THE EVOLUTION OF PROTON-TRIGGERED OXYGEN PUMPS: ROOT EFFECT HAEMOGLOBINS IN BASAL ACTINOPTERYGIAN FISHES

by

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ABSTRACT

It has recently been proposed that the Root effect (reduction of blood oxygen carrying capacity at low pH) evolved in the basal actinopterygian lineage of fishes in the absence of red blood cell (RBC) pH1-protecting βNHE activity. Consequently, there is the potential for these species to experience a reduction in blood O2 carrying capacity, and thus O2 uptake at the gills, during a generalized acidosis associated with exercise, hypoxia, and hypercarbia. I analyzed the haemoglobins (Hbs) of seven species within the basal actinopterygian lineage (from basal to derived: American paddlefish (Polyodon spatula), white sturgeon (Acipenser transmontanus), spotted gar (Lepisosteus oculatus), alligator gar (Atractosteus spatula), bowfin (Amia calva), mooneye (Hiodon tergisus), and pirarucu (Arapaima gigas)) based on the hypothesis that RBC pH1 is not reduced to low enough levels in the general circulation to activate their Root effects. This may result from either RBC buffering by Hb, or onset pH values of the Root effect that are lower than those produced by these generalized acidoses. The former was investigated via Hb titrations, which simultaneously assessed intrinsic Hb buffer capacities and oxylabile Haldane effects, while the latter was investigated by spectrophotometric analyses of Hb O2 saturation over a pH spectrum of pH 5.5 to 8.5. The results suggest that the Hb proton-binding properties of these species’ Hbs unlikely play a major role in the buffering of the intracellular compartment of the RBC when compared with those Hb proton-binding properties of Root effect species with βNHE. However, the onset pH values of these seven species’ variably-sized Root effects were considerably lower than those expected to be produced by a hypoxia- or exercise-induced generalized acidosis. There was also a correlation between the presence of countercurrent choroid retia and
low Hb buffer values, large Bohr/Haldane effects, large Root effects, and high Root
effect onset pH values, which collectively suggest a different process of Root effect
evolution than is currently assumed. Taken together, it appears as though O₂ uptake is
not jeopardized in these early Root effect species, despite their relatively low Hb proton-
binding properties and lack of βNHE.
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LIST OF ABBREVIATIONS

ATP  Adenosine triphosphate
AE   Anion exchanger
βNHE Adrenergically-activated sodium/proton exchanger
CA   Carbonic anhydrase
Cl−  Chloride
CO2  Carbon dioxide
GTP  Guanosine triphosphate
H+   Proton
Hb   Haemoglobin
Hb P50 buffer value Mean of oxygenated and deoxygenated Hb buffer values
HCO3− Bicarbonate
His  Histidine residue
NTP  Nucleoside triphosphate
O2   Oxygen
Pcc2 Partial pressure of carbon dioxide
pHe  Extracellular pH
pHi  Intracellular pH
pK   Negative logarithm of dissociation constant
Po2  Partial pressure of oxygen
PwCO2 Partial pressure of carbon dioxide in water
PwO2 Partial pressure of oxygen in water
RBC  Red blood cell
R-state Relaxed (oxygenated) state of haemoglobin
SEM  Standard error of mean value
T-state Tense (deoxygenated) state of haemoglobin
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STATEMENT OF CO-AUTHORSHIP

Chapters 2 and 3 of this thesis are co-authored. The research questions, experimental designs, laboratory research, and data analyses were conducted by Matthew Regan under the supervision of Dr. Colin J. Brauner. Following the conclusion of the experiments, the manuscripts were written solely by Matthew Regan in consultation with Dr. Colin J. Brauner.
CHAPTER 1: GENERAL INTRODUCTION

Oxygen (O₂) is a prerequisite of vertebrate life. However, as it is not sufficiently soluble in water, the oxygen carrying capacity of the plasma does not allow supply to meet metabolic demand at the respiring tissue in multicellular organisms. To account for this, vertebrate species have evolved to use haemoglobin (Hb), a tetrameric protein that reversibly binds O₂ molecules to each of four iron-containing haem groups, allowing for the efficient transport of necessary quantities of O₂ from the environment to the cells. Furthermore, Hb is responsible for the transport of waste carbon dioxide (CO₂) from the cell to the environment, through both the direct binding of CO₂, as well as the protons yielded from the hydration of CO₂ to bicarbonate. Due to these vital roles in the organism, Hb has been thoroughly studied for well over a century and has yielded critical information not only on the respiratory physiology of vertebrates, but the structure/function relationships of proteins in general.

*Haemoglobin's function in blood gas transport*

In the blood of fishes, Hb plays a central role in the tight coupling of O₂ and CO₂ transport (Brauner and Randall, 1996; 1998). At the gill, O₂ diffuses into the bloodstream where it binds to one of four haem groups on the Hb molecule within the red blood cell (RBC). This occurs in a cooperative fashion, whereby the binding of the first O₂ molecule to Hb increases the O₂ affinity of the subsequent three haem groups due to conformational changes in the tetrameric protein resulting from Hb O₂ binding (Jensen et al., 1998). Oxygen is then transported to the tissues, where, as a result of O₂ consumption, blood Po₂ decreases and O₂ is subsequently released from Hb for passive
diffusion into the respiring cell. This process is made more efficient in the presence of CO$_2$. A metabolic waste, CO$_2$ diffuses out of the respiring cell and into the tissue capillaries where it is quickly transferred to the RBC cytosol (Fig. 1.1). Here, in the presence of the catalyst carbonic anhydrase (CA), it is converted to its hydrated form (bicarbonate; HCO$_3^-$) through the following the reaction:

$$\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+$$

Approximately 95% of the CO$_2$ produced by the body is transported in the blood in this hydrated form (Tufts and Perry, 1998; Fig. 1.1). Bicarbonate is excreted from the RBC in exchange for chloride via band 3 anion exchangers, while the protons remain in the RBC to be taken up by Hb (Tufts and Perry, 1998). This can occur in either of two fashions. One method is through the intrinsic buffering capacity of the Hb molecule, which is a function of the number of proton-binding sites (histidine (His) residues) exposed on the surface of the molecule, the pK values of these residues, and the molecular microenvironments in which they reside (Jensen et al., 1989); or, the Haldane effect, an oxylabile method of Hb proton-binding that results from the exposure of additional His residues and the elevation of their pK values when Hb is converted from its oxygenated to its deoxygenated conformation (Brauner and Randall, 1998). Both methods effectively account for the protons produced in the hydration reaction, and as such, both force the reaction in favour of more HCO$_3^-$ production (i.e., increased CO$_2$ transport). However, due to its mechanistic linkage with the Bohr effect, the Haldane effect allows for the proficient delivery of O$_2$ to the tissue through a method that couples it with the efficient removal of CO$_2$ from the tissue (Brauner and Randall, 1996; 1998; Fig. 1.1). The binding of protons to the newly-exposed His residues on Hb (called Bohr
groups) leads to the formation of intramolecular salt bridges which stabilize the deoxygenated \( T(ense) \)-state of the protein, decreasing its affinity for \( O_2 \) and thus releasing it for diffusion into the tissue (Jensen, 2004). This is the Bohr effect, and is defined as a reduction in Hb \( O_2 \) affinity with a reduction in blood pH (Bohr et al., 1904). The more \( CO_2 \) that is released into the blood, the more protons that are produced in the RBC, and thus, the more \( O_2 \) that is released from Hb for diffusion into the tissue. In this way, through the oxylabile binding of protons to particular groups on the Hb molecule, \( O_2 \) supply can elegantly meet \( O_2 \) demand at the metabolically active tissue.

*The basal actinopterygians: A transitional group in Hb evolution I*

There has been shown to be an inverse relationship between intrinsic Hb buffer capacity and the Haldane effect among the fishes. Defined as "plesiomorphic" due to the possession of primitive morphological or molecular characters (Janvier, 2007; McKenzie et al., 2007), species such as elasmobranchs possess Hbs of high buffer capacities and small Haldane effects, while the more derived teleost species possess Hbs of low buffer capacities and large Haldane effects (Jensen, 1989; Berenbrink et al., 2005). Thus, there are two different but equally effective strategies by which fishes bind protons within the RBC, each resulting in the efficient transport and subsequent removal of \( CO_2 \). However, there exists a paucity of information on the nature of this transition in Hb proton-binding. As the elasmobranchs and teleosts straddle the basal actinopterygians on the phylogenetic spectrum (Janvier, 2007), it is possible that this transition occurred within this group of fishes. Living "primitive" species such as the basal actinopterygians are the few extant remnants of taxa that dominated periods of the fossil record, and as such, their
physiological functions are believed to mirror those of their fossil anatomical contemporaries (Janvier, 2007; McKenzie et al., 2007). There are, of course, limitations to this idea. Extant members of plesiomorphic lineages have likely been subjected to any number of selective pressures since diverging from their last common ancestors, and depending on the nature of these selective pressures, the physiology of the species in question may well have been altered in the process. By and large, however, these physiological characters remain in a relatively similar state, to the point where some believe that they may contribute to the patterning of phylogenetic relationships in the same way that morphological and molecular characters can (Janvier, 2007). So, by analyzing the Hb proton-binding characteristics of extant members of the basal actinopterygian lineage, we may gain an understanding of how the Hb proton-binding properties changed over evolutionary time.

The Root effect

Haemoglobin’s proton sensitivity has been very much amplified in the most derived class of fishes, the teleosts. This group constitutes roughly 27 000 of 28 000 species of extant fishes (Nelson, 1994) and accounts for approximately half of all the earth’s known living vertebrates (Baillie et al., 2004). The increased proton sensitivity of their Hbs, a phenomenon called the Root effect, is believed to have played a major role in facilitating this explosive species radiation (Moyle and Cech, 1996; Berenbrink et al., 2005). At the level of Hb, the Root effect is much like the Bohr effect in that the binding of protons to particular groups on the Hb (Root groups, in this case) stabilizes the T-state of the protein. However, the bonds formed upon proton-binding are so strong that
complete saturation with oxygen cannot be achieved, even at Po2s of up to 140 atmospheres (Root, 1931; Root and Irving, 1943; Scholander and Van Dam, 1954). The Root effect is therefore capable of decreasing not just the blood’s O2 affinity (as results from the Bohr effect), but also its total O2 carrying capacity. Furthermore, the physiological implications of this large reduction in Hb O2 carrying capacity are very much different than those that result from the Bohr effect’s decreased Hb O2 affinity. Together with an acid-producing gas gland and a countercurrent capillary network (rete) that allows for the diffusion of O2 and protons from the venous supply back into the arterial supply, teleosts use the Root effect to deliver vast quantities of O2 to two sites in particular: the eye and the swimbladder (Pelster and Weber, 1991; Pelster and Randall, 1998; Pelster and Decker, 2004). This ensures the retinal cells remain saturated with O2 despite their avascularization, and allows for the swimbladder to be filled with pure molecular O2 directly from the bloodstream despite high Po2s and large hydrostatic pressures. But as with most things physiological, with the benefits of the Root effect come a potential trade-off. Certain conditions, including hypoxia, exercise, and hypercarbia, are capable of eliciting a generalized acidosis of the blood. In conjunction with pH sensitive Root Hbs, this generalized acidosis could jeopardize O2 uptake at the gill during a time when the fish is most in need of O2. To compensate, teleost fishes have the ability to regulate RBC pHi through the use of Na+/H+ exchangers on the surface of the RBC membrane (βNHE; Nikinmaa, 1992; Pelster and Randall, 1998). These exchangers are activated through the binding of catecholamines that are released into the blood under stressful conditions, and are capable of keeping RBC pHi high in the face of a generalized blood acidosis. As a result, O2 loading at the gill is not jeopardized.
The basal actinopterygians: A transitional group in Hb evolution II

The evolutionary relationships of the Root effect and its related processes have recently been shown by Berenbrink et al. (2005) in a comparative study across a broad range of fish species. Their findings suggest that the Root effect first appeared in the last common ancestor of the polypteriformes and the acipenseriformes (Fig. 1.2). This was eventually followed by the appearance of the choroid rete in the last common ancestor of the amiiformes and the osteoglossiformes, which maximized and localized the acidosis necessary to drive O₂ off the Root Hbs and into the retinal cells. And eventually, when the Root effect had already reached roughly 50% in the elopomorphs, βNHE evolved to protect RBC pH during generalized blood acidoses (Berenbrink et al., 2005; Fig. 1.2).

