance is monitored for a period of 5–10 s.

4.2.2.4 Determining C18 activity inside the red blood cell

Data from parts 4.2.2.2 and 4.2.2.3 were reported as molar concentration of RBC CA ([CA]), absorbance of CA solutions (A) calculated as above, and $K_{cat}$, calculated as $A/([CA]$ over the 5-10s reaction period. Solutions were diluted 1:10 for the assay, therefore final concentrations were reduced 10-fold for calculations. Catalytic activity was assumed 100% at time zero (t=0), i.e. prior to C18 exposure; therefore subsequent $K_{cat}$ values were expressed as a ratio to t=0 and reported as % activity. Activity, mean ±S.E.M. (%), is presented graphically as a function of time following addition of C18 to RBCs, and activity at each sampling time was analyzed statistically via ANOVA and a post-hoc Holm-Sidak multiple comparisons test. All analyses were interpreted using $\alpha < 0.05$ to determine statistical significance.

4.2.3 Series 2: Effect of hypercarbia and the membrane-impermeant carbonic anhydrase inhibitor, C18, on real-time red muscle PO$_2$ in rainbow trout

4.2.3.1 Experimental animals, surgical procedures

Fish were netted, anaesthetized in a bath containing 0.1 mg l$^{-1}$ MS-222 buffered with 0.1 mg l$^{-1}$ NaHCO$_3$, and then immediately transferred to a surgery table. Gills were continuously irrigated with appropriately chilled, aerated, dechlorinated water containing diluted anaesthetic (0.075 mg l$^{-1}$ MS-222 and NaHCO$_3$). An indwelling cannula (PE50) was surgically implanted into the dorsal aorta (DA) according to Soivio et al. (1975), filled with heparinized (50,000 IU l$^{-1}$
lithium heparin, Sigma-Aldrich cat. no. H0878; St. Louis, MO, USA) Cortland’s saline (Wolf, 1963), and attached to a 1 ml tuberculin syringe so that blood pressure could be monitored while a fibre-optic sensor was implanted into the red muscle (RM) as described below.

An O$_2$-sensitive optical chemical fibre sensor (PreSens; Precision Sensing GmbH, Loligo Systems ApS, Denmark), with a 10 mm tapered Teflon-coated tip was connected to an Oxy-4 micro four-channel DAQ-TEMP oxygen meter and signal amplifier (cat #OX11700 Loligo Systems ApS, Denmark) via RS-232 serial cable and calibrated in humidified 12°C air (100% saturation) and a saturated sodium sulfite (Na$_2$SO$_3$, Sigma-Aldrich cat. no. 239321; St. Louis, MO, USA) solution (0% saturation). Phase angles were recorded and integrated into the algorithms provided with the manufacturer’s software package, FibSoft™, for PC Windows.

Guide marks were made with a permanent marker on the coated portion of the optode at 5 mm as well as 20 mm beyond the exposed glass, and the entire lead was secured inside a sterile glass Pasteur pipette (Figure 4.2). Each calibrated optode remained soaking in heparinized Cortland’s saline (100 IU ml$^{-1}$) until it was surgically implanted in to the fish as described below and similar to McKenzie and colleagues’ protocol (McKenzie et al., 2004).

The fish, DA cannulae in place, was repositioned on the surgery table such that the left lateral side was exposed (Figure 4.2). Moist sponges and paper towels were used to cover the entire fish, aside from the site of optode insertion, and irrigation tubes were moved from the opercula/gills into the buccal cavity. A small incision was made in the skin just dorsal to the lateral line and 40 mm posterior to the periphery of the opercular plate to reveal the underlying thin layer of RM. Two sutures were prepared along the same line, still dorsal to the lateral line, but 20 mm and 80 mm posterior to this incision. A third suture was prepared on the dorsal side of the fish, immediately anterior to the dorsal fin. The glass Pasteur pipette, optode encased, was advanced under the skin, parallel to the lateral line, for approximately 10 mm, ensuring that the blunted end was flush with the underside of the skin to avoid damaging the musculature. Then
the glass pipette was held at an angle approximately 45° to the skin, and the tip of the optode was advanced into the musculature for approximately 15 mm. Gentle pressure was applied to the skin atop the implanted optode, and the glass pipette was withdrawn along the coated lead, secured near the opposite end, and the optode secured in position with sutures to the skin (Figure 4.2). The position of the optode in the RM was confirmed post-mortem under a dissection microscope upon finishing the experiment, and each optode was removed and recalibrated to account for drift over the experimental period.

Following surgery, fish were transferred to a black Perspex box to recover, during which time red muscle PO\(_2\) (RMPO\(_2\)) was continuously monitored using the manufacturer’s software. The DA cannula was flushed with heparinized (10 IU ml\(^{-1}\)) Cortland’s saline (Wolf, 1963) and then attached to a pre-calibrated (as described above) stainless steel flow-through fiber optic O\(_2\) sensor (FTCH-MICRO Loligo Systems ApS, Denmark) to measure arterial blood PO\(_2\) (P\(_a\)O\(_2\)) during each blood sampling interval as specified below. The open end of the DA cannula was filled with heparinized Cortland’s saline, and sealed for the overnight recovery period. Both sensors, RMPO\(_2\) and P\(_a\)O\(_2\) were reconnected to the Oxy-4 micro four-channel system, and RMPO\(_2\) was logged every 2 min overnight, during which time fish were allowed to recover in black Perspex boxes in normoxic water (Figure 4.2).

**4.2.3.2 Sampling procedures**

For each sampling interval throughout the remainder of Series 2 and at the end of experimentation, blood was sampled according to the same protocol described below.

Approximately 1.5 ml of whole blood was withdrawn from the DA through a flow-through O\(_2\) optode to measure arterial PO\(_2\) (P\(_a\)O\(_2\)) and collected in a pre-gassed Eppendorf™ tube. Total O\(_2\) (TO\(_2\)) was measured in duplicate on 50 µl aliquots taken via Hamilton™ syringe according to
Whole blood [Hb] (mM per tetramer) was measured by adding blood to Drabkin’s solution (Sigma-Aldrich cat. no. D5941; St. Louis, MO, USA), measuring absorbance at 540 nm, and applying a millimolar extinction coefficient of 11. Haematocrit was determined by centrifuging 60 µl whole blood in heparinized micro-capillary tubes for 3 min at 12,000 rpm. Blood pH (pH_e) was measured using a thermostated capillary pH electrode (model BMS 3 MK 2 Blood Microsystem), in conjunction with a PHM 84 meter (Radiometer, Denmark). The remaining blood was analyzed for whole blood TCO\textsubscript{2} (model 965 Analyzer; Corning), and then centrifuged (3 min at 10,000 rpm), and plasma was removed to measure plasma TCO\textsubscript{2}. The freeze-thaw technique (Zeidler and Kim, 1977) was used to measure pH\textsubscript{i}, also using the BMS 3 Mk2 Blood Microsystem and PHM 84 meter. Plasma was flash frozen in liquid N\textsubscript{2} and stored at -80°C for later catecholamine analysis (see below). Unless noted, all assays were performed in triplicate.

4.2.3.3 Neuromuscular blockade immobilization procedure

Prior to experimentation, a baseline blood sample was collected from resting fish as described above. Then, via the DA, fish were injected with a skeletal muscle relaxant, tubocurarine ((+)-tubocurarine chloride hydrate, Sigma-Aldrich cat. no. T2379; St. Louis, MO, USA) an anti-nicotinic, neuromuscular blocking drug (Campbell and Gannon, 1976; Johnson et al., 1991; Oswald, 1978; Reilly and Thompson, 2007). Tubocurarine was used to keep the fish calm and maintain the integrity and placement of the implanted optode during experimentation. It is understood that surgical procedures but not anesthesia will elevate plasma catecholamines (Le Bras, 1982). Therefore it was assumed that these fish prior to, during, and following neuromuscular blockade would exhibit resting catecholamine levels because they were allowed
overnight recovery from surgery. This was later confirmed upon plasma catecholamine analyses (see below).

