PATTERNS OF PLASMA ACCESSIBLE CARBONIC ANHYDRASE LOCALISATION IN DERIVED, BASAL, AND DEVELOPING FISHES

by

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Abstract

Teleosts comprise half of all vertebrates and have successfully invaded every aquatic habitat on Earth. They owe this success, at least in part, to their unique oxygenation system. This system is comprised of highly pH sensitive hemoglobins (Hb; large Bohr and Root effects), red blood cell (RBC) intracellular pH (pHi) protection by a beta adrenergically stimulated sodium proton exchanger (β-NHE), and a heterogeneous distribution of plasma accessible carbonic anhydrase (paCA; absence at the gills and presence at the tissues). Together, these components enhance oxygen O₂ unloading to the tissues while protecting oxygen uptake at the gills during a generalised acidosis. While the first two components have received much research interest, the third component, a heterogeneous distribution of paCA, has not and is the focus of my thesis. It has been hypothesised that the absence of paCA in the gills of teleosts is due to the presence of the large Root effect and the associated risks to O₂ loading. In my thesis I aimed to address this principal hypothesis with three research chapters. I firstly confirmed the presence of paCA in most tissues, but absence in all four gill arches of the rainbow trout, which until now had been assumed to be true based on data exclusively from gill arch 2. I then investigated rainbow trout through ontogeny, and found that pre-hatch, during embryonic Hb expression branchial paCA is expressed while β-NHE is not; a pattern that exhibits an abrupt switch with hatching. Finally, I investigated several CA characteristics in three basal actinopterygian species for which nothing was known previously. I demonstrated that paCA in the gill was likely lost earlier than predicted by my principal hypothesis, suggesting instead that the loss of branchial paCA is related to the increasing magnitude of the Bohr coefficient and corresponding decrease in Hb buffer capacity. Collectively, my findings suggest that the presence of a large Root effect is unlikely to be the single selective...
factor for the loss of branchial paCA in teleosts, with implications for our understanding of a group that represents half of all vertebrates.
Lay Summary

Teleost fishes are one of the most successful and diverse groups on Earth. Their success may be due to unique adaptations allowing them to enhance oxygen supply to their tissues compared to other vertebrates. Teleosts have very pH sensitive haemoglobins, but by actively regulating their red blood cell pH, they can safeguard oxygen uptake at their gills, and localise plasma accessible carbonic anhydrase (paCA) to enhance oxygen delivery to the tissues. I confirmed that adult rainbow trout possess paCA in most tissues, and entirely lack paCA in the gills, but that this is not the case in these fish before hatching. By investigating fishes from groups ancestral to the teleosts, I discovered that the loss of gill paCA likely occurred earlier than previously thought. This system is expected to be found in all teleosts and so the results of my thesis have implications for half of all vertebrates.
Preface

A version of Chapter 2 has been published as: Nelson, C., Dichiera, A. M., Jung, E. H., & Brauner, C. J. (2023). An atlas of plasma accessible carbonic anhydrase availability throughout the vasculature of the model teleost, the rainbow trout. *Journal of Comparative Physiology B*. DOI: 10.1007/s00360-023-01484-7. I designed the study with input from Dr. Brauner. I carried out all analyses with sampling assistance from Dr. Jung, and primer design assistance from Dr. Dichiera. I wrote the manuscript with editorial input from Dr. Jung, Dr. Dichiera, and Dr. Brauner.

A version of Chapter 3 has been prepared for publication as: Nelson, C., Dichiera, A. M., & Brauner, C. J. (in submission). Branchial plasma accessible carbonic anhydrase is an embryonic trait in rainbow trout (*Oncorhynchus mykiss*) that is lost with development and the onset of the Root effect. I designed the study with input from Dr. Brauner. I carried out all analyses with primer design assistance from Dr. Dichiera. I wrote the manuscript with editorial input from Dr. Dichiera, and Dr. Brauner.

A version of Chapter 4 has been prepared for publication as: Nelson, C., Standen, A. M., Allen, P. J. & Brauner, C. J. (in submission). Basal actinopterygians provide insight into the evolution of the unique teleost oxygenation system: an analysis of the loss of branchial plasma accessible carbonic anhydrase. I designed the study with input from Dr. Brauner. I carried out all analyses with materials provided by from Dr. Standen and Dr. Allen. I wrote the manuscript with editorial input from Dr. Standen, Dr. Allen, and Dr. Brauner.
All animal husbandry and experiments were conducted according to the policies and guidelines of The Canadian Council on Animal Care and approved by the UBC Animal Care Committee (Protocol no. A19-0284-A009). Animals sourced from The University of Ottawa (samples supplied by Dr. Standen, chapter 4) were also subject to approval by The University of Ottawa Animal Care Committee (protocol no.: BL-3625 and BL3671). Animals sourced from Mississippi State University (samples supplied by Dr. Allen, chapter 4) were also subject to approval by the Institutional Animal Care and Use Committee at Mississippi State University (protocol no: 21-161).
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List of Symbols and Abbreviations

A  gill surface area
AE-II  type II anion (Cl\(^-\)/HCO\(_3^\)-) exchanger
ANOVA  analysis of variance
atm  atmospheres
ATP  adenosine triphosphate
ATUs  accumulated thermal units
bl s\(^{-1}\)  body lengths per second
CA  carbonic anhydrase
CA-II  carbonic anhydrase II isoform
CA-IV  carbonic anhydrase IV isoform
CA-IVa  carbonic anhydrase IVa isoform
CA-IVb  carbonic anhydrase IVb isoform
CA-IVc  carbonic anhydrase IVc isoform
cat.  catecholamines
CaCO\(_3\)  calcium carbonate
Ca\(^{2+}\)  calcium
cDNA  complimentary DNA
Cl\(^-\)  chloride
CO\(_2\)  carbon dioxide
C18  membrane impermeable CA inhibitor
dpf  days post fertilisation
dph  days post hatch
G         g-force
GPI       glycophosphatidylinositol
GTP       guanosine triphosphate
H+        proton
Hb        hemoglobin
HbA       adult Hb isoforms
HbE       embryonic Hb isoforms
Hb-O2     hemoglobin bound O₂
HCO₃⁻      bicarbonate
His       histidine
H₂O       water
H₂CO₃      carbonic acid
ILOS       incipient lethal O₂ concentration
K          diffusion coefficient
K⁺        potassium
Mg²⁺       magnesium
mM        millimolar
mmol      millimoles
MO₂       oxygen consumption rate
mRNA      messenger RNA
MYA       million years ago
n         sample size
N₂        nitrogen
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Term</th>
</tr>
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<tbody>
<tr>
<td>Na⁺</td>
<td>sodium</td>
</tr>
<tr>
<td>NH₃⁺</td>
<td>ammonia</td>
</tr>
<tr>
<td>OEC</td>
<td>oxygen equilibration curve</td>
</tr>
<tr>
<td>OH⁻</td>
<td>hydroxide</td>
</tr>
<tr>
<td>O₂</td>
<td>oxygen</td>
</tr>
<tr>
<td>paCA</td>
<td>plasma accessible carbonic anhydrase</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pH</td>
<td>- log [H⁺]</td>
</tr>
<tr>
<td>pHe</td>
<td>extracellular pH</td>
</tr>
<tr>
<td>pHᵢ</td>
<td>intracellular pH</td>
</tr>
<tr>
<td>PICA</td>
<td>plasma inhibitors of CA</td>
</tr>
<tr>
<td>PI-PLC</td>
<td>phosphatidylinositol specific phospholipase C</td>
</tr>
<tr>
<td>PCO₂</td>
<td>partial pressure of CO₂</td>
</tr>
<tr>
<td>PO₂</td>
<td>partial pressure of O₂</td>
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<tr>
<td>PO₂w</td>
<td>PO₂ in water</td>
</tr>
<tr>
<td>PO₂b</td>
<td>PO₂ in blood</td>
</tr>
<tr>
<td>PO₂w – PO₂b</td>
<td>PO₂ difference between water and blood</td>
</tr>
<tr>
<td>P₅₀</td>
<td>partial pressure of oxygen at which Hb molecules are half saturated</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative PCR</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
</tr>
<tr>
<td>RQ</td>
<td>respiratory quotient (= CO₂ produced / O₂ consumed)</td>
</tr>
<tr>
<td>R²</td>
<td>coefficient of determination</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulphate</td>
</tr>
</tbody>
</table>
SEM  standard error of the mean

\( t \)  thickness

TMS  tricaine methanesulfonate

\( t_{1/2} \)  half time

\( U \)  units

\( Zn \)  zinc

\( \alpha \)  alpha

\( \beta \)  beta

\( \beta \text{Hb} \)  hemoglobin buffer capacity

\( \beta \text{-NHE} \)  beta adrenergically stimulated \( \text{Na}^+ / \text{H}^+ \) exchanger

\( \Delta \)  delta

\( \Delta \text{a-vO}_2 \)  arterio-venous difference in \( \text{O}_2 \) concentration

\( \Delta \text{a-vpH} \)  arterio-venous pH difference

\( \Delta \log P_{50} \)  log \( P_{50} \) difference

\( \Delta \text{pH} \)  pH difference

\( \mu \text{L} \)  microlitre

\( \mu \text{mol} \)  micromole

\( \Phi \)  Bohr coefficient

\([ \] \)  concentration of (symbol inside square brackets)

\( \pm \)  plus or minus
Glossary

Acidosis: the decrease in pH (corresponding increase in [H\(^+\)]) in a fluid.

Bohr effect: the decrease in Hb-O\(_2\) affinity with an increase in [H\(^+\)], which is quantified by the Bohr coefficient (\(\Phi = \Delta \text{log } P_{50}/\Delta \text{pH}\)). Reciprocal: Haldane effect.

Buffer capacity: the ability of a solution to resist pH change.

Embryonic: describes a trait or phenotype that exists during early stage development, primarily before hatching.

Endogenous: a substance produced internally by the body.

Hypercapnia: increased blood CO\(_2\) concentration.

Hypoxia: decreased blood O\(_2\) concentration.

Isoform: a member of a set of functionally similar proteins that originate from a single gene family and are the result of genetic differences.

Lamellae: thin multi-tissue structures of fish gills through which blood flows in a countercurrent orientation to water to maximise O\(_2\) uptake.

Ontogeny: the process of individual development from a single cell, an egg cell or a zygote, to an adult organism.

Paedomorphic: an embryonic or juvenile trait that is retained into adulthood.

Physoclistous: swimbladder without a connection to the external environment.

Physostomous: swimbladder with a pneumatic duct connecting the bladder to the external environment.

Retia mirable: vascular structures made up of counter-current flowing blood vessels, in close proximity, which are used to exchange and concentrate heat, ions or...
gases

Root effect the reduction in Hb carrying capacity for O₂ due to a reduction in pH and is accompanied by reduced cooperativity of Hb O₂ binding

Tetrameric a molecule that is comprised of four structural subunits
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I owe the utmost gratitude to my supervisor, Dr. Colin Brauner, for his continued patience and guidance throughout my PhD. Thank you for this opportunity to ask all the questions and explore all the possibilities. Thank you for the opportunities to travel, collaborate, and learn, and for sharing your love of carbonic anhydrase with me. I am so grateful to Dr. Bill Milsom who has also mentored and guided me throughout this process. I will always fondly remember our Wednesday afternoon discussions of all things animal physiology. Special thank you also to my other committee members, Dr. Philip Matthews and Dr. Eric Taylor, for your vast knowledge and copious feedback. To Eric Lotto and Patrick Tamkee, thanks for all your help in the aquatics facility – I certainly wouldn’t have made it here without you. Thanks to Benjamin Mills for illustrations used throughout my thesis and many presentations. I am also thankful for all the wonderful collaborators and scientists I have had the pleasure of working with and who will always inspire me.

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It would be remiss of me not to acknowledge the land on which this work and play occurred. I conducted my PhD on the traditional, ancestral, and unceded territory of the Coast
Salish People: the xʷməθkʷəy̓əm (Musqueam), Sḵwx̱wú7mesh (Squamish), and səlilwətaɬ (Tsleil-Waututh) Nations. I came here as an uninvited visitor and I will be forever grateful for the opportunity to have learned, worked, played, and lived in this beautiful part of the world.

Finally, thanks must go to my family. I appreciate your unwavering support of my pursuit into a world foreign to your own experiences. One day I’ll get better at explaining what it is that I do! Without your endless planning of holidays to Canada, the past five years would have felt much longer and harder. Benjamin, how you have sweetened this whole experience – words cannot express how grateful I am for you.
Chapter 1: Introduction

Respiration comprises a suite of characteristics essential to all life. Aerobic metabolism requires the ability to take up O\textsubscript{2} from the environment, transport it to the respiring cells, produce ATP, and excrete metabolic CO\textsubscript{2}. These basic steps display various modifications and specialisations across groups, but their primary functions are preserved. Animals breathe air, water, or both, using lungs, gills, accessory air breathing organs, the skin, or some combination (Carter, 1931). Life evolved in water (Brack, 1993) and as such, water breathing is thought to be ancestral to air breathing (Bayley et al., 2018); fishes therefore represent an ideal system in which to study the evolution of respiratory characteristics.

The teleost fishes comprise half of all extant vertebrate species (Helfman et al, 2009; Ravi & Venkatesh, 2018). They belong to the ray-finned fishes and underwent explosive adaptive radiation in the wake of the Permian Period mass extinction event (Glasauer & Neuhauss, 2014; Randall et al., 2014). They exhibit vast degrees of variation in all aspects of morphology, physiology, and life history traits (Randall et al., 2014) which has allowed them to inhabit a wide variety of aquatic environments. Modern teleosts are highly derived with many specialisations compared to the ancestral vertebrate condition. Arguably one of the most notable specialisations this group exhibits, and the focus of my thesis, is their unique enhanced O\textsubscript{2} delivery system (Rummer, 2010; Randall et al., 2014; Harter & Brauner, 2017).

The teleost system of enhanced O\textsubscript{2} unloading is comprised of three major components: a highly pH sensitive hemoglobin (Hb), red blood cell (RBC) pH\textsubscript{i} protection, and a heterogeneous distribution of plasma accessible carbonic anhydrase (paCA; Harter & Brauner, 2017). This suite of characteristics apparently evolved under conditions of low environmental O\textsubscript{2} (fig. 1.1; Clack, 2007) and enables the teleosts to increase O\textsubscript{2} unloading to the tissues by up to 65% with no change.
in blood perfusion (Rummer et al., 2013). Enhanced O₂ unloading briefly functions as follows.

The presence of highly pH sensitive Hbs effectively couples O₂/CO₂ transport through the RBC (Brauner & Rummer, 2011). Possession of highly pH sensitive Hbs selected for the ability to control RBC pH to protect O₂ uptake at the gills (Nikinmaa, 1982). The teleosts protect RBC pH via a Na⁺/H⁺ exchanger that is activated by catecholamines and is therefore triggered by stress (for example after exhaustive exercise; Nikinmaa, 1982; Berenbrink et al., 2005). At the respiring tissues paCA transfers acid loads into the RBC and effectively short circuits the RBC pH protective mechanism (Harter & Brauner, 2017). Certain tissues (for example, the eye and swimbladder) possess retia, in which a counter current vascular arrangement and gas gland magnify this effect (Berenbrink, 2011). This short circuiting is beneficial as it enhances O₂ unloading and is thought to have originally evolved to ensure adequate O₂ delivery to the avascular retina (Berenbrink et al., 2005; Berenbrink, 2007; Damsgaard et al., 2020). The RBC pH short circuiting represents, however, a potentially dangerous situation at the gills and paCA is absent in the branchial vasculature of teleosts (Harter & Brauner, 2017) ensuring that environmental O₂ uptake is not compromised during a generalised acidosis. Therefore, the absence of paCA in the gills appears to be as essential as its presence in the respiring tissues for the maintenance of O₂ transport.

Together the existence of highly pH sensitive Hbs, RBC pH protective mechanism, and a heterogeneous distribution of paCA comprise the system of enhanced O₂ unloading and make adult teleosts truly unique compared to other vertebrates. While the majority of work investigating this system has been conducted in salmonids (Rummer & Brauner, 2011; Rummer et al., 2013; Alderman et al., 2016; Harter & Brauner, 2017; Harter et al., 2019), it appears likely that this system may be widely available in teleosts (Berenbrink et al., 2005; Dichiera & Esbaugh, 2020;
Shu et al., 2022). We know very little about the functioning of this system in developing teleosts; however, we may expect it to be reminiscent of that seen in other, non-teleostean vertebrates. Elasmobranchs, other basal fishes, and air-breathing vertebrates have somewhat pH sensitive Hbs, lack RBC pH protection, and possess paCA throughout the vasculature, including at the gas exchange surface (Berenbrink et al., 2005). Berenbrink et al. (2005) plotted the evolution of Hb pH sensitivity and RBC pH protection through a teleost lineage (see section 1.4) but we do not yet know how the evolution of heterogeneous expression of paCA evolved, an area central to my thesis.

In my thesis I sought to address the following principal hypothesis: **hemoglobins with a large, physiologically relevant Root effect have imposed constraints on branchial plasma accessible carbonic anhydrase availability within teleost fishes.** I addressed this principal hypothesis via three observational studies. Chapter 2 of my thesis focuses on establishing an atlas of paCA localisation in 14 separate tissues of the rainbow trout (*Oncorhynchus mykiss*), filling various knowledge gaps that have prevailed in the literature for some time. Chapters 3 and 4 address my principal hypothesis by focussing on scenarios where the potential constraint of highly pH sensitive Hbs on branchial paCA availability may be lifted: in early life stage teleosts (chapter 3), and in basal ray-finned fishes (chapter 4). The remainder of chapter 1 will now review the above concepts in detail and discuss how they led to the development of my thesis' principal hypothesis and the experimental design employed. This introductory chapter will then conclude by discussing the specific hypotheses and predictions of each study chapter.
1.1 Fishes

Life evolved in the primordial oceans (Miller, 1953; Brack, 1993; Eichenseer, 2019) and has since experienced at least 3.5 billion years of evolution (Mojzsis et al., 1996). Early vertebrate life evolved in the Cambrian Period and the jawed fishes first appear in the fossil record during the mid-Silurian Period (Shu et al., 2003). The Devonian Period was significant for aquatic vertebrate evolution and saw the appearance of the first fishes (Ilves & Randall, 2007). The lobe-finned fishes (Sarcopterygii) were highly successful in the early Devonian and gave rise to the first tetrapods which colonized land (González & Northcutt, 2011). The ray-finned fishes (Actinopterygii) diverged from the lobe-finned fishes ~ 400-450 million years ago and today comprise half of all extant vertebrates (Nelson et al., 2016). The diversity of lobe-finned fishes collapsed during the Permian Period mass extinction event and today’s extant representatives comprise six species of lungfishes and two species of coelacanths (Lopez et al., 2017). During the Permian Period mass extinction event, the actinopterygians were able to take advantage of this new niche availability (due to loss of sarcopterygian species) and underwent explosive adaptive radiation (Randall et al., 2014). Extant ray-finned fishes are often subdivided into basal non-teleosts (four major lineages: polypteriformes, acipenseriformes, lepisosteiformes, and amiiformes) and the teleosts (Venkatesh, 2003), which represent more than 98% of extant fish species (Helfman et al., 2009; Nelson et al., 2016; Ravi & Venkatesh, 2018).