Although sensible on a number of levels, this evolutionary picture of Berenbrink et al.'s (2005) begs a question as to how species with a Root effect are capable of maintaining O₂ uptake during a generalized acidosis in the absence of βNHE. These species include the acipenseriformes (sturgeons and paddlefish), the ginglymods (gars), the amiiformes (bowfin), and the osteoglossiformes (the bony-tongues; the basal teleosts), all of whom, it seems, are at risk of losing up to 50% of their blood O₂ carrying capacity during times of hypoxia, exercise, or hypercarbia (Fig. 1.2). That they are some of the very few “primitive” fishes to survive to the present day is likely a testament to their ability to compensate in such situations. How they manage this, however, is unresolved.
Hypothesis

Despite the presence of a significant Root effect and a lack of protective βNHE activity, the basal actinopterygian fishes occupying the transitional phase in Root effect evolution must surely remain capable of securing O₂ at the gills during a generalized blood acidosis. I hypothesize this would occur only if RBC pHᵢ remained higher than the pH values required to activate these species' Root effects. This could result from either of two scenarios: either these species are capable of regulating their RBC pHᵢ by some means other than βNHE; or, the Root effect onset pH values of these species are lower than those produced by exercise, hypoxia, or hypercarbia.

Due to its high concentration in the RBC and status as the blood’s predominant non-bicarbonate buffer, Hb would be the likely candidate for protecting RBC pHᵢ in these species during a generalized acidosis. This proton-binding ability would be a function of both intrinsic Hb buffer capacity and the oxylabile Haldane effect, both of which can be measured simultaneously through Hb-H⁺ titrations. As well as Hb’s role in pHᵢ protection, these experiments could also reveal the nature of the transition in Hb proton-binding among fishes from its ancestral state of high buffer value/small Haldane effect to low buffer value/large Haldane effect, particularly if done on the Hbs of basal actinopterygian species.

Regarding the second scenario, Root effect onset pH values have been shown to vary interspecifically, from pH 6.15 in elephantnose fish (Berenbrink, 2007) to pH 7.35 in rainbow trout (Pelster and Weber, 1990). It is therefore possible that the onset values of these Root effect species lacking βNHE vary in such a way that they all occur at pH values lower than those produced by exercise, hypoxia, or hypercarbia. This would
prevent the Root effect from ever being activated under these conditions, and therefore, O₂ uptake would not be jeopardized. The Root effect is commonly just measured as its maximum value, comparing Hb O₂ saturation at pH 8.5 (where there is no Root effect) to that at pH 5.5 (where there is maximal desaturation). However, measuring Hb O₂ saturation at many points along a pH spectrum will elucidate the exact pH value at which a species' Root effect is activated. Comparing this onset pH value to those of a generalized acidosis will make clear whether this Hb characteristic is indeed playing a role in the preservation of O₂ uptake at the gill.

*Thesis objectiv 2s*

The objective of this thesis is to determine the Hb proton-binding properties of species within the basal actinopterygian lineage of fishes. Expressed in order of most basal to most derived, the species analyzed in this thesis include (Fig. 1.3): American paddlefish (*Polyodon spathula*), white sturgeon (*Acipenser transmontanus*), spotted gar (*Lepisosteus oculatus*), alligator gar (*Atractosteus spatula*), bowfin (*Amia calva*), mooneye (*Hiodon tergisus*), and pirarucu (*Arapaima gigas*). This selection constitutes two representative species from each of the four orders outlined by Berenbrink et al. (2005) to be particularly demonstrative of transitions in Hb-H⁺ sensitivity (Amiiformes excepted, *Amia calva* being the only extant member). Combined with thorough methodologies (Hb titrations of oxygenated and deoxygenated Hbs; Hb O₂ saturation over detailed pH spectra; all experiments done in the presence and absence of organic phosphates), I hope to gain a comprehensive understanding of the Hb proton-binding properties of species within this interesting evolutionary. If I can assume the Hb
characteristics of these extant species to be representative of the ancestral states of Hb 
(Janvier, 2007; McKenzie et al., 2007), these analyses may allow me to understand the 
ways in which these Hb properties changed over evolutionary time, and how they may 
have allowed for the adaptive radiation of the largest group of vertebrates on the planet 
today – the teleosts.
Figure 1.1. A diagrammatic representation of gas exchange at the tissue. Carbon dioxide (CO₂) diffuses from the tissue into the red blood cell (RBC) where it is hydrated to bicarbonate (HCO₃⁻) and protons (H⁺) in the presence of carbonic anhydrase (CA; the small amount of CO₂ which binds directly to Hb is not represented in this diagram). Bicarbonate is exchanged for chloride (Cl⁻) via the band 3 anion exchanger (AE), while the protons are bound by haemoglobin (Hb), stabilizing its deoxygenated T-state and thereby releasing its bound O₂ for diffusion into the tissue. The reverse scenario occurs at the gills.
Figure 1.2. Changes in specific Hb buffer value (circles), Root effect magnitude (squares), and βNHE activity (triangles) of extant fishes shown in phylogenetically progressive order, from basal to derived. Light grey and dark grey fields indicate the presence of choroid and swimbladder retia, respectively. Hb buffer values refer to deoxygenated, organic phosphate-free haemolysates in 0.1 M KCl at physiological temperature and RBC pH. Root effect values represent the maximal percentage decrease in O₂ saturation of Hb in air-equilibrated haemolysates in pH 5.5 buffer relative to > pH 8.0 buffer. βNHE activities were measured on deoxygenated red blood cells in physiological salines at arterial pH and physiological temperature according to the isoproterenol-activated rate of ²²Na uptake. Modified from Berenbrink et al., 2005.
Figure 1.3. Phylogeny of the living vertebrates, depicting topology that is most widely accepted by morphologists and paleontologists. Main clades and selected characters applying to living taxa: 1, craniates; 2, vertebrates; 3, gnathostomes; 4, chondrichthyans; 5, elasmobranchs; 6, osteichthyans; 7, actinopterygians; 8, actinopterans; 9, acipenseriformes; 10, neopterygians; 11, halecostomes; 12, teleosts; 13, elopomorphans; 14, sphenacanthids; 15, lungfishes; 16, lepidosirenidae; 17, rhipidistians. Figure taken from Janvier, 2007; illustrations for terminal taxa from Janvier, 1996.
1. The Root effect is believed to have evolved in the basal actinopterygian lineage of fishes in the absence of RBC pH₁-protecting βNHE.

2. Oxygen uptake may therefore be threatened during generalized acidoses of the blood brought on by exercise, hypoxia, or hypercarbia.

3. Two hypotheses as to how these fishes maintain O₂ uptake during these conditions are:
   - Significant Hb proton-binding properties (intrinsic Hb buffering and oxylabile Haldane effect) that protect RBC pH₁, and thus, O₂ uptake.
   - Root effect onset pH values that are lower than those produced by a generalized acidosis of the blood brought on by exercise, hypoxia, or hypercarbia.

4. The objectives of this thesis were:
   - To simultaneously measure the Hb buffer values and the Haldane effects of species within the basal actinopterygian lineage of fishes through the H⁺ titrations of their oxygenated and deoxygenated Hbs.
   - To characterize the Root effect properties of these species’ Hbs in a comprehensive fashion so as to determine their Root effect onset pH values.
References


CHAPTER 2: HAEMOGLOBIN-PROTON BINDING PROPERTIES OF BASAL ACTINOPTERYGIAN FISHES

Introduction

Haemoglobin (Hb) is intimately involved in the transport and delivery of oxygen (O₂) in all vertebrate species (Antarctic icefishes excepted), binding O₂ at the respiratory surface and releasing it at the metabolically active tissue. Furthermore, it plays an important role in the transport of carbon dioxide (CO₂) in the reverse direction, binding the protons produced by the hydration of CO₂ to bicarbonate and therefore increasing the blood’s carrying capacity of CO₂. These dual roles of Hb in O₂ and CO₂ transport are coupled through the Bohr/Haldane effect (Brauner and Randall, 1996; 1998). The protons produced by the hydration of CO₂ in the red blood cell (RBC) bind to specific groups (Bohr groups) on the oxygenated (relaxed) R-state Hb which stabilize its deoxygenated (tense) T-state, decreasing its affinity for O₂ and subsequently releasing it for diffusion into the tissue (the Bohr effect; Riggs, 1988; Jensen et al., 1998; Jensen, 2004). This R to T transition exposes more histidine residues (His) on the surface of the Hb molecule which bind additional protons, further stabilizing the T-state as well as aiding in proton-buffering and the transport of CO₂ (the Haldane effect; Brauner and Randall, 1998).

Variation in Hb-H⁺ binding among fishes

It has recently been proposed that the Bohr effect increased in relative proportion with the Root effect (decreased O₂ carrying capacity with low pH; Root, 1931; Pelster and Randall, 1998) among the basal actinopterygian lineage of fishes (Berenbrink et al.,
The Root effect, something of an exaggerated Bohr effect used to deliver large amounts of O₂ to the swimbladder and eye of fishes (Brittain, 1987), has been shown to be correlated with low intrinsic Hb buffer values (Jensen, 1989; Brauner and Weber, 1998; Jensen, 2001; Berenbrink et al., 2005; Berenbrink, 2006). This reduced intracellular proton-binding ability could left-shift the equilibrium of the CO₂ hydration reaction, thereby hindering the formation of bicarbonate and the subsequent transport of CO₂. However, there appears to be an inverse relationship between intrinsic Hb buffer capacity and oxylabile Hb proton-binding ability (Haldane effect) in the few species that have been studied. It has been shown that plesiomorphic fishes such as elasmobranchs possess Hbs of high buffer capacities and small Haldane effects, while the more derived teleosts possess Hbs of some of the lowest buffer capacities and largest Haldane effects seen among all vertebrate species (Jensen, 1989; Berenbrink et al., 2005). This suggests two different, but equally effective, mechanisms for intracellular proton-binding and subsequent CO₂ transport in the blood. Assuming the Hb properties of extant plesiomorphic species to be representative of their respective ancestral states (Janvier, 2007; McKenzie et al., 2007), measuring the Hb buffer capacities and Haldane effects of those fishes intermediate to the elasmobranchs and teleosts on the vertebrate phylogeny may potentially shed light on the evolution of this Hb proton-binding transition.

The potential cost of the Root effect

The evolution of the Root effect, presumably among the basal actinopterygians (Berenbrink et al., 2005), raises another interesting question in regards to Hb proton binding. Having evolved in the absence of RBC pH₁-protecting βNHE (Berenbrink et al.,
2005), the presence of a Root effect in these fishes comes with the potential of losing a significant proportion of the blood’s O2 carrying capacity during a generalized acidosis, and thus may compromise O2 uptake at the gills. However, it is likely that these fishes experience generalized acidoses as a result of hypoxia and exercise in their natural environments (Nelson, 1994; Clack, 2007; Brauner and Berenbrink, 2007; Ilves and Randall, 2007). Together with the fact these basal actinopterygian fishes have successfully survived over 250 million years to the present day (Janvier, 2007), it is likely they are capable of coping with such situations. Oxygen carrying capacity would be preserved in these intermediate species so long as their RBC pHi remained higher than those pH values resulting from a generalized acidosis. This may be the result of either RBC pHi-protection by some means other than βNHE, or Root effect onset pH values in these species that are lower than those produced by a generalized acidosis in vivo. The former hypothesis is addressed in the present study, while the latter is addressed in the subsequent study (Chapter 3). Haemoglobin’s pivotal role in blood buffering and its overall abundance within the RBC cytosol suggest it as the potential protector of RBC pHi during generalized blood acidoses in these intermediate species. Although it has been shown that those species with a Root effect generally have Hbs of low overall buffer value, this may be a trait exclusive to those Root effect species with protective βNHE. Berenbrink et al. (2005) reported buffer values for the deoxygenated Hbs of some basal actinopterygian species at a single physiological pH value, the results suggesting a gradual decrease in buffer value with phylogenetic progression among these species. However, as Hb buffer value varies significantly as a function of both oxygenation status and pH (Jensen et al., 1998), accounting for these variables in the context of a species’
particular Root effect onset pH value may shed additional light on whether or not the
buffering properties of Hb are in fact playing a key role in protecting RBC pHi, and with
it, O2 uptake, during a generalized acidosis.

It is pertinent, therefore, to measure the proton-binding properties of the
oxygenated and deoxygenated Hbs of species within the basal actinopterygian lineage
over a broad pH range. Both the intrinsic buffering capacity of Hb and the Haldane
effect can be measured simultaneously through the acid-base titrations of oxygenated and
deoxygenated Hbs. In the present study, these titrations were performed on isolated Hbs
of the following species, expressed in order of most basal to most derived: American
paddlefish (Polyodon spathula), white sturgeon (Acipenser transmontanus), spotted gar
(Lepisosteus oculatus), alligator gar (Atractosteus spatula), bowfin (Amia calva),
mooneye (Hiodon tergisus), and pirarucu (Arapaima gigas), all species which occupy a
transitional phase in the evolution of both the Bohr/Haldane effect and the Root effect
(Berenbrink et al., 2005). The objective of this study was to perform Hb titrations on the
haemolysates of these fishes (with and without saturating levels of organic phosphates) to
characterize the Hb proton binding properties of a particularly interesting group in the
evolution of vertebrates, as well as to help understand how a large Root effect could have
evolved in the absence of RBC βNHE.