The concentration of tubocurarine used was chosen because it is known to cause ganglionic blockade in vagal pathways, sufficient to cause complete immobilization (Reilly and Thompson, 2007), while leaving afferent nerve traffic intact (Oswald, 1978). Tubocurarine was prepared with Cortland’s saline and administered for a final concentration of 0.1 mg ml\(^{-1}\) in the fish (assuming 5% blood volume). This concentration also resulted in apnoea, and therefore fish were force-ventilated in the black Perspex boxes (Figure 4.2) during the experiments, which also controlled for any changes in ventilation patterns that may have otherwise occurred during experimental treatments. Upon re-establishment of baseline physiological parameters (which occurred as early as 22 min, but usually ranged from 70-110 min) as described below, the experimental protocol commenced.

4.2.3.4 Experimental protocol: hypercarbia and C18 exposure

Once baseline RMPO\(_2\) measurements had been recorded, rainbow trout were exposed to flow-through hypercarbia (1.5% CO\(_2\), balance air), which was regulated with a Cameron gas-mixer (Cameron, prototype U.S.A.). Water PCO\(_2\) (P\(_w\)CO\(_2\)) was monitored continuously using a Clark-type PCO\(_2\) electrode (Model #E201, Loligo Systems ApS, Denmark) and Radiometer PHM 71 Acid-Base Analyzer (Radiometer, Denmark). Preliminary experiments revealed that exposure to 2% CO\(_2\) elicited an arterial blood acidosis that proved too severe for the fish, which is why 1.5% CO\(_2\) was chosen for this treatment. Fish were exposed to hypercarbia for approximately 20 min, during which real-time RMPO\(_2\) was monitored and logged every second. Following a 20-min hypercarbia exposure, blood samples were taken and analyzed as described above, and then water flowing into the Perspex boxes was replaced with normocarbic water.
Fish were exposed to normocarbia for at least 20 min, during which time RMPO$_2$ was monitored for recovery, and upon recovery, P$_a$O$_2$ was measured, and a blood sample was taken and analyzed as described above. Then, a bolus of C18 prepared in Cortland’s saline and DMSO (20% by volume) was injected via 1 ml syringe into the DA for a final concentration in the blood (assuming 5% blood volume) of 4 mg kg$^{-1}$ or 200 µM (404.87 g mol$^{-1}$ molecular weight) (Hilvo et al., 2008; Hisar et al., 2005), during which time RMPO$_2$ was monitored, and after 20 min had elapsed, a blood sample was taken and analyzed as described above. Fish were then re-exposed to hypercarbia, as mentioned above, RMPO$_2$ monitored, and samples collected and analyzed. All blood samples withdrawn were replaced with an equal volume of Cortland’s saline, and all injections were done slowly, over the course of 1 min.

4.2.3.5 Plasma catecholamines

Catecholamine levels, noradrenaline (NA), adrenaline (AD), and combined, were measured in rainbow trout plasma samples collected as described above (n = 7 to 10). To compare these with true resting catecholamine levels, an additional group of fish (300-600 g wet body mass) was equipped with a DA cannula, as described above, and allowed to recover for 24-48 h (Bourne and Cossins, 1982; Randall and Perry, 1992; Tetens et al., 1988). After this time, 1.5 ml of blood was collected from each fish into heparinized syringes, withdrawn into an Eppendorf™ tube, centrifuged at 6,000 rpm for 3 min, plasma removed and expired into a separate Eppendorf™ tube, and immediately frozen in liquid N$_2$ (n=13). All samples were shipped on dry ice to be analyzed by Dr. S.F. Perry at the University of Ottawa.

Plasma catecholamine levels were determined on alumina extracted samples (75-200 µl) using HPLC with electrochemical detection (Woodward, 1982) and 3,4-dihydroxybenzylamine hydrobromide (DHBA) as an internal standard. The HPLC incorporated a Varian ProStar 410
solvent delivery system (Varian Chromatography Systems, Walnut Creek, CA) coupled to a Decade II electrochemical detector and VT-03 electrochemical flow cell (Antec USA, Hanover MD).

4.2.3.6 Calculations and statistical analyses

Data are presented as mean ± S.E.M. Mean corpuscular haemoglobin concentration (MCHC) was calculated as Hb/(Hct/100). Haemoglobin saturation (SO₂) was calculated by dividing TO₂ (after subtracting physically dissolved O₂ according to Boutilier et al. (1984) by the theoretical maximum carrying capacity of the rinsed RBCs based upon the tetrameric Hb concentration obtained spectrophotometrically according to Tucker (1967). Blood PCO₂ was calculated from plasma TCO₂ and pHₑ as described previously by Brauner et al. (2004) using the CO₂ solubility coefficient and pK published for rainbow trout (Boutilier et al., 1984) and by rearranging the Henderson-Hasselbalch equation. All PO₂ (RM and arterial) data were saved as text files and analyzed using Acqknowledge® Data Acquisition Software (Version 3.7.3, BIOPAC Systems, Inc.). Catecholamine concentrations for both noradrenaline (NA) and adrenaline (AD) were reported for each individual fish at each sampling interval and statistically compared to the mean resting levels derived from the additional groups of resting, cannulated fish. All other data were compared statistically between treatments, and statistical differences were detected via paired t-test or repeated measures ANOVA and, when necessary, a post-hoc Holm-Sidak multiple comparisons test. All statistical analyses were conducted using SigmaStat for Windows 3.5.0.54 (Systat Software, Inc., 2006), and all analyses were interpreted using α < 0.05 to determine statistical significance.
4.3 Results

4.3.1 Series 1: Characterizing C18 for use in rainbow trout

The rate of CO$_2$ hydration was significantly slower in the absence of CA compared to when CA was present (P<0.001). This is indicated by the significantly shallower slope over the first 30 s and significantly lower absolute pH value over the first 6 min following the addition of CO$_2$-saturated water (P<0.001; Figure 4.3A). C18, benzolamide, and acetazolamide all inhibited CA, as indicated in Figure 4.3B, and a concentration of 200 µM C18 most closely resembled the inhibition profiles for benzolamide and acetazolamide at concentrations commonly used to inhibit CA *in vivo* and *in vitro* using rainbow trout. All inhibitors resulted in significantly shallower slopes over the first 30 s (P<0.001) and significantly lower absolute pH values (P<0.001) within 3–4 min following the addition of CO$_2$-saturated water to the CA assay compared to the CA-catalyzed reaction (Figure 4.3B).

Prior to C18 exposure, the average RBC CA concentration in rainbow trout RBCs was 153.6 ±9.1 µM (data not shown) and its catalytic rate was 7.2 x 10$^4$ s$^{-1}$ (expressed as K$_{cat}$; data not shown), which was assumed to represent 100% RBC CA activity (Figure 4.4). Following a 30-min incubation period in the presence of C18, K$_{cat}$ decreased 86% to 6.1 x 10$^4$ s$^{-1}$ (data not shown), which was not statistically different from initial activity values (Figure 4.4). After 60-, 150-, 210-, and 260-min exposures to C18, K$_{cat}$ significantly decreased to 5.1 x 10$^4$, 4.5 x 10$^4$, 3.7 x 10$^4$, and 2.9 x 10$^4$ s$^{-1}$ (respectively, data not shown), which resulted in a significant decrease in RBC CA activity by 27, 37, 47, and 67%, respectively (Figure 4.4).
4.3.2 Series 2: Effect of hypercarbia in the presence and absence of the membrane-impermeant carbonic anhydrase inhibitor, C18, on real-time red muscle PO$_2$ in rainbow trout

Baseline blood variables, following overnight recovery, are reported in Figures 4.5 and 4.6 and Table 4.1 as starting conditions for the nine experiments. The skeletal muscle relaxant, tubocurarine, calmed fish and, while RMPO$_2$ often initially fell to ~10 mm Hg, all variables were restored as fast as 22 min, but on average 90 ± 21 min. Figure 4.5 displays a representative trace of the entire experimental sequence of Series 2 on RMPO$_2$, and Figure 4.6 presents mean values for pH$_i$, pH$_e$, P$_a$O$_2$, and RMPO$_2$. Table 4.1 presents all other baseline and treatment-induced changes in physiological variables. Post-tubocurarine recovery values represent the baseline for comparison of all variables measured during the subsequent experimental treatments.