1.2 The teleosts

Teleost fishes are a diverse and varied group comprising over 30,000 species (Helfman et al., 2009; Nelson et al., 2016; Ravi & Venkatesh, 2018). Modifications enhancing swimming and feeding, along with an additional genome duplication event increased their rate of evolution (Ilves
& Randall, 2007; Opazo et al., 2012) and led to them becoming one of the most successful groups on Earth. For example, morphological specialisation of jaw structures in cichlids are considered fundamental innovations as they allowed extreme specialisation of food source use and lineage diversification within individual lakes (Liem, 1973; Kocher, 2004; Singh et al., 2017). Similarly, the physoclistous swimbladder (filled by diffusion of gas from the bloodstream, as opposed to the physostomous swimbladder that is filled by gulping air at the surface) allowed species to invade deeper habitats yet maintain hydrostatic equilibrium during vertical migrations (Steen, 1970; Bayley et al., 2018).

A pre-requisite for the evolution of the physoclistous swimbladder is the development of the unique teleost oxygen (O₂) delivery system (Berenbrink et al., 2005; Randall et al., 2014). This system evolved during a period of low atmospheric O₂ in the 100 million years following the Permian Period mass extinction event (fig. 1.1; Clack, 2007; Randall et al., 2014), and is thought to be associated with the high degree of hypoxia tolerance that exists among extant teleosts. This has, at least in part, enabled the teleosts to become one of the most successful vertebrate groups, allowing them to exploit almost every aquatic habitat including those characterised by low O₂, high carbon dioxide (CO₂), high hydrostatic pressure, and variable salinity (Wootton, 1990; Hwang, 1987). The following sections of this introduction explore the mechanisms, relative importance and evolutionary development of the physiological adaptations associated with this unique O₂ delivery system in the context of the adaptive radiation that occurred in the teleost lineage. Without any one of these adaptations, the teleosts may not be the speciose group they are today (Randall et al., 2014).
1.3 Respiration

Aerobic respiration encompasses an essential suite of processes in all living organisms which are involved with the provision of O$_2$, and so energy, for vital cellular processes (Hughes, 1963). The O$_2$ transport cascade (section 1.3.2) describes how environmental O$_2$ and CO$_2$ are transported between the gas exchange surface and the tissues. Vast modifications of form and function exist across vertebrate life, and the following sections will focus on the components of the suite of respiratory processes most relevant to the teleosts, and my thesis.

1.3.1 The multifunctional fish gill

Most fishes use gills as the primary gas exchange surface. These gills are constructed of two gill baskets (one on each side of the head) each containing four gill arches, comprised of filaments and lamellae (fig. 1.2A). These structures exhibit various adaptations that maximise the ability to extract O$_2$ from the environment and excrete CO$_2$. Due to the lower solubility of O$_2$ relative to CO$_2$ in water, fishes primarily regulate their ventilation to ensure O$_2$ uptake rather than CO$_2$ removal (as is the case in air-breathing vertebrates; Evans et al., 2005). While all fish gills possess a similar basic structure, there are a range of adaptations specific to the environment inhabited by different species. For example, the remodelling of gill filaments and lamellae is a common response exhibited by fishes inhabiting low O$_2$ or ion rich environments (Shartau & Brauner, 2014; Scott et al., 2016) and such gill remodelling is usually accompanied by reduction in reliance on the gills for O$_2$ uptake (Bayley et al., 2018; Damsgaard et al., 2019a). Indeed, air-breathing in fishes has evolved as many as 80 times independently (Damsgaard et al., 2019a) often in hypoxic and/or hypercapnic environments and consequently a range of air-breathing apparatuses have evolved (Bayley et al., 2018; Damsgaard et al., 2019a).
While gills are crucial for the exchange of respiratory gases in all water breathing fishes, they also play vital roles in ion exchange, acid-base regulation, and ammonia excretion (Evans et al., 2005). Ionocytes, concentrated on the trailing edge of the lamellae (Christiansen et al., 2012), exchange ions (primarily sodium (Na\(^+\)), chloride (Cl\(^-\)), and ammonia (NH\(_3\))) with the external water, with the specifics of this transport depending on the salinity of the environmental water (fresh, brackish or saltwater). Species that can move between environments differing in ionic composition and salinity (for example, salmonids) possess various physiological mechanisms to cope with challenges associated with this transition (Björnsson et al., 1989). While it is important to remember that the fish gill is a multi-functional organ, these roles are not central to the focus of my thesis and so the following sections will predominantly consider the role of the gills in relation to gas exchange.

### 1.3.2 The oxygen transport cascade

The oxygen transport cascade (fig. 1.2) describes the physiological processes of acquiring O\(_2\) from the environment and transporting it to the tissues where it is used in the production of ATP. This cascade includes five major steps: 1) gill ventilation (fig. 1.2B), 2) gill diffusion (1.2C), 3) gill and tissue perfusion, 4) tissue diffusion, and 5) mitochondrial O\(_2\) delivery. This suite of processes exists without any one rate limiting step (theory of symmorphosis; Weibel et al., 1991) thereby maximising the efficiency of the system. As stated above, the cascade begins with gill ventilation: the process of moving water over the gills, facilitating the diffusion of O\(_2\) into the blood (step 2). The gills are ventilated by buccal cavity musculature to provide almost continuous water flow across the lamellae (Evans et al., 2005), thereby, maintaining partial pressure gradients between the external and internal environments.
Step two of the oxygen transport cascade is the diffusion of gases across the gills, which can be described using the Fick equation:

$$\text{MO}_2 = \dot{Q} \times \beta_b (P_{aO_2} - P_{vO_2})$$

where $\text{MO}_2$ describes the rate of $O_2$ consumption, $\dot{Q}$ is the cardiac output, $\beta_b$ is the blood $O_2$ capacitance, $P_{aO_2} - P_{vO_2}$ is the partial pressure gradient between the arterial and venous blood. The Fick equation can be applied to explain features of gas exchange surfaces generally; a short diffusion distance, thin gas exchange membrane, and large surface area (as are all true of fish gills) maximise the ability of gases to diffuse across the membrane. Counter-current flow of water and blood maintains the $P_{O_2}$ gradient and allows fishes to extract more than 80% of the dissolved $O_2$ from the water (Cameron & Davis, 1970; Lomholt & Johansen, 1979).

Perfusion of the gills with blood via a vast capillary network allows this dissolved $O_2$ to move into the RBCs where it reversibly binds to Hb (step 3). Blood flow through the vasculature is dependent on cardiac output, which is a function of stroke volume and heart rate. Both parameters are highly plastic allowing the animal to increase $O_2$ delivery during stress (for example to facilitate predator escape) and minimise expenditure when demand is reduced (at rest). $O_2$ transport and delivery is also controlled through changes in [Hb] and $O_2$-affinity that vary depending on a multitude of factors (section 1.3.3). The same processes that regulate gill perfusion also apply to tissue perfusion.

At the respiring tissues Hb releases its bound $O_2$, which diffuses into adjacent cells down its partial pressure gradient (step 4; Riley & Cournand, 1951). Similarly to the gills, a short diffusion distance, large partial pressure gradient, and large capillary surface area enhance the rate of diffusion of $O_2$ into the cells (Piiper, 2000). Oxygen in the cells diffuses into the mitochondria
where it acts as the final electron acceptor in the electron transport chain resulting in the production of ATP (Babcock, 1999). This represents the final step in the O₂ transport cascade.

By considering the O₂ transport cascade steps in reverse, it is possible to visualize CO₂ movement from the respiring tissues to excretion across the gills (fig. 1.2). Carbon dioxide is freely diffusible across cell membranes and moves down its partial pressure gradient from the tissues into the blood. In the RBCs it is converted into HCO₃⁻ and H⁺ by carbonic anhydrase (CA; section 1.3.7). HCO₃⁻ is transported out of the RBC in exchange for Cl⁻ by a type-II anion exchanger (AE-II) and is transported through the vasculature in this form. Upon reaching the gills, HCO₃⁻ moves back into the cell and is rehydrated to CO₂, which can then diffuse across the branchial epithelium (Perry, 1986) and into the water.

Although the O₂ transport cascade is thought to exist without any one rate limiting step (Weibel et al., 1991), the movement of CO₂ appears to be limited by the rate of HCO₃⁻ uptake into the RBC by AE-II (Swenson, 1990; Perry & Gilmour, 1993). Perhaps the most important component of the O₂ transport cascade to my thesis is Hb, and particularly the characteristics of teleost Hbs that are explored in detail in the following sections (section 1.3.3 and 1.3.4).

### 1.3.3 Hemoglobin

Teleosts, along with most vertebrates, possess tetrameric Hbs encapsulated within the RBCs, consisting of two alpha (α) and two beta (β) subunits. Hemoglobin is one of the most intensively studied proteins and numerous comprehensive reviews describe its structure and function in detail (for example, Jensen et al., 1998; Weber & Fago, 2004; Brittain, 2005; Storz et al., 2013; Storz, 2019). Each subunit contains a haem group with a central iron atom that is responsible for reversibly binding and transporting O₂ (Weber & Jensen, 1988). Upon oxygenation
Hb undergoes a conformational change from the T-state of deoxy-Hb to the R-state of oxy-Hb (Cameron, 1989). Most teleosts possess various Hb isoforms, and several are typically expressed simultaneously within an individual (Witeska, 2013), a characteristic that is thought to be adaptive in waters with variable O₂ content (Jensen et al., 1998). Hb isoforms differ in their O₂ affinity which is commonly assessed using P₅₀: the PO₂ required to achieve saturation of 50% of each Hb molecule (fig. 1.3; Weber & Jensen, 1988). A low P₅₀ value indicates high Hb-O₂ affinity which facilitates the binding of O₂ at the gills. Conversely, a high P₅₀, facilitates O₂ unloading and is beneficial at the respiring tissues. P₅₀ can be affected by temperature, pH, and heterotrophic ligand binding, for example, organic phosphates (ATP or GTP) and Cl⁻ (Nikinmaa & Boutiler, 1995; Jensen et al., 1998; Nelson et al., 2019). In the presence of heterotrophic ligands, P₅₀ increases due to increased formation of salt bridges between dimers (Perutz, 1970). Through spatial and/or temporal modulation of heterotrophic ligand concentrations within the RBC, O₂ affinity of Hb is modifiable, allowing control of uptake and release of O₂ throughout the vasculature (Jensen et al., 1998). pH is particularly important for the modification of Hb-O₂ affinity in teleosts due to their increased pH sensitivity relative to other vertebrates (large Bohr/Root effects; section 1.3.4).

In addition to the increased pH sensitivity observed in teleost Hbs (explored further in section 1.3.4), they also show reduced Hb buffer capacity (βHb) compared to that found in mammalian Hbs due to the reduction in the number of conserved Histidine (His) residues, and the acetylation of N-terminal amide groups (Jensen, 1989). A low βHb may be vital to the teleosts unique O₂ delivery system as it affects the buffering dynamics between the plasma and the RBC compartments and its strength is related to the magnitude of Hb pH sensitivity (Berenbrink, 2006; explored in further detail in section 1.3.4).
### 1.3.4 The Bohr and Root effects

The Bohr and Root effects represent characteristics of teleost Hbs that allow the modulation of Hb-O$_2$ affinity based on changes in pH and represent one of the three major components of the unique teleost oxygenation system. These effects can be visualized through alterations of shape in the oxygen equilibration curve (OEC; fig. 1.3). The (alkaline) Bohr effect is defined as the decrease in Hb-O$_2$ affinity with an increase in [H$^+$], which is quantified by the Bohr coefficient ($\Phi = \Delta \log P_{50}/\Delta \text{pH}$; Bohr et al., 1904; West, 2019). Respiring tissues produce CO$_2$ which reduces blood pH in the tissue capillaries (localized acidosis). Due to the presence of Bohr effect Hbs, this pH change causes an increase in $P_{50}$, and facilitates O$_2$ unloading (Nikinmaa, 1990). Therefore, the Bohr effect greatly increases the capacitance of the blood for O$_2$ by enabling changes in $P_{50}$ throughout the circulation, preserving conditions that benefit O$_2$ loading at the gills and unloading at the tissues.

While the Bohr effect is found in almost all vertebrates, teleosts display a larger Bohr coefficient compared to other vertebrates (human $\Phi = -0.48$ (Severinghaus, 1966), trout $\Phi = -0.91$ (Rummer & Brauner, 2015), which plays a significant role in gas exchange (Maren & Swenson, 1980), increasing the magnitude of Bohr induced tissue oxygenation by an order of magnitude (Rummer & Brauner, 2011). The increased magnitude of the Bohr coefficient in teleosts is associated with a reduced number of histidine residues (Jensen, 1989; Lukin & Ho, 2004) and corresponds to a decreased $\beta$Hb. Hbs with a large Bohr coefficient therefore require a reduced acid load to trigger Hb deoxygenation (Berenbrink, 2007). The Haldane effect is the mirror image of the Bohr effect (Christiansen et al., 1914) and as such describes the displacement of CO$_2$ and H$^+$ from Hb by oxygenation. Thermodynamic evidence that Bohr and Haldane coefficients are numerically the same was demonstrated by Wyman (1964).
The large Bohr coefficient observed in teleosts appears to have evolved once and was associated with the evolution of the Root effect (Root, 1931; Berenbrink et al., 2005); a trait exclusive to teleosts. The Root effect is defined as the reduction in Hbs carrying capacity for \( \text{O}_2 \) in response to a reduction in pH (Nikinmaa, 1986) and is accompanied by reduced Hb dimer cooperativity (changes in Hill coefficient) at low pH (Root, 1931). Similar to the Bohr effect, the Root effect arises from quaternary changes in Hb structure as a result of additional bonds between dimers (Perutz & Brunori, 1982) and is often described as an exaggerated form of the Bohr effect (Perutz & Brunori, 1982; Brittain, 2005). A ‘normal’ teleost Root effect is considered to cause reductions in Hb-\( \text{O}_2 \) saturation of at least 40% (this definition is used throughout; Berenbrink et al., 2005). Teleosts, which exhibit both an alkaline Bohr effect as well as the Root effect, do not exhibit an acid Bohr effect (increased Hb-\( \text{O}_2 \) binding at low pH; Berenbrink, 2006). The absence of the acid Bohr effect allows for the continued decrease in Hb-\( \text{O}_2 \) affinity at low pH (Brittain, 2005). Therefore, Root effect Hbs always have a large Bohr effect, presenting challenges in teasing apart the contributions of these mechanisms to the unique teleost oxygenation system. Throughout my thesis I will refer to them cumulatively, either as the large Bohr/Root effect or the highly pH sensitive Hbs found in teleosts.

The large Bohr/Root effects of teleost Hbs may be of greater benefit to \( \text{CO}_2 \) transport, than to \( \text{O}_2 \) unloading in the general circulation. Lapennas (1983) proposed that the optimal Bohr/Root coefficient for oxygen delivery would be half the respiratory quotient (\( \text{RQ} = \text{CO}_2 \) produced/\( \text{O}_2 \) consumed; classically 0.7-1.0) of the respiring tissue. Most air breathing vertebrates that have a Bohr effect have a Bohr coefficient about half the value of RQ (0.35-0.5) and therefore fall within Lapennas’ (1983) range for optimization of \( \text{O}_2 \) delivery. By contrast, teleosts typically possess much larger Bohr/Root coefficients, reaching above the range of the optimal RQ proposed by
Lapennas (1983) suggesting that the Bohr coefficient in teleosts is instead optimised for CO$_2$ transport (Morris, 1967). Despite the theory of Lapennas (1983), more research effort has focussed on elucidating the role of the Bohr/Root effect on enhanced O$_2$ unloading in teleosts tissues, particularly in tissues with vascular counter current exchangers (section 1.3.5).

1.3.5 Retia mirabilia and enhanced O$_2$ unloading

Retia mirabilia are vascular structures made up of counter-current flowing, closely packed blood vessels, which are used to exchange and concentrate heat, ions or gases (Witternberg & Witternberg, 1974). Many teleosts possess retia in the eye (choroid rete) and as part of the physoclistous swimbladder (swimbladder rete). The choroid rete allows for enhanced O$_2$ delivery to the eye, which permits reduced capillary density and improved visual acuity (Damsgaard et al., 2019b). These characteristics likely lifted constraints of aerobic respiration in the avascular eye and enabled teleosts to invade deeper, darker waters with their improved vision (Pelster & Weber, 1991; Berenbrink et al., 2005). The physoclistous swimbladder is not connected to the external environment and instead is filled by O$_2$ that is released from the blood due to a localized acidosis that is generated and maintained by the swimbladder rete and associated gas gland (Steen, 1970).

These highly specialised tissues generate much larger acidoses than are ordinarily observed in the circulation (Kurn & Marti, 1966). Via the Bohr/Root effect present in teleost Hbs, the retia allow the sequestration of large amounts of O$_2$ from circulating Hb (Ingermann, 1982); retia can generate PO$_2$ values in excess of 50 atm thereby establishing the large partial pressure gradients required for the rapid diffusion of O$_2$ across large distances (Scholander, 1954; Scholander & Van Dam, 1954). This enables the energetically expensive activities of the eye (Damsgaard et al., 2019b) and provides O$_2$ for buoyancy maintenance via the physoclistous swimbladder (Steen,
Together with the increased visual capabilities provided by the choroid rete, the physoclistous swimbladder allowed teleosts to invade deep ocean environments without the need to surface to fill a physostomous swimbladder. Furthermore, the ability to maintain neutral buoyancy at depth reduces the energetic constraints of locomotion and leads to improved swimming performance in modern teleosts (Lauder & Liem, 1983; Berenbrink, 2007).

The increased pH sensitivity of teleost Hbs appears to be an important factor in the development of the retia involved in oxygenating both the eye and the swimbladder. The evolution of both the Bohr and Root effects preceded the evolution of the retia (fig. 1.1, 1.4); however, it is the retia that localizes the acidosis that can exploit the Bohr/Root effect (Berenbrink et al., 2005; Damsgaard et al., 2019b). Indeed, it appears that the presence of the choroid rete may be associated with the presence of the Root effect (Berenbrink et al., 2005; Berenbrink, 2007). According to Berenbrink et al. (2005), the only extant groups in which a large secondary decrease in Root effect magnitude is detected is in those that also lack the choroid rete, and there are several examples of species that have reduced Root effect magnitude but maintain the swimbladder rete. Berenbrink et al. (2005) suggest that the RBC β-NHE (section 1.3.6) evolved specifically to protect O\textsubscript{2} secretion to the eye as impairment of O\textsubscript{2} secretion in this tissue leads to blindness extremely quickly (Witternberg & Witternberg, 1974; Wells, 2009).

While the Bohr/Root effect in combination with an enhanced localized acidosis in tissues such as the choroid and swimbladder retia can be highly beneficial in unloading large amounts of O\textsubscript{2}, it can also have negative consequences during a generalized acidosis. The reduced O\textsubscript{2} affinity and carrying capacity of Bohr/Root Hbs under such acidic conditions could impede full Hb-oxygenation during passage through the gills and lead to hypoxemia. Such conditions are not uncommon and can be triggered by several factors including anoxia, hypercarbia, and exhaustive
exercise (Randall, 1982b; Perry, 1986, Val et al., 2005). Teleosts therefore need a mechanism by which to protect RBC pH to ensure O₂ uptake at the gill is not compromised, and they do this through the RBC β-NHE.