Materials and methods

Animal acquisition

White sturgeon (Acipenser transmontanus; 1-2 kg) and bowfin (Amia calva; 0.5-
1.0 kg) were kept in large flow-through outdoor tanks (dechlorinated city water; PwO2 >
130 torr; $P_{w_c}O_2 < 0.1$ torr; $T = 11-17^\circ$C; fish density < 25 kg fish per m$^3$ water) at the University of British Columbia, Vancouver, Canada, prior to sampling. Pirarucu (Arapaima gigas; 1-2 kg) were maintained in outdoor static tanks at the National Institute for Research of the Amazon (INPA). American paddlefish (Polyodon spathula), spotted gar (Lepisosteus oculatus), alligator gar (Atractosteus spatula) and mooneye (Hiodon tergisus) were all sampled immediately after being caught in their respective natural habitats. Samples for each species consisted of blood from at least three adult fish of either sex, with the exception of spotted gar which consisted of the blood of a single adult.

**Haemolysate preparation**

Whole blood was drawn from the caudal vein of anaesthetized animals (1 ml of 200 mmol l$^{-1}$ benzocaine per litre of water; Sigma E1501) into heparinized syringes, where red cells were then separated by centrifugation and washed three times in cold Cortland’s physiological saline (Wolfe, 1963). The red cells were lysed by addition of two-times volume cold deionized water and subsequent freezing, and cell debris was removed by ten minutes of chilled (4°C) centrifugation at 14 000 r.p.m. (Thermo Electron Corporation 21000R, Waltham, MA, USA). The haemolysates were purified by removing cell solutes and organic phosphates by repeated passage through mixed-bed ion exchange columns (Amberlite IRN-150 mixed bed ion exchange resin), and were then divided into 0.5 ml aliquots and frozen at -80°C until use.
**Haemoglobin titrations**

Haemoglobin titrations were conducted on concentrated stripped haemolysates that were thawed and diluted to a final concentration of 40 μmol l\(^{-1}\) Hb\(_4\) and 0.1 mol l\(^{-1}\) KCl according to the methods of Jensen (1989). Haemoglobin concentration was confirmed after conversion to cyanomethaemoglobin using a millimolar extinction coefficient of 11 at 540 nm, and methaemoglobin content was assessed on identical sub-samples using the spectrophotometric method of Benesch et al. (1973). Samples where methaemoglobin exceeded 10% of total Hb were discarded. A 2 ml volume of the haemolysate (in the absence or presence of GTP at a molar ratio of 3:1 relative to the tetrameric haemoglobin (GTP:Hb\(_4\))) was then transferred to a chilled (12°C), magnetically stirred glass titration vessel where the it was equilibrated with humidified oxygen (100%) for 90 minutes. After reaching a stable pH reading, hydrogen ion titrations were performed with an automated Radiometer (Copenhagen, Denmark) TitraLab 90 titration apparatus, where 0.01 mol l\(^{-1}\) NaOH was added in 10 μl increments to raise pH from isoionic to approximately pH 9.2. After five minutes of equilibration at a pH of 9.2, titration with 0.01 mol l\(^{-1}\) HCl (10 μl increments) was initiated and continued until pH 5.2 was reached, allowing seven minutes of equilibration time between injections. Total amount of NaOH or HCl to reach these end points was recorded. The same procedure was performed on a separate 2 ml sample from the same stock haemolysate, this time equilibrated in humidified nitrogen (100%). Three to four separate H\(^{+}\) titrations were performed on the haemolysates of each species, yielding reproducible results.
Data analysis

The Hb buffer values were derived from the negative slope between adjacent points on the oxygenated and deoxygenated Hb titration curves for each species. Because there are large differences in Hb proton binding over small pH ranges (Jensen et al., 1998), as well as large differences in proton binding between oxygenated and deoxygenated Hb at a constant pH (Jensen et al., 1998), the Hb P₅₀ buffer value was used to derive a single value for each species that would be more representative of in vivo Hb buffer properties. These Hb P₅₀ buffer values were calculated for each of three different, physiologically relevant pH ranges: pH 6.8-7.0, pH 7.0-7.2, and pH 7.2-7.4. The resulting Hb P₅₀ buffer value for (example) pH 6.8-7.0 therefore accounted for variation in proton binding brought about by both oxygenation status and minor fluctuations in pH between 6.8 and 7.0.

The fixed acid Haldane effects of these species were determined by calculating the vertical distances between their oxygenated and deoxygenated titration curves (ΔZ_H, mol H⁺ taken up per mol Hb₄ upon deoxygenation at constant pH). And by plotting the inverse negative slope (-ΔpH/ΔZ_H) of the titration curves as a function of Z_H, the inflection points on the titration curves become localized at two peaks. Quantification of the ΔZ_H between these peaks (Z_H being a molar ratio of protons to Hb₄) gives an accurate measurement of the number of groups being titrated in the physiological pH range (De Bruin and van Os, 1968; Jensen, 1989) and was determined for bowfin, mooneye, and pirarucu. The peaks on the differentiated curves of the other four species could not be accurately resolved due to the linear nature of their titration curves.
Results

Haemoglobin titrations

Representative H⁺ titration curves of oxygenated and deoxygenated haemoglobin from American paddlefish, white sturgeon, spotted gar, alligator gar, bowfin, mooneye, and pirarucu are drawn to the same scale in Fig. 2.1, showing how net proton charge (\(Z_{\text{H}}\), mol H⁺ mol⁻¹ Hb₄) of stripped Hbs in 0.1 M KCl changes as a function of pH in the absence and presence of GTP (3:1 molar ratio of GTP:Hb₄). Zero net proton charge refers to the isoelectric pH and is used as the reference point (Tanford, 1962).

Haemoglobin Buffer Values

The slope of each Hb titration curve indicates the Hb buffer value at that pH (mol H⁺ mol⁻¹ Hb₄ pH unit⁻¹), which varies according to pH, oxygenation status, GTP concentration and species (Fig. 2.2). The Hb P₅₀ buffer values in the presence of GTP varied among the seven species at physiological pH (pH 7.2-7.4), showing a decreasing trend with phylogenetic progression that becomes particularly pronounced starting with bowfin (Fig. 2.3). Pirarucu is a notable exception to this, its Hb exhibiting a relatively high buffer value at physiological pH compared to the other more derived species studied (bowfin and mooneye). This trend of decreasing Hb buffer value with phylogenetic progression becomes less apparent under more acidic conditions (pH 7.0-7.2 and pH 6.8-7.0). Whereas the Hb P₅₀ buffer values of the four more plesiomorphic species tended to decrease at the more acidic pH ranges, those of the three more derived, rete-bearing species showed a marked increase (Fig. 2.3). The same general trends were apparent in...
the stripped Hbs, with lower overall Hb P50 buffer values for a given pH range due to a lack of allosteric modification through GTP binding (data not shown).

Haldane effects

The vertical distance, or \(\Delta Z_H\), between the oxygenated and deoxygenated titration curves indicates the fixed acid Haldane effect (\(\Delta Z_H\) mol H\(^+\) taken up per mol Hb\(_4\) upon deoxygenation at constant pH), which also varies according to pH, GTP concentration, and species (Fig. 2.4). The magnitude of oxylabile proton binding varied among the studied species, with the three rete-bearing species (bowfin, mooneye, pirarucu) exhibiting considerably larger maximum Haldane effects than the four more plesiomorphic species (paddlefish, sturgeon, spotted gar, alligator gar), particularly in the presence of GTP (Fig. 2.4a). These maxima were slightly less for each species in the absence of GTP, as well as left shifted on the pH axis by approximately 0.2-0.4 pH units (Fig. 2.4b).

Titratable groups

Due to a lack of conspicuous inflection points on the Hb titration curves of paddlefish, sturgeon, spotted gar, and alligator gar, this analysis could only be objectively performed for the Hbs of bowfin, mooneye, and pirarucu. The number of groups titrated on the surfaces of their Hbs were 16, 14, and 17, respectively (Fig. 2.5). These numbers were determined from the titration curves of deoxygenated, stripped Hbs in accordance with the methods of Jensen (1989).
Discussion

In fish Hbs, an inverse relationship has been shown to exist between the magnitudes of Hb buffer capacities and oxylabile Haldane effects, such that plesiomorphic species (elasmobranchs) tend to have high Hb buffer values and small Haldane effects, while derived species (teleosts) tend to have low Hb buffer values and large Haldane effects (Jensen et al., 1998). This transition in Hb proton-binding strategy therefore likely occurred among the intermediate group, the basal actinopterygian fishes, the same group in which the Root effect evolved (Berenbrink et al., 2005). The objective of the present study was to determine the nature of this Hb proton-binding transition among the basal actinopterygian fishes, and to see whether these Hb properties may help preserve O₂ uptake in these intermediate Root effect species during a generalized blood acidosis, despite a lack of RBC βNHE activity. The findings suggest that the nature of the Hb proton-binding transition was not completely gradual, but rather punctuated, with Hb buffer value decreasing and Haldane effect increasing markedly starting with the rete-bearing bowfin from fairly steady ancestral levels in the four basal species. However, none of the Hb proton-binding properties of these species suggest they play any significant role in the preservation O₂ uptake during generalized acidoses.

Influence of GTP

Nucleoside triphosphates (NTPs) have been shown to influence Hb function by reducing Hb O₂ affinity through the stabilization of the protein’s T-state (Weber et al., 1975; Pelster and Weber, 1990; Brauner and Weber, 1998). Guanosine triphosphate (GTP) and adenosine triphosphate (ATP) are the predominant NTPs in the RBCs of
fishes (Val, 2000), with GTP exerting a greater effect on Hb O2 affinity due to an
For this reason, all of the experiments in the present study were performed in both the
presence and absence of saturating levels of GTP (3:1 GTP:Hb4). As well as reducing Hb
O2 affinity, the allosteric effects of GTP expose more proton-binding groups (His
residues) on the surface of the Hb molecule, in addition to altering the molecular
microenvironments of existing His residues in such a way that increases their pK values
(Pelster and Weber, 1990; Jensen et al., 1998). This results in a respective increase in
overall proton-binding ability and a right shift of the titration curve (and resulting buffer
curve) along the pH scale for a GTP-bound Hb when compared to a stripped Hb (Figs.
2.1, 2.2). The degree to which these effects are manifest varies among species. For
instance, bowfin Hbs showed much greater GTP-dependent variation in buffer capacity
than did sturgeon Hbs. This is likely the result of the conformational changes to Hb
resulting from GTP binding exposing more titratable groups in bowfin Hb relative to
sturgeon Hb.

The Haldane effect is also influenced by GTP in that it is manifest to a greater
degree and over a narrower, higher pH range when GTP is bound to Hb. Again, this
effect varies interspecifically, but among the species studied here it is particularly
apparent in the three most derived species with the largest Haldane effects – bowfin,
mooneye, and pirarucu (Figs. 2.1, 2.4).

The remainder of the discussion will focus primarily on those experiments done in
the presence of GTP, as we believe these to be more representative of the in vivo
conditions of these fishes.
Haemoglobin buffer curves

The capacity of Hb as a buffer is a function of the type and number of amino acid residues capable of binding protons over a given pH range, as well as the molecular microenvironments in which these residues are located. These titratable amino acid residues are divided into three pH-dependent classes (Tanford, 1962): the guanidyl group of arginine and the amino group of lysine are titrated at basic pH values (> pH 9.0); the carboxyl groups of glutamic acid and aspartic acid are titrated at acidic pH ranges (< pH 6.0); and the imidazole group of histidine residues (His) and the terminal α-amino groups are titrated in the "physiological" pH range (pH 6.0-9.0). It is the disparity in pK values of the titratable groups on the Hb molecule that leads to the variation in proton binding ability as a function of pH (Fig. 2.2). Furthermore, the T- and R-states of a Hb molecule come with changes in the protein conformation, leading to variation in the exposed titratable groups and their microenvironments. This results in intra-molecular differences in oxygenated and deoxygenated Hb buffer curves (Fig. 2.2), a phenomenon which becomes more apparent with phylogenetic progression due to increases in the Haldane effect and, subsequently, variation in the oxygenated and deoxygenated Hb titration curves for a given species (Fig. 2.1). Similarly, the allosteric effects of GTP influence the proton-binding abilities of Hb for the reasons outlined above (Figs. 2.1, 2.2).

The microenvironment of Hb within the RBC is in a constant state of flux, and, as outlined in the foregoing discussion, the ability of Hb to effectively bind protons is subject to this microenvironment. When alluding to the general ability of a particular Hb to bind protons, it is therefore important to account for such variation brought about by
microenvironmental fluctuations within the RBC. Hence, the Hb P₅₀ buffer value. Describing the ability of a Hb molecule to bind protons in this manner accounts for variation in both oxygenation status and pH fluctuation, two variables which it is likely to encounter in circulation.