Post-tubocurarine recovery, hypercarbia (1.5% CO$_2$) resulted in a significant increase in RMPO$_2$ from 47.1 ± 6.4 to 75.2 ± 2.2 mm Hg and significantly decreased pH$_i$ from 7.23 ± 0.01 to 7.13 ± 0.02 (Figure 4.5 and 4.6) and pH$_e$ from 8.09 ± 0.04 to 7.79 ± 0.04 (Figure 4.6). Baseline variables were reestablished when fish were returned to normocarbia (Table 4.1 and Figure 4.6).

Injection of C18 during normocarbia had no significant effect on either pH$_e$ or RMPO$_2$ relative to normocarbia values. Exposure to hypercarbia (C18+1.5%CO$_2$) then significantly reduced pH$_i$ (to 7.14 ± 0.02), but not pH$_e$ or RMPO$_2$ (Figure 4.6). Thus, C18 abolished the large increase in RMPO$_2$ observed during hypercarbia.

Arterial blood PO$_2$, which was initially at 132.1 ± 5.5 mm Hg, did not change significantly at any sampling interval (Figure 4.6), and Hb-O$_2$ saturation remained high, ranging from 86.8 to 99.0%. These saturations were consistent with those interpolated from the OEC (Chapter 2) for the respective P$_a$O$_2$. There was a significant increase in [Hb] at the return to normocarbia sampling interval (P<0.01) relative to starting conditions but no significant changes in Hct or MCHC (Table 4.1). RBC CO$_2$ content (TCO$_2$) was lowest in post-tubocurarine
recovery, and was significantly elevated over this value upon exposure to C18 + 1.5% CO₂ (P<0.01; Table 4.1). There were no significant changes in plasma TCO₂ (Table 4.1), but blood PCO₂ was significantly elevated during both hypercarbia treatments (with and without C18) (P<0.01) relative to post-tubocurarine recovery values (Table 4.1).

The ΔPO₂ depicted in a representative trace (Figure 4.5) and as mean values in Figure 4.7 refers to the change in RMPO₂ upon exposure to hypercarbia (1.5% CO₂). Hypercarbia alone elevated ΔPO₂ by 30.9 ±7.6 mm Hg (65% increase). As hypothesized, the elevation in ΔPO₂ was eliminated when fish were exposed to hypercarbia following injection of C18 (ΔPO₂ = -1.2 mm Hg) (P<0.001) (Figure 4.7).

Plasma catecholamines in resting fish 24 to 48 h following DA cannulation surgery were 4.9 ±2.8 and 0.4 ±0.2 nM for noradrenaline (NA) and adrenaline (AD) respectively. Because of high individual variation in plasma catecholamine levels, individual results are displayed in Figures 4.8A and 4.8B along with published NA and AD levels in resting rainbow trout, values for fish exercised to exhaustion, and the levels that will half-maximally stimulate the βNHE on the RBC included for reference (Motais et al., 1987; Niina et al., 1988; Nikinmaa, 1992; Salama and Nikinmaa, 1989). Except for the two individuals that had significantly elevated plasma NA levels following the overnight recovery (P<0.001), plasma NA and AD were not significantly elevated throughout the rest of the experiment. No relationships could be detected between the two individuals that exhibited elevated NA or AD at any sampling interval and any other physiological variable or RMPO₂ (P>0.05) (Figure 4.8).

**4.4 Discussion**

The level of hypercarbia used in this study resulted in a mild acidosis (-0.1 pHᵢ and -0.3 pHₑ units) but was associated with a dramatic 30 mm Hg elevation in RMPO₂, corresponding to
a 65% increase over control levels. When fish were re-exposed to hypercarbia, following injection of a relatively membrane-impermeant CA inhibitor, C18, this response was abolished. This finding supports my initial hypothesis that CA-mediated RBC βNHE short-circuiting is operational in vivo in rainbow trout, and results in a localized acidosis at the level of the RBC elevating RMPO₂ through exploitation of the combined Bohr-Root effect. Furthermore, it highlights the importance of extracellular (membrane-associated) CA to this response. Interestingly, exposure to this level of hypercarbia did not significantly elevate catecholamine levels, suggesting that β-adrenergic activation of the NHE may not be a pre-requisite for this response. A more general RBC NHE may be involved, as was observed in vitro (Chapter 3). The increase in RMPO₂ has large implications to O₂ delivery and far-exceeds anything previously observed in vertebrates. The present study, which represents the first to use C18 in a teleost, also confirmed the effectiveness of this new membrane-impermeant CA inhibitor for use in rainbow trout.

4.4.1 Critique of techniques

The response to hypercarbia (elevated water PCO₂) exposure in this study was as predicted, and changes in blood PCO₂ and pH were relatively modest and largely consistent with those reported in previous studies. The pH changes (from 7.99 to 7.79) that Smith and Jones (1982) demonstrated in hypercarbic rainbow trout were similar to the pH changes observed here after 20-30 min. However, a 20-30 min hypercarbia exposure decreased blood pHc from 8.09 to 7.79 and increased blood PCO₂ from 1.7 to 4.0 mm Hg. McKendry and Perry (2001) observed arterial pH decreasing to 7.43 and blood PCO₂ increasing to 6.15 mm Hg during a similar 20-min 1.5% CO₂ hypercarbic exposure (in the water). Overall, the magnitude of the changes in pH and PCO₂ induced in this study were consistent with previous studies on non-paralyzed fish and only
slightly below those expected at 90% $U_{crit}$ or post-exercise for rainbow trout (Brauner, 1995; Perry and Wood, 1989). Thus, disturbances to blood acid-base status of the magnitude chosen in this study are likely encountered \textit{in vivo} in normocarbic rainbow trout and therefore could enhance tissue O$_2$ delivery under such conditions.

While hypercarbia typically induces hyperventilation in elasmobranchs and teleosts (Graham et al., 1990; Heisler et al., 1988; Kinkead and Perry, 1991; Perry and Gilmour, 1996; Wood and Munger, 1994), I chose to control for this by paralyzing and force-ventilating the fish to monitor changes in RMPO$_2$ with greater sensitivity during the experimental treatments. For example, in trout exposed to similar levels of hypercarbia as employed here, ventilation volume nearly doubled within 5 min of exposure (Smith and Jones, 1982). In this study, fish were ram-ventilated at a water flow rate of approximately 1,600 ml min$^{-1}$ kg$^{-1}$. This volume is up to 6-fold greater than the ventilatory volume calculated in non-paralyzed hypercarbia-exposed fish (Smith and Jones, 1982), but was necessary to recover tissue PO$_2$ (which fell to near zero) following tubocurarine injection. Thus, force ventilation was required to ensure recovery and monitor changes in RMPO$_2$ without the confounding effects of changes in ventilation evident in the aforementioned studies.

Hypercarbia was also chosen as the treatment to induce an acid-base disturbance because it has only minor affects on metabolic rate. Therefore, any changes in RMPO$_2$ could easily be detected over an unchanging background O$_2$ consumption rate. Thus, changes in RMPO$_2$ observed here are likely higher than that would occur during exposure to other stressors or exercise. However, it is likely that a substantial $\Delta$PO$_2$ would still be evident during other stressors, especially if changes to blood pH are more pronounced than were detected here. Ultimately, this preparation was chosen to maximize the chances of observing the proposed phenomenon.
4.4.2 The role of catecholamines and enhanced oxygen delivery

Hypercarbia elevates catecholamine levels indirectly through its effects on O₂ transport, but neither NA nor AD levels were elevated in hypercarbia-exposed fish in this study. Despite such marked changes in pH, McKendry and Perry did not observe increases in plasma NA in hypercarbia-exposed fish either, consistent with these findings, but they did observe significant elevations in plasma AD (McKendry and Perry, 2001). In many studies, a strong correlation has been noted between catecholamine levels and arterial blood PCO₂ in rainbow trout (Kinkead et al., 1993; Perry and Gilmour, 1996; Perry and Kinkead, 1989; Thomas, 1994), but that was not the case here. This suggests that the mechanism proposed to create a large pHₐ-v to permit enhanced general tissue O₂ delivery via the Bohr-Root effect may also be operational during exposure to a mild stressor, in the absence of β-adrenergic activation of βNHE. It is not known whether another NHE isoform on the RBC membrane is activated, or an alternate pathway for activating the βNHE exists, but the findings are consistent with those of Chapter 3 (in vitro studies). The NHE1 isoform in particular is activated via other pathways, such as volume or osmotic disturbances and HIF1α, to name a few (Claiborne et al., 1999; Deigweiher et al., 2008; Shimoda et al., 2006; Wakabayashi et al., 1997; Yun et al., 1995). Clearly additional experiments are required to identify the specific mechanism involved. While this was a surprising finding, the fact that the system is operational in the absence of elevated catecholamine levels suggests a broader range of conditions for enhanced general O₂ delivery by this mechanism than originally envisaged.