1.3.6 RBC β-NHE pH protection

The teleost ‘solution’ to avoiding the adverse effects of highly pH sensitive Hbs during a generalized acidosis (i.e., compromised environmental O₂ uptake) is RBC pH regulation and thereby control of the pH that Hb experiences (Nikinmaa, 1982; Nikinmaa, 1992; Berenbrink et al., 2005). The β-NHE system of RBC pH protection represents the second major component of the unique teleost oxygenation system. During a generalized acidosis (decrease in whole blood pH) catecholamines (adrenaline and noradrenaline) are released into the circulation (Wood & Perry, 1985) that bind to β-adrenergic receptors on the RBC membrane (Primmett et al., 1986). This causes an excitation cascade resulting in net excretion of H⁺ from the RBC (in exchange for Na⁺), thereby increasing pH (fig. 1.2D; Brauner & Berenbrink, 2007). The reduced intracellular [H⁺] (increased pH) favours the carbonic anhydrase (CA) catalysed CO₂ hydration reaction resulting in the production of HCO₃⁻ and H⁺. As HCO₃⁻ is transported out of the cell by the AE-II at a relatively slow rate compared to H⁺ excretion by β-NHE, HCO₃⁻ will accumulate, alkalinizing the cell and increasing the Hb-O₂ affinity, and consequently safeguarding Hb-O₂ uptake at the gill (Perry, 1986). Due to transport of Na⁺ and Cl⁻ into the cell, in exchange for H⁺ and HCO₃⁻ extrusion respectively, cellular osmolality increases leading to water movement into the cell and consequential cell swelling (Nikinmaa, 1982). Extruded H⁺ recombine with HCO₃⁻ to form CO₂ in the plasma, however teleosts do not possess circulating plasma CA (unlike elasmobranchs) so the
formation of CO$_2$ occurs at the extremely slow uncatalyzed rate ($t_{1/2} \approx 90$ seconds; Kern, 1960; Heming, 1984; Nikinmaa, 1992; section 1.3.7). Thus, pH$_i$ protection in the absence of plasma accessible CA (paCA) uncouples plasma/extracellular pH (pHe) and pH$_i$, thereby protecting Hb-O$_2$ uptake at the gills (Randall et al., 2014). In contrast to the gills (where paCA is absent), paCA is available at the respiring tissues and plays an important role in CO$_2$ excretion. By facilitating CO$_2$ movement into the RBC, paCA will maintain high plasma PCO$_2$. Once within the RBC, H$^+$ released by CO$_2$ hydration will be bound to Hb via the Haldane effect as O$_2$ is released to the tissues (section : 1.3.4; Brauner & Randall, 1998), promoting CO$_2$ movement from the plasma into the RBC and aiding in the maintenance of low RBC PCO$_2$. Short-circuiting of the $\beta$-NHE pH$_i$ protective system acts to temporarily impair CO$_2$ removal from the tissues but promotes O$_2$ delivery (fig. 1.2E).

Whilst the $\beta$-NHE system provides RBC pH$_i$ protection during a generalized acidosis, it can also limit O$_2$ unloading by stabilising Hb in its high O$_2$ affinity state. In compensation, $\beta$-NHE activity is correlated to O$_2$ availability and its activity decreases at very high O$_2$ tensions, as are found in the choroid and swimbladder retia (Motais et al., 1987; Berenbrink et al., 2005). Clearly a key component to the functioning of the $\beta$-NHE system of RBC pH$_i$ protection is the role, localisation, and availability of CA enzymes (section 1.3.7).

### 1.3.7 The carbonic anhydrases

Carbonic anhydrase is a zinc metalloenzyme responsible for the reversible de/hydration reaction vital for the timely excretion of CO$_2$. In the absence of CA the reaction proceeds:

$$\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{HCO}_3^- + \text{H}^+$$
however, catalysis by CA reduces the reaction to the following:

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

Carbonic anhydrase is one of the oldest (Tashian, 1989) and fastest known enzymes, with a reaction time of 0.18 s\(^{-1}\), compared to \(\sim 90\) s\(^{-1}\) for the uncatalyzed reaction (Kern, 1960; Itada & Forster, 1977). It is expressed ubiquitously in living organisms (Gilmour, 2010) with various isoforms found in animals, plants, algae, bacteria, archaea, and fungi (Moroney et al., 2001; Supuran, 2008a; Elleuche & Pöggeler, 2009; Banerjee & Deshpande, 2016). The fast reaction rate of CA allows for the rapid excretion of CO\(_2\) during passage of the RBC through the gill capillaries which typically does not exceed 2.5 seconds (Gilmour et al., 1994) and can be much faster during exercise or stress (Randall, 1982a; Brauner et al., 2000).

Although CA clearly plays a vital role in the excretion of CO\(_2\) in vertebrates, it is important to remember that this is not its only role. The reaction that CA catalyses is vital to various other physiological process (Tashian, 1989; Henry, 1996; Chegwidden et al., 2000) including: acid-base and ion regulation (Henry & Swenson, 2000; Georgalis et al., 2006b), environmental CO\(_2\) sensing (Qin et al., 2010), lactate and ammonia efflux (Ebaugh & Tufts, 2006), HCO\(_3^-\) reabsorption in the kidney lumen (Pelis et al., 2003; Georgalis et al., 2006b), and acid excretion into the swimbladder and intestines (Pelster, 1995; Gervais & Tufts., 1998; Würtz, 1999; Georgalis et al., 2006b; Grosell et al., 2007). While these roles are also of great physiological importance, they are not the focus of my thesis and so will not be discussed in further detail.

The mechanism by which CA catalyses CO\(_2\) hydration is illustrated as follows for the RBC CA-II isoform: CO\(_2\) produced by the respiring tissues diffuses into the RBC where it encounters the intracellular pool of CA-II. The catalytic Zn ion of CA is located in an active site cavity that is composed of specific His residues. A H\(_2\)O molecule is recruited that rapidly dissociates to form a
OH\textsuperscript{−} and a H\textsuperscript{+}. A further His residue binds the H\textsuperscript{+}, producing a strong nucleophile. CO\textsubscript{2} enters a hydrophobic pocket on the active site and is positioned next to the OH\textsuperscript{−}. Due to proximity of the Zn and CO\textsubscript{2}, nucleophilic attack occurs forming HCO\textsubscript{3}\textsuperscript{−} (Silverman & Lindskog, 1988). This direct formation of HCO\textsubscript{3}\textsuperscript{−} bypasses the formation and subsequent dissociation of H\textsubscript{2}CO\textsubscript{3} involved in the slow uncatalyzed reaction (Esbaugh & Tufts, 2006). Initially bound to Zn, the HCO\textsubscript{3}\textsuperscript{−} diffuses out of the active site allowing entry of another H\textsubscript{2}O molecule, briefly inactivating the enzyme. Transfer of the His bound H\textsuperscript{+} to the surrounding solution occurs via the H\textsuperscript{+} shuttling mechanism (Tu & Silverman, 1989) regenerating the enzyme (Silverman & Lindskog, 1988). The H\textsuperscript{+} shuttling mechanism appears to be the rate limiting step (Gilmour, 2010) and is highly dependent on the buffering capacity in the surrounding solution (Lindskog & Silverman, 2000). The HCO\textsubscript{3}\textsuperscript{−} produced is transferred out of the cell in exchange for Cl\textsuperscript{−} by AE-II where it is transported in the plasma for excretion at the gill (fig. 1.2D).

Various different isoforms of CA exist, including RBC, cytosolic and membrane bound forms (Gilmour, 2010). Mammals generally possess 16 CA isoforms (Gilmour, 2010), however teleosts have varying numbers of isoforms due to additional whole genome duplication events (Gilmour, 2012). Despite the diversity of CA isoforms found in vertebrates, the catalytic mechanism described above is conserved (Lindskog, 1997; Banjeree & Deshpande, 2016). The most abundant pool of CA in vertebrates, and the isoform that has received the most attention is CA-II, a cytoplasmic isoform contained within the RBCs (Maren, 1967; Esbaugh et al., 2004). The RBC is a favourable environment for the hydration of CO\textsubscript{2} due to the high [CA] (100 times that in the plasma) and the high buffering capacity of Hb (compared to plasma; Henry & Swenson, 2000). The latter ensures availability of H\textsuperscript{+} for HCO\textsubscript{3}\textsuperscript{−} dehydration, maximizing the catalytic efficiency of the enzyme (Henry & Swenson, 2000).
In addition to the RBC, cytoplasmic isoforms are also found in high concentrations in various tissues, including in the gills. Intracellular CA in the gill epithelium is not thought to be directly involved in CO₂ excretion as it is inaccessible to the HCO₃⁻ in the plasma (Gilmour, 2012), however, it plays various important roles in acid-base balance and ion exchange (Gilmour & Perry, 2009; Gilmour, 2012).

Recently, research effort has focussed on membrane bound, plasma accessible isoforms in fishes (Harter & Brauner, 2017), particularly CA-IV. CA-IV has a high catalytic rate (similar to RBC CA-II; Esbaugh & Tufts, 2006) and is bound to cellular membranes by a glycoprophosphatidylinositol (GPI) anchor and so is typically apically oriented (Zhu & Sly, 1990). CA-IV is the major plasma accessible isoform and as such is the most relevant isoform to the unique system of enhanced O₂ unloading in teleosts.

Carbonic anhydrase nomenclature reflects the vast amount of research on CA in mammalian systems, generating some confusion when discussing isoforms in non-mammalian species (Tufts et al., 2003; Esbaugh et al., 2005). Further complications arise from the additional whole genome duplication events in fishes, resulting in CA isoforms that have no mammalian counterpart (Esbaugh et al., 2004; Esbaugh et al., 2005; Lin et al., 2008). Such issues have now been well resolved for cytoplasmic isoforms (Esbaugh et al., 2004; Esbaugh et al., 2005; Esbaugh & Tufts, 2006; Lin et al., 2008; Gilmour & Perry, 2009; Gilmour, 2010; Santovito et al., 2012) but work remains to be done in establishing the organisation and diversity of isoforms with alternative cellular localisation. Throughout my thesis, I use CA-II and/or RBC CA to refer to the high-activity cytoplasmic CA isoform found in the RBCs of teleost fishes, and CA-IV and/or paCA to refer to the major membrane bound isoform.
1.3.7.1 **A heterogeneous distribution of paCA in the vasculature of teleosts**

Recent studies have provided evidence for a heterogeneous distribution of paCA in the teleost circulation: the third component (alongside highly pH sensitive Hbs and RBC pHi protection) comprising the unique teleost oxygenation system. Through the heterogeneous availability of paCA, O₂ and CO₂ transport are coupled in specified locations (fig. 1.2E; Rummer & Brauner, 2011). The presence of paCA leads to short circuiting of RBC pHi protection (as the extruded H⁺ are now combined in the plasma with HCO₃⁻ to form CO₂ at the fast catalysed rate, which diffuses back into the cell and reduces pHi), ultimately resulting in enhanced O₂ unloading due to the high pH sensitivity of teleost Hbs (fig. 1.2E). Short circuiting of the RBC β-NHE would be highly problematic in the branchial capillaries during a generalized acidosis as low RBC pHi confers low O₂ affinity and reduced carrying capacity in the highly pH sensitive Hbs of teleosts, compromising the ability to bind atmospheric O₂. It is generally accepted that in teleosts there must be an absence of paCA in the branchial and venous vasculature (fig. 1.2E; Henry et al., 1997; Perry & Laurent, 1990; Harter & Brauner, 2017) for the RBC β-NHE system of pHi protection to function. In contrast to the situation at the gills, availability of paCA at the respiring tissues can be harnessed to deliberately short circuit RBC β-NHE, thereby reducing the pH that Hb experiences, greatly increasing the arterial-venous pH difference (Δa-νₚH) and causing a large increase in O₂ unloading. This scenario characterises the system of enhanced O₂ unloading in teleosts (fig. 1.2E).

Although there has not been systematic investigation of paCA distribution within the teleost circulatory system (this knowledge gap is the focus of chapter 2 of my thesis) functional evidence for some tissues has been obtained. paCA has been indicated in various tissues of salmonid fishes including: white muscle (Rummer & Brauner, 2011), red muscle (Rummer et al., 2013), heart (Randall et al., 2014; Alderman et al., 2016), brain, and kidney (Georgalis et al., 2016).
Functional work has also been conducted indicating the physiological importance of this system of enhanced O\textsubscript{2} unloading; Rummer et al. (2013) suggested that paCA in red muscle of rainbow trout could double tissue O\textsubscript{2} delivery with no change in blood flow. \textit{In vivo} support for the role of paCA in enhanced tissue O\textsubscript{2} unloading is available from studies employing exercise and hypoxia as physiological stressors; Harter et al. (2019) showed that in normoxia, at a swimming speed of 1 bl s\textsuperscript{-1}, when paCA was selectively inhibited (using a membrane impermeable CA inhibitor, C18) swimming speed and metabolic rate were maintained, but this was associated with almost a 30\% increase in cardiac output. Fish that were forced to swim at 1.5 bl s\textsuperscript{-1} collapsed following C18 injection (Harter et al., 2019). Similarly, Atlantic salmon (\textit{Salmo salar}) exposed to a hypoxia challenge test (incipient lethal O\textsubscript{2} saturation: ILOS) were able to tolerate 30 \% lower PO\textsubscript{2} values when paCA was available (control fish; ILOS 13.3 \% environmental O\textsubscript{2}) compared to those that had paCA selectively inhibited (C18 injection; ILOS 19.5 \% environmental O\textsubscript{2}; Carless & Brauner, unpublished observation). These studies suggest that the role of paCA may be not restricted to stressful conditions. It is possible that salmonids, and teleosts generally, are routinely relying on RBC β-NHE short circuiting to enhance tissue O\textsubscript{2} unloading even during routine exercise or exposure to routine hypoxia (Rummer et al., 2010; Rummer & Brauner, 2011).

It is the heterogeneous distribution of paCA that makes the teleosts truly unique. Certain aspects of the teleost enhanced O\textsubscript{2} unloading system exist in other vertebrates but no other groups possess the suite of characteristics that allow functional RBC pH\textsubscript{i} protection at rest and short-circuiting during exercise or stress. In air breathing vertebrates and elasmobranchs, paCA at the gas exchange surface (and throughout the entire vasculature) is the norm (Swenson et al., 1995; Henry & Swenson, 2000; Wilson et al., 2000; Gilmour et al., 2001; Gilmour et al., 2002; McMillan, 2018). Availability of paCA isoforms at the gas exchange surface is thought to be the ancestral
vertebrate condition that has been secondarily lost in the teleosts (Harter & Brauner, 2017). Teleosts are a highly derived group that represent the exception to the vertebrate rule in terms of paCA localisation, which prompts the question: why? One way to investigate why the teleosts are so divergent (in terms of paCA localisation) is by looking at scenarios where we see a return to a condition reminiscent of the ancestral state. One example of this is the Antarctic icefishes (family: channichthyidae) – a family of fishes found exclusively in the Southern Ocean (Kock, 2005). They are unique among adult vertebrates in that they completely lack Hb and largely lack RBCs (Ruud, 1954; Barber et al., 1981), characteristics thought to be adaptations compensating for increased blood viscosity at low temperatures (Egginton, 1996). One result of the natural knockout of Hb is the retention of branchial paCA in the icefishes (Harter et al., 2018a). The icefishes possess various traits that are thought to be paedomorphic (embryonic traits retained in adulthood) and Harter et al. (2018a) suggest that branchial paCA may represent another such trait. paCA availability at the gas exchange surface may be a trait that is functionally constrained in adult teleosts due to their highly pH sensitive Hbs, a constraint that has been subsequently released in the icefishes. Embryonic teleosts, which lack highly pH sensitive Hbs would be an extremely interesting study system in which to investigate this hypothesis and represent the focus of chapter 3 of my thesis.

1.3.7.2 Diversity of CA-IV isoforms

Recent work suggested that further specialisation of paCA isoform activity may exist, based on presence of multiple CA-IV isoforms. The zebrafish genome exhibits at least nine CA-IV like isoforms with variable tissue distributions (Lin et al., 2008). Dichiera et al. (2023) also found evidence for three distinct isoforms in the genome of rainbow trout (CA-IVa, CA-IVb, and CA-IVc) with CA-IVa and CA-IVb having divergent tissue distributions and CA-IVc suggested
to be expressed exclusively in early life stages. Multiplication in isoform numbers likely resulted from whole genome duplication events (Holland, 2013), but the divergent tissue distributions observed suggests the isoforms may play differing roles. Whole genome duplication events are thought to play major roles in promoting the evolution of traits, with numerous examples of duplicated genes having divergent roles in vertebrates (Kassan et al., 2009; Hoffmann et al., 2012; Storz, 2016). Dichiera et al. (2023) suggested that CA-IVa may play a greater role in gas exchange due to its expression in heart and muscle tissues, and CA-IVb may be of greater importance for ion and acid-base balance due to its localisation to the kidney and intestines, however this hypothesis is yet to receive functional confirmation. While such functional validation for differing roles of CA-IV isoforms is unfortunately beyond the scope of my thesis, expression of CA-IVa and CA-IVb throughout the vasculature of adult rainbow trout is investigated in chapter 2, and in the gills during early development in chapter 3.

1.3.7.3 Plasma inhibitors of carbonic anhydrase

Various teleosts have been shown to have endogenous plasma inhibitors of carbonic anhydrase (PICA) with salmonids possessing the most potent enzymes examined to date (Henry et al., 1997, McMillan et al., 2019). The primary role of these inhibitors remains unresolved, however there are several explanations suggested in the literature that relate to protection against the negative effects of RBC CA release due to haemolysis, and Zn recycling (Dimberg, 1994; Henry et al., 1997; Henry & Heming, 1998). While the selection pressures for the presence of PICA in teleosts is unknown, it is known that: 1) PICA are selective in their inhibition of cytosolic isoforms; in mammals endogenous PICA are ineffective against paCA, yet cause significant inhibition of cytoplasmic isoforms (Hill, 1986; Roush & Fierke, 1992), and 2) that PICA are highly
species specific, with reduced inhibition observed when PICA are tested across species (Henry et al., 1997). The Antarctic icefishes also possess PICA (despite lacking RBC and so the RBC pool of CA-II), which, similarly to mammals, are highly effective at inhibiting cytoplasmic CA but not isoforms found in the microsomal fraction of gill homogenates (Tufts et al., 2002). In mammals, and likely also in teleosts, PICA and paCA do not appear to be mutually exclusive (Maren et al., 1993; Geers & Gros, 2000). The combined presence of paCA and PICA may be vital to ensure tight control of CA availability to catalyse reactions in the plasma. This compartmentalisation appears to be an important component of the teleost oxygenation system.

In the elasmobranchs, no endogenous PICA have been detected, and presence of circulating plasma CA activity has been measured (Henry et al., 1997; Gilmour et al., 2002; McMillan et al., 2019). The elasmobranchs use a rather different respiratory strategy to the teleosts in this respect, and do not regulate the availability of CA for plasma reactions by means of inhibitors. While the evolutionary trajectory of several teleost respiratory traits has been well characterised (section 1.4), others, such as the presence of PICA have not been investigated in the basal actinopterygians.