*Haemoglobin P₅₀ buffer values*

There was a general trend of decreasing Hb P₅₀ buffer values with phylogenetic progression among the seven studied species (Fig. 2.3). This trend was especially apparent in the physiological pH range (pH 7.2-7.4), and became less pronounced as conditions became more acidic (pH 7.0-7.2 and pH 6.8-7.0; Fig. 2.3). It has repeatedly been observed that the Hb buffer capacities of teleost fishes are significantly lower than those of almost all other vertebrate species (Jensen, 1989; Brauner and Weber, 1998; Jensen et al, 1998; Jensen, 2001; Berenbrink et al, 2005; Berenbrink, 2006; Brauner and Berenbrink, 2007), a trait ultimately attributable to a lower number of proton-binding groups (i.e., His residues) on the molecule itself (Riggs, 1970; Jensen, 1989; Berenbrink et al, 2005; Berenbrink, 2006). For instance, human HbA contains 38 His residues per tetramer (Braunitzer et al., 1961), while that of the elasmobranch, spiny dogfish, contains 40 His residues (Aschauer et al., 1985). In comparison, the tetramers of two teleost species, trout and carp, contain only 16 and 18 His residues, respectively (Hilse and Braunitzer, 1968; Bossa et al., 1978). Appropriately, the Hb buffer capacities of these species reflect the number of His residues that make them up, with both human and dogfish displaying high Hb buffer capacities, and trout and carp displaying low Hb buffer capacities (Siggaard-Anderson, 1975; Jensen, 1989). However, the overall His content of
a Hb molecule does not fully account for its ability to buffer protons, as some of these residues may have pK values too low to be operational in vivo, or, more regularly, be buried within the protein moiety and unavailable for titration. Thus, the true reflection of a Hb's ability to buffer protons is the number of titratable groups available on its surface. For the aforementioned Hbs of human and dogfish, these numbers are 26 and 30, respectively, while trout and carp display 7 and 6 groups on the surface of their respective Hbs that are available for titration (Berenbrink, 2006). Through the differential plotting of the Hb titration curves (i.e., -ΔpH/ΔZ_H) as a function of Z_H, the inflection points on the regular titration curves become localized as two separate peaks (Fig. 2.5), the difference between which gives an accurate measure of the number of groups being titrated (De Bruin and van Os, 1968; Jensen, 1989). Due to the linear shape of their Hb titration curves, well-resolved peaks were hard to discern in the differentially-plotted titration curves of the ancestral species (paddlefish, white sturgeon, spotted gar, and alligator gar). However, the differential plots of the deoxygenated, stripped Hbs of bowfin, mooneye, and pirarucu indicated approximately 16, 14, and 17 titratable groups per tetramer, respectively (Fig. 2.5). These numbers agree well with the predictions of Berenbrink et al. (2005) based on a correlation of the buffer values of Hbs with known numbers of titratable groups on their surfaces.

The Haldane effect

It is believed that the ancestral state of tetrameric Hb among fishes is that of a high buffer value, while the Hbs of more derived teleost fishes display much lower buffer values (Jensen, 1989; Jensen et al., 1998). With a low Hb buffer value comes the
potential of jeopardized CO$_2$ transport in the blood. Teleost fishes compensate for this by way of a large Haldane effect, a different, but equally viable strategy for accounting for the protons produced by CO$_2$ hydration and resulting in a tight interaction between O$_2$ and CO$_2$ exchange (Brauner and Randall, 1996; 1998). By and large, this trend was illustrated in the present study, where it was shown that those species with low Hb $P_{50}$ buffer values were characterized by large Haldane effects, while those with high Hb $P_{50}$ buffer values had small Haldane effects (Figs. 2.3, 2.4). The exception to this is pirarucu, a species that displays both a high Hb buffer capacity and a large Haldane effect (possible reasons for this are discussed below). In the absence of this anomalous species, there is a significant negative correlation between Hb $P_{50}$ buffer value and the Haldane effect in the physiological pH range (data not shown; $r^2 = 0.805; P < 0.05$). Therefore, despite the lower intrinsic Hb buffer capacities of the more derived fishes in this study, their large Haldane effects likely ensure CO$_2$ transport and removal is not jeopardized.

*Transitions in Hb-$H^+$ binding in light of the Root effect*

The data for these transitional species do not display a gradual progression towards a low Hb $P_{50}$ buffer value and a large Haldane effect so much as they do a dichotomy between the four basal species (paddlefish, sturgeon, spotted gar, alligator gar) and the three derived species (bowfin, mooneye, pirarucu; Figs. 2.1 – 2.4). This suggests a more punctuated evolution of these Hb traits as opposed to a more gradual evolution, and appears to be associated with the presence of a choroid rete in bowfin, mooneye, and pirarucu. The choroid rete is a countercurrent capillary network located at the eye that is used to both localize and maximize the acidosis necessary to activate the Root effect, an
arrangement these fishes use to deliver high levels of oxygen to the avascularized retinal tissue. This tissue therefore allows for the exploitation of the Root effect, itself a Hb property shown to be correlated with low Hb buffer capacity and large Haldane effect (Jensen et al., 1998; Jensen, 2001; Berenbrink et al., 2005; Berenbrink, 2006). Together with the findings of the related study (Chapter 3) which show a similar dichotomy between rete-bearing and non-rete species in relation to their Root effect characteristics, it may be sensible to expect traits associated with the Root effect (i.e., low Hb buffer capacity; large Haldane effect) to vary correspondingly.

The Root effect is believed to have evolved among the basal ray-finned fishes in the absence of RBC pH1-protecting βNHE activity (Berenbrink et al., 2005). Combined with the low Hb buffer capacities of these fishes, O2 transport could be jeopardized during a generalized blood acidosis resulting from exercise, hypoxia, or hypercarbia. The seven species of the present study all occupy this transitional group, and it was hypothesized that their Hb proton-binding properties may deviate from those of βNHE-equipped Root effect species in such a way that would protect RBC pH1 under such conditions, and with it, O2 uptake at the gill. This would be manifest as higher intrinsic buffer capacities and larger Haldane effects. However, the results suggest that this is not the case. The buffer capacities and Haldane effects of the four basal species’ Hbs (paddlefish, sturgeon, spotted gar, alligator gar) do not deviate in any significant way from those of the more plesiomorphic elasmobranchs (Jensen, 1989) that lack the Root effect altogether (Berenbrink et al., 2005). Likewise, the Hb proton-binding properties of the rete-bearing bowfin, mooneye, and pirarucu do not differ from those of more derived βNHE-equipped Root effect species such as trout, carp, eel, and tuna (Jensen, 1989;
Brauner and Weber, 1998; Jensen, 2001; pirarucu excepted in the case of intrinsic Hb buffering for reasons stated below). This suggests that O₂ uptake during generalized blood acidoses is not likely preserved in these species by way of RBC pH₁ protection on the part of Hb. As the related study shows (Chapter 3), this is instead attributed to relatively low Root effect onset pH values.

Although the reasons for it are not fully resolved, a low Hb buffer value may help to optimize the utilization of the Root effect insomuch as fewer protons need transferring to the RBC to drive the cytosol down to Root effect onset pH. This would allow for optimal oxygen delivery via both Bohr and Root effects, but could potentially come at the detriment to CO₂ transport. However, the correlation of large Haldane effects with low Hb buffer values and the presence of a Root effect ensures CO₂ transport is not jeopardized. In this way, a species may take advantage of efficient proton-dependent O₂ delivery without any cost to their ability to transport and remove CO₂.

Postulating pirarucu

The Hb proton binding properties of pirarucu are somewhat anomalous in that they do not display the low intrinsic buffer capacity typical of other teleost Hbs. Rather, pirarucu displays Hb buffer capacities equal to, or even greater than, those of more plesiomorphic species (Figs. 2.2, 2.3). Moreover, unlike the presumably ancestral-state Hbs of the more plesiomorphic species, the Hbs of pirarucu display a large Haldane effect (Figs. 2.1, 2.4), uncharacteristic of a Hb with a low intrinsic buffer value (Jensen, 1989; Brauner and Weber, 1998). Taken together, these two phenomena suggest pirarucu
as having an exceptional ability to bind protons in the blood, and subsequently, an exceptional ability to transport CO₂. But why?

Pirarucu are obligate air-breathers, securing roughly 80% of their O₂ at their air-breathing organ (Randall et al., 1978). In fact, if denied access to air for more than 10 minutes, pirarucu will actually drown (Val and Almeida-Val, 1995). This breathing strategy results in high levels of blood CO₂, with a Pco₂ measured at approximately 27 mmHg in arterial blood (Randall et al, 1978) compared to the arterial Pco₂ values of trout (2.9 mmHg; Perry et al, 1996), eel (2.4 mmHg; Perry et al, 1996), turbot (2.5 mmHg; Perry et al, 1996), and dogfish (1.1 mmHg; Perry et al, 1996). As roughly 90% of CO₂ is transported in its hydrated form in the blood of fishes (Tufts and Perry, 1998), the high levels of CO₂ measured in the blood of pirarucu has the potential to produce a large quantity of acid once hydrated and dissociated into HCO₃⁻ and H⁺. In this situation, having a large Hb buffer capacity to account for these protons and, subsequently, facilitate CO₂ transport and protect against a generalized blood acidosis would be beneficial. This is especially true for pirarucu, who, unlike many other air-breathing fishes, do not display haematocrits and whole blood Hb concentrations considerably higher than those of water-breathing fishes with regular Pco₂ values (Graham, 1997). The occurrence of this high intrinsic Hb buffer capacity may then be an adaptation paralleling the increased aerial dependence of this species. As air-breathing is thought to have evolved as many as 67 times among the fishes (Graham, 1997), there is no shortage of species among which to compare Hb buffer values to test this hypothesis.
Conclusions

The findings of the present study suggest that the transition in Hb proton-binding strategy from large intrinsic buffering/small Haldane effect to low intrinsic buffering/large Haldane effect occurred not by gradual succession among the primitive ray-finned fishes, but rather a more punctuated method. A marked decrease in buffer value and increase in the Haldane effect appears to have occurred in the last common ancestor of bowfin and the teleosts, and is correlated with the first appearance of the choroid rete. As the choroid rete has also been shown to be correlated with a significant increase in the magnitude and onset pH of the Root effect (Chapter 3), it is possible that this transition in Hb proton-binding is in some way related to the optimization and/or utilization of the Root effect in these fishes – a low intrinsic Hb buffer value would reduce the number of necessary protons shifted to the RBC cytosol to activate the Root effect, while a large Haldane effect would ensure CO₂ transport did not suffer despite the decreased Hb buffer capacity. Furthermore, being linked mechanistically to the Bohr effect, a large Haldane effect would allow for the tight coupling of O₂ and CO₂ transport in the blood of these fishes (Brauner and Randall, 1996; 1998). However, this transition in Hb proton-binding would not necessarily increase the overall proton-binding capacity of Hb in these basal ray-finned species to the extent that it would protect O₂ uptake in the case of a generalized blood acidosis as hypothesized. In light of the findings of the related study, however, this is likely not a necessary characteristic of these species’ Hbs despite their lack of RBC βNHE, as the onset pH values of their respective Root effects all appear to be lower than those resulting from even a severe generalized acidosis of the blood (Chapter 3).
Figure 2.1. Representative Hb H⁺ titration curves, $Z_H$ (mol H⁺ mol⁻¹ Hb₄) as a function of pH, for oxygenated (●) and deoxygenated (○) Hbs of American paddlefish (A), white sturgeon (B), spotted gar (C), alligator gar (D), bowfin (E), mooneye (F), and pirarucu (G) in the presence (black) and absence (grey) of organic phosphates (3:1 GTP:Hb₄). Titrations were performed on haemolysates at a [Hb₄] of 0.04 mmol l⁻¹ and a [KCl] of 0.1 mol l⁻¹. The presence of a choroid rete within a species is indicated by ®.
Figure 2.2. Representative buffer curves (-ΔZH/ΔpH) as a function of pH for oxygenated (●) and deoxygenated (○) Hbs of American paddlefish (A), white sturgeon (B), spotted gar (C), alligator gar (D), bowfin (E), mooneye (F), and pirarucu (G) in the presence (black) and absence (grey) of organic phosphates (3:1 GTP:Hb4). Titrations were performed on haemolysates at a [Hb4] of 0.04 mmol l⁻¹ and a [KCl] of 0.1 mol l⁻¹. The presence of a choroid rete within a species is indicated by ®.
Figure 2.3. Haemoglobin P₅₀ buffer values in the presence of GTP (3:1 GTP:Hb₄) for seven basal actinopterygian species. Values were determined by averaging all points along the oxygenated and deoxygenated buffer curves (Fig. 2.2) within each of the three physiologically-relevant pH ranges (pH 6.8-7.0; pH 7.0-7.2; pH 7.2-7.4) for each species. The presence of a choroid rete within a species is indicated by °.
Figure 2.4. Fixed-acid Haldane effects ($\Delta Z_H$, the number of protons taken up per Hb$_4$ upon deoxygenation at constant pH) as a function of pH for seven basal actinopterygian species in the absence (A) and presence (B) of GTP (3:1 GTP:Hb$_4$). $\Delta Z_H$ was calculated from the vertical distance between the oxygenated and deoxygenated titration curves (Fig. 2.1) for a given species. The presence of a choroid rete within a species is indicated by ®.
Figure 2.5. Representative differential titration curves (-$\Delta$H/$\Delta$ZH; the inverse of buffer value) as a function of ZH, taken from the deoxygenated titration curves of bowfin (A), mooneye (B), and pirarucu (C) in the absence of GTP, according to the methods of Jensen (1989). The horizontal distance between the inflection points indicates the number of titratable groups in the neutral pH range (approx. pH 6.0-9.0).
CHAPTER SUMMARY

1. Assuming the Hb properties of these seven species to be representative of their respective ancestral states, the transition in Hb proton-binding from a high Hb buffer value/small Haldane effect to a low Hb buffer value/large Haldane effect appears to have occurred in a somewhat punctuated manner in the last common ancestor of the amiiformes and the teleosts. This is correlated with the first appearance of the choroid rete, as well as a significant increase in the magnitude and onset pH value of the Root effect (Chapter 3).