4.4.3 Enhanced oxygen delivery in teleost fishes

To date, only two studies have ever monitored real-time red muscle PO₂ in a teleost, and both support enhanced tissue O₂ delivery relative to mammals. McKenzie and colleagues
measured RMPO$_2$ prior to, during, and following mild hypoxia exposure and during both sustained and exhaustive exercise (McKenzie et al., 2004). The RMPO$_2$ values that they measured (between 50 and 60 mm Hg) are consistent with the values reported here, but are higher than in mammals (25-35 mm Hg; Behnke et al., 2001; Gutierrez et al., 1989; Hutter et al., 1999; Jung et al., 1999; Suttner et al., 2002), despite similar starting arterial and mixed venous PO$_2$ values (Farrell and Clutterham, 2003; Holeton and Randall, 1967; Hutter et al., 1999; McKenzie et al., 2004). Beyond this, RMPO$_2$ is consistently high in teleost fishes (Egginton et al., 2002; Johnston, 1987) and remains elevated during stress (hypoxia, exercise, and hypercarbia) (this study and McKenzie et al., 2004) and above P$_a$O$_2$ (approximately 20 mm Hg; Farrell and Clutterham, 2003) despite decreases in P$_a$O$_2$, as well as above tissue PO$_2$ in rats and humans (Behnke et al., 2001; Gutierrez et al., 1989; Hutter et al., 1999; Jung et al., 1999; Suttner et al., 2002). My preparation allowed me to advance this state of knowledge because I could monitor P$_a$O$_2$ while directly observing the effects of a hypercarbia-induced general acidosis on RMPO$_2$ in the presence and absence of a plasma-accessible CA inhibitor allowing characterization of the mechanism proposed to be operating in vivo.

In this study, an increase in RMPO$_2$ of approximately 30 mm Hg following exposure to hypercarbia was observed, which is large, considering that Bohr effect alone systems would not realize even a 2 mm Hg PO$_2$ increase to facilitate tissue O$_2$ delivery (Chapter 2). In this study, it is clear that a mild acidosis (increase in blood PCO$_2$ to 4 mm Hg) can potentially greatly benefit red muscle O$_2$ delivery, which could clearly benefit general tissue metabolism and even locomotion. It would also be interesting to consider the teleost cardiac system, as many teleosts, unlike birds and mammals, lack coronary arterial O$_2$ supply to the heart and rely strictly on cardiac circulation supplied by deoxygenated venous blood (Farrell and Clutterham, 2003; Farrell and Jones, 1978). During stress or exercise, tissue O$_2$ demand could drastically decrease venous content, allowing for limited supply to the hard-working heart. If teleosts have a
mechanism in place, where locomotory tissue oxygenation is enhanced via the Bohr-Root effect and plasma-accessible CA, \( P_{O_2} \) may be protected from sharp decreases, thus ensuring adequate \( O_2 \) to the myocardium (Farrell and Clutterham, 2003), another potential level at which the combined Bohr-Root effect may facilitate general \( O_2 \) delivery.

4.4.4 Verifying the role of plasma-accessible carbonic anhydrase

Monitoring tissue \( O_2 \) status in real-time while exposing fish to a stressor and a new pharmaceutical, C18, has proven a powerful combination to validate the mechanism proposed for permitting enhanced general \( O_2 \) delivery via the Bohr-Root effect. Hypercarbia increased RMPO\(_2\) with a plasma-accessible CA-dependent mechanism that was unlikely associated with effects on RBC CA (Figure 4.7). The C18 inhibitor does not enter the RBC for at least 30 min following \textit{in vitro} exposure to incubated rinsed and resuspended rainbow trout RBCs (Figure 4.4). Following exposure for 60 min, at least 75% of RBC CA activity remains functional (Figure 4.4), which was well within the duration over which experiments were completed following C18 injection in Series 2. Until this study, C18 had only been used in rats, and with great success. Not only was C18 a potent CA inhibitor and relatively impermeant to RBC membranes, it did not affect other physiological variables \textit{in vivo} (Scozzafava et al., 2000).

Compared to C18, benzolamide, another low-molecular weight CA inhibitor that has been commonly used in fish, easily permeates the rat RBC such that after 2 h, the final concentration in the rat RBCs was 15,500-times the trace concentration of C18 (Scozzafava et al., 2000).

Successful use of C18 in fish is a great advance because to date localizing and determining function of plasma-accessible CA isoforms has been difficult in fish, despite their ubiquity among vertebrates (Gilmour and Perry, 2009; Nikinmaa, 1990). Results from this study support the presence of a plasma-accessible CA isoform in rainbow trout RM with at least one purpose
potentially related to enhanced tissue oxygenation. Further research is necessary to localize and characterize these functionally significant isoforms, but the successful, selective inhibition of a plasma-accessible isoform and association with a function as important as O₂ delivery, as determined in this study is a significant contribution.

4.4.5 Conclusions

Teleost fish evolved an extraordinary O₂ delivery mechanism that greatly enhances O₂ delivery in select locations provided a large pH\(_{a-v}\) can be established, which I have now demonstrated possible in the general circulation. Thus, previous speculation has been given empirical and quantitative support. Here, rainbow trout red muscle exhibited O₂ delivery dependent upon plasma-accessible CA, which short-circuits the βNHE or possibly a general “housekeeping” NHE on the RBC membrane. In doing so, an acidosis is recycled back into the RBC, and a sufficient pH\(_{a-v}\) required to permit the Bohr-Root effect to enhance O₂ delivery \textit{in vivo} is realized.

Root effect Hbs evolved 150 MY prior to the first appearance of the choroid \textit{rete} at the eye in teleosts, one of the two known anatomical structures (\textit{retia}) associated with this exceptional O₂ delivery system evident in half of all extant vertebrate species. If the Bohr-Root effect system is facilitating general O₂ delivery during stress or exercise, as supported here and by McKenzie et al. (2004), this would help shed insight into early selection for Root effect Hbs 150 MY before their use at the teleost retina.
4.5 Chapter Summary

1. When rainbow trout are exposed to elevated water CO$_2$ (hypercarbia), a mild acidosis develops, and PO$_2$ at the red muscle (RMPO$_2$) is dramatically elevated from an average of 47 to 75 mm Hg, an increase averaging 31 mm Hg. This corresponds with a 65% elevation in RMPO$_2$ over resting conditions and a finding with great implications to an enhanced O$_2$ delivery system in teleosts.

2. The CA inhibitor, C18, completely and significantly eliminated the increase in hypercarbia-induced RMPO$_2$ in rainbow trout, demonstrating the importance of extracellular (membrane-associated) CA to the proposed mechanism.

3. Adrenergic stimulation of the RBC NHE may not be a critical component, as catecholamine levels were not consistently elevated above resting values. There may be non-adrenergic activation of RBC NHE.

4. In rainbow trout red muscle, PO$_2$ was consistently higher than has been reported in humans and rats, suggesting a fundamental difference between teleost and mammalian O$_2$ delivery systems.
4.6 Tables

Table 4.1. Haematological and CO$_2$-related variables measured at each sampling interval in Series 2, where the effect of hypercarbia on real-time red muscle PO$_2$ in rainbow trout, and the effects of the membrane-impermeant carbonic anhydrase inhibitor (C18) were tested.