1.4 Temporal evolution of the unique teleost oxygenation system

The mechanisms discussed in previous sections all represent vitally important aspects to the unique teleost oxygenation system and without any one of them, the benefits of enhanced O\textsubscript{2} unloading cannot be realised. Berenbrink et al. (2005) described the sequential evolution of several components of this system along the percomorph lineage (fig. 1.4), assuming that traits observed in extant animals are representative of their respective ancestral conditions. The ancestral state of the teleosts appears to be one that included a high $\beta$Hb value, no RBC $\beta$-NHE, no choroid or swimbladder retia, a small Bohr coefficient, no Root effect (Berenbrink et al., 2005), and a
homogenous distribution of paCA (Harter & Brauner, 2017). This condition remains in the elasmobranchs (Gilmour et al., 2002; Gilmour et al., 2006; McMillan et al., 2019). The following paragraphs in this section discuss in further detail the accepted evolutionary trajectory of several teleost respiratory characteristics based on the work of Berenbrink et al. (2005).

The Root effect likely evolved in the basal actinopterygians during the Devonian period, sometime after the divergence of the teleosts from the lobe-finned fishes (Randall et al., 2014). The magnitude of the Root effect increased in the polypteriformes and acipenseriformes (Berenbrink et al., 2005) but pH values required for Root effect onset were low relative to blood pH values that would likely occur in vivo, and thus the Root effect in these groups is thought to lack physiological relevance (Regan & Brauner, 2010). Hemoglobin buffer capacity is inherently linked to Bohr/Root magnitude (section 1.3.4) and consequently decreased in concert with the increasing Bohr/Root effect magnitude, reaching the modern low value in the common ancestor with Amia (fig. 1.4; Berenbrink et al., 2005).

The choroid rete evolved once around 250 MYA in the last common ancestor of Amia and the teleosts (fig. 1.4; Berenbrink et al., 2005), well after the increase in the magnitude of the Root effect (Berenbrink et al., 2005). It is crucial that the Root effect evolved before the retia as without the Root effect, the retia mirabile would shunt O₂ away from the arterial supply, bypassing tissue delivery (Berenbrink et al., 2005). Berenbrink et al. (2005) suggest that this system evolved to protect O₂ secretion in the eye, a tissue that undergoes cell death imminently when deprived of O₂. Thus, the choroid rete is associated with highly visual species.

The β-NHE system of RBC pH protection evolved approximately 150 MYA (Randall et al., 2014), after the divergence of the Osteoglossomorpha (fig. 1.4) and was followed by an increase in exercise capacity through the evolution of the percomorph lineage (Berenbrink et al.,
The increased exercise capacity observed after the appearance of RBC pHi protection was likely due to the movement of the onset pH for Root Hb O₂ unloading into the physiological range and thus exploitation of this increased pH sensitivity. Regan and Brauner (2010) illustrated that in species with a large Root effect but no β-NHE, the pH onset of the Root effect was too low to be physiologically relevant. The β-NHE, Root Hbs, and localised availability of paCA for short circuiting, may have enabled this increase in exercise capacity as has been indicated by in vivo studies (Rummer et al., 2013; Harter et al., 2019; Carless & Brauner, unpublished observation).

The earliest origins of the swimbladder rete (4 separate evolutions in teleosts) are 130 and 140 MYA in the Elopomorpha and Euteleostei respectively (fig 1.4) and both groups subsequently experienced significant adaptive radiations (Berenbrink et al., 2005). The adaptive drive for the maintenance of the Root effect to fill the physoclistous swimbladder appears insufficient to maintain this trait, probably due to the risk posed by a large Bohr/Root effect compromising O₂ uptake during a generalized acidosis (section 1.3.5). This view is supported by the various species that have secondarily lost the Root effect and choroid rete but maintain a physoclistous swimbladder (Berenbrink et al., 2005).

Clearly the unique teleost oxygenation system represents a complex scenario with numerous important mechanisms. There are several examples of species that have secondarily lost or reduced one or several of these mechanisms suggesting scope for plasticity within the system. Berenbrink et al. (2005) report losses of the swimbladder and choroid retia five times independently, however only the choroid rete losses are associated with a loss or reduction in magnitude of the Root effect. These secondary reductions in Root effect magnitude occurred on the phylogenetic branches leading to Gnathonemus, Anguilla, the loaches (Misgurnus and Pangio), the catfishes (Silurus and Pelteobagrus), and Monopterus (Berenbrink et al., 2005). In addition,
the β-NHE has also been lost four times independently in the same groups (except *Gnathonemus*; Berenbrink et al., 2005) and a fifth teleostean loss was reported in the sablefish (Rummer et al., 2010).

Until recently, the role of CA in this system (and particularly paCA isoforms) has been overlooked, despite its vital role in the localization of enhanced O$_2$ unloading. Several recent studies have begun to elucidate the role and importance of paCA within the teleost system, however many questions remain. While Berenbrink et al.’s (2005) analysis of the evolution of βHb, Root effect magnitude, and β-NHE activity through the evolution of the percomorph lineage represents an important advancement of our understanding of the teleost oxygenation system, the role of paCA is notably absent from this work. The loss of paCA in the gills of teleosts appears essential to the functioning of the teleost system of enhanced O$_2$ unloading, yet paCA is usually present at the gas exchange surface of air breathing vertebrates (Zhu & Sly, 1990) and elasmobranchs (McMillan et al., 2019). How paCA was lost from the gills of teleosts as they evolved from the ancestral vertebrate condition is so far undocumented. I can however make predictions based on the known evolutionary trajectory of several other key characteristics. Dichiera et al. (2020) recently indicated a correlation between the presence of the high activity RBC CA-II isoform, as is found in teleost RBCs, and an absence of branchial paCA: conversely, elasmobranchs have a low activity RBC CA-II isoform and presence of paCA in the gills (McMillan et al., 2019). Second, an association appears to exist between a large, physiologically relevant Root effect and the absence of branchial paCA (Harter & Brauner, 2017). Therefore, based on the presence of these two factors, it may be possible to predict whether any individual species is likely to have branchial paCA availability. Such correlations can become important tools for
hypothesising which respiratory strategies may be employed by species yet to receive investigation.

1.5 Diversity in experimental systems

In comparative physiology, the rainbow trout is held as a model species upon which much research effort is directed (Thorgaard et al., 2002). While focussing on a model species enables easy comparison of results between studies and/or laboratories (Müller, 1997; Fields & Johnston, 2005; Hunter, 2008), it also restricts our knowledge of a system that may exist in most teleosts, thus representing half of all vertebrates (Nelson et al., 2006; Helfman et al., 2009; Ravi & Venkatesh, 2018). In keeping with the published literature, in my thesis I refer to the rainbow trout as a model teleost.

It appears that the enhanced O\textsubscript{2} unloading discussed above likely applies broadly to the teleosts (Berenbrink et al., 2005; Shu et al., 2022), yet many exceptions to the requirements for this system have been noted. There are at least five instances where the magnitude of the Root effect has significantly decreased and several examples of the loss of β-NHE (Berenbrink et al., 2005; Rummer et al., 2010; section 1.4). Similarly, the ancestral vertebrate condition is often equated with the suite of characteristics observed in extant elasmobranchs, with most research interest having been focussed on dogfish species (\textit{Squalus} spp.). As a result, rainbow trout and dogfishes are often used as examples of two groups with divergent solutions to the same challenge: ensuring adequate O\textsubscript{2} uptake and delivery over a variety of environmental conditions. Over-generalisation and a lack of knowledge of how respiratory characteristics exist in transitionary groups may mean that rainbow trout and dogfishes should be thought of as opposite ends of a spectrum, rather than as adequate representatives of two highly divergent and derived groups.
1.5.1 Diversity across life stages

The majority of our knowledge of respiratory systems, and comparative animal physiology in general, comes from adult individuals of model species (explored in section 1.5). However, in most cases juveniles or other developmental stages possess different characteristics compared to their adult counterparts and respiratory characteristics are no exception from this pattern. It is known that salmonids express different respiratory pigments pre-hatch (embryonic Hb: HbE; Rombough, 1988). These HbE do not exhibit a large Bohr/Root effect (Iuchi, 1973), and are exclusively expressed until hatch (Iuchi & Yamamoto, 1983) when adult Hb polymorphs (HbA) become available (Bianchini & Wright, 2013). HbE also have greater O₂ affinity compared to HbA, a characteristic that is thought to protect O₂ uptake under the hypoxic conditions in the egg (Rombough & Drader, 2009). Despite the existence of Hbs specific to pre-hatch life stages, the gills do not play a dominant role in gas exchange until ~ 23 days post hatch (dph; Fu et al., 2010). Various questions remain surrounding the presence and roles of the gills and Hb during development, and other components of the unique teleost O₂ unloading system in developing fishes have yet to be investigated. Two major components that remain absent from our knowledge of the developing teleost respiratory system are 1) the distribution of paCA throughout the circulatory system, particularly its availability in the gills, and 2) the presence of RBC β-NHE and thus rapid RBC pH regulation. Firstly, it has been proposed that paCA presence in the gills may represent an embryonic trait that is then lost in some species during development (see section 1.3.7.1). Secondly, if early life stage fishes lack highly pH sensitive Hbs, they may be expected to also lack RBC β-NHE. It would be of great interest to know if these characteristics progress along a similar
trajectory in the developing fishes as has been identified across the evolutionary trajectories of the teleosts (Berenbrink et al., 2005) and will be the focus of chapter 3 of my thesis.

### 1.6 Thesis objective and structure

My thesis builds on a growing collection of recent work elucidating the importance of a lack of paCA in the gills for the unique enhanced oxygenation system observed in teleosts (Rummer et al., 2010; Harter & Brauner, 2017; Harter, 2018). By selectively maintaining the absence of paCA in the branchial vasculature, the teleosts can enhance O₂ unloading to tissues during an acidosis where paCA is present by short circuiting RBC β-NHE, while ensuring conditions for O₂ uptake at the gills are not compromised. Previous work has indicated that highly pH sensitive Hbs may be incompatible with the presence of branchial paCA and so the principal hypothesis of my thesis is: **hemoglobins with a large, physiologically relevant Root effect have imposed constraints on branchial plasma accessible carbonic anhydrase availability within teleost fishes.** As discussed in detail above, there are three main components to the teleost system of enhanced O₂ unloading: 1) pH sensitive Hbs (large, physiologically relevant Bohr/Root effect), 2) rapid RBC pHi protection by way of β-NHE, and 3) a heterogeneous distribution of paCA, with absence in the gills. The present work focuses on 1) confirming the current assumption of a heterogeneous expression of paCA within the teleost vasculature (chapter 2), and 2) utilising scenarios where one or more of these components is absent (chapters 3 and 4) to test the above principal hypothesis. The remainder of this introductory chapter will outline work conducted in chapters 2 (section 1.6.1), 3 (section 1.6.2), and 4 (section 1.6.3) followed by a general discussion of my results (chapter 5).
1.6.1 An atlas of rainbow trout paCA

Chapter 2 explores the heterogeneous localization of paCA within the vasculature of a model teleost, the rainbow trout. The unique oxygenation system illustrated in teleosts is reliant on availability of paCA at the respiring tissues where it can short-circuit RBC β-NHE leading to enhanced O₂ unloading, and an absence of paCA in the branchial vasculature to ensure O₂ uptake is not compromised during a generalised acidosis.

Chapter 2 tested the hypothesis that paCA is absent from the branchial vasculature of all four gill arches but present in all other tissues in rainbow trout. Lack of paCA has previously been shown in the branchial vasculature of gill arch 2 of rainbow trout (Henry, 1991) and this has been assumed to apply to all four arches. Here I address this assumption and assess 10 other tissues for paCA availability using a suite of validated biochemical and molecular methods. Compiled results from this methodological suite provide evidence that paCA is lacking in the vasculature of all four gill arches but is present in all other tissues tested (anterior-, mid-, posterior-intestine, pyloric caeca, liver, kidney, heart, brain, white muscle), except for the stomach. These data suggest that enhanced O₂ unloading is available to almost all rainbow trout tissues, and not restricted to those with elevated O₂ demands as has been previously thought.

1.6.2 The availability of branchial paCA in developing rainbow trout

Developing rainbow trout represent an interesting scenario in which to study respiratory physiology as they exhibit several physiological changes during the first 50 days post fertilization (dpf). Rainbow trout possess specialised HbE until hatch when the transition to HbA isoforms occurs. These embryonic isoforms do not have a large Bohr/Root effect (Iuchi, 1973) and their high O₂ affinity is thought to facilitate O₂ uptake in the hypoxic egg (Rombough & Drader, 2009)
despite the lack of fully functional gills at this time (Fu et al., 2010). Studying these developing fish, with their relatively pH insensitive Hbs represents a scenario where I can assess the impact of the absence of highly pH sensitive Hbs on the presence of the other components of the unique teleost oxygenation system, namely branchial paCA and RBC β-NHE.

Chapter 3 tested the hypothesis that during expression of HbE in developing rainbow trout, paCA will be present in the branchial vasculature and there will be a lack of RBC β-NHE activity. Developing rainbow trout were sampled from 25 to 50 dpf and mRNA expression of CA-IVa, CA-IVb, and β-NHE was measured. Data from previous research (Bianchini & Wright, 2013) was utilised to inform the major transitionary period from HbE to HbA isoform availability. mRNA expression data clearly showed a switch in respiratory strategy with hatch: pre-hatch fish had significant expression of CA-IVa, but no expression of CA-IVb or β-NHE. Post-hatch, with the transition to HbA, no expression of CA-IVa or CA-IVb was identified, but there was a significant increase in β-NHE expression. While I did not measure paCA activity in this chapter, support for the assumption that the mRNA expression is representative of protein availability comes from chapter 1 where I found strong correlation between mRNA expression and microsomal CA activity. Pre-hatch rainbow trout therefore appear to employ a respiratory strategy more similar to that observed in extant elasmobranchs, and representative of the ancestral vertebrate condition. These data suggest that the unique teleost oxygenation system appears to be restricted to adult individuals, at least in the rainbow trout.
1.6.3 Mapping the respiratory characteristics of basal ray-finned fishes

Chapter 4 explored how the lack of a physiologically relevant Root effect impacts other respiratory characteristics in basal ray-finned fishes. As described above (section 1.4) the evolution of certain respiratory characteristics (including Root effect magnitude and β-NHE activity) has been plotted through the percomorph lineage (Berenbrink et al., 2005) but the evolution of other characteristics (for example, branchial paCA availability, plasma CA activity, RBC CA activity, and presence of endogenous PICA) remains undocumented.

Chapter 4 tested the hypothesis that transitionary groups (phylogenetically located between the elasmobranchs and teleosts) possess intermediary respiratory characteristics with respect to the elasmobranch and teleost models of $O_2$ unloading. Alligator gar (*Atractosteus spatula*), white sturgeon (*Acipenser transmontanus*), and Senegal bichir (*Polypterus senegalus*) were chosen to investigate the availability of branchial paCA, circulating plasma CA, RBC CA activity, and the presence of PICA for several reasons; they represent species from the groups (lepisosteiformes, acipensiformes, and polypteriformes, respectively) identified by Berenbrink et al. (2005) as having intermediary respiratory characteristics in terms of Root effect magnitude and RBC β-NHE activity and were satisfactorily abundant for the biochemical analyses employed in this project. My data reveal that these respiratory characteristics likely evolved in a stepwise fashion, much like previous research has shown for Root effect magnitude and RBC β-NHE activity (Berenbrink et al., 2005). My work therefore supports the concept that the teleost and elasmobranch models for $O_2$ unloading should be viewed as opposite ends of a spectrum of respiratory characteristics, rather than two groups with distinct solutions to the same environmental challenge.
Figure 1.1 Fluctuations in atmospheric oxygen (%) over geological time periods, from 443 million years ago (MYA) to present day. Red and blue indicate global periods of warm and cold temperatures, respectively. The Permian Period mass extinction event is indicated by the red line and occurred approximately 252 MYA. Characteristics of the unique teleost oxygenation system (Root effect hemoglobins, choroid rete, RBC β-NHE, and swimbladder rete) are indicated at the time of first appearance. Reproduced from Randall et al. (2014).
Figure 1.2: Schematic illustrating various steps in the oxygen transport cascade in teleosts.

A) O$_2$ moving from the environment to the tissues where it acts as the final electron acceptor in the production of ATP, and CO$_2$ from the tissues to the environment. B) flow of water through from the buccal to opercular cavity in the gill. C) the countercurrent flow of water and blood across the gill lamellae, thereby maximising gas exchange. D & E) Mechanism by which the beta adrenergically stimulated sodium proton exchanger (β-NHE) protects intracellular pH (pHi) during a generalized acidosis in the absence of plasma accessible carbonic anhydrase (paCA); catecholamines (cat.) in the circulation bind to β-adrenergic receptors on RBC membrane causing an excitation cascade resulting in net intrusion of sodium (Na$^+$) and extrusion of protons (H$^+$). This shifts the equilibrium of the carbonic anhydrase (CA) catalysed CO$_2$ de/hydration reaction towards the production of bicarbonate (HCO$_3^-$) and H$^+$ that alkalinizes the cell due to the slow rate of HCO$_3^-$ extrusion by the type II anion exchanger (AE-II) thereby protecting pHi from a generalized acidosis and effectively uncoupling pHi from extracellular pH (pHe). In the presence of plasma accessible CA (paCA) this system is short-circuited (E), and the RBC is no longer protected from the acidosis as pH$i$ and pH$e$ are effectively recoupled. This leads to enhanced unloading of oxygen (O$_2$) to the respiring tissues.
Figure 1.3: Oxygen equilibration curve (OEC) showing the relationship between partial pressure of oxygen (PO$_2$) and hemoglobin saturation (%) for different hemoglobins. Black line indicates a ‘normal’ curve for tetrameric hemoglobin exhibiting cooperative binding of O$_2$ molecules. The red ‘Bohr’ line indicates how the curve changes in animals whose hemoglobin exhibits pH sensitivity – the curve is right shifted (a greater PO$_2$ is required to achieve the same saturation). In addition to a large Bohr coefficient, the teleosts also exhibit a large Root effect, indicated by the blue line. In Root hemoglobins, the curve is also down shifted, signifying an inability to completely saturate hemoglobin molecules, even at very high PO$_2$ values. Dashed lines in corresponding colours indicate P$_{50}$ for each.
Figure 1.4: Timing of evolutionary changes in hemoglobin (Hb) buffer value, Root effect magnitude, and β-adrenergically stimulated sodium proton exchanger (β-NHE) activity across the trajectory of the percomorph lineage. Reproduced from Berenbrink et al. (2005).
Chapter 2: An atlas of rainbow trout plasma accessible carbonic anhydrase availability throughout the vasculature of a model teleost, the rainbow trout

2.1 Summary

Teleost fishes possess a unique oxygenation system that permits enhanced oxygen unloading during stress. It is comprised of three main characteristics: highly pH sensitive hemoglobin, red blood cell (RBC) intracellular pH (pHi) protection, and a heterogeneous distribution of plasma accessible carbonic anhydrase (paCA). A heterogeneous distribution of paCA is essential; its presence permits enhanced oxygen unloading during stress, while its absence at the gills maintains conditions for oxygen uptake by pH sensitive hemoglobins. I hypothesised that paCA would be absent from all four gill arches, as has been previously indicated for arch two, and that paCA would be present in all other tissues. Using a suite of biochemical and molecular methods I confirmed the absence of paCA from all four arches. I also found evidence for paCA in nine other tissues and a lack of paCA availability in the stomach. Expression was highly variable between tissues and suggests these differences may be associated with their respective metabolic activities. Additionally, I analysed the specific CA-IV isoform expressed within each tissue and showed almost complete separation of expression between tissues: CA-IVa was detected in the heart, brain, anterior intestine, and liver, whereas CA-IVb was detected in all intestine sections, pyloric caeca, kidney, and white muscle. This adds to a growing collection of work suggesting CA-IVa and b play divergent roles in gas exchange and ion/acid-base balance, respectively. The current study represents the first comprehensive atlas of paCA availability within a teleost, the rainbow trout, and fills important gaps in our knowledge of this unique oxygenation system.
2.2 Introduction

Teleosts represent half of all living vertebrates and more than 98% of extant fishes, comprising over 30,000 species (Nelson et al., 2006; Helfman et al., 2009; Ravi & Venkatesh, 2018; section 1.2). This group displays a vast degree of variation, various modifications of form, and have invaded almost every aquatic habitat including those characterised by low oxygen (O$_2$), high carbon dioxide (CO$_2$), high hydrostatic pressure, and variable salinity (Wootton, 1990). While the Permian Period mass extinction event is commonly associated with the loss of marine fish species, teleosts were able to exploit the new niche availability and underwent explosive adaptive radiation (Helfman et al., 2009). It has been suggested that modifications in swimming and feeding, along with an additional genome duplication event, enhanced their rate of evolution during this time (Ilves & Randall, 2007). In the 100 million years following the Permian Period mass extinction event, atmospheric O$_2$ levels were much lower than today (fig. 1.1), providing a driver for the evolution of hypoxia tolerance and the suite of respiratory characteristics that make the teleosts truly unique (Randall et al., 2014). These unique adaptations are paramount to the success of the teleosts and likely enabled them to become one of the most successful vertebrate groups on Earth.