2. When compared to those of more plesiomorphic species lacking the Root effect, as well as more derived species with both the Root effect and protective βNHE activity, it is unlikely the Hb proton-binding properties of these seven species as playing a significant role in protecting RBC pH, and with it, O₂ uptake, during generalized blood acidoses.
References


CHAPTER 3: ROOT EFFECT HAEMOGLOBIN CHARACTERISTICS IN BASAL ACTINOPTERYGIAN FISHES

Introduction

Over its 450 million year history (Dickerson, 1971), haemoglobin (Hb) has been the subject of countless selective pressures in countless species due to its functional position at the interface of the organism and its environment (Powers, 1980; Berenbrink, 2006). This has resulted in a remarkable degree of heterogeneity in protein structure and function, with fishes in particular displaying extraordinary variation in Hb multiplicity and function (Pelster and Weber, 1990; Weber, 1990; Jensen et al., 1998; Bonaventura et al., 2004). These molecular characteristics have been shaped by the selective pressures brought about by new niches and environments and, in some cases, are believed to have contributed to the relative success of whole classes of species. One such example is the Root effect, a characteristic of certain fish Hbs allowing for the enhanced delivery of oxygen (O₂) to particular tissues. It is believed that this Root effect has played a major role in the explosive adaptive radiation of a group of fishes that accounts for half of all living vertebrate species in the world today – the teleosts (Weber, 2000; Bonaventura et al., 2004; Berenbrink et al., 2005; Berenbrink 2007).

The Root effect

At the level of Hb, the Root effect is thought to be an exaggerated Bohr effect (Brittain, 1987), with Root Hbs showing a large decrease in both O₂ affinity and cooperativity at low pH (Pelster and Randall, 1998). Protons bound to particular groups
(Root groups) on the Root Hb stabilize the deoxygenated T(ense)-state to such an extent that complete saturation with O\textsubscript{2} is unattainable (Root, 1931; Root and Irving, 1943), even at Po\textsubscript{2}s of up to 140 atmospheres (Scholander and Van Dam, 1954). Physiologically, however, the Root effect has very different implications for gas transport than the Bohr effect. In conjunction with a gas gland and rete, tissues that respectively create and localize an acidosis within capillaries, fishes use these Root Hbs to offload large quantities of O\textsubscript{2} to the swimbladder and eye (Brittain, 1987; Pelster and Randall, 1998). This O\textsubscript{2} multiplication system allows fishes to regulate their swimbladder volume, and thus their buoyancy in the water column, as well as to ensure adequate O\textsubscript{2} delivery to the retinal cells despite the avascularization of the fish eye (Bridges et al., 1998; Pelster, 2001). However, the Root effect comes with potentially unfavorable consequences. Certain conditions, including hypoxia and exercise, are capable of producing generalized blood acidoses through the production of CO\textsubscript{2} and metabolic acid which, in conjunction with pH-sensitive Root Hbs, may jeopardize O\textsubscript{2} uptake at the gill. To compensate, teleost fishes have the ability to regulate RBC pH\textsubscript{i} through the use of adrenergically-stimulated Na\textsuperscript{+}/H\textsuperscript{+} exchangers on the RBC membrane (\(\beta\)NHE). These exchangers are activated through the binding of catecholamines released to the blood under stressful conditions, and with the resulting proton extrusion, are capable of keeping red cell pH\textsubscript{i} high (Nикинмаа, 1983; Thomas and Perry, 1992; Nikinmaa and Salama, 1998). In this way, teleosts preserve O\textsubscript{2} uptake during generalized blood acidoses despite possessing proton-sensitive Root Hbs.
The basal actinopterygian fishes: A transitional group in Root effect evolution

The evolutionary relationships of the Root effect and its associated mechanisms have been recently investigated by Berenbrink and colleagues (2005) in a comparative study across a broad range of fishes. Their results indicate that the protective βNHE did not evolve until long after the appearance of the Root effect Hb traits (increased proton sensitivity; decreased Hb buffer capacity) and the choroid rete (Berenbrink et al., 2005). From this, it can be assumed that those intermediate species possessing a Root effect but lacking βNHE are at risk of jeopardizing up to 50% of their O₂ carrying capacity in the case of a generalized acidosis. These include species within the acipenseriformes (sturgeons and paddlefish), ginglymods (gars), amiiformes (bowfin), and osteoglossomorphs (the bony-tongues; the basal teleosts). That these species are some of the very few 'primitive' fishes to have successfully survived to the present day (Janvier, 2007) is likely a testament to their ability to compensate in such situations. How they are accomplishing this, however, is unresolved, as relatively little is known about the blood properties of this most interesting group (Brauner and Berenbrink, 2007).

We hypothesize that despite a significant Root effect and lack of βNHE, O₂ binding at the gills of these transitional species during a generalized blood acidosis must be maintained. This would only occur if RBC pHᵢ did not get low enough to activate their Root effects in the general circulation, a likely result of either RBC pHᵢ regulation by some means other than βNHE, or onset pH values of the Root effect in the Hbs of these species that are lower than those brought about by hypoxia or exercise. The previous chapter addressed the former, where it was shown that these species' Hb buffer capacities are not uncharacteristically high so as to preserve O₂ binding through the
protection of RBC pH; (Chapter 2). The latter scenario is addressed in the present study. Expressed in order from most basal to most derived, the species investigated in this study include: American paddlefish (*Polyodon spathula*), white sturgeon (*Acipenser transmontanus*), spotted gar (*Lepisosteus oculatus*), alligator gar (*Atractosteus spatula*), bowfin (*Amia calva*), mooneye (*Hiodon tergisus*), and pirarucu (*Arapaima gigas*). Not only do these species all belong to the transitional group in the evolution of the Root effect, but they also straddle the first appearance of the choroid rete, a countercurrent network of capillaries that, in conjunction with the Root effect, plays a key role in the generation of high O2 tensions in the eye (Wittenberg and Wittenberg, 1974; Wittenberg and Haedrich, 1974; Bridges et al., 1998). The analysis of Root effect characteristics in species that both lack (paddlefish, white sturgeon, spotted gar, and alligator gar) and possess (bowfin, mooneye, and pirarucu) choroid retia may shed light on its influence on these Hb properties. The objective of this study was to measure the onset pH of the Root effect in the haemolysates of these species in the absence and presence of organic phosphates (GTP). Assuming the Hb properties of these seven species to be representative of their ancestral states (Janvier, 2007; McKenzie et al., 2007), this may allow us to gain insight into how a large Root effect could have evolved in this group of fishes, despite the absence of RBC βNHE.

**Materials and methods**

**Animal acquisition**

White sturgeon (*Acipenser transmontanus*; 1-2 kg) and bowfin (*Amia calva*; 0.5-1.0 kg) were kept in large flow-through outdoor tanks (dechlorinated city water; \( P_{wO_2} > \))
130 torr; $P_{CO_2} < 0.1$ torr; $T = 11-17^\circ C$; fish density $< 25$ kg fish per m$^3$ water) at the University of British Columbia, Vancouver, Canada, prior to sampling. Pirarucu (*Arapaima gigas*; 1-2 kg) were maintained in outdoor static tanks at the National Institute for Research of the Amazon (INPA). American paddlefish (*Polyodon spathula*), spotted gar (*Lepisosteus oculatus*), alligator gar (*Atractosteus spatula*) and mooneye (*Hiodon tergisus*) were all sampled immediately after being caught in their respective natural habitats. Samples for each species consisted of blood from at least three adult fish of either sex, with the exception of spotted gar which consisted of the blood of a single adult.

**Haemolysate preparation**

Whole blood was drawn from the caudal vein of anaesthetized animals (1 ml of 200 mM benzocaine per litre of water; Sigma E1501) into heparinized syringes, where red cells were then separated by centrifugation and washed three times in cold Cortland’s physiological saline (Wolfe, 1963). The red cells were lysed by addition of two-times volume cold deionized water and subsequent freezing, and cell debris was removed by ten minutes of chilled (4°C) centrifugation at 14 000 r.p.m. (Thermo Electron Corporation 21000R, Waltham, MA, USA). The haemolsates were purified by removing cell solutes and organic phosphates by repeated passage through mixed-bed ion exchange columns (Amberlite IRN-150 mixed bed ion exchange resin), and were then divided into 0.5 ml aliquots and frozen at -80°C until use. Upon thawing, Hb concentration was determined after conversion to cyanomethaemoglobin using a millimolar extinction coefficient of 11 at 540 nm, and methaemoglobin content was
assessed on identical sub-samples using the spectrophotometric method of Benesch et al. (1973). Samples where methaemoglobin exceeded 10% of total Hb were discarded.

**Root effect quantification**

The magnitude of the Root effect was determined by measuring oxygen saturation of Hb spectrophotometrically at atmospheric Po$_2$ (157 mmHg) in Tris buffers (50 mmol l$^{-1}$; Trizma base, Sigma T6066) ranging in pH from 5.5 to 8.5. Air-equilibrated concentrated haemolysates, diluted to a final concentration of 160 μmol l$^{-1}$ Hb$_4$ and 0.1 mol l$^{-1}$ KCl, were then mixed with the buffers in a 1 ml cuvette. This procedure was conducted for haemolysates in both the absence and presence of GTP (GTP:Hb$_4$ ratio of 3:1; guanosine 5'-triphosphate sodium salt hydrate, Sigma G8877). Absorption at wavelengths of 540, 560 and 576 nm were measured and recorded using a Shimadzu (Columbia, MD, USA) UV-160 spectrophotometer, and were used to calculate percent Hb O$_2$ saturation according to Benesch et al. (1973) as described below.

**Data analysis**

Percent Hb O$_2$ saturation at each pH was calculated using the following equations according to Benesch et al. (1973),

\[
[\text{Oxy Hb}] = (1.4747A_{576} - 0.6820A_{560} - 0.5329A_{540}) \quad (1)
\]

\[
[\text{Deoxy Hb}] = (1.4749A_{560} + 0.2141A_{576} - 1.1042A_{540}) \quad (2)
\]
where $A$ is the optical density at the peak absorption wavelength for oxygenated Hb (540 and 576 nm) and for deoxygenated Hb (560 nm). Equations (1) and (2) were then added together to give total Hb in solution, by which the oxygenated Hb concentration was divided to yield the percent oxygenation status of the haemolysate at each pH.

The pH of Root effect onset was determined for four different degrees of Hb O$_2$ desaturation: 5%, 10%, 15% and 20%. These values were subtracted from the average fully oxygenated percentage (between pH 7.5 and 8.5, where no Root effect is present), and the pH at the respective Hb O$_2$ saturation percentages was determined using the sigmoidal line of best fit generated for each sample. A sample size of three to four was used for each species.

Statistical analysis

Statistical $t$-tests were performed when comparing the mean values of both maximal Root effect and Root effect onset pH values among the rete-bearing species and the non-rete species, as well as each species’ Root effect onset pH values in the presence and absence of GTP. Data were transformed in the few instances where equal variance tests were not passed.

Statistical tests were done using SigmaStat 3.0.

Results

Percent Hb O$_2$ saturation as a function of pH for the stripped haemolysates in aerated Tris buffer of American paddlefish, white sturgeon, spotted gar, alligator gar, bowfin, mooneye and pirarucu is shown in Fig. 3.1. The Hb O$_2$ saturation for each
species decreased with a decrease in pH, attributable to the Root effect. The magnitude of the Root effect varied among the species, with the Hb of the three rete-bearing species (bowfin, mooneye, and pirarucu) showing a significantly greater mean level of Hb O₂ desaturation than that of the four more plesiomorphic species (paddlefish, white sturgeon, spotted gar and alligator gar; Fig. 3.1; without GTP: \( t = -13.441; d.f. = 19; P < 0.001 \); with GTP: \( t = -9.690; d.f. = 18; P < 0.001 \)).