<table>
<thead>
<tr>
<th></th>
<th>Starting conditions</th>
<th>Post-tubocurarine recovery</th>
<th>1.5% CO$_2$</th>
<th>Return to normocarbia</th>
<th>C18 exposure</th>
<th>C18 + 1.5% CO$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Hb] (mM)</td>
<td>1.0$^a$</td>
<td>1.4$^{ab}$</td>
<td>1.1$^{ab}$</td>
<td>1.6$^b$</td>
<td>1.3$^{ab}$</td>
<td>1.1$^{ab}$</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>25.9</td>
<td>31.2</td>
<td>31.6</td>
<td>34.6</td>
<td>25.9</td>
<td>28.3</td>
</tr>
<tr>
<td>MCHC (mM)</td>
<td>4.3</td>
<td>4.4</td>
<td>3.6</td>
<td>4.5</td>
<td>4.3</td>
<td>3.7</td>
</tr>
<tr>
<td>RBC TCO$_2$ (mM)</td>
<td>3.2$^{ab}$</td>
<td>0.9$^a$</td>
<td>2.8$^{ab}$</td>
<td>1.9$^{ab}$</td>
<td>1.8$^{ab}$</td>
<td>4.4$^b$</td>
</tr>
<tr>
<td>Plasma TCO$_2$ (mM)</td>
<td>9.9</td>
<td>9.7</td>
<td>11.2</td>
<td>9.8</td>
<td>10.0</td>
<td>11.4</td>
</tr>
<tr>
<td>Blood PCO$_2$ (mm Hg)</td>
<td>2.4$^{ab}$</td>
<td>1.7$^{a}$</td>
<td>4.0$^c$</td>
<td>2.5$^{ab}$</td>
<td>1.8$^a$</td>
<td>3.3$^b$</td>
</tr>
</tbody>
</table>

Note: Lower-case letters that differ demarcate significant differences between treatment groups within a given physiological variable. As a reference, post-tubocurarine recovery data are outlined with a heavy black line, as these data represent the new baseline values for each variable to which subsequent treatment values are compared. All data are presented as means ±S.E.M, and all analyses were interpreted using $\alpha < 0.05$ to determine statistical significance.
4.7 Figures

Figure 4.1. Structure of compound-18 (C18), the low molecular weight (404.87 g mol$^{-1}$), membrane-impermeant, sulphonamide first characterized by Scozzafava et al. (Scozzafava et al., 1999). The CA inhibitor, C18, has been demonstrated to inhibit human (cloned) CA I and II isozymes as well as CA IV isolated from bovine lung microsomes (Inhibition, $K_i =$ 3, 13, and 10 nM respectively) (Scozzafava et al., 1999).
**Figure 4.2** A schematic and photographic representation of the surgical procedure employed to implant the fibre optic O$_2$ probe into the red muscle (RM) of rainbow trout and the recovery apparatus. An O$_2$-sensitive optical chemical fibre sensor with a 10 mm tapered Teflon-coated tip (A) and an additional 25 mm of the coated portion of the optode (B) with guide marks made at 5 mm as well as 20 mm beyond the exposed glass, was secured inside a sterile glass Pasteur pipette (D). Two sutures (E) were prepared dorsal to the lateral line, approximately 20 and 80 mm posterior to the periphery of the operculum. A third suture was prepared on the dorsal side of the fish, immediately anterior to the dorsal fin (E). The exposed glass tip of the sensor (A) as well as 10 mm of the coated part of the lead (B) were collectively (C) advanced under the skin into the RM and secured in place with aforementioned three sutures (E) (as seen in upper photograph). The previously implanted DA cannula (G) was connected to a 1cc tuberculin syringe and flushed periodically with heparinized Cortland’s saline. The entire lead and fiber optic O$_2$ sensor (F) was connected to an Oxy-4 micro four-channel DAQ-TEMP oxygen meter and signal amplifier via RS-232 serial cable (not shown). Post-surgery, fish were transferred to a black Perspex box to recover overnight, during which time they were ram ventilated (bottom photograph) until regular opercular movement resumed. This ram-ventilation apparatus (bottom photograph) was used again on fish following exposure to the neuromuscular blockade just prior to the experimental procedure.
Figure 4.3 The electrometric delta pH carbonic anhydrase (CA) assay reporting the change in pH over time following addition of CO₂ equilibrated water in the presence (solid circles) and absence (red inverted triangles) of carbonic anhydrase (CA) (Panel A) and with CA in the presence of CA inhibitors, C18 (blue squares), benzolamide (yellow triangles), and acetazolamide, (green diamonds), final concentrations 200 µM, 94 µM, and 100 mM respectively (Panel B). Data are mean ±S.E.M. Upon the addition of CO₂-equilibrated water at t = 3 min (x-axis), asterisks represent significant differences from the CA-catalyzed reaction at 3.5, 4, 5, 6, 7, 8, 9, and 10 min.
Figure 4.4 Red blood cell (RBC) CA activity in rinsed RBCs exposed to C18 (final concentration 200 µM) *in vitro*. Letters that differ indicate significant differences, symbols indicate mean ±S.E.M. Note: RBC CA activity is not significantly reduced until 60 min following exposure to C18.
Figure 4.5 Representative trace illustrating on-line changes in rainbow trout RMPO$_2$ immediately following an overnight recovery from surgery (implanting of DA cannula and RM tissue O$_2$ sensor) and throughout the experimental procedure of Series 2. The y-axis represents RMPO$_2$ in mm Hg, and the x-axis indicates elapsed time in min with experimental treatments indicated by vertical lines. The change in PO$_2$ upon hypercarbia exposure with and without C18 in circulation is represented as ΔPO$_2$. In the interest of space, and in some cases clarity, intervals of time demarcated by hash marks were removed.
Figure 4.6 Graphs representing means ±S.E.M. for intracellular pH (pH$_i$) (panel A), extracellular pH (pH$_e$) (panel B), arterial PO$_2$ (P$_a$O$_2$ in mm Hg, panel C), and red muscle PO$_2$ (RMPO$_2$ in mm Hg, panel D) upon recovery from surgery (starting conditions), recovery from neuromuscular blockade (post-tubocurarine recovery), and upon each experimental treatment in rainbow trout. Lower-case letters that differ demarcate significant differences between treatment groups within a given physiological variable. As a reference, post-tubocurarine recovery data are outlined with a heavy black line, as these data represent the new baseline values for each variable to which subsequent treatment values are compared. Statistical significance is indicated with p-values in the upper right corner of each panel, if applicable (non-significance is indicated “NS”). All data are presented as means ±S.E.M, and all analyses were interpreted using $\alpha < 0.05$ to determine statistical significance.
Figure 4.7 The mean ±S.E.M. absolute change in RMPO$_2$ ($\Delta$RMPO$_2$, y-axis) relative to post-tubocurarine recovery (see Figure 4.6) following exposure to hypercarbia in the absence (1.5% CO$_2$) and presence of the CA inhibitor C18 (C18+ 1.5% CO$_2$) in circulation in rainbow trout. An asterisk demarcates a significant difference from post-tubocurarine recovery values (P<0.001).
**Figure 4.8** Plasma noradrenaline and adrenaline (nM) levels in individual rainbow trout (a single colour represents the same animal) upon recovery from surgery (starting conditions), post-tubocurarine recovery, and following each experimental treatment in Series 2. Published noradrenaline and adrenaline levels for resting fish (indicated by the bottom gray bar), following exhaustive exercise, and values associated with βNHE half maximal response are indicated by gray lines as indicated on the right y-axis for both panels (see text for references and further details). Statistical significance (relative to resting levels measured in this study: 4.9 ±2.85 nM and 0.42 ±0.23 nM respectively) is indicated in the top right corner of each panel. Please refer to text for further details.
4.8 References


Le Bras, Y. M. (1982). Effects of anaesthesia and surgery on levels of adrenaline and noradrenaline in blood plasma of the eel (*Anguilla anguilla* L.). *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology** 72**, 141-144.