The unique teleost oxygenation system that permits enhanced O$_2$ unloading during stress is comprised of several major components highly pH sensitive hemoglobin (Hb), red blood cell (RBC) intracellular pH (pHi) protection, and a heterogeneous distribution of plasma accessible carbonic anhydrase (paCA; Rummer et al., 2013, Randall et al., 2014, Harter & Brauner, 2017). The majority of teleosts possess the first component (highly pH sensitive Hbs), which includes a large Bohr coefficient (the reduction in O$_2$ affinity due to a reduction in pH), and evolved once
along with the concomitant evolution of the Root effect, the reduction in Hb carrying capacity for
O₂ (Nikinmaa, 1990) and decreased cooperativity (changes in Hill coefficient) at low pH (Root,
1931; fig 1.3; see section 1.3.4 for in depth description). In certain tissues, such as the retia of the
eye and swimbladder, the pH sensitivity of Root effect Hbs may be exploited via a counter-current
capillary arrangement (section 1.3.5). This is thought to increase the unloading of O₂ from Hb,
thereby permitting the improved visual acuity (Damsgaard et al., 2020) and buoyancy control that
is characteristic of teleosts (Berenbrink, 2007). Indeed, it is hypothesised that the Root effect may
have originally evolved to enhance general O₂ delivery (Rummer et al., 2013). Under conditions
of a generalised acidosis, however, these characteristics could prevent complete O₂ uptake at the
gills and result in hypoxemia (Nikinmaa, 1986). Therefore, early teleosts needed a mechanism to
protect RBC pH to ensure O₂ loading would not be compromised during a generalised acidosis
such as is seen after exhaustive exercise (Wood et al., 1991) or exposure to hypoxia (Perry &
Kinkead, 1989). This represents the second component, a beta-adrenergically stimulated sodium-
proton exchanger (β-NHE) that provides this pH protection (fig. 1.2D; Nikinmaa, 1982;
Nikinmaa, 1992; Berenbrink et al., 2005). RBC β-NHE effectively uncouples extracellular pH
(pHe) from pH, therefore protecting the pH environment that Hb experiences and maintaining
Hbs ability to effectively bind O₂ at the gills.

The final component of this unique system, a heterogeneous distribution of paCA, has
received much less attention (section 1.3.7). Carbonic anhydrase is a zinc metalloenzyme
responsible for the reversible hydration of carbon dioxide (CO₂) to bicarbonate (HCO₃⁻). Since its
discovery almost 100 years ago (Meldrum & Roughton, 1933; Stadie & O’Brien, 1933), it has
been shown to play various roles in essential cellular processes including, but not limited to, gas
exchange, ion balance, and acid-base regulation (Chegwidden et al., 2000; Georgalis et al., 2006a;
Georgalis et al., 2006b; Gilmour, 2010). Its role in gas exchange (primarily focussed on CO₂ excretion) has been extensively studied across vertebrate taxa (Henry & Swenson, 2000) and various isoforms have been identified, including RBC, cytosolic, plasma, and membrane bound forms (Gilmour, 2010). Similar to mammals, teleost RBCs comprise the largest pool of CA with a high concentration of the high activity CA-II isoform (Maren & Swenson, 1980). The role of paCA (predominantly the CA-IV isoform) in gas exchange, and specifically its presence at the gas exchange surface, has been well characterised in air breathing vertebrates, where it is shown to play a modest but significant role in CO₂ excretion (Heming et al., 1994; Henry & Swenson, 2000; Gilmour & Perry, 2004), by rapidly catalysing HCO₃⁻ dehydration in the plasma. This trait is also present in elasmobranchs (Whitney & Briggle, 1982; Stabenau et al., 1996; Henry & Swenson, 2000; McMillan et al., 2019) but is notably absent in most teleosts (reviewed by Harter & Brauner, 2017). As a result, it has been hypothesised that homogeneous expression of paCA throughout the vasculature represents the ancestral vertebrate condition (Harter & Brauner, 2017) and that paCA has been secondarily lost in the gills of teleosts.

It is hypothesised that the lack of paCA in the gas exchanger of teleosts is associated with the evolution of Root effect Hbs and the resulting RBC pHi protection mechanism (Randall et al, 2014; Harter & Brauner, 2017). For the RBC β-NHE to protect pHi during an acidosis, paCA must be absent from the gas exchange surface (Henry et al., 1988; Heming, 1984) so that RBC pHi is effectively uncoupled from pHe at this location. Where paCA is available, however, during a generalised acidosis protons extruded from the RBC via β-NHE rapidly recombine with HCO₃⁻ in the plasma to form CO₂, short-circuiting RBC β-NHE and acidifying the RBC (fig. 1.2E). This greatly enhances O₂ unloading (Rummer & Brauner, 2011; Shu et al., 2018) which may be beneficial to respiring tissues but is disadvantageous for O₂ loading at the gills. In contrast to
teleosts, elasmobranchs and most other vertebrates possess paCA at the gas exchange surface but importantly do not have Root effect Hbs or RBC β-NHE (Morrison et al., 2015; McMillan et al., 2019). It has been assumed that teleosts possess paCA in all tissues except the gills due to the benefits conferred by increased O$_2$ delivery to the respiring tissues (Rummer & Brauner, 2011). This allows teleosts to maintain O$_2$ uptake at the gills during stress when RBC β-NHE is activated, while also enhancing O$_2$ unloading to the respiring tissues.

The distribution of paCA throughout the vasculature of teleosts has received relatively little research interest in comparison to the other components of the teleost oxygenation system (fig. 1.4; Berenbrink et al., 2005), and so the assumption of heterogeneous availability remains. To date, paCA has been implicated in several salmonid tissues including; heart (Georgalis et al., 2006a; Alderman et al., 2016), red muscle (Rummer et al., 2013), posterior kidney (Georgalis et al., 2006a), choroid rete (Damsgaard et al., 2020), white muscle (Henry et al., 1997) and brain (Georgalis et al., 2006a); however, a comprehensive atlas of paCA tissue localisation in a model species, for example the rainbow trout (Walbaum, 1792), remains unavailable. Recently Dichiera et al. (2023) showed that there are at least two genes encoding CA-IV in adult teleosts: CA-IVa, CA-IVb. Differences in the localisation patterns of expression across tissues suggest they are involved primarily in gas exchange and ion/acid-base balance respectively; heart and red muscle showed highest expression of CA-IVa whereas CA-IVb showed greatest expression in the kidney (Dichiera et al., 2023). While this has yet to be supported by functional evidence, such divergence of expression suggests further compartmentalisation of the different roles of CA.

In this study I aimed to fill the knowledge gaps surrounding tissue specific paCA availability in teleosts by constructing an atlas of paCA localisation in 14 different rainbow trout tissues (lamellae from gill arches 1, 2, 3, 4, stomach, pyloric caeca, anterior-, mid- and posterior-
intestine, heart, brain, liver, kidney, and white muscle). I used a suite of biochemical and molecular methods as a single diagnostic test for paCA is unavailable. These tests consist of 1) ultracentrifugal washing and measurement of CA activity of different membrane fractions. CA activity that is truly associated with the membrane will not be significantly reduced after ultracentrifugal washing (Harter et al., 2018a); 2) specific inhibition of CA activity by sodium dodecyl sulphate (SDS). SDS is a detergent to which the disulphide bonds present in mammalian CA-IV have been shown to be resistant (Whitney & Briggle, 1982; Waheed et al., 1996) and therefore CA activity resulting from paCA isoforms will not show significant inhibition; 3) specific inhibition of CA activity by plasma. Salmonids have potent plasma inhibitors of CA (PICA; Henry et al., 1997) that are thought to protect against unwanted CA release due to RBC lysis (Dimberg, 1994) and are ineffective against paCA isoforms (Roush & Fierke, 1992; Peters et al., 2000); and 4) mRNA expression of two distinct CA-IV isoforms: CA-IVa and CA-IVb (Dichiera et al., 2023). By using this suite of tests to construct an atlas of rainbow trout paCA distribution, I was able to address two main hypotheses: 1) paCA is absent from the branchial capillaries of all four gill arches, and 2) paCA is present in all other rainbow trout tissues studied here. This study fills an important gap in our current knowledge of the teleost oxygenation system and will help us better understand its potential in half of all extant vertebrates.

2.3 Materials and Methods

2.3.1 Animal housing and tissue collection

Female rainbow trout (mean size: 401.43 g, 34 cm) were obtained from Miracle Springs hatchery, Mission, BC, Canada and transferred to The University of British Columbia (UBC)
where they were reared in 2100 L recirculating freshwater tanks (dechlorinated Vancouver tap water: \(\text{Na}^+ = 0.09, \text{Cl}^- = 0.10, \text{Ca}^{2+} = 0.10, \text{Mg}^{2+} = 0.011, \text{K}^+ = 0.004 \text{ mmol L}^{-1}\), hardness as \(\text{CaCO}_3 = 3.3 \text{ mg L}^{-1}\), \(\text{pH} = 7.0\)) at 10 \(^\circ\text{C}\). Fish were fed to satiation with commercial dry pellets (Skretting, Stavanger, Norway) three times per week. All procedures were conducted according to the policies and guidelines of The Canadian Council on Animal Care and approved by the UBC animal care committee (protocol no.: A19-0284-A009). Sample sizes for all measurements available in table A1.

At time of sampling, individual fish were netted and euthanized using pH buffered Aqualife TMS (100 mg/L; Syndel) in a 10 L bucket. Upon cessation of opercular beating, fish were injected with 100 U sodium heparin (500 U/mL; Sigma, H3393, St Louis, MO, USA) into the caudal vein to prevent clotting before being returned to anaesthetic for one additional minute to ensure deep anaesthesia. Blood samples (approx. 1 mL) were collected using a heparinized syringe from the caudal vein. Blood was separated by centrifugation at 3000 G. Plasma was transferred into a separate tube and both plasma and packed RBCs were flash frozen using liquid nitrogen (\(\text{N}_2\)) before storage at \(-80\ ^\circ\text{C}\).

Tissue samples collected for ultracentrifugal washing and specific inhibition were taken from perfused fish to reduce potential RBC CA contamination. Perfusion was conducted via cardiac puncture. PE-100 tubing was inserted into the aorta and blood was replaced with heparinized Cortland’s saline (in mM: 124.1 NaCl, 5.1 KCl, 2.9 NaH\(_2\)PO\(_4\), 1.9 MgSO\(_4\), 1.4 CaCl\(_2\), 11.9 NaHCO\(_3\), 10 U/mL sodium heparin (Sigma, H3393)). Perfusion was continued until gills appeared visually free of RBCs (~500 mL saline per fish). Tissue samples were collected (gills (lamellae from arches 1-4 were sampled individually), stomach, pyloric caeca, anterior-, mid-, posterior-intestine, heart, brain, liver, kidney, and white muscle), rinsed with Cortland’s saline to
remove any blood clots, wrapped in aluminium foil, and flash frozen in liquid N₂. All samples were stored at -80 °C until further use.

For mRNA expression analysis, fish were held and euthanized as previously described and tissues were collected from un-perfused animals. Tissue samples were immediately immersed in at least 5 volumes of RNA later (ThermoFisher, AM7021, Waltham, MA, USA). Samples were placed in the fridge overnight to allow penetration of the tissue, before being transferred to -80 °C for storage for up to three months prior to analyses.

2.3.2 CA activity measurement

Carbonic anhydrase activity was measured in all samples using the electrometric ΔpH assay (Henry, 1991). Tissue samples were thawed on ice and cut into fine pieces by hand to allow for easy homogenisation. Approximately 2 g of tissue (mean ± SEM: 2.1 ± 0.02 g) was added to 2 mL of ΔpH assay buffer (in mmol l⁻¹: 225 mannitol, 75 sucrose and 10 Tris base, adjusted to pH 7.3 with 10% phosphoric acid) and homogenised using a Polytron PT1200 (Lucerne, Switzerland) on ice. The crude homogenate underwent differential centrifugation at 4 °C following protocol previously described (800 G for 20 min, 8000 G for 20 min – centrifuge: Beckman Coulter Allegra X-22R, 100,000 G for 90 min – centrifuge: Beckman Coulter Optima L-100XP; see Henry et al., 1993, Harter et al., 2018a for further detail). Aliquots were frozen and stored at -80 °C after each step of centrifugation; this additional freeze-thaw cycle has been previously validated to have no significant effect on activity (T. S. Harter, unpublished data). From here on, aliquots frozen from the supernatant and pellet after spin three are referred to as cytoplasm and pre-wash microsomes respectively, and those from the pellet after spin four are post-wash microsomes. The reaction medium consisted of 6 mL ΔpH assay buffer in a 10 mL glass reaction vessel submerged
in water in a 35 mL thermostatted vessel maintained at 4 °C. pH was measured using an InLab Cool combined electrode (Mettler Toledo, 51343174, Columbus, OH, USA) with pH amplifier (ADInstruments, FE165, Colorado Springs, CO, USA) and PowerLab (ADInstruments, PL2602), and data were visualised in LabChart (version 8, ADInstruments). Enzyme source was added (100 μL tissue fraction) and the reaction was initiated via the addition of 200 μL of CO₂ saturated water (also maintained at 4 °C) from a 250 μL gas tight Hamilton syringe. The reaction velocity, as indicated by change in pH, was continuously recorded over a pH change of 0.15 pH units. To obtain the true rate of reaction, the uncatalyzed rate (without sample addition; measured separately) was subtracted from the observed rate and the absolute buffer capacity (measured in separate titrations and calculated from the buffer curve of the assay buffer over the tested pH range) was taken into account to convert from pH units min⁻¹ to μmol H⁺ min⁻¹. Finally, protein content was measured spectrophotometrically at 595 nm using the Bradford assay (Sigma, B6916) and bovine serum albumin standards (Sigma, A4612) to give a final activity value in μmol H⁺ min⁻¹ mg⁻¹. Activity measurements after treatment with phosphatidylinositol specific phospholipase C (PI-PLC) – an enzyme that severs the glycosylphosphatidylinositol (GPI) anchor by which CA-IV is membrane bound - were also conducted, however, preliminary studies revealed problems with the assay and so this avenue was not pursued further.

2.3.2.1 Ultracentrifugal washing

Membrane pellets were washed using an additional step of ultracentrifugation to remove CA isoforms not truly associated with the membrane (100 000 G, 90 mins, 4 °C – centrifuge: Beckman Coulter Optima L-100XP). Pellets were resuspended in 2 mL ΔpH assay buffer using mild sonication. Pre-wash refers to CA activity data collected before the ultracentrifugal washing
step, and post-wash refers to that measured after ultracentrifugal washing. The effect of washing was quantified by comparing CA activity between pre- and post-wash microsomes. Raw data can be found in table A2.1.

2.3.2.2 Specific inhibition – SDS

Washed microsome samples were exposed to 0.005% sodium dodecyl sulphate (SDS; Sigma, 436143) during the ΔpH assay. Carbonic anhydrase activity measured in the presence of SDS was compared to that without SDS to assess inhibitory effect of SDS as a percentage of original activity.

2.3.2.3 Specific inhibition - plasma

Rainbow trout plasma first underwent validation (RBC lysate activity was measured with and without plasma – see fig. A2.1) to confirm PICA presence. Washed microsomal samples were exposed to 100 μL rainbow trout plasma during the ΔpH assay and CA activity was measured as described above. Carbonic anhydrase activity in the presence of plasma was compared to that measured in the absence of plasma to calculate percentage inhibition.

2.3.3 mRNA expression

Samples were thawed on ice and homogenised using a Bullet Blender Storm 24 bead beater (Next Advance, Troy, NY, USA) with Precellys ceramic (zirconium oxide) beads (Bertin Corp., Rockville, MD, USA). mRNA was extracted according to the recommended protocol using Qiagen RNeasy mini kit (Qiagen, 74106, Germantown, MD, USA). RNA concentration (450 ± 39 ng/μL) and quality (260/280 ratio: 2.03-2.17, 260/230 ratio: 1.41-2.39) were measured
spectrophotometrically using a Nanodrop 2000 (ThermoFisher, ND-2000). Samples were treated with DNase I (ThermoFisher, EN0521) and cDNA was synthesized from 0.5 μg RNA using RevertAid Reverse Transcriptase (ThermoFisher, EP0441) and random hexamer primers (ThermoFisher, SO142). cDNA was diluted 5 times in molecular water (ThermoFisher, BP28191) before use in the qPCR reaction.

Relative mRNA expression was assessed using 2X Maxima SYBR Green/ROX qPCR Master Mix (ThermoFisher, K0221) on a Bio-Rad CFX96 RT-PCR Detection system (BioRad, Hercules, CA, USA). Primer sequences (table 2.1) for qPCR were either obtained from published literature (β-actin; Georgalis et al., 2006a) or custom designed using Primer3Plus software from published NCBI sequences (CA-IVa: XM_021624267.2; CA-IVb: AY514871.1). The qPCR conditions (final volume = 12.5 μL) were as follows: cDNA template: 1 μL, forward and reverse primers: 200-400 nM, 2X SYBR Green master mix: 6.25 μL. The annealing and extension temperatures over 40 cycles were 58 °C (60 s) and 72 °C (30 s), respectively. To ensure that SYBR green was not being incorporated into non-specific amplicons or primer-dimers during qPCR, products were analysed using gel electrophoresis and single bands of the expected sizes were achieved in all cases. In addition, the construction of dissociation curves for SYBR green after 40 cycles qPCR revealed the presence of single amplicons for each primer pair. To confirm that residual genomic DNA was not being amplified, control samples were included in which no reverse transcriptase was added during cDNA synthesis.