Both Root effect magnitude and onset pH were elevated in the presence of allosterically-modifying GTP (Figs. 3.1, 3.2), with the onset pH values of the rete-bearing species in particular increasing significantly over those of their stripped haemolysates [at 10% desaturation: bowfin (\( t = -3.561; d.f. = 4; P = 0.024 \)); mooneye (\( t = -7.211; d.f. = 4; P = 0.002 \)); pirarucu (\( t = -2.990; d.f. = 4; P = 0.04 \))]. In both cases, there was a general dichotomy between the four more plesiomorphic species and the three rete-bearing species on the Root effect traces (Fig. 3.1).

The pH at which four different levels of Hb O₂ desaturation (5, 10, 15 and 20% of total blood haemoglobin) were observed for each species' stripped haemolysates in the presence and absence of GTP is shown in Fig. 3.2. Due to relatively small Root effects, the pH values at which 20% (and even 15% in the case of paddlefish) of total blood Hb was desaturated were indeterminable for three of the four more plesiomorphic species (a single spotted gar sample excepted). The relatively modest desaturation levels that were reached in these species were observed only at very low pH values, even for a 5% decrease in oxygen carrying capacity (Fig. 3.2). The three rete-bearing species, however, all exhibited large Root effects, and the mean pH values at which each of 5, 10, and 15% desaturation (20% not achieved in non-rete species) were observed at were significantly
higher than those of the four more plesiomorphic species (e.g. 5% desaturation without GTP: $t = -6.382; \text{d.f.} = 13; P < 0.001$; 5% desaturation with GTP: $t = -6.240; \text{d.f.} = 21; P < 0.001$). All seven species' Root effect onset pH values were considerably lower than the lowest average RBC pH, that might be expected (based upon literature values) during hypoxia and exercise. Furthermore, the variation in pH onset between the four different levels of desaturation was less for each of the rete-bearing species than for the more plesiomorphic species (Fig. 3.2), evident by the shallower slopes of their Root effect curves (Fig. 3.1).

Root effect onset pH (5% Hb O$_2$ desaturation) was positively correlated with the maximal Root effect of that species ($r^2 = 0.700; \text{d.f.} = 21; P < 0.001$; Fig. 3.3). Again the dichotomy of the four more plesiomorphic species and the three rete-bearing species is evident, with the former segregated on the lower left of the graph signifying a low Root effect maximum and a low pH of onset, and the latter segregated on the upper right portion of the graph signifying a high Root effect maximum and a high pH of onset (Fig. 3.3).

**Discussion**

It has been proposed that the Root effect evolved in the basal actinopterygian lineage of fishes in the absence of βNHE activity (Berenbrink et al., 2005), a situation that comes with the potential of losing a significant proportion of the blood's O$_2$ carrying capacity during times of generalized acidoses. In light of the recent finding that the proton-binding abilities of these species' Hbs (intrinsic buffering and oxylabile Haldane effect) do not likely compensate for a lack of βNHE activity (Chapter 2), the objective of
The present study was to gain insight into how a sizeable Root effect could evolve in the absence of βNHE. The findings indicate that the onset pH of the Root effect is below even the lowest predicted pH values that would be elicited in the general circulation of these fishes following severe exercise or exposure to hypoxia.

**Influence of GTP**

Adenosine triphosphate (ATP) and guanosine triphosphate (GTP) are the predominant organic phosphates in fish RBCs and have been shown to act as major cofactors in Hb function by reducing Hb O₂ affinity (Weber et al., 1975; Pelster and Weber, 1990; Brauner and Weber, 1998; Val, 2000). Of these, GTP exerts the stronger effect on Hb O₂ saturation due to an additional stabilizing bond within the T-state of the Hb molecule (Pelster and Weber, 1990; Val, 2000). For this reason, all of the experiments in the present study were performed in both the presence and absence of saturating levels of GTP (3:1 GTP:Hb₄). As well as reducing Hb O₂ affinity, the allosteric effects of GTP alter the molecular microenvironments of the Root groups of Hb in such a way that increases their pK values (Pelster and Weber, 1990; Jensen et al., 1998). These allosteric effects become manifest as increases in both Root effect magnitude and onset pH (Figs. 3.1, 3.2), although the degree to which GTP influences these Hb characteristics is species-specific. For instance, the Hbs of carp and eel each show a Root effect of 40% in the presence of saturating levels of organic phosphates, but no Root effect in their absence (Pelster and Weber, 1990). Conversely, trout Hbs display large Root effects of 40% in the absence of organic phosphates, increasing to 55% when they are added to the haemolysate (Pelster and Weber, 1990). All of the experiments in
the present study were performed in both the presence and absence of saturating levels of GTP (3:1 GTP:Hb₄), where it was shown that the influence of GTP on these Hb characteristics varied interspecifically. Root effect magnitude was generally increased upon GTP binding, ranging from very slight (e.g., alligator gar; pirarucu; Fig. 3.1) to up to a 7% increase in overall Hb O₂ desaturation (sturgeon; Fig. 3.1), while onset pH was increased in all species by up to 0.5 pH units (mooneye; Figs. 3.1, 3.2).

The remainder of the discussion will focus primarily on those experiments done in the presence of GTP, as we believe these to be more representative of the in vivo conditions of these fishes.

Root effect onset pH

Although believed to be a trait almost exclusive to the haemoglobins (Hbs) of teleosts (Pelster and Randall, 1998), this study supports the findings of Berebrink and colleagues’ (2005) that the Root effect is also a characteristic of the Hbs of more plesiomorphic fishes. Each of the seven species studied here possesses Hbs that display a Root effect (Fig. 3.3), ranging from approximately 7% of total blood oxygen carrying capacity in paddlefish to 40% in mooneye. Variation also exists in the onset pH values of these species’ Root effects (Figs. 3.1, 3.2). To our knowledge, few, if any, studies have looked at the mechanism underlying the variation in Root effect onset pH. However, it is likely that this pH value is a function of the pK values of the particular amino acid residues responsible for binding the protons that bring about the R to T conformational change in the Hb. As it has been shown that the mechanistic basis of the Root effect varies interspecifically, that is, different amino acid residues on different Hbs have been
shown to be involved in the Root effect-associated conformational changes (Perutz and Brunori, 1982; Nagai et al., 1985; Mylvaganam et al., 1996; Jensen et al., 1998; Tsuneshige et al., 2002), one may also expect the pK values of these different groups to vary. This would be manifest as interspecific differences in Root effect onset pH, as are shown in Fig. 3.2. The onset pH value for each species was determined at four different levels of Hb O₂ desaturation (5, 10, 15, and 20% of total Hb), although not all seven species had Root effects capable of achieving 20% desaturation. Of the four plesiomorphic species, only the haemolysates of spotted gar became 20% desaturated (a single sample), while those of paddlefish, white sturgeon, and alligator gar showed at most 10 to 15% desaturation (Fig 2). Moreover, the pH values at which these levels of desaturation were achieved were very low relative to in vivo conditions in the general circulation, all of them occurring well below pH 6.3 (5% desaturation in white sturgeon excepted; Fig. 3.2). Conversely, the haemolysates of the rete-bearing bowfin, mooneye, and pirarucu all displayed Root effects larger than 20%, and the pH values at which the four desaturation levels were achieved were in a more physiologically-relevant range. Five percent desaturation occurred in these three species between pHs 6.8 and 7.0, while 20% was achieved between pHs 6.4 and 6.7 (Fig. 3.2).

Each of the seven species' Root effect onset values were quite low compared to those that would be expected during a generalized acidosis. Exercise (acute and exhaustive) and hypoxia are ways of generating severe acidoses of the blood. However, little information on the effect of these challenges on blood-gas transport exists for these species (Brauner and Berenbrink, 2007). A survey of the responses to exhaustive exercise in a broad range of fishes (Squalus acanthias; Lepisosteus osseus; Amia calva;
Oncorhynchus mykiss) indicates that blood pH$_e$ may be reduced to between 7.3 and 7.7 (Milligan and Wood, 1986; Burleson et al., 1998; Gonzalez et al., 2001; Richards et al., 2003). Assuming a ΔpH$_i$/ΔpH$_e$ value of 0.47 (0.471 in rainbow trout, Heming et al., 1986; 0.469 in white sturgeon, Baker et al., in press), these extracellular pH values translate to RBC pH$_i$ values between 7.1 and 7.3. These are considerably higher than those pH values that would result in even a 5% Root effect in the studied species (Fig. 3.2). Similarly, hypoxia acidifies the blood to pH 7.4-7.7 in a comparable range of fishes (Holeten and Randall, 1967; Butler and Taylor, 1975; Randall et al., 1992), translating to intracellular pH values between 7.15 and 7.3 (Heming et al., 1986; Baker et al., in press), which again are considerably higher than the Root effect onset pH values in any of the studied species (Fig. 3.2). It therefore appears as though O$_2$ uptake is not likely compromised in these species following exhaustive exercise or hypoxia, despite relatively large Root effects and no βNHE activity.

There are conditions which may have posed larger threats to these species’ O$_2$ uptake throughout evolutionary time, however. Stem actinopterygian species, which gave rise to the species studied here, date back over 300 million years (Janvier, 2007) to a time when the earth’s atmosphere and water systems were significantly higher in CO$_2$, lower in O$_2$, and much more acidic (Algeo et al., 2001; Berner, 2006; Clack, 2007). Animals from this era dealt with these challenges in different ways – some secured O$_2$ from the air; others abandoned their aquatic habitats altogether; while still others became more robust at dealing with their harsh environments (Clack, 2007; Brauner and Baker, 2008). An example of the latter is found in the acipenseriformes, whose extant sturgeon species have been shown to be among the most CO$_2$ tolerant of fishes (Crocker and Cech,
In conditions of hypercarbia that may be similar to those of its proposed ancestral habitats, white sturgeon has been shown to survive despite a blood acidosis resulting in a RBC pH$_i$ of 6.8 (Baker et al., in press; Brauner and Baker, 2008). Taken as the extreme example of a potential generalized blood acidosis, pH$_i$ 6.8 would not be capable of activating the Root effect in the four ancestral species (Fig. 3.2b). However, it may be low enough to reduce blood oxygen carrying capacity by 10% in the three rete-bearing species (15% in the case of mooneye; Fig. 3.2b). Whether this degree of desaturation would pose a threat to these animals is not known. However, βNHE first appeared in the elopomorphs, the order of fishes immediately following the osteoglossomorphs on the phylogenetic spectrum (Berenbrink et al., 2005; Berenbrink, 2007). Taken together, the potential for incomplete Hb O$_2$ saturation under potentially hypercarbic ancestral conditions may have potentially provided the necessary selective pressure for RBC βNHE evolution.

**Root effect without retia**

There is a dichotomizing trend in the data set between those species with a rete and those lacking one. These significant differences in both Root effect magnitude and onset pH suggest a potential role of the rete in manifesting a Root effect that is operational in vivo. All of the non-rete species analyzed show relatively small Root effects elicited at pH values that appear too low to be seen in vivo, while the rete-bearing species show significantly larger Root effects elicited at pH values that may occur at the eye in the presence of a gas gland and rete. This suggests that although the non-rete species do technically have a Root effect, it is not one that is likely operational at pH
values seen *in vivo*. Its presence within, and benefit to, the organism is therefore questionable.

There is a highly significant correlation between Root effect and Bohr effect magnitude among the ray-finned fishes starting around the acipenseriformes (Berenbrink et al., 2005). It has been suggested that this correlation stems from the Root effect being the extreme byproduct of the mechanism by which these early ray-finned fishes activated their Bohr effects (Berenbrink et al., 2005). The Root effect may therefore not have evolved specifically for enhanced O₂ delivery itself, but rather as the extreme manifestation of a selection-favoured Bohr effect in the early ray-finned fishes. When the choroid rete eventually evolved at a particularly beneficial site (i.e., the avascular eye) in the last common ancestor of bowfin and the teleosts, the Root effect could then be used and selected for in and of itself, as the necessary activating acidoses were now capable of being generated by the organism. The findings of the present study strongly support this idea. The significant differences in both Root effect magnitude and onset pH between species with and without retia (Figs. 3.1, 3.2) suggest the rete as playing an important role in the optimization of the Root effect. Furthermore, the extremely low onset pH values of those species lacking retia (Figs. 3.1, 3.2) suggest the rete as a necessary mechanism in the utilization of the Root effect within the organism.