CHAPTER 5: GENERAL DISCUSSION

The combined Bohr-Root effect is an extraordinary \( \text{O}_2 \) delivery mechanism that evolved only in teleost fishes, and is well known for oxygenating the eye and inflating a swimbladder at depth. In this thesis, I hypothesized that a mechanism exists in teleosts where a generalized acidosis can be localized and recycled at the level of the RBC, thus permitting the combined Bohr-Root effect to enhance general \( \text{O}_2 \) delivery. For this hypothesis, I first determined that the potential for \( \text{O}_2 \) delivery via a combined Bohr-Root effect was an order of magnitude greater than with a Bohr effect alone. I then proposed a novel mechanism through which a large arterial-venous red blood cell pH difference (\( \Delta \text{pH}_{\text{a-v}} \)) could be achieved in the general circulation to permit enhanced \( \text{O}_2 \) delivery via the Bohr-Root effect. I tested and supported this mechanism where RBC \( \beta \text{NHE} \) is adrenergically-activated during stress and subsequently short-circuited by plasma-accessible CA at the tissue, at both the in vitro and in vivo levels. At the red muscle in rainbow trout in vivo, this mechanism was associated with a 65% increase in tissue \( \text{PO}_2 \). Findings from each of the three primary studies of my thesis greatly advance the understanding of how teleosts potentially cope with environmental and exercise-induced respiratory stress. On a broader scale, this research may help explain one of the most successful adaptive radiations of an animal group in evolutionary history, that of the teleost fishes. Relevant findings from each chapter, and their respective implications and significance are elaborated upon below.
5.1 The influence of pH on haemoglobin-oxygen binding: A comprehensive investigation of the Bohr-Root effect system in rainbow trout, *Oncorhynchus mykiss*

In Chapter 2, the objectives were as follows:

i) Generate complete $O_2$ equilibrium curves (OECs) for rainbow trout blood over a predetermined pH range and calculate the increase in the $O_2$ partial pressure ($\Delta P_{O_2}$) that would be associated with the right-shift in the OEC for each pH change over the entire range of Hb-$O_2$ saturations.

ii) Compare this with the $\Delta P_{O_2}$ calculated from models developed for a vertebrate possessing a Bohr effect alone

iii) Model the degree to which the Bohr-Root effect system could enhance $O_2$ delivery potential, relative to the Bohr effect alone, for a given expected $\Delta pH_a$ over the entire range of Hb-$O_2$ saturations.

iv) Validate three independent protocols for generating OECs.

I performed the most comprehensive investigation to date on the effect of pH on the OEC in a teleost fish possessing the combined Bohr-Root effect. Three independent protocols generated consistent results. Surprisingly, such a data set did not previously exist for rainbow trout, one of the best-studied animals in comparative physiology.

The pH-mediated $\Delta P_{O_2}$ in rainbow trout is non-linear and far exceeds (by 2.5-8 fold) that possible with a linear Bohr-only system (human) given a -0.2 pH unit change. At Hb-$O_2$ saturations of 40%, the $\Delta P_{O_2}$ in a Bohr-Root effect system exceeds that of a Bohr effect alone by 2.5 to 4.2-fold, depending on the pH change. At Hb-$O_2$ saturations
of 80% or higher, the ΔPO₂ is far greater (>8-fold), independent of the pH changes examined. Thus, with a pH change of -0.2 units, which can be realized in vivo, the Bohr-Root effect has potential to enhance general O₂ delivery by almost an order of magnitude more than that of a Bohr effect system alone. This is especially evident near full Hb-O₂ saturation, which is also realistic in vivo.

For this potential to be realized at the capillary, however, a large ΔpHₐ₋ᵥ must be established in the general circulation, which may occur during exercise or exposure to environmental hypoxia. However, when blood returns to the gill, Hb would not effectively bind O₂ because of the reduced affinity and carrying capacity (due to the acidosis), which would also reduce subsequent O₂ delivery (Brittain, 1987; Ingermann, 1982; Milligan and Wood, 1987; Pelster et al., 1992; Salama and Nikinmaa, 1988; Tufts and Randall, 1989). To limit the impact of a generalized acidosis, teleosts release catecholamines that activate βNHE on the RBC membrane to protect intracellular pH and O₂ binding at the gill (Berenbrink and Bridges, 1994). This presumably eliminates the potential for the Bohr-Root effect system to be exploited for O₂ delivery beyond one pass through the circulatory system. However, in Chapters 3 and 4, I proposed and validated a mechanism where short-circuiting of RBC βNHE via plasma-accessible CA creates a large ΔpHₐ₋ᵥ, thus permitting the Bohr-Root effect system for enhanced general tissue O₂ delivery. This mechanism was characterized in vitro in Chapter 3 and in vivo in Chapter 4.
5.2 A MECHANISM AT THE LEVEL OF THE RED BLOOD CELL FOR ENHANCED OXYGEN DELIVERY IN RAINBOW TROUT (Onchorhynchus mykiss): SHORT-CIRCUITING βNHE IN VITRO

In Chapter 3, the objectives were as follows:

i) Create an *in vitro*, closed-system to determine if plasma-accessible CA short-circuited the βNHE in the presence of an acidosis to facilitate O₂ release from the Hb of rainbow trout blood.

ii) Determine the extent to which PO₂ was elevated (ΔPO₂) as a result of βNHE short-circuiting in a closed-system *in vitro* and estimate the degree to which it might influence O₂ delivery if operational *in vivo*.

The primary requirement for O₂ delivery to be enhanced via the Bohr-Root effect system is a localized, recycled acidosis, such as that created in the vascular architecture associated with the eye and swimbladder. But until this study, this was not thought possible in the capillaries of the general circulation. One way to achieve a large ΔpHₐᵥ in the general circulation is when teleosts release catecholamines that activate βNHE on the RBC membrane to protect intracellular pH and O₂ binding at the gill (Berenbrink and Bridges, 1994), which is short-circuited by plasma-accessible CA. I validated this mechanism in an *in vitro* closed-system using rainbow trout RBCs. Carbonic anhydrase short-circuited the βNHE, effectively recycling H⁺ removed via the βNHE back into the RBC to maintain the intracellular acidosis. In doing so, PO₂ was elevated in the closed-system, resulting in a large ΔPO₂. The resulting ΔPO₂ was as great as 30 mm Hg, which when compared to the ΔPO₂ values interpolated from Chapter 2 (Figure 3.7) following
similar levels of blood acidification, indicates that the majority of the initial acidosis was recycled back into the RBC via this mechanism. Therefore, Chapter 3 demonstrated in vitro, proof-of-principle for a mechanism whereby a large pH shift could be created at the capillary level by recycling an acidosis back into the RBC to exploit the potential of the Bohr-Root effect. Harnessing a large ΔPO₂ with the Bohr-Root effect by short-circuiting the βNHE with CA has great implications in vivo. If operational at select locations such as muscle endothelia, plasma-accessible CA could create a very large RBC ΔpHₐ-v and increase in the driving force for general O₂ delivery.

My results from Chapter 3 require many assumptions and validations to apply this mechanism to in vivo conditions. First, tissues are not a closed-system. Oxygen is continuously consumed by tissues, and so the magnitude of ΔPO₂ would likely be diminished in vivo. Second, βNHE short-circuiting must be sufficiently rapid to operate in vivo. The βNHE short-circuiting occurred within seconds (as implied by changes in PO₂), faster than the initial acid-induced Root-off response in the sequence, and up to 10-times faster than the decrease in PO₂ and pHₑ associated with catecholamine addition and activation of the βNHE. The PO₂ optodes that I used have a response time of <1 s (as discussed in Chapter 3), but if the changes in PO₂ occurred faster than could be tracked with this technology (Forster and Steen, 1969; Roughton, 1964), the response time would be well within a realistic time frame for capillary blood transit, which is typically 1-3 s (Bhargava et al., 1992; Honig et al., 1977; Pelster and Scheid, 1992; Randall, 1982; Tetens and Lykkeboe, 1981).