Expression of each gene of interest was standardised to β-actin expression using the Pfaffl method (Pfaffl, 2001). The β-actin gene was selected as a suitable reference gene (Shekh et al., 2017) and validated as part of the current study. Data were log transformed for statistical analysis and presentation. Amplification efficiencies were determined by standard curves generated by
serial dilution of pooled rainbow trout cDNA. Primer pair efficiencies were within 90-110 % and R² > 0.99 for all samples. The qPCR non-detects were incorporated by manually assigning a relative expression value of zero.

2.3.4 Statistical analyses

All analyses and figure production were conducted in R Studio (version 1.2.5042). Carbonic anhydrase activity data are presented in μmol H⁺ min⁻¹ mg⁻¹. Inhibition data are presented as % inhibition compared to control value. Data were assessed for normal distribution using Shapiro-Wilk test, and non-normally distributed data were log transformed to meet test requirements or non-parametric equivalents were used (indicated in the text). Comparisons between gill arches were conducted using two-way ANOVA with Tukey pairwise comparisons used to elucidate differences where required. Paired t-tests were used to analyse the effects of ultracentrifugal washing, SDS, and plasma on rainbow trout tissues. Statistical tests on gene expression data were performed on log transformed data; one sample t-tests were used to assess for significant mRNA expression in each tissue. p-values are reported throughout, and significant differences are indicated on figures by asterisks: * = p< 0.05, ** = p< 0.01, *** = p< 0.001.

2.4 Results

2.4.1 Gill arches

The CA activity in individual gill arches was measured using the ΔpH assay (arch 1 being the most anterior and arch 4 the most posterior) to assess potential differences in paCA activity
between each arch. No significant differences in activity in either the cytoplasmic (data not shown; p > 0.05) or washed microsome compartments were detected between the four arches (fig. 2.1; p > 0.05). There were no significant differences between the four gill arches in sensitivity to inhibition by plasma (fig. 2.2D; p > 0.05). There were no differences between gill arches in the effect of SDS on the cytoplasmic fraction (fig. 2.2A; p > 0.05); however, there were significant differences between gill arches when microsomal fractions were exposed to SDS with Tukey pairwise comparisons revealing significantly reduced inhibition in arch 3 compared to arch 1 (fig. 2.2A; p < 0.05).

In all gill arches, ultracentrifugal washing caused a significant decrease in CA activity in the microsomal fraction (fig. 2.1A; arch 1: p < 0.01, arch 2 (log transformed): p < 0.01, arch 3: p < 0.001, arch 4: p < 0.01) suggesting the catalytic activity measured is not a result of a membrane bound isoform. The significant inhibitory effect of SDS on both the washed microsomes (fig. 2.2A; arch 1(log transformed): p < 0.01, arch 2: p < 0.01 arch 3: p < 0.01 arch 4: p < 0.01) and cytoplasmic fractions (fig. 2.2A; arch 1: p < 0.01, arch 2: p < 0.01, arch 3: p < 0.01, arch 4: p < 0.05) in the homogenates from all four gill arches implies the lack of paCA. Treatment with rainbow trout plasma containing endogenous PICA had a significant inhibitory effect on both the washed microsomes (fig. 2.2D; arch 1: p < 0.01, arch 2: p < 0.01, arch 3: p < 0.001, arch 4: p < 0.05) and cytoplasmic (fig. 2.2D; arch 1: p < 0.001, arch 2: p < 0.001, arch 3: p < 0.001, arch 4: p < 0.01) fractions in the gill homogenates, further implying the lack of paCA. mRNA expression analysis revealed no significant expression of either CA-IVa or CA-IVb in any of the four gill arches (fig. 2.3, p > 0.05).
2.4.2 Gut tissues

Ultracentrifugal washing caused a significant decrease in CA activity in the stomach (p<0.01) and posterior intestine (p<0.05) suggesting the catalytic activity measured is not a result of a membrane bound isoform (fig. 2.1B). In contrast, ultracentrifugal washing did not significantly decrease CA activity in the pyloric caeca, anterior-, or mid-intestine suggesting the catalytic activity measured in these tissues is likely a result of a membrane bound isoform (fig. 2.1B; p>0.05). SDS had no significant inhibitory effect on the washed microsomes of any of the gut tissues tested (fig. 2.2B; posterior intestine log transformed; p>0.05) but did significantly inhibit CA activity in the cytoplasmic fraction of stomach, pyloric caeca, anterior-, and mid-intestine (fig. 2.2B; p<0.05) confirming the sensitivity of cytoplasmic CAs to this detergent. Treatment with rainbow trout plasma had a significant inhibitory effect on the washed microsomes in stomach (log transformed to meet test assumptions; p<0.001) and pyloric caeca (p<0.05) but did not significantly inhibit CA activity in the washed microsomes of anterior-, mid-, or posterior-intestine (fig. 2.2E; p>0.05). Similarly, CA activity from cytoplasmic fractions of stomach and pyloric caeca were significantly reduced by addition of plasma (both p<0.01) but anterior-, mid-, and posterior-intestine were not significantly reduced (fig. 2.2E; p>0.05) suggesting the latter tissues possess paCA. mRNA expression analysis revealed significant expression of CA-IVa (fig. 2.3A) only in the anterior intestine (p<0.01), and significant expression of CA-IVb (fig. 2.3B) in pyloric caeca (p<0.01), anterior- (p<0.001), mid- (p<0.001), and posterior-intestine (p<0.05) but no significant expression of either isoform was detected in the stomach (p>0.05).
2.4.3 Other tissues (heart, brain, liver, kidney, and white muscle)

Ultracentrifugal washing had no significant effect on CA activity in the heart, brain, or liver (fig. 2.1C; p > 0.05) suggesting the catalytic activity measured is a result of a membrane bound isoform. However, a significant decrease in activity was measured in the kidney and white muscle as a result of washing (fig. 2.1C; p < 0.01), suggesting that paCA isoforms are not present in these tissues. SDS did not have a significant inhibitory effect on the washed microsomes of the heart, brain, liver, or white muscle (fig. 2.2C; p > 0.05). In contrast, a significant effect of inhibition was measured in the kidney (p < 0.01) suggesting no paCA presence. In the cytoplasmic fraction, the heart (log transformed to meet test assumptions) and white muscle exhibited lack of sensitivity to SDS (fig. 2.2C; p > 0.05); however, all other tissues experienced significantly reduced activity (brain & kidney: p < 0.01; liver: p < 0.05; fig. 2.2C). Treatment with rainbow trout plasma caused significant inhibition in the washed microsomal samples from brain and kidney (fig. 2.2F; both p < 0.001) but not in heart, liver, or white muscle (p > 0.05; fig. 2.2F). In the cytoplasmic fraction all tissues displayed a significant inhibitory effect by plasma (heart, liver, kidney & white muscle: p < 0.01, brain: p < 0.001; fig. 2.2F). Analysis of mRNA expression revealed significant expression of CA-IVa in heart (p < 0.01), brain (p < 0.01), and liver (p < 0.001; fig. 2.3A) and significant expression of CA-IVb in kidney and white muscle (both p < 0.05; fig. 2.3B).

2.5 Discussion

In this study, I tested the hypotheses that paCA is absent from the branchial capillaries of all four gill arches and present in all other rainbow trout tissues. To address this, I used a suite of biochemical and molecular methods to measure activity and illustrate specific isoform presence. No evidence was found for paCA availability in the branchial capillaries of any of the four gill
arches (table 2.2). This confirms the long-held assumption that rainbow trout completely lack branchial paCA, which until now was presumed based solely on studies of gill arch 2 (Henry, 1991). While paCA appears to be generally present throughout the circulatory system of the rainbow trout, there was one exception: the stomach, where paCA appears to be absent (table 2.2, fig. 2.4). In addition, mRNA expression analysis revealed almost complete separation among tissues of CA-IVa and CA-IVb isoform expression with only the anterior intestine expressing both CA-IVa and b isoforms (fig. 2.3). These data fully support my first hypothesis that paCA would be absent from all four gill arches, but only partially supports the second, that paCA would be present in all other rainbow trout tissues. The following discussion will centre the current results within our established knowledge of the unique teleost oxygenation system.

### 2.5.1 Implications of paCA for enhanced O₂ unloading

As outlined in the introduction (and discussed at length elsewhere, see review by Harter & Brauner, 2017), the unique teleost oxygenation system is comprised of three major components that allow for the enhanced unloading of O₂ to the tissues during stressful conditions: highly pH sensitive Hb, RBC pH-buffer protection, and a heterogeneous distribution of paCA (figs. 1.2, 1.3). In the current study I confirmed a lack of paCA in all four gill arches. This finding represents an important extension of our knowledge and supports current understanding of how teleosts ensure a suitable environment for O₂ binding in the gills during a generalised acidosis. I also showed homogenous localisation of paCA in all other rainbow trout tissues with the exception of the stomach (fig. 2.4, table 2.2). These findings provide insight into how this system functions in a variety of tissues. It has previously been shown that RBC pH-buffer short-circuiting by paCA enhances O₂ unloading to the red muscle (Rummer et al., 2013) and that inhibition of paCA impairs O₂
unloading and swimming performance in Atlantic salmon (Harter et al., 2019), illustrating its importance to the strenuous migrations of salmonids (Shu et al., 2018). Until now, however, evidence for the presence of paCA has been restricted to certain tissues, with a comprehensive atlas of availability lacking. With the results of the current study, I can speculate that the benefits of enhanced O₂ unloading during stress are likely available to all rainbow trout tissues (with the exception of the stomach) due to the availability of paCA.

Assuming that paCA availability will enable enhanced O₂ unloading during stress in all tissues where it is present, the extent of the benefit conferred to each individual tissue would then be a function of relative blood flow, metabolic activity, the magnitude of an acid-base disturbance, RBC adrenergic stimulation, and the amount of paCA available in the capillaries of that tissue to short circuit RBC β-NHE. I measured varying levels of CA-IV mRNA expression across the tissues investigated with the greatest expression measured in the heart (fig. 2.3); however, further functional studies are required to investigate and quantify tissue specific enhanced O₂ unloading. The benefits of high levels of paCA in the heart would be enhanced O₂ unloading during an acidosis, such as that induced by exhaustive exercise, thereby enabling cardiac output and thus blood flow to metabolically active tissues to be maintained (Alderman et al., 2016). However, the heart represents a potentially risky location for paCA availability due to its proximity to the gills and consequently the short time available for pHi recovery. Harter et al. (2018b) showed that RBC β-NHE is sufficiently rapid to restore appropriate RBC pHi for O₂ uptake within venous transit time (t₁/₂ 17 seconds). However, blood leaving the heart flows through the ventral aorta straight to the branchial arteries (Helfman et al., 2009), a distance that may take considerably less than the half time for pHi recovery identified by Harter et al. (2018b). It is likely that only a small proportion of the total blood volume pumped through the heart comes into contact with the lumen
walls of the myocardium, and thus a relatively small percentage of RBCs are short-circuited to enhance $O_2$ unloading to the heart, permitting the majority of RBC’s to recover appropriately high pH. For other tissues the benefit of enhanced $O_2$ unloading during stress appears less obvious. For example, in the gut of many animals blood flow is reduced during exercise to prioritise $O_2$ delivery to the muscles (Thorarensen et al., 1993). Under such a scenario, paCA could be important in increasing blood $O_2$ extraction for a given tissue and ensuring sufficient conditions for aerobic metabolism during stress.

Considering the hypothesis that paCA is ancestrally ubiquitous among tissues in vertebrates and selectively lost in the gills of teleosts (Harter and Brauner, 2017), I speculate that the lack of paCA in the stomach may be associated with the need to excrete acid and thus remove $CO_2$ at the expense of $O_2$ unloading during stress. After a meal, an alkaline tide ensues (Niv & Fraser, 2002; Cooper & Wilson, 2008) where a relative increase in plasma $HCO_3^-$ results from the secretion of $H^+$ from the blood into the stomach lumen to aid in digestion. The functional significance of a lack of paCA in the stomach remains to be investigated, however it is possible that the absence of paCA plays a role in ensuring adequate $H^+$ secretion (Fleming et al., 1995; Agarwal et al., 2019). Interestingly, paCA is absent from the stomach in a number of mammals (Lönnerholm, 1983; Fleming et al., 1995, Purkerson & Schwartz, 2005). Therefore, it is plausible that the absence of paCA in the stomach may be a broadly vertebrate trait (Fleming et al., 1995; Kivelä et al., 2005) to enhance acid excretion and may be independent of the Root effect, clearly an area worthy of further study.
2.5.2 Implications of multiple CA-IV isoforms

Carbonic anhydrase is vital to numerous cellular processes related to O$_2$ and CO$_2$ exchange, ion balance, and acid-base regulation (Chegwidden et al., 2000; Georgalis et al., 2006; Gilmour, 2010). The presence of tissue specific isoforms, however, may indicate a different primary role for a given isoform. Recently Dichiera et al. (2023) found evidence for at least two CA-IV (a and b) isoforms that are present in the vasculature of several teleosts, consistent with previous data from zebrafish (Lin et al., 2008). While the functional differences between these isoforms remains to be investigated, there was almost complete separation of tissue expression leading to the hypothesis that these isoforms may play primarily different roles in different tissues (Dichiera et al., 2023).

Due to its major expression in the heart, CA-IVa has been suggested to play a primary role in gas exchange (both in CO$_2$ removal and enhanced O$_2$ unloading) while due to major expression in the kidney, CA-IVb has been suggested to play a primary role in acid-base balance and ion exchange (Dichiera et al., 2023). Due to the lack of functional evidence, this area remains purely speculative and an exciting avenue for further research; however, even if these different isoforms perform a different primary role, their function - catalysing the reversible de/hydration of CO$_2$ and enhancing O$_2$ unloading - remains the same.

The results of the current study align with those of Dichiera et al. (2023) showing almost complete separation of CA-IV isoform expression; I confirm the highest expression of CA-IVa and CA-IVb in the heart and anterior intestine respectively (fig. 2.3). However, in the anterior intestine I detected significant expression for both CA-IV isoforms (fig. 2.3). While paCA plays a role in maintaining the acid-base equivalents required for digestion and osmoregulation, it also likely aids in O$_2$ unloading in this tissue. In the intestines, high luminal HCO$_3^-$ concentration is essential for digestion and osmoregulation (Grosell et al., 2007; Grosell et al., 2009). Luminal
CA-IV has been shown to play an important role in maintaining the HCO$_3^-$ and H$^+$ gradients required for the apparently active secretion of HCO$_3^-$ into the lumen in marine teleosts (Grosell et al., 2007); however, it is unknown if this system is also employed by freshwater teleosts due to their differences in osmoregulatory strategy, ion balance, and drinking rates (Wilson, 1999). If CA-IV also has luminal expression, then the functions of this isoform may not be exclusively linked to roles in the plasma and require further research. Tissues such as the intestines, which comprise both capillary and luminal spaces, represent particularly interesting locations regarding the relevance of CA-IV availability and would be excellent candidates for future research.

2.5.3 Previous research and future directions

Overall, the presence or absence of paCA in various rainbow trout tissues reported here is generally in agreement with previous work but greatly expands upon the previous literature to provide the first comprehensive atlas of paCA availability in a teleost (fig. 2.4). Georgalis et al. (2006b) previously demonstrated paCA in the brain of rainbow trout, which I confirm to be the CA-IVa isoform (fig. 2.3, table 2.2). Georgalis et al. (2006b), however, also found strong evidence for CA-IV availability in the posterior kidney, a conclusion I was unable to fully support. I found no evidence for paCA in the kidney in any of the biochemical tests conducted (figs. 2.1, 2.2) although I was able to detect a significant but low level of mRNA expression of CA-IVb (fig. 2.3). This suggests that paCA activity in rainbow trout kidney in the current study may have been below the detection threshold of the biochemical assays used, highlighting a limitation of the use of these assays in tissues with very low paCA availability. It is also possible that the conflicting biochemical data between the current study and that of Georgalis et al. (2006b) results from differences in the environment experienced by the fish used in each study; rainbow trout housed
in Ottawa tap water (Georgalis et al., 2006b) experienced much harder water (higher calcium carbonate concentration) than those from the current study. Fish subjected to exhaustive exercise in soft water present higher levels of blood lactate (Kieffer et al., 2002; Dussault et al., 2008) and extracellular CAs play an important role in lactate transport (Svichar & Chesler, 2003). Therefore, localisation of CA-IV isoforms in the kidney may be a plastic trait associated with environmental conditions. Whether water composition (or other environmental variables) affects CA-IV expression levels would be experimentally testable and reveal interesting insight into the potential plasticity of CA-IV in different tissues. Indeed, differences in water composition between studies may also explain conflicting results in other tissues; Georgalis et al. (2006b) found no evidence for paCA in the white muscle or intestines whereas the current study consistently demonstrated expression of CA-IVb in these tissues.