The degree to which a species is capable of offloading O₂ to the tissue via the Bohr effect is determined by the product of its Bohr coefficient and the arterio-venous pH change (Lapennas, 1983). As the Bohr effect is directly proportional to the Haldane effect (proton binding of Hb upon deoxygenation), the larger the Bohr coefficient, the smaller the arterio-venous pH change. In fact, it has been shown that the optimal Bohr
coefficient for O₂ delivery to the aerobically respiring tissue is between 0.35 and 0.5, while coefficients greater than that are ideal for blood buffering/CO₂ transport (Lapennas, 1983). The Hb titrations performed in the previous study (Chapter 2) revealed the Bohr coefficients of these seven basal actinopterygian species. Those species lacking retia had maximal Bohr coefficients between 0.35 and 0.5 (sturgeon excepted at 0.65), suggesting a Hb adaptation for O₂ delivery via the Bohr effect, while those species with retia displayed maximal Bohr coefficients at or around unity, suggesting Hb adaptation for proton buffering and CO₂ transport. This is sensible given the fact that the non-rete species had relatively high Hb buffer values, while those of the rete-bearing species were generally lower (Chapter 2). Furthermore, although a large Bohr coefficient may not be ideal for O₂ delivery under regular aerobic conditions, it allows for a greater right shift of the O₂ equilibrium curve if provided with a sufficient acidosis (Lapennas, 1983). In bowfin, mooneye, and pirarucu, such an acidosis is produced by the choroid rete at the eye, and thus their large Bohr coefficients allow for larger quantities of O₂ to be delivered to the retinal cells.

There is a slight exception to the rete/non-rete dichotomy in sturgeon. Sturgeon Hbs display buffer values markedly lower than those of the other non-rete species (Chapter 2), a maximal Bohr coefficient of 0.65 (Chapter 2), and a 5% Root effect onset pH not significantly different than that of bowfin (pH 6.63; Fig. 3.2). These together suggest that sturgeon may in fact be capable of using their modest Root effect to some degree. Although there is no known gas gland/rete analogue at the eye or swimbladder of sturgeon, pH values approaching 6.6 may still be generated in capillaries where large quantities of CO₂ could potentially have profound influence on the minute blood
volume’s pH, and thus, its capacity to deliver O₂. Once in the efferent venous supply, this acidified blood would be quickly diluted so as to not negatively influence pH in the general circulation. And as roughly 98% of the O₂ in the blood is Hb-bound, even a modest 5% decrease in Hb O₂ saturation could result in large increases in blood Po₂. Although no evidence of Root effect utilization is known to exist for sturgeon, it would be interesting to analyze the O₂ tension of certain tissues (namely retinal tissue) to see if they are in fact using their modest Root effect to drive Po₂s at these sites above those of arterial blood.

Conclusions

It was shown that the Root effect is indeed a feature of the Hbs of the basal actinopterygian fishes, in agreement with the recent findings of Berenbrink et al. (2005). However, the in vivo relevance of these Root effects varies interspecifically in a manner that appears dependent on the presence or absence of the counter-current rete. Paddlefish, white sturgeon, spotted gar, and alligator gar all lack retia, and although they possess Hbs that display Root effects, they are not likely capable of utilizing them under in vivo conditions by reason of low onset pH values and a lack of any known tissue capable of generating the appropriate acidosis. Bowfin, mooneye, and pirarucu all possess choroid retia, however, and their higher onset pH values indicate they are likely operational in vivo at the eye. Taken together, the fact that the onset pH values of all seven species’ Root effects are lower than even the lowest pH values likely to occur following exposure to hypoxia or exercise renders it unlikely that the presence of the Root effect in the absence of βNHE would pose a threat to these species’ O₂ uptake. This
would allow for the positive selection of the Root effect as a mechanism of $O_2$ delivery to override any negative selection for it brought about by its potentially adverse effects on $O_2$ uptake. In this way, the low onset pH values of the species within this transitional phase of Root effect evolution may explain how this important Hb characteristic was capable of becoming almost ubiquitous among fishes thereafter, despite a lack of protective RBC βNHE activity.
Figure 3.1. The magnitude of the Root effect at different pHs in the presence (●) and absence (○) of 3:1 GTP:Hb₄ in American paddlefish (A), white sturgeon (B), spotted gar (C), alligator gar (D), bowfin (E), mooneye (F), and pirarucu (G). Values were determined spectrophotometrically on hemolysates at a [Hb₄] of 0.16 mmol l⁻¹ and a [KCl] of 0.1 mol l⁻¹. The presence of a choroid rete within the species is indicated by ®.
Figure 3.2. Root effect onset pH values in haemolysates of seven basal actinopterygian fishes in the absence (A) and presence (B) of saturating GTP (3:1 GTP:Hb₄). Root effect onset was determined for 5, 10, 15, and 20% Hb O₂ desaturation. Data are mean values ± SEM. Bars lacking SEM represent the lone sample to reach the respective desaturation percentage from that group. The presence of a choroid rete within the species is indicated by ®. Horizontal grey bars are indicative of predicted lowest RBC pHᵢ range that would occur during hypoxia or exercise, based upon literature values (see discussion for further details). Average onset value for the three rete-bearing species is significantly greater than that of the four non-reté species (P < 0.001; see Results section for further details).
Figure 3.3. Maximal Root effect as a function of Root effect onset pH (5% Root effect) for each haemolysate sample of American paddlefish, white sturgeon, spotted gar, alligator gar, bowfin, mooneye, and pirarucu. Haemolysates, in the presence of GTP (3:1 GTP:Hb₄), were brought to a final [Hb₄] of 0.16 mmol l⁻¹ and [KCl] of 0.1 mol l⁻¹. Individual points represent individual measurements. $r^2 = 0.700$; d.f. = 21; $P < 0.001$. 
CHAPTER SUMMARY

1. The Root effect onset pH values of all seven species were shown to be lower than even the lowest pH values likely to occur in the RBC following exposure to hypoxia or exercise. The presence of a Root effect in the absence of βNHE therefore does not likely pose a threat to O₂ uptake.

2. Assuming the Hb properties of these seven species to be representative of their respective ancestral states, a significant increase in Root effect magnitude and onset pH value appears to have occurred in the last common ancestor of the amiiformes and the teleosts. This is correlated with the first appearance of the choroid rete, as well as a punctuated increase and decrease in the Haldane effect and Hb buffer value, respectively (Chapter 2).

3. The low onset pH values of the plesiomorphic non-rete species, the optimality of their Bohr coefficients for O₂ delivery, and the positive correlation of the Bohr effect and Root effect together suggest that the ancestral state of the Root effect may have been nothing more than the extreme manifestation of their Bohr effects, only becoming of physiological relevance itself once a tissue capable of producing the required acidosis to elicit it (i.e., the rete) evolved at a particularly beneficial site (i.e., the avascularized retina).
References


The Hb system of fish is a curious entity by relative standards. Within it, there exists a degree of functional variation that is unseen in the Hb systems of most, if not all, other vertebrate groups. This is made manifest in a number of ways – Hb O₂ affinity is high in some fishes and low in others; Bohr/Haldane effects are large in some fishes and virtually non-existent in others; Hb multiplicity results in up to 15 isoforms per individual in some fishes, while others lack Hb altogether; and present in some fishes and not in others is a Hb-based O₂ multiplication system (the Root effect) that is believed to have played a key role in the explosive radiation of the teleosts, a group of fishes which accounts for more than half of the world’s extant vertebrate species (Weber, 2000; Baillie et al., 2004; Bonaventura et al., 2004; Berenbrink et al., 2005; Berenbrink, 2007). This assortment of Hb traits has contributed to the successful survival of fishes in habitats of all sorts, from hypoxic to hyperoxic, acidic to basic, and everything in between. And what is more, thanks to a robust fish phylogeny and increasing knowledge of the Hbs of many species within it, we can start to gain an understanding of how some of these diverse Hb characteristics arose.

The objective of this thesis was to determine the Hb proton-binding properties of species within the basal actinopterygian lineage of fishes, including their Hb buffer capacities, Haldane effects, and Root effect characteristics. The pertinence of this question lies in the fact that many of the aforementioned variations in Hb function are pH-dependent, in that they are the result of conformational changes to Hb that come about with proton-binding. This is of specific relevance to the Bohr/Haldane effects, the Root effect, and their respective influences on Hb O₂ affinity. Furthermore, it is among
the basal actinopterygian fishes that the Root effect is believed to have evolved, eventually coming to play a significant role in a respiratory physiological strategy that is unique to the teleosts (Pelster and Randall, 1998; Berenbrink et al., 2005). By analyzing the characteristics of these species' Hb proton-binding properties, light can be shed on the nature of the evolution of these Hb traits.

Synthesis

The Root effect is one component of a multi-faceted physiological system used to deliver large quantities of O₂ to the eye and swimbladder of fishes. This O₂ delivery is enabled through the cohesive functioning of these proton-sensitive Root Hbs with an acid-producing gas gland, a countercurrent rete, a low Hb buffer capacity, and protective βNHE activity. As such, it is a fascinating model by which to help understand the evolution of physiological systems. The lone study that has addressed this question suggests an evolutionary sequence that is rational on a number of levels (Berenbrink et al., 2005). However, it begs a further question insomuch as those species in which the Root effect and its related processes originally evolved appear to be at risk of a diminished O₂ carrying capacity during generalized blood acidoses due to a lack of βNHE activity. These species include those of the basal actinopterygian lineage, the fishes situated intermediate to the more plesiomorphic elasmobranchs and the more derived teleosts on the fish phylogeny. There are 36 non-teleost members of this group found living in the world today (Nelson, 1994), and while the vast majority of their contemporaries have long since gone extinct (Janvier, 2007), these 36 species have been able to successfully adapt to their ever-changing environments and squeak through the
sieve that is natural selection. Their survival is likely, in part, a testament to their adaptability, and certainly not excluded from this list of “dealt-with” selective pressures is their ability to acquire O₂ amidst generalized acidoses of the blood, as the extant members are known to deal with instances of exercise, hypoxia, and hypercarbia in their natural environments (Nelson, 1994; Brauner and Berenbrink, 2007; Ilves and Randall, 2007). I hypothesized that this would result from RBC pHᵢ remaining higher than the pH required to trigger the Root effect in these species during generalized blood acidoses. This could occur as a result of RBC pHᵢ protection by some means other than βNHE (likely Hb, for reasons discussed in Chapter 2), or Root effects in these species with onset pH values lower than those produced by the generalized blood acidoses resulting from exercise, hypoxia, or hypercarbia. The results yielded by the Hb titration experiments reported in Chapter 2 suggest the Hb proton-binding properties (intrinsic Hb buffering and oxylabile Haldane effects) of seven species within the basal actinopterygian lineage as not likely playing substantial roles in protecting the intracellular pH of the RBC. However, the results of the Hb O₂ saturation experiments reported in Chapter 3 suggest Root effect onset pH values in these same species as being lower than those likely to be produced by generalized blood acidoses brought on by exercise, hypoxia, or hypercarbia (Holeton and Randall, 1967; Butler and Taylor, 1975; Heming et al., 1986; Milligan and Wood, 1986; Randall et al., 1992; Burleson et al., 1998; Gonzalez et al., 2001; Richards et al., 2003; Baker et al., in press; Brauner and Baker, 2008). It would therefore appear as though O₂ uptake is not jeopardized in these species during generalized blood acidoses on account of Root effect onset pH values that are lower than those produced by these conditions.
The methods employed in Chapter 2, combined with the particular species analyzed, allowed me to assess another pertinent question – the nature of the transition in Hb proton-binding strategy among fishes. There are two methods by which the Hbs of fishes take up the protons yielded by CO₂ hydration within the RBC: intrinsic buffering, and the oxylabile Haldane effect (see Chapter 2). An inverse relationship has been shown to exist in fishes between the magnitudes of their Hb buffer values and their Haldane effects, such that plesiomorphic species such as elasmobranchs tend to have high Hb buffer values and small Haldane effects, while derived species such as teleosts tend to have low Hb buffer values and large Haldane effects (Jensen, 1989; Jensen et al., 1998). This transition in Hb proton-binding strategy therefore likely occurred among the basal actinopterygians, the group of fishes intermediate to the elasmobranchs and the teleosts on the phylogenetic spectrum (Janvier, 2007). As the Hb titrations employed in Chapter 2 simultaneously assess both the intrinsic buffer capacity and Haldane effect of the Hbs being titrated, so long as the Hb properties of these seven species are representative of their respective ancestral states (Janvier, 2007; McKenzie et al., 2007), titrating these Hbs could elucidate the nature of this transition in Hb proton-binding. The results suggest that the transition in Hb proton-binding strategy from large intrinsic buffering/small Haldane effect to low intrinsic buffering/large Haldane effect occurred not by gradual succession among the primitive ray-finned fishes, but rather a more punctuated process. A marked decrease in buffer value and increase in the Haldane effect occurred in the last common ancestor of bowfin and the teleosts, and is correlated with what is believed to be the first appearance of the choroid rete (Berenbrink et al., 2005). As the findings of Chapter 3 show the presence of a choroid rete to be associated with a significant increase
in the magnitude and onset pH of the Root effect, it is possible that this transition in Hb proton-binding is in some way related to the optimization and/or utilization of the Root effect in these fishes – a low intrinsic Hb buffer value would reduce the number of necessary protons shifted to the RBC cytosol to activate the Root effect, while a large Haldane effect would ensure CO₂ transport did not suffer despite the decreased Hb buffer capacity. Furthermore, being linked mechanistically to the Bohr effect, a large Haldane effect would allow for the elegant and tight coupling of O₂ and CO₂ transport in the blood of these fishes (Brauner and Randall, 1996; 1998).