I also found that enhancing basal activity of NHE, which is an important component to the proposed mechanism for the combined Bohr-Root effect for general O₂
delivery, may not require catecholamines *in vitro*, suggesting even broader implications when applying this mechanism *in vivo*. The ΔPO₂ observed in this study also occurred, albeit to a lesser extent, without adrenergic stimulation. In a teleost such as the rainbow trout, catecholamine release and βNHE activation occurs when arterial blood Hb-O₂ saturation is reduced below 50% (Motais et al., 1989; Perry and Gilmour, 1996; Perry and Thomas, 1991), which typically occurs in rainbow trout when water PO₂ falls below 60 mm Hg or arterial PO₂ falls below 20 mm Hg. However, as illustrated in Chapter 2 and in previous studies, in rainbow trout and many teleosts, the majority of Bohr H⁺ are released above 50% Hb-O₂ saturation (Brauner et al., 1996; Brauner and Jensen, 1999; Brauner and Randall, 1996; Brauner et al., 2001). Therefore, a large ΔPO₂ following βNHE short-circuiting may not be realized in a system operating at the lower end of the OEC. Rainbow trout possess a highly-sensitive βNHE (Borgese et al., 1987; Motais et al., 1987; Nikinmaa, 1983; Nikinmaa et al., 1990), but other (non-adrenergic) NHEs exist on the RBC, acting as “housekeeping” H⁺ exchangers to regulate pH and cell volume (Claiborne et al., 1999; Deigweiher et al., 2008; Wakabayashi et al., 1997; Yun et al., 1995) and may be involved. A CA-mediated increase in ΔPO₂ was not detected in additional experiments conducted in Chapter 3 in the presence of a general NHE inhibitor (EIPA), suggesting the importance of an NHE isoform. The isoform may not require β-adrenergic activation pathways associated with catecholamines, but this requires further investigation. If the mechanism I proposed operates in the absence of catecholamines in the upper regions of the OEC where the majority of H⁺ are released, the Bohr-Root effect could greatly influence tissue PO₂ during a minor acidosis or even at rest. This may have implications toward selection pressures associated with the evolution of the Root effect
Hbs, which evolved in teleosts approximately 250 million years before the βNHE evolved as a paralog to the adrenergic-insensitive NHE1 found in other vertebrates (Brett et al., 2005; Nickerson et al., 2003).

In the absence of adrenergic stimulation, increased RBC [HCO$_3^-$] could activate NHE via sAC and cAMP as it does in the dogfish and toadfish. Tresguerres and colleagues recently determined that, in Pacific spiny dogfish (Squalus acanthias L.), soluble adenylyl cyclase (sAC), a signaling enzyme that is activated by elevated [HCO$_3^-$], via cAMP activates the H$^+$ pumps associated with acid-base regulation at the gill (Tresguerres et al., 2010a). They recently also confirmed a role for sAC in a teleost, the Gulf toadfish (Opsanus beta) (Tresguerres et al., 2010b). In my study, CO$_2$ diffuses into the RBC where CA catalyzes hydration to H$^+$ and HCO$_3^-$, the former binding to Hb, displacing O$_2$, and therefore elevating PO$_2$, and the latter removed for Cl$^-$ via anion exchange. In support of this possibility, I found that inhibiting protein kinase A (PKA) abolished the CA-mediated increase in PO$_2$. Because the cAMP-activated PKA pathway is characteristic of the βNHE, this finding suggests that the βNHE isoform was involved. Then, upstream of PKA and cAMP, sAC was inhibited, and again, the increase in PO$_2$ was abolished, provisionally supporting the role of sAC as a possible activation pathway involved in this mechanism (Rummer et al., unpublished data). Therefore, sAC may play a role in regulatory control of ion transport and pH regulation in the RBC, acting as a HCO$_3^-$ sensor.

In Chapter 3, I proposed and investigated in vitro, a mechanism in rainbow trout RBCs whereby, under certain circumstances, an acidosis could be localized and recycled via CA-mediated βNHE or NHE short-circuiting, resulting in a large increase in ΔPO$_2$ in
a closed-system. If realized in vivo, such a system could greatly enhance tissue O\textsubscript{2} delivery, which was the focus of Chapter 4.

### 5.3 A MECHANISM FOR ENHANCED OXYGEN DELIVERY TO MUSCLE TISSUE IN RAINBOW TROUT IN VIVO

In Chapter 4, the objectives were as follows:

i) Characterize a new isoform-specific, relatively membrane-impermeant CA inhibitor (C18) for the first time in a teleost.

ii) Determine if CA-mediated RBC βNHE short-circuiting was operational in vivo in rainbow trout by observing changes in red muscle PO\textsubscript{2} during a generalized blood acidosis in the absence and presence of a CA inhibitor (C18) specific to plasma-accessible isoforms.

When rainbow trout were exposed to hypercarbia, the mild blood acidosis ensuing resulted in an unprecedented 65% increase in RMPO\textsubscript{2} over resting conditions. From Chapter 2, I know that this increase in RMPO\textsubscript{2} was in line with what I would expect from a combined Bohr-Root effect system subjected to a similar pH change, which may be an order of magnitude over what could be observed in a Bohr effect alone system. As predicted in Chapter 3, this ΔPO\textsubscript{2} was abolished when plasma-accessible CA was inhibited. Therefore, I concluded that the short-circuiting mechanism developed and tested in the in vitro studies of Chapter 3 is likely operational in vivo.

Notably, the hypercarbia used in vivo did not increase plasma catecholamines, suggesting βNHE could not have been activated. Therefore, consistent with the in vitro
studies of Chapter 3, catecholamines may not be a pre-requisite for exploitation of the Bohr-Root effect. Consequently, the benefit to the fish in terms of enhanced $O_2$ delivery would not be restricted to the extreme conditions that typically result in catecholamine release (as described above) and the potential for it to be occurring under resting conditions needs further study. Additional in vivo experiments similar to those conducted in Chapter 4 but in resting and exercising fish would be illuminating.

My in vitro findings in Chapter 3 required a plasma-accessible CA isoform. Information regarding distribution and function of various CA isoforms in teleosts is limited, compared with higher vertebrates where they are known to be ubiquitous (Gilmour and Perry, 2009; Henry and Swenson, 2000; Hilvo et al., 2008; Siffert and Gros, 1982). Here, a new isoform-specific membrane-impermeant CA inhibitor, C18, allowed me to demonstrate a role for plasma-accessible CA short-circuiting the $\beta$NHE in rainbow trout red muscle. The C18 did not cross the RBC membrane for at least 15 min in rainbow trout RBCs in vitro, making C18 a very useful compound for in vivo studies because in the in vitro characterization in Chapter 3, I determined that the $t_{1/2}$ for the CA-mediated increase in $PO_2$ was approximately 7 s. However, RBC capillary transit times are thought to be much shorter (Bhargava et al., 1992; Honig et al., 1977; Pelster and Scheid, 1992; Randall, 1982; Tetens and Lykkeboe, 1981). Thus, the RBC could be interacting with plasma-accessible CA upstream of the capillary, potentially priming the blood before it reaches the capillary. In glass catfish (Kryptopterus bicirrhis), plasma-accessible CA has been localized to the endothelial cells lining the dorsal aorta but not the ventral aorta (J. Wilson, personal communication), where it would inhibit gill $O_2$ uptake if present. Future studies should focus on isolating and perfusing sections of
arterial and venous circulation to address the degree to which the RBC NHE is short-circuited upstream of the capillary by plasma-accessible CA isoforms. Furthermore, future studies will result in a more thorough understanding of how CA-mediated RBC NHE short-circuiting may benefit O$_2$ delivery to tissues other than the red muscle, such as the heart (Farrell and Clutterham, 2003) or the metabolically active gut (Grosell et al., 2009), and whether it is a global phenomenon throughout rainbow trout and other teleost species as well.