Conducting the suite of analyses used in the current study to determine the presence or absence of paCA is both time and resource consuming. While mRNA expression analysis arguably provides the most valuable insight into the presence of specific CA-IV isoforms, such measurements are challenging in other species due to the need to design, develop, and validate species specific primer pairs. In contrast, biochemical analyses can be easily conducted across a wide range of species but may give a false negative result for tissues with very low paCA availability. Unfortunately, the biochemical tests used here illustrate that the CA measured is extracellular but do not definitively provide evidence that it is plasma accessible; however, many previous studies have used these tests in conjunction with others that do directly assess plasma availability, for example immunohistochemistry (Georgalis et al., 2006a; Alderman et al., 2016; Harter et al., 2018a) and find good consistency with biochemical results which increases my confidence in the likely plasma accessible localisation of the CA activity measured here.
Encouragingly, I observed a high degree of consistency among tests with the likelihood of any one test result matching the overall result from the entire suite, being around 80%. This suggests that any one test can give a reliable indicator or paCA presence or absence. In cases where I see disagreement between the different tests for a given tissue, biological context may help provide explanation. For example, in the white muscle, the CA-IVb expression detected here may be associated with the interstitial fluid compartment rather than being accessible to the plasma (Wang et al., 1998). Another example is the brain where ultracentrifugal washing, specific inhibition by SDS, and mRNA expression all resulted in evidence for the presence of paCA and specifically the CA-IVa isoform; however, CA activity in the microsomal fraction did not appear to be inhibited by rainbow trout plasma. The brain represents a unique location where the functional mechanism may not be as clear cut as the established role of paCA in enhanced O₂ unloading, CO₂ excretion, and acid-base balance. Instead of lining the capillaries, paCA present in the brain tissue may be in contact with the extracellular fluid (Jones, 1979). The presence of CA-IV has been demonstrated both within the brain tissue (localised to astrocytes; Svichar et al., 2005) and in the capillaries in mammals (Ghandour et al., 1992), as well as being previously indicated in the brain of rainbow trout (Georgalis et al., 2006a). Presence in both regions suggests there may be two CA-IV pools with differing roles; CA-IV within the capillaries at the blood brain barrier would be expected to aid in O₂ delivery to this metabolically active tissue, whereas CA-IV on the astrocytes likely plays a role in modulating brain pH (Chesler, 2003) or CO₂ chemoreception (Hanson et al., 1981). Therefore, the major role of CA-IV in the brain likely depends on its specific location and requires further research to elucidate.
2.5.4 Conclusions

The current study represents the first comprehensive atlas of paCA availability within a teleost model species, the rainbow trout, and fills important gaps in our knowledge of this unique oxygenation system (table 2.2, fig. 2.4). I suggest that the absence of paCA in specific tissues (gills and stomach) is the result of secondary losses that may have evolved to protect other mechanisms vital to the functionality of the tissue in question (for example, acid excretion into the stomach lumen, and O₂ uptake in the gills). I also highlight the potential plasticity of CA-IV availability in certain tissues (for example, kidney), an area that deserves further study. Finally, I add to a growing collection of work suggesting CA-IVa and CA-IVb may play divergent roles in gas exchange and ion/acid-base balance respectively by confirming almost complete separation of expression amongst tissues.
CA activity (µmol H⁺ min⁻¹ mg⁻¹)

Tissue

Fraction
- post-wash
- pre-wash

** CA activity (µmol H⁺ min⁻¹ mg⁻¹)
Figure 2.1 Carbonic anhydrase activity of microsomal fractions prior to and following high speed ultracentrifugation. A) gill arches 1-4, B) different regions of the gut; stomach, pyloric caeca, anterior-, mid-, and posterior intestine, and C) heart, brain, liver, kidney, and white muscle. Solid points with bars represent mean ± SEM, translucent points represent individual data values. A significant decrease in activity (as indicated by asterisks: * p< 0.05, ** p< 0.01, *** p< 0.001) between pre- (CA activity data measured prior to additional ultracentrifugal spin) and post-wash (CA activity data measured after additional ultracentrifugal spin) samples indicates a lack of paCA as CA activity in the respective tissue was reduced by ultracentrifugal washing, suggesting the CA was not truly membrane bound. Conversely, the lack of a significant change between pre- and post-wash activity indicates the presence of paCA.
Figure 2.2 Percent carbonic anhydrase activity inhibition of homogenates by sodium dodecyl sulphate (SDS) - panels A, B, C, and plasma - panels D, E, F. (A, D) gill arches 1-4, (B, E) different regions of the gut; stomach, pyloric caeca, anterior-, mid-, and posterior-intestine, and (C, F) heart, brain,, liver, kidney, and white muscle of rainbow trout. Solid points with bars represent mean ± SEM, translucent points represent individual data values. A significant inhibitory effect (comparison of control and SDS/plasma treated samples; indicated by asterisks: * p< 0.05, ** p< 0.01, *** p< 0.001; only displayed for washed microsomal fractions) indicates a lack of paCA. Washed microsomes refer to the same fraction as ‘post-wash’ in figure 1.
Figure 2.3 Log mRNA expression of A) CA-IVa and B) CA-IVb in gill arches 1-4, stomach, pyloric caeca, anterior-, mid-, and posterior-intestine, heart, brain, liver, kidney, and white muscle of rainbow trout. Data are standardised to β-actin expression within each sample and are analysed and presented as log expression ± SEM. Significant expression (compared to zero) is indicated by the presence of asterisks above each bar: * = p< 0.05, ** = p< 0.01, *** = p< 0.001.
Figure 2.4 Summary figure for rainbow trout plasma accessible carbonic anhydrase atlas study. Tissues coloured in light grey in top row (all four gill arches and stomach) showed no evidence for plasma accessible carbonic anhydrase. All other tissues, coloured in dark grey in bottom row (pyloric caeca, anterior-, mid-, and posterior-intestine, heart, brain, liver, kidney, and white muscle) showed evidence for plasma accessible carbonic anhydrase. Red outlines indicate the CA-IV isoform present is likely involved in primarily gas exchange roles (CA-IVa) and blue outlines indicate the CA-IV isoform present is likely involved in primarily acid-base/ion exchange roles (CA-IVb).
Table 2.1 Primer sequences used in qPCR analysis. β-actin primer pairs were sourced from and previously validated by Koldkjær et al. (2004). CA-IVa and CA-IVb primer pairs were designed and validated as part of the current study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>Forward</td>
<td>5′-CCAACAGATGTGGATCAGCAA-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-GGTGGGCACAGAGCTGAAGTGGA-3′</td>
</tr>
<tr>
<td>CA-IVa</td>
<td>Forward</td>
<td>5′-CCCCATGGGAGCTGCATATTGCTTCA-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-AGGTCTGCTGGAGGAATCA-3′</td>
</tr>
<tr>
<td>CA-IVb</td>
<td>Forward</td>
<td>5′-TGCTACCAGTGCCAGGTATCAT-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-GCTACCAGTGATGTAATGGG-3′</td>
</tr>
</tbody>
</table>
Table 2.2 Summary data for all test results for all fourteen tissues. Evidence from a given test for the presence of plasma accessible carbonic anhydrase (paCA) in the respective tissue is indicated by “+” and absence by “-”. The final column (“paCA?”) summarises the overall result of paCA presence or absence for each tissue analysed based on the test outcomes from previous columns. Tissues were labelled “Yes” (as having paCA) if they exhibited at least three positive results from the four tests or labelled “No” (as not having paCA) if they exhibited less than three positive results. The kidney represents an exception to this rule and is labelled “Yes” and is justified in more detail in the discussion.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Ultracentrifugal washing</th>
<th>SDS</th>
<th>Plasma</th>
<th>mRNA expression</th>
<th>paCA?</th>
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<tbody>
<tr>
<td>Gill arch 1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>No</td>
</tr>
<tr>
<td>Stomach</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>No</td>
</tr>
<tr>
<td>Pyloric Caeca</td>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>Ant. Intestine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Yes</td>
</tr>
<tr>
<td>Mid Intestine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>Post. Intestine</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>Heart</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Yes</td>
</tr>
<tr>
<td>Brain</td>
<td>+</td>
<td>+</td>
<td>-</td>
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</tr>
<tr>
<td>Liver</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Yes</td>
</tr>
<tr>
<td>Kidney</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>White Muscle</td>
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<td>+</td>
<td>+</td>
<td>-</td>
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</tr>
</tbody>
</table>
Chapter 3: Branchial plasma accessible carbonic anhydrase is an embryonic trait in rainbow trout (Oncorhynchus mykiss) that is lost with development and correlated with the onset of the Root effect

3.1 Summary

The teleosts possess a unique respiratory system comprised of three major components (highly pH sensitive hemoglobin, red blood cell (RBC) intracellular pH (pHi) protection, and a heterogeneous distribution of plasma accessible carbonic anhydrase (paCA) – absence at the gills) which allows them to enhance oxygen delivery to the respiring tissues whilst protecting oxygen uptake at the gills. The lack of paCA at the gills in teleosts is thought to be associated with the evolution of highly pH sensitive hemoglobins and RBC pHi protection as the opposite pattern is observed in elasmobranchs and other vertebrates. Our knowledge of this system is detailed in adults, but little is known about its development during ontogeny. Developing rainbow trout (Oncorhynchus mykiss) express embryonic RBCs containing Hb that is not highly pH sensitive; however, the availability of gill paCA and RBC pHi protection is unknown. I show that pre-hatch fish express paCA in the gills, which is abruptly lost with the circulation of highly pH sensitive adult hemoglobins and concurrent rise of RBC pHi protection. I conclude that gill paCA likely represents an embryonic trait in rainbow trout and is likely reminiscent of the ancestral vertebrate condition.
3.2 Introduction

Salmonids are a highly specialised family of fishes renowned for the strenuous upstream migrations conducted by various species. They are a member of the Teleostei, a group which comprises half of all vertebrates (Nelson et al., 2006; Helfman et al., 2009; Ravi & Venkatesh, 2018; section 1.2). They owe their success, at least in part, to their unique respiratory system (Helfman et al., 2009; Randall et al., 2014; Harter & Brauner, 2017). This system comprises various physiological specialisations including: highly pH sensitive hemoglobins (including a large Bohr/Root effect (the reduced affinity and carrying capacity of Hb for O_2 under low pH conditions (Bohr, 1904; Root, 1931); section 1.3.4), intracellular pH (pHi) protection by way of a beta-adrenergically stimulated sodium proton exchanger (β-NHE) on the red blood cell (RBC; Henry et al., 1988; section 1.3.6), and a heterogeneous distribution of plasma accessible carbonic anhydrase (paCA; section 1.3.7), where paCA is present in many tissues but absent in the gills (chapter 2 - Nelson et al., 2023; Harter et al., 2019). During a generalized acidosis, this collection of respiratory characteristics enables these fishes to enhance O_2 unloading to the respiring tissues whilst also protecting O_2 uptake at the gills. This system has been extensively studied in the salmonids where it likely plays an important role in supporting their renowned swimming performance, central to their unique life history (Harter et al., 2019), but has recently been shown to be functional in several other teleost species (Shu et al., 2022). Berenbrink et al. (2005) plotted the evolution of the Root effect and β-NHE through the percomorph lineage (fig. 1.4) showing that these characteristics exist in most teleosts and Randall et al. (2014) suggest that these respiratory traits are associated with the tremendous adaptive radiation seen in teleosts today.

The roles of the Root effect and RBC β-NHE in salmonids have been well studied and reviewed (sections 1.3.4-1.4; Berenbrink et al., 2005, Harter & Brauner, 2017; Nikinmaa et al.,
however, much of our knowledge of how CAs function in respiratory processes comes from work on mammalian systems. The CAs are a family of zinc metalloenzymes responsible for catalysing the reversible hydration of CO$_2$ to HCO$_3^-$.

CAs are ubiquitous across all taxa (Moroney et al., 2001; Supuran, 2008a; Elleuche & Pöggeler, 2009; Banerjee & Deshpande, 2016), and in vertebrates the greatest concentration of CA is found inside the RBC: salmonids possess a high activity RBC cytosolic isoform most similar to mammalian CA-II (Gilmour, 2010). Red blood cell CA has received much attention since its discovery and its roles in O$_2$ and CO$_2$ transport have been well described (see reviews by: Chegwidden et al., 2000; Henry & Swenson, 2000). A myriad of other roles (including ion regulation, acid base balance, and lactate transport) and different isoforms (for example, cytosolic, RBC, plasma, secreted, mitochondrial, and membrane-bound forms) have been well documented across vertebrate taxa (Gilmour, 2010). Cellular-level localisation of CA isoforms allows the animal to restrict catalysis to certain locations and permits the compartmentalisation of the various roles in which CA is essential (Henry, 1988; Ochrietor et al., 2005; Tiwari et al., 2005). While the reaction that CA catalyses remains the same across the range of physiological process, the direction of the reaction depends on the specific isoform involved and its corresponding cellular localisation (section 1.3.7).

A key component of the unique respiratory system in salmonids is the heterogeneous distribution of paCA within the vasculature (fig. 1.2); paCA is present in all tissues except the gills and stomach (chapter 2 - Nelson et al., 2023). Therefore, in the vasculature of most tissues there are two major pools of CA available for gas exchange: paCA (major isoform: CA-IV) and RBC CA-II. CO$_2$ released from the respiring tissues diffuses into the RBC where it encounters CA-II and is converted into HCO$_3^-$ and H$^+$. The HCO$_3^-$ is transported out of the RBC into the plasma by a type II anion exchanger (AE-II; Jensen & Brahm, 1995). Under resting conditions, the H$^+$ load
can be adequately buffered by Hb, but during stress (for example after exhaustive exercise) catecholamines are released that activate the β-NHE and export H⁺ into the plasma in exchange for sodium (Na⁺; Nikinmaa, 1990) thereby safeguarding RBC pHi. In areas of the vascular system where paCA is available, HCO₃⁻ and H⁺ can recombine at the rapid catalysed rate, reforming CO₂ that can then re-acidify the RBC and short-circuit β-NHE (Motais et al., 1989; Nikinmaa et al., 1990; Lessard et al., 1995). As a result of the large Bohr/Root effect found in many teleosts (Berenbrink et al., 2005), this positive feedback system leads to enhanced O₂ unloading to the respiring tissues (fig. 1.2E; Lessard et al., 1995; Rummer et al., 2013). While this is beneficial to tissues with high metabolic demands (such as the heart (Alderman et al. 2016) and red muscle (Rummer et al., 2013; chapter 2 - Nelson et al., 2023), the lack of paCA is crucial to ensuring O₂ uptake during this acidosis (Harter & Brauner, 2017). Interestingly, Dichiera et al. (2023) found that most teleosts have evolved multiple isoforms of CA-IV, and in a few representative species, these CA-IVs are expressed in different tissues implying divergent roles. Multiple CA-IV isoforms were recently confirmed in rainbow trout (*Oncorhynchus mykiss*; Walbaum, 1792), where CA-IVa expression was observed in tissues where its role is expected to be involved in gas exchange (for example in the heart and brain) and CA-IVb localisation in tissues where acid-base/ion regulation is predicted to be its primary role (for example in the digestive tract; chapter 2 - Nelson et al., 2023). Nelson et al. (2023; chapter 2) also confirmed lack of paCA in all four gill arches and found there to be a lack of paCA in the stomach (fig. 2.4, table 2.2), consistent with patterns seen in air breathing vertebrates (Lönnerholm, 1983; Fleming et al, 1995, Purkerson & Schwartz, 2005). While functional evidence is lacking, these distribution patterns further indicate the importance of tissue and cellular level organisation in regulating the roles of CAs.
The system of enhanced O\textsubscript{2} unloading in teleosts is a highly derived trait (section 1.4). It has been well documented that elasmobranchs and air breathing vertebrates do not possess highly pH sensitive Hbs or RBC β-NHE but do possess paCA in the gills and lungs (Brittain, 1987; Cossins & Gibson, 1997; Henry et al., 1997; Geers & Gross, 2000; Henry & Swenson, 2000; Gilmour et al., 2001; McMillan et al., 2019). Thus, it has been proposed that the presence of paCA represents the ancestral vertebrate condition (Harter & Brauner, 2017). Membrane-bound isoforms have been shown to play an important but small role in gas exchange at the respiratory epithelium in elasmobranchs and air breathing vertebrates, predominantly in CO\textsubscript{2} excretion (Henry & Swenson, 2000, Gilmour & Perry, 2004; McMillan et al., 2019). Examples of teleost species that have secondarily lost one or more of these traits led to the hypothesis that the Root effect is associated with the loss of paCA in the gills of teleosts (Berenbrink et al., 2005; Rummer et al., 2013). Evidence from Antarctic icefishes, which have secondarily lost the Root effect via almost complete loss of RBCs and Hb, supports this hypothesis; icefishes have significant expression and activity of CA-IV in the branchial vasculature (Harter et al., 2018a). Despite being a highly derived teleostean group, branchial paCA appears crucial for CO\textsubscript{2} excretion in the absence of RBCs and their high CA activity (Gilmour, 2010). Harter et al. (2018a) suggest that the secondary loss of the Root effect in the Antarctic icefishes resulted in selection for the retention of paCA in the branchial epithelium, and possibly represents another example of paedomorphosis (larval traits retained into adulthood) in this group (Montgomery & Clements, 2000).

The respiratory components previously described have been well studied in adults, however, much less is known about how these characteristics change through development (section 1.5.1). It is known that salmonids display respiratory pigments pre-hatch (Rombough, 1988) and that these unique embryonic Hb (HbE) polymorphs do not present a large Bohr/Root
effect (Iuchi, 1973). HbE polymorphs are the primary available Hb until 14 days post hatch (dph) although HbA polymorphs start to become available immediately post-hatch (Iuchi & Yamamoto, 1983; Bianchini & Wright, 2013). Gilmour et al. (2009) showed that cytoplasmic CA isoforms are crucial for CO₂ excretion in developing zebrafish as early as 24 hours post fertilisation. It is not known, however, whether the embryonic RBCs that contain HbE (Iuchi & Yamamoto, 1983; Bianchini & Wright, 2013) possess β-NHE, or whether the distribution of paCA is different in the gill vasculature of developing fishes relative to adults.

In the current study I aimed to fill several major gaps in our understanding of the developing teleost respiratory system by investigating CA-IV mRNA expression in the branchial vasculature and RBC β-NHE expression during early rainbow trout development, particularly during the transition from major HbE to HbA isoform expression. Rainbow trout were chosen for this analysis as their suite of respiratory characteristics in adults has been highly studied. I hypothesised that 1) during major HbE expression there will be no expression of RBC β-NHE, 2) during major HbE expression branchial CA-IV will be expressed, and 3) during the transitional period where both HbE and HbA are in circulation there will be an associated increase in RBC β-NHE and decrease in branchial CA-IV expression, respectively. These data will enable us to assess the overarching hypothesis that branchial paCA is an embryonic trait in rainbow trout that is lost in adults with the presence of Root effect Hbs.

3.3 Materials and Methods
3.3.1 Experimental animals and tissue collections

Rainbow trout eyed eggs were obtained at 17 days post fertilisation (dpf; 199 accumulated thermal units (ATUs)) from Freshwater Fisheries Society of BC (FFSBC) from their Vancouver Island Trout Hatchery in Duncan, British Columbia, Canada. Fish were maintained in a Heath tray system for the duration of the experiment which was shielded from light and supplied with continuous flow (maximum flow rate: 2 L min\(^{-1}\)) of local dechlorinated tap water (Na\(^+\) = 0.09, Cl\(^-\) = 0.10, Ca\(^{2+}\) = 0.10, Mg\(^{2+}\) = 0.011, K\(^+\) = 0.004 mmol l\(^{-1}\), hardness as CaCO\(_3\) = 3.3 mg l\(^{-1}\), pH = 7.0) at 10 °C. Daily water quality checks were conducted (pH, ammonia, temperature, flow rate, removal of dead animals) and fish were sampled every day or every other day depending on age (see fig. 3.1 for details). Fish were staged according to Vernier (1969). Sample sizes for all measurements available in table A1.

Fish were sampled between 25 and 50 dpf to cover the transition from major embryonic to major adult expression of Hb isoforms. All fish were euthanised using pH buffered Aqualife TMS (100 mg/L; Syndel) prior to sampling. Once euthanized, fish were immersed in at least 5 volumes RNAlater (ThermoFisher, AM7021, Waltham, MA, USA) and stored at 4 °C overnight before storage at -80 °C for later use. Pre-hatch embryos were dechorionated before immersion in RNAlater to ensure sufficient penetration. Ten larger fish were sampled from a different pool of animals (also obtained from FFSBC Vancouver Island Hatchery, Duncan, British Columbia, Canada) to allow a comparison to adult control animals (5 x 20 g fish and 5 x 100 g fish).