Finally, the actinopterygian species analyzed in my thesis straddle the original appearance of the choroid rete in the last common ancestor of bowfin and the teleosts (Berenbrink et al., 2005). The rete is believed to be an integral part of the Root effect-related O₂ delivery system (Pelster and Scheid, 1992; Pelster and Randall, 1998; Pelster, 2001; Berenbrink et al., 2005), allowing for the diffusion of O₂, CO₂, and protons from the venous capillaries back into the arterial supply. Analyzing the Hbs of these particular species allowed me to gain an understanding of how the presence of a rete may influence the manifestation of Root effect-related Hb characteristics. Indeed, the results of Chapters 2 and 3 suggest the rete as being associated with these four related Hb characteristics, the relationships of which are made clear when the data are portrayed on the same graph (Fig. 4.1). Haemoglobin buffer capacity, Haldane/Bohr effect, and Root effect maximum and onset pH all change markedly in accordance with the appearance of the choroid rete. What is more, Fig. 4.1 suggests a somewhat different manner by which the Root effect and its related Hb properties may have evolved compared to that of our current understanding based on the findings of Berenbrink and colleagues (2005). Their
data show a gradual increase in maximal Root effect and a gradual decrease in Hb buffer value with phylogenetic progression among the basal actinopterygians, suggestive of directional selection for these Hb traits (Endler, 1986). However, as my data show Root effects in the basal orders of the actinopterygians (i.e., acipenseriformes; ginglymod) that are elicited only at very low pHs, and as these species possess no known mechanisms of generating such extreme acidoses in the blood (i.e., gas gland; rete), a question arises as to how a seemingly in vivo-irrelevant Hb trait can be selected for in a directional fashion. My data, on the other hand, show these traits as changing markedly only once an acid-producing mechanism appears at a particularly beneficial site (i.e., the avascularized eye; Fig. 4.1). This is sensible, as it is presumably only with the help of a rete that a species is capable of generating the appropriate acidosis to allow it to make use its modest Root effect. Once this countercurrent system was in place in the last common ancestor of bowfin and the teleosts, those individuals with larger Root effects elicited at more in vivo-relevant pHs would have been capable of offloading larger amounts of O₂ to their avascularized retinas. This may have allowed for improved vision, assisting in such things as foraging and mate selection, and ultimately leading to increased survival and/or reproductive success of those individuals possessing these particular Hb traits. It is in this fashion that favourable selection may have originally been placed on the Root effect and its onset pH value, as it was now capable of being utilized to its own end due to the species’ ability to generate the appropriate activating acidoses through the choroid rete. Furthermore, the theoretical negative consequences of the Root effect (i.e., jeopardized O₂ uptake during generalized blood acidoses in the absence of βNHE) were rendered negligible in these species owing to a low Root effect onset pH value (Chapter 3).
Hence, we see a marked increase in these Root effect properties starting with bowfin, the most basal of extant species to possess a choroid rete (Fig. 4.1). But if the Root effect seemingly only became physiologically relevant to organisms with the appearance of the choroid rete, why would it exist, albeit modestly, in the more basal orders (acipenseriformes; ginglymods) that lack retia? This is especially pertinent with regards to the potential negative consequences that come with a Root effect in the absence of βNHE activity (i.e., jeopardized O₂ uptake during generalized acidoses). The reason for this may lie in the positive correlation between Root effect and Bohr effect magnitudes, and the hypothesis of the Root effect being the extreme expression of the mechanism by which these early ray-finned fishes activated their Bohr effects (Berenbrink et al., 2005). The Root effect may therefore not have evolved specifically for enhanced O₂ delivery itself, but rather as the byproduct of a selection-favoured Bohr effect in the early ray-finned fishes (Berenbrink et al., 2005). The Bohr coefficients determined for the four non-rete species in Chapter 2 suggest their Bohr effects as being selected-for as a mechanism of O₂ delivery (Lapennas, 1983), as the magnitude of their Bohr coefficients are consistent with the optimal value determined for oxygen delivery under steady state conditions. This, therefore, may explain the presence of a seemingly in vivo-irrelevant Root effect in these species.

_Future directions_

I do not assume I am anomalous among graduate students in terms of the lack of satiation I feel upon reaching the end of my MSc, for, in many ways, a completed thesis project raises as many questions as it answers. Once a difficult task, posing new and
interesting research questions has become much easier with the knowledge acquired over the course of a masters degree. I will use this section to briefly expand on some interesting ideas and questions that relate to my thesis project that I believe would be worthwhile pursuits for a future masters student.

As physiological traits do not tend to leave their mark in the fossil record, the study of the evolution of physiological systems hinges in part on the comparative phylogenetic approach (Bradely and Zamer, 1999; McKenzie et al., 2007; Garland et al., 2005; Berenbrink, 2006). There are limitations to this approach in the form of assumptions that the physiology of an extant member of a "primitive" order is representative of the ancestral physiological state of that species' (normally extinct) ancestors. This is a legitimate concern, one that is particularly relevant to the study of the evolution of a protein like Hb where, it has been shown, single amino acid substitutions on a relatively large 65,000 dalton protein can have marked effects on the protein's function (Hochachka and Somero, 2002). For this, and other, reasons, it is therefore difficult to infer evolutionary relationships of species based on physiological function alone. An effective way of increasing the robustness of the comparative phylogenetic approach, however, is to include in the study as many species as possible so as to allow for a more highly resolved picture of the evolution of the physiological trait in question. Although the seven species included in this study were fairly representative of the basal actinopterygian lineage, it would be interesting to see if the inclusion of as many of the species within this group as possible would alter our understanding of how these Hb traits came about. It could also illuminate the degree of intra-order variation in these traits. If there did in fact exist a large degree of variation within a particularly variable order (e.g.,
the osteoglossids), this would possibly be an indication of adaptations of Hb to particular environments, and could be tested using phylogenetically independent contrasts.

There was an anomaly found in pirarucu with regards to its high Hb buffer value. Pirarucu's Hb buffer value is significantly higher than those of any other teleost measured (Jensen, 1989; Brauner and Weber, 1998; Jensen, 2001; Berenbrink et al., 2005), and is much more similar in magnitude to those Hb buffer values of more plesiomorphic species such as elasmobranchs (Jensen, 1989). However, it displays a Haldane effect that is markedly higher than those species, one that is not dissimilar from the Haldane effects of other teleosts (Jensen, 1989; Brauner and Weber, 1998; Jensen, 2001), suggesting a remarkable ability to buffer protons in the blood, and therefore, transport large quantities of CO₂. This is perhaps attributable to pirarucu being an obligate air-breathing fish, with arterial Pco₂ levels ten times greater than those of virtually all other fishes’ measured (Randall et al, 1978; Perry et al, 1996). It would be interesting to see if this phenomenon was paralleled in the Hbs of other obligate air-breathing fishes, namely those with a Root effect which should, presumably, possess Hbs of low buffer capacity. A likely candidate for this could be found in the climbing perch (Anabas testudineus), an obligatory air-breather (Graham, 1997) of the highly derived Perciformes order whose species have been shown to possess a large Root effect (Berenbrink et al., 2005).

One of the conclusions of Chapter 3 involves the Root effect coming into physiological relevance only when an acid-producing mechanism (i.e., the gas gland and rete) evolved at a particularly beneficial site (i.e., the eye) in the last common ancestor of bowfin and the teleosts. This is a sensible idea, but it is based on the assumption that all
fisheyesareavascularized, and thus, would benefit from an $O_2$ delivery mechanism like the Root effect. However, I am unable to find any information in the literature suggesting that any fish eyes other than those of teleosts are avascularized. If this is the case, that only teleost fishes possess avascularized eyes, then perhaps the reason for this is attributable to the fact that they have a Root effect and a choroid rete that together are capable of saturating the retinal cells with $O_2$, and thus, negate the need for a high degree of retinal vascularization. Retinal cells are highly sensitive to low $O_2$ levels (Pelster and Randall, 1998), and a way of ensuring sufficient $O_2$ saturation is by increasing their vascularization. However, this could come at the detriment to visual acuity (Chang et al., 2001). So, if a different mechanism capable of saturating these cells with $O_2$ evolved (i.e., the Root effect and choroid rete), perhaps it would be selected-for over the potentially vision-limiting high vascularization mechanism of retinal oxygenation. If this were the case, one could hypothesize that avascularization of the fish eye only began with the evolution of the choroid rete in the last common ancestor of bowfin and the teleosts, while those more plesiomorphic species lacking choroid retia would retain eyes of high vascularization so as to ensure their sensitive retinal cells remained $O_2$-saturated. Although speculative, this hypothesis is supported by the fact that the eyes of dogfish ($Squalus acanthias$), an elasmobranch lacking choroid retia, appear to be highly vascularized (Chris Wood, personal communication), as do those of white sturgeon (personal observation). It would therefore be interesting to determine the vascularization in a number of species lacking choroid retia, including those species plesiomorphic to bowfin as well as those derived teleosts that have secondarily lost their choroid retia (e.g., $Silurus glanis$, $Monopterus albus$; Berenbrink et al., 2005). Because it is currently
believed the selective pressures acting on the Root effect were in place so as to saturate an avascularized retina with O₂, if it was found that only those eyes of Root effect/retia-equipped fishes were avascularized, our understanding of Root effect evolution and the selective pressures driving it may be altered.

Possibly related to this evolutionary idea of the Root effect is the case of white sturgeon. Sturgeon Hbs display buffer values markedly lower than the other non-rete species (Chapter 2), a maximal Bohr coefficient of 0.65 (Chapter 2), and a 5% Root effect onset pH not significantly different than that of bowfin (pH 6.63; Chapter 3). Together, these suggest that the white sturgeon may be capable of using its modest Root effect in some capacity, despite its lack of known retia or acid-producing tissue. It would therefore be of interest to measure the Po₂ values at certain “candidate” tissues (i.e., those that are highly metabolically active). If these Po₂ values were higher than those of the arterial blood supply, it is likely that the Root effect is involved in O₂ delivery (Pelster and Randall, 1998). “Candidate” tissues may include the eye and the exercising muscle, where the diffusion of large quantities of CO₂ into the minute blood volume of the supply capillaries may potentially drive pH down to 6.6. Indeed, preliminary experiments looking at the ocular Po₂ values of white sturgeon have shown that the O₂ levels of their vitreous humor immediately proximal to their retinas are higher than are their arterial O₂ levels (Regan et al., unpublished). If this in fact is the case, it has the potential to modify some of the ways we view the evolution of the Root effect, and could perhaps even compliment the ideas mentioned above related to retinal vascularization.
Figure 4.1. Changes in mean values of haemoglobin (Hb) P₅₀ buffer value (circles), Haldane effect (diamonds), maximal Root effect (squares), and Root effect onset pH (triangles) in seven species belonging to four orders of basal actinopterygian fishes. In phylogenetically progressive order, these include: acipenseriformes (American paddlefish; white sturgeon); ginglymod (spotted gar; alligator gar); amiiformes (bowfin); osteoglossiformes (mooneye; pirarucu). Grey field indicates the presence of a choroid rete, with species belonging to the amiiformes believed to be the first in which this system originally evolved. All values determined from experiments performed on haemolysates at [Hb₄] of 0.04 mmol l⁻¹ (buffer value and Haldane effect) or 0.16 mmol l⁻¹ (Root effect maximum and onset pH) and a [KCl] of 0.1 mol l⁻¹, in the presence of organic phosphates (3:1 GTP:Hb₄).
1. Although their Hb proton-binding properties (intrinsic buffering and Haldane effect) do not appear to be playing any major role in protecting RBC pH_i during generalized acidoses of the blood, the basal actinopterygian fishes appear to protect O_2 uptake during these conditions by way of Root effect onset pH values below those produced by the generalized acidoses themselves. This is despite these fishes displaying relatively large Root effects and lacking RBC pH_i-protecting βNHE.

2. Assuming the Hb properties of these seven species to be representative of their respective ancestral states, numerous Hb properties changed in marked, and even significant ways, in correlation with the appearance of the choroid rete in the last common ancestor of the amiiformes and the teleosts. This Hb traits include: buffer value (decreased); Haldane effect (increased); Bohr effect (increased); Root effect magnitude (increased); Root effect onset pH value (increased).

3. The results yielded by this thesis have provided numerous further questions, and with them, research avenues that would be interesting to pursue in the future.
References