### 5.4 Broader Synthesis

Aerobic respiration is a prerequisite for all vertebrate life, and so it follows that atmospheric O$_2$ levels have been a primary selective pressure on the molecular, biochemical, physiological, and behavioral aspects of respiration. Geological records suggest that the greatest trend toward increasing environmental O$_2$ (and decreasing environmental CO$_2$) began in the late Silurian (~410 MYA) period and continued through the Devonian (reviewed in Graham et al., 1995). This period coincided with the evolution of actinopterygians. During this time, the actinopterygians evolved quickly in order to compete with placoderms and sharks until such large predators began receding in dominance. Devonian fishes evolved gas-filled swimbladders connected to the mouth via the pneumatic duct (physostomous) that allowed them to gulp and release air at the surface, an early means for regulating buoyancy that conferred increased mobility but still restricted these fishes to shallow waters (Figure 5.1). Devonian fishes also evolved hinged-jaws, and beyond feeding strategies, this new ability to widely open and close the mouth also had clear respiratory implications. A more efficient means to pass water over
the gills could allow high gill surface area, enhanced capacity for O\textsubscript{2} extraction, and high aerobic scope. Food sources widened as well, as jawed fishes could be active, agile hunters as opposed to relying largely on passive filter feeding or sucking mechanisms (Helfman et al., 1997). With increasing activity and mobility, muscle density also increased in many of the jawed fishes in comparison to some of the earlier, more sluggish fishes. The above advances all hinged on improvements to O\textsubscript{2} extraction from water to blood, but my research suggests that enhanced O\textsubscript{2} delivery from the blood to the tissues may have been fulfilled by the evolution of a large Bohr-Root effect.

Root effect Hbs evolved in teleost fish ~400 MYA, after the jaws and physostomous (open) swimbladder, but at least 150 MY before the appearance of either anatomical structure associated with Root Hbs, specifically the choroid rete at the eye or rete mirabile at the phyoclistous (closed) swimbladder (Berenbrink et al., 2005; Berenbrink, 2007) (Figure 5.1). I provided a theoretical mechanism and supplied experimental support for the key components whereby an acidosis produced by CO\textsubscript{2} released from respiring tissues can be localized and recycled within the general circulation at the level of the RBC.

Perhaps early teleosts utilized the combined Bohr-Root effect for general O\textsubscript{2} delivery for 150 MY before the appearance of the choroid rete (Figure 5.1). If this was the case and the driving force for O\textsubscript{2} delivery could be elevated by orders of magnitude over what is possible in vertebrates possessing a Bohr effect alone, as demonstrated both in vitro and in vivo in this study, Root effect Hbs may be considered an exaptation for O\textsubscript{2} delivery, as proposed by Berenbrink et al. (2005). By definition, this trait therefore may have evolved to serve a particular function, but subsequently came to serve another.
Thus, an incipient function of Root Hbs – general O$_2$ delivery – may have been co-opted to give rise to the complex physiological system at the eye and swimbladder 150-270 MY later (Figure 5.1).

My findings open doors to further understanding the selection pressures that may have been involved with the evolution of enhanced O$_2$ delivery in fish and vertebrates in general. The Bohr effect evolved independently three times in vertebrates, but only once was this associated with the Root effect (Berenbrink, 2007; Berenbrink et al., 2005). In the other groups, the Bohr coefficient does not exceed Lapennas's proposed optimal value (50% RQ) for O$_2$ delivery (Lapennas, 1983), as it does in the fish with a combined Bohr-Root effect. A progressively larger Bohr coefficient, ultimately leading to the Root effect, could be selected for without disadvantage to O$_2$ delivery with the mechanism I have proposed throughout this thesis. Three main components are required: the presence of the combined Bohr-Root effect at the level of the Hb, the βNHE or NHE activity at the level of the RBC, and distribution and activity of CA in the capillary endothelia of the tissues, all of which may be dependent on phylogeny or life history and activity of the species, or both. Berenbrink et al. (2005) suggests that a pattern for the appearance and disappearance of choroid and swimbladder retia as well as the magnitude of the Root effect, βNHE activity, and Hb buffer values. They used simple measurements for each of the characteristics to generate a general model to overlay on a phylogeny consisting of Euteleostei, Otocephala, Elopomorpha, Osteoglossomorpha, early ray-finned fishes, Tetrapods, Dipnoi, and Chondrichthyes. Of these eight groups, the first five groups display characteristics such as a pronounced Root effect, RBC βNHE activity, and low Hb buffer values (Berenbrink et al., 2005), a suite of characteristics most likely to support
β-adrenergic short-circuiting. Short-circuiting the β-adrenergic response or the NHE must be rapid for this mechanism to operate in vivo. Therefore, it will be important in future studies to understand how the magnitude and onset pH of the combined Bohr-Root effect and level of NHE activity may influence how the Bohr-Root effect enhances general O₂ delivery.

This study was executed on a derived species, O. mykiss. My preliminary experiments with a basal actinopterygian, white sturgeon (Acipenser transmontanus), which has a small combined Bohr-Root effect and lacks RBC βNHE, show a qualitatively similar phenomenon at the RBCs. The same experimental system used in Chapter 3 was applied to sturgeon RBCs. As in rainbow trout, acidification of blood in a closed-system resulted in an increase in PO₂, and subsequent addition of CA resulted in a further increase in PO₂, albeit less pronounced than in rainbow trout. There is a relationship between the potential of this mechanism to elevate PO₂ and the NHE type (adrenergic or non-adrenergic), however this clearly requires further investigation. As the actinopterygians evolved (those derived from and including the last common ancestors of teleosts and Amia calva), the Root effect onset pH moved into the in vivo RBC pHᵢ range and Hb buffering capacity greatly decreased (Berenbrink, 2007; Berenbrink et al., 2005; Regan and Brauner, 2010a; Regan and Brauner, 2010b). This was also associated with an increase in the magnitude of the Root effect (Berenbrink, 2007; Berenbrink et al., 2005b; Regan and Brauner, 2010a; Regan and Brauner, 2010b), which would allow this system to facilitate O₂ delivery to specialized structures where an acidosis could be localized, definitely an area worth pursuing further.

Overall, this study represents the first proposed and validated (both in vitro and in
vivo) model where the Bohr-Root effect could have very large effects on tissue O₂ delivery outside of the specialized systems of the eye and swimbladder, and far greater than anything previously observed in other vertebrates. If this represents the process through which the Root effect was initially selected for, before improved vision and better buoyancy control associated with a vascular rete and localized acidosis (Figure 5.1), this may shed insight into the early evolution of the Root effect, one of the most amazing adaptations among vertebrates.
5.5 Figures

Figure 5.1. A timeline illustrating the approximate first appearances of structures and systems related to Root effect Hbs in teleost fishes (MYA, million years ago).
5.6 References


Perry, S. F. and Gilmour, K. M. (1996). Consequences of catecholamine release on ventilation and blood oxygen transport during hypoxia and hypercapnia in an elasmobranch (Squalus acantias) and a teleost (Oncorhynchus mykiss). Journal of Experimental Biology 199, 2105-2118.

Perry, S. F. and Thomas, S. (1991). The effects of endogenous or exogenous catecholamines on blood respiratory status during acute hypoxia in rainbow trout (Oncorhynchus mykiss). Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 161, 489-497.


APPENDIX A

Animal care certificate for all experimental studies
ANIMAL CARE CERTIFICATE

Application Number: A07-0080
Investigator or Course Director: Colin Brauer
Department: Zoology

Animals:
- Fish White Sturgeon 105
- Trout Rainbow trout 144
- Fish Sable fish "Anopopoma fimbria" 36
- Turtles Macquarie turtle, Emydura macquarii and the saw shelled turtle, Elseya latisternum 12
- Fish eggs Rainbow trout
- Fish mudskippers, Scartelaos histophorus 50
- Hagfish Pacific hagfish "Eptatretus stoutii" 105

Start Date: March 1, 2007  Approval Date: June 8, 2010

Funding Sources:

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<tr>
<th>Funding Agency</th>
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<tr>
<td>British Columbia Ministry of Environment</td>
<td>Lab analysis of fish eradication with carbon dioxide</td>
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<tr>
<td>Natural Sciences and Engineering Research Council of Canada (NSERC)</td>
<td>Gas exchange and acid-base regulation in fish</td>
</tr>
<tr>
<td>Natural Sciences and Engineering Research Council of Canada (NSERC)</td>
<td>Hemoglobin and red blood cell function in fish; physiological aspects related to the root effect</td>
</tr>
<tr>
<td>UBC Dean of Science</td>
<td>New faculty start up grant</td>
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Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093