3.3.2 mRNA expression analysis

Fish were thawed on ice and whole gill baskets from both sides were carefully excised under a microscope using miniature forceps and scissors. Measurement of RBC β-NHE mRNA
expression was inferred from excised gill tissue that was perfused with blood, which was confirmed visually. Gill tissue was homogenised using a Bullet Blender Storm 24 bead beater (Next Advance, Troy, NY, USA) with Precellys ceramic (zirconium oxide) beads (Bertin Corp., Rockville, MD, USA). mRNA was extracted using Qiagen RNeasy mini kit (Qiagen, 74106, Germantown, MD, USA) according to the recommended protocol. RNA quality (260/280 ratio: 2.0-2.1, 260/230 ratio: 1.4-2.4) and concentration (228 ± 8.9 ng/μL) were measured spectrophotometrically using a Nanodrop 2000 (ThermoFisher, ND-2000). Samples were treated with DNase I (ThermoFisher, EN0521) and cDNA was synthesized from 0.5 μg RNA using RevertAid Reverse Transcriptase (ThermoFisher, EP0441) and random hexamer primers (ThermoFisher, SO142). cDNA was diluted 5 times in molecular water (ThermoFisher, BP28191) before use in qPCR reaction.

Relative mRNA expression was assessed using 2X Maxima SYBR Green/ROX qPCR Master Mix (ThermoFisher, K0221) and real-time qPCR on a Bio-Rad CFX96 RT-PCR Detection system (BioRad, Hercules, CA, USA). Primer sequences were either obtained from published literature (β-actin: Georgalis et al., 2006a; CA-IVa & CA-IVb: chapter 2 - Nelson et al., 2023) or custom designed (β-NHE) using Primer3Plus software (table 3.1). The qPCR conditions (final volume = 12.5 μL) were as follows: cDNA template: 1 μL, forward and reverse primers: 200-400 nM, 2X SYBR Green master mix: 6.25 μL. The annealing and extension temperatures over 40 cycles were 58 °C (60 s) and 72 °C (30 s), respectively. To confirm that SYBR green was not being incorporated into non-specific amplicons or primer-dimers during qPCR, products were analysed using gel electrophoresis and single bands of the expected sizes were achieved in all cases. Additionally, the construction of dissociation curves for SYBR green after 40 cycles qPCR revealed the presence of single amplicons for each primer pair. To ensure that residual genomic
DNA was not being amplified, control samples were included in which no reverse transcriptase was added during cDNA synthesis.

Expression of each gene of interest was standardised to β-actin expression using the delta-delta ct method (Pfaffl, 2001) and data is displayed as relative expression throughout. β-actin was selected as a suitable reference gene (Shekh et al., 2017) and validated as part of the current study (fig. A3.1). Amplification efficiencies were calculated by standard curves generated by serial dilution of pooled rainbow trout cDNA. Primer pair efficiencies were within 90-110 % and R² > 0.99 for all samples. qPCR non-detects were incorporated by manually assigning a relative expression value of zero.

### 3.3.3 Statistical analyses

All statistical analyses and data visualisation were conducted in R Studio (version 1.2.5042). mRNA expression data are presented as relative expression to housekeeping gene (β-actin), and analyses compared each developmental stage value to the adult value. Analyses of variance (ANOVAs) were performed on log transformed data and used to test for differences in expression between stages with post-hoc Tukey multiple comparisons of means used to reveal where differences existed. Data (fig. 3.2) are presented as relative expression to stage 27 for CA-IV and adults for β-NHE. Significant differences are indicated on figures by asterisks: * = p <0.05, ** = p <0.01, *** = p <0.001 and only shown for differences found between a given developmental stage and the adult value. All data are presented as mean ± SEM and sample sizes are available in table A1.
3.4 Results

There were no significant differences in expression levels of any of the genes measured between the 20 g and 100 g fish (p >0.05; fig. A3.2). As a result, these groups were combined for all further analyses and referred to as “adult” fish. Developing rainbow trout differed significantly from adult fish in several of the respiratory characteristics measured and importantly, these differences were no longer apparent following hatch indicating embryonic rainbow trout employ a significantly different respiratory strategy compared to their adult counterparts (see table A3.1 for detailed expression data).

3.4.1 CA-IVa

As expected, no significant level of CA-IVa mRNA expression was detected in the gill tissue of adult fish (fig. 3.2A); however, significant mRNA expression was detected for CA-IVa in the gills of all fish prior to hatch (ANOVA: p <0.001; post-hoc Tukey analysis; stage 27 (25dpf): p <0.001, stage 28/29 (27 dpf): p <0.01, and stage 30 (29-33 dpf): p <0.001) relative to adult mRNA expression (fig. 3.2A). No significant expression of CA-IVa was detected in any stage post hatch (p >0.05; fig. 3.2A).

3.4.2 CA-IVb

As expected, no significant level of CA-IVb mRNA expression was detected in the gill tissue of adult fish. In addition, no significant expression was detected for CA-IVb in the gills of any fish at any developmental stage (ANOVA: p >0.05; fig. 3.2B).
3.4.3 β-NHE

Significant expression of β-NHE mRNA was detected in adult animals (fig. 3.2C). Expression of β-NHE was significantly lower in pre-hatch fish compared to adult fish (ANOVA: p <0.01; post-hoc Tukey analysis; stage 27 (25 dpf): p <0.001, stage 28/29 (27 dpf): p <0.05, and stage 30 (29-33 dpf): p <0.01). Fish at stage 27 had more than tenfold lower β-NHE expression compared to adult individuals. Expression levels in post-hatch developing fish were not significantly different from adult levels (p >0.05; fig. 3.2C). Although non-significant, fish at stages 31 and 32 showed intermediate expression for β-NHE between pre-hatch and adult fish.

3.5 Discussion

In the current study I addressed three major hypotheses: 1) during major HbE expression (pre-hatch) there will be no expression of RBC β-NHE, 2) during major HbE expression branchial CA-IV will be expressed, and 3) during the transitionary period where both HbE and HbA are expressed there will be an associated increase in RBC β-NHE and decrease in branchial CA-IV expression respectively. I measured mRNA expression for branchial paCA and RBC β-NHE in rainbow trout throughout early development from pre-hatch to 16 days post-hatch (dph) and found significant expression of CA-IVa (gas exchange linked paCA isoform; Dichiera et al., 2023) in pre-hatch rainbow trout that was abruptly lost with hatching, but no expression of CA-IVb (ion/acid-base regulation linked paCA isoform) at any stage of development (figs. 3.2, 3.3). Prior to hatch, there was very little expression of RBC β-NHE, but expression levels increased greatly following hatch (figs. 3.2, 3.3). These data support my hypotheses and indicate that pre-hatch rainbow trout use a respiratory strategy more similar to elasmobranchs compared to their adult counterparts. These data also provide preliminary support for the overarching hypothesis that
branchial paCA is an embryonic trait in teleosts that is retained or lost in adults in correlation with the increased pH sensitivity of their Hbs (figs. 3.3, 3.4). The following discussion will centre the current results within our knowledge of the adult teleost and elasmobranch respiratory systems.

An interesting pattern observed in the current data is the difference in changes in expression of branchial CA-IVa and RBC β-NHE with hatching. While CA-IVa expression dropped to zero immediately following hatch, β-NHE expression exhibited a more gradual transition towards the adult phenotype (figs. 3.2A, 3.2C). Although non-significant, fish at stages 31 and 32 (34-40 dpf, 0-7 dph) showed intermediate expression of β-NHE (fig. 3.2C). Previous work (Bianchini & Wright, 2013) showed that during this time there is expression of both HbE and HbA and circulation of both embryonic and adult RBCs, with HbA and adult RBCs predominating as development continues. I speculate that β-NHE is only present on the membrane of adult RBCs hence its apparently intermediate expression during this transitionary period where the proportion of adult RBCs is increasing (Iuchi, 1973; Iuchi & Yamamoto, 1983). While changes in β-NHE activity have been demonstrated in response to various factors (for example: temperature, season, RBC age, and social status; Cossins & Kilbey, 1989; Koldkjær et al., 2004; Thomas & Gilmour, 2006), the presence (or absence) of β-NHE on larval RBCs has yet to be investigated. Although beyond the scope of this study, the hypothesis that β-NHE is exclusively expressed on adult RBCs is clearly worthy of further investigation.

Despite measuring significant CA-IVa expression pre-hatch, at no point was CA-IVb expression detected (fig. 3.2B). Dichiera et al. (2023) suggested that these two CA-IV isoforms support different functions due to their tissue expression patterns: CA-IVa in gas exchange and CA-IVb in ion and acid-base balance. Despite the evidence for divergent expression patterns for CA-IVa and CA-IVb, both isoforms are predicted to be apically bound, and so plasma facing (Zhu
& Sly, 1990), and have identical mechanisms of catalysis (Lindskog, 1997). Consequently, both CA-IV isoforms are expected to be equally capable of short-circuiting the RBC β-NHE system of pHi protection. Based on this I speculate that the significant expression of CA-IVa in pre-hatch fish in the current study may be indicative of a role for this paCA isoform in gas exchange, possibly related to CO₂ excretion as is seen in elasmobranchs (Morrison et al., 2015). The developing teleost gill first plays a role in ion exchange and as such this is thought to be the driving force in early gill development (Rombough, 2002; Fu et al., 2010); however, while the skin is the major organ for both gas and ion exchange until several weeks post-hatch, the gills were responsible for a greater proportion of total O₂ uptake compared to Na⁺ uptake until approximately 7 dph (Fu et al., 2010).

The expression of branchial CA-IVa pre-hatch, suggests that whilst the magnitude of the role of the gills in gas exchange in early rainbow trout development may be limited, it should not be dismissed. It would be interesting to further investigate the role of the gills during early development to CO₂ excretion to determine whether branchial CA-IVa contributes. Branchial CA-IVa may allow the developing rainbow trout to maximise the ability of its developing gills to exchange gases. Knockout experiments would provide interesting insight into the role of CA-IVa to gas transport during early development, especially if further studies support the hypothesis that paCA at the gas exchange surface is an embryonic trait in all vertebrates.

With these new data elucidating temporal changes in branchial paCA and RBC pHi protection in pre-hatch rainbow trout, I can speculate upon the likely developmental trajectory. When embryonic RBCs containing HbE are in exclusive circulation, CA-IVa is expressed in the branchial vasculature allowing CO₂ to be hydrated by CA both inside the RBC and in the plasma. During this time there is no requirement for the β-NHE system of RBC pHi protection due to the lack of large Bohr/Root effects of HbE (fig. 3.3; Bianchini & Wright, 2013). During the transition
from HbE to HbA, paCA in the branchial vasculature is rapidly downregulated to zero presumably to protect O₂ uptake in the event of an acidosis in the newly circulating adult RBCs that do possess large Bohr/Root effects. As the proportion of adult RBCs in the circulation increases, β-NHE pHi protection becomes increasingly important to maintain a suitable intracellular environment and protect O₂ uptake at the gills. During the transition from embryonic to adult RBCs β-NHE expression is low (fig. 3.2C; stages 31 and 32) and fish at this stage may be particularly vulnerable to the negative effects of a generalised acidosis on O₂ loading. During this time, however, fish still express some HbE, which have increased O₂ affinity compared to adult isoforms (Bianchini & Wright, 2013; Iuchi 1973). It is possible, and testable, that the mixture of Hb isoforms expressed during this time allows the fish to cope with such acidotic conditions (Weber, 1990; Brauner & Weber, 1998). In adult teleosts, branchial paCA is unfavourable as its presence would potentially limit O₂ uptake during a generalised acidosis (due to the inability to rapidly adrenergically regulate pHi resulting from β-NHE short circuiting; Harter & Brauner, 2017).

While the results of the current study provide further insight and expansion of our understanding of how the unique teleost oxygenation system develops, protein level investigation would be a useful addition to this dataset to confirm the mRNA expression I have measured is translated into enzyme availability. Due to the small size of these developing animals, and thus limited volume of tissue available, methods regularly used to measure CA activity (ΔpH assay – Henry, 1991) were not feasible. Previous work by Nelson et al. (2023; chapter 2) using the same CA-IVa/b primer pairs showed biochemical evidence that paCA activity matched expression data in adult rainbow trout. A similar relationship between expression and activity for RBC β-NHE was also shown by Koldkjaer et al. (2004). These data from previous studies permit confidence that the mRNA expression data presented here would be supported at the protein level, despite such
analyses being unfeasible in the current study. Furthermore, while salmonids are frequently used as model species for studies investigating the unique teleost oxygenation system, they are inadequate representatives of the vast diversity seen in teleosts due to their highly specialised, extremely active life histories. Collecting data on these characteristics through development for other, relatively unrelated, teleost species would greatly add to the relevance and power of the current analysis.

In conclusion, the current study illustrates that pre-hatch rainbow trout use a significantly different respiratory strategy compared to their adult counterparts (figs. 3.3, 3.4). This strategy appears more similar to that seen in elasmobranchs, with branchial paCA availability, no RBC pHi protection, and no large Bohr/Root effect. A necessary next step will be to expand this work to investigate these characteristics in additional teleost species. These data directly address and support the hypothesis that branchial paCA represents an embryonic trait, and that this trait is lost in adult teleosts in correlation with the development of a large Bohr/Root effect. paCA at the gas exchange surface may be a trait broadly available in vertebrates and as such is likely reminiscent of the ancestral vertebrate condition, an area clearly worthy of further study.
Figure 3.1: Graphic illustrating sampling protocols used in chapter 3. A) Schematic illustrating age (days post fertilisation; dpf), developmental stage according to Vernier (1969), sampling events (indicated by open arrows), hatching time, and sample sizes (n) of embryonic, larval, and adult rainbow trout (Oncorhynchus mykiss) sampled for mRNA expression analysis, and B) illustrations (not to scale) detailing morphological changes used to categorise fish by developmental stage (from left to right: stages 27, 29, 30, 31, 32, 33, and 35; redrawn from Vernier, 1969), and indicating that fish at stages 27-30 represent dechorionated, pre-hatch animals.
Figure 3.2: Relative mRNA expression of A) CA-IVa, and B) CA-IVb in the gills, and C) β-NHE in the blood of rainbow trout through embryonic development and in adult fish. Expression is displayed relative to stage 27 for CA-IVa and CA-IVb and adults for β-NHE. Developmental stages are according to Vernier (1969). Hatching is indicated by black dashed line prior to stage 31. Significance relative to adult expression levels for each gene (assessed using ANOVA and post-hoc Tukey analyses) is indicated by asterisks: * = p<0.05, ** = p<0.01, *** = p<0.001. Data are presented as mean ± SEM (n= 5-27). Sample sizes for each gene are available in table A1 and raw expression data can be found in table A3.1.
**Figure A**

- **Root effect** (% max. Hb desaturation)
- **Bohr Effect** ($\Delta \log P_{50}$ / $\Delta pH$)

**Figure B**

- **Relative mRNA expression**

**Legend**
- Blue: Bohr effect
- Green: Root effect
- Purple: CA-IVa
- Orange: HbE β-1
- Gray: β-NHE

**Developmental stage**
Figure 3.3: Patterns of rainbow trout respiratory characteristics through development: A) Root effect magnitude - green (visualised as maximal % Hb desaturation during exposure to 1.2 kPa CO₂ in normoxia), Bohr coefficient (ΔlogP₅₀/ΔpH; note reversed y axis) during exposure to 1.2 kPa CO₂ in normoxia, B) relative mRNA expression of branchial CA-IVa (purple), HbE β-1 (red), and β-NHE (grey). All data in panel A) and HbE β-1 data in panel B) were replotted from Bianchini & Wright (2013) and are included here to illustrate changing patterns of respiratory characteristics. Curves were chosen based on statistically improved fit to the data. Raw data for mRNA expression of CA-IVa and β-NHE can be found in table A3.1.
Figure 3.4: Summary figure of findings of chapter 3 investigating the system of enhanced oxygen unloading found in some teleosts through development in rainbow trout. Left group of fish/characteristics are pre-hatch (indicated by egg below) and right group of fish/characteristics are post-hatch. From left to right characteristics are as follows: large Bohr/Root effect, red blood cell (RBC) intracellular pH (pHi) protection by a beta adrenergically stimulated sodium proton exchanger (β-NHE), and presence of plasma accessible carbonic anhydrase (paCA). Grey scale diagrams indicate an absence of the given characteristic, and full colour diagrams indicate the presence of the given characteristic.
Table 3.1: Primer sequences used in qPCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>Forward</td>
<td>5′-CCAACAGATGTGGATCAGCAA-3′</td>
<td>Georgalis et al. (2006b)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-GGTGGACAGGCTGAAGTGTA-3′</td>
<td></td>
</tr>
<tr>
<td>CA-IVa</td>
<td>Forward</td>
<td>5′-CCCCATGGAGCTGATATTGTCA-3’</td>
<td>Chapter 2 (Nelson et al., 2023)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-AGGTTCTGCTGGAGGAATCA-3’</td>
<td></td>
</tr>
<tr>
<td>CA-IVb</td>
<td>Forward</td>
<td>5′-TGCTACCAGTCCAGGTATTCAT-3’</td>
<td>Chapter 2 (Nelson et al., 2023)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-GGTACCCAGTGAATGTGATGG-3’</td>
<td></td>
</tr>
<tr>
<td>β-NHE</td>
<td>Forward</td>
<td>5′-GGACCTTGTGACCACATGCTT-3’</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-CTGCCTTCTCCCTTTGATC-3’</td>
<td></td>
</tr>
</tbody>
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Chapter 4: Basal actinopterygians provide insight into the evolution of the unique teleost oxygenation system: an analysis of the loss of branchial plasma accessible carbonic anhydrase

4.1 Summary

The teleosts possess a unique set of respiratory characteristics that allow this group to enhance oxygen unloading to the tissues during stressful conditions. This system is comprised of three major components: highly pH sensitive hemoglobins (large Bohr/Root effects), rapid red blood cell (RBC) intracellular pH (pHi) protection, and a heterogeneous distribution of plasma accessible carbonic anhydrase (paCA; absence in the gills and presence in most other tissues). The first two components have received considerable research effort and their evolution throughout a teleost lineage has been described; however, the evolutionary loss of branchial paCA has received little attention and its evolutionary trajectory through the basal actinopterygians remains unknown. In the current study I investigated for the presence or absence of branchial paCA, along with circulating plasma CA, RBC CA activity, and availability of endogenous plasma inhibitors of CA (PICA) in species belonging to three basal actinopterygian groups: lepisosteiormes, acipensiformes, and polypteriformes. I present the first evidence for branchial paCA in a basal actinopterygian species: the Senegal bichir (*Polypterus senegalus*) and show that like the teleosts, white sturgeon (*Acipenser transmontanus*) and alligator gar (*Atractosteus spatula*) do not possess branchial paCA. I discuss the varying respiratory strategies for these species, and suggest that the evolution of branchial paCA, circulating plasma CA, RBC CA activity, and endogenous PICA exhibited sequential development as has been shown to be the case for Hb pH sensitivity and RBC
pHi protection. Further studies are required to confirm the patterns indicated here across a broader range of species with varying life histories within these groups. The current study adds to a growing collection of data suggesting that the elasmobranch and teleost respiratory models should be thought of as opposite ends of a spectrum, rather than representative of divergent solutions to a common challenge.

4.2 Introduction

Vertebrates transport oxygen (O$_2$) from the environment to their tissues via the O$_2$ transport cascade (fig. 1.2), with the reverse process occurring for excretion of carbon dioxide (CO$_2$). The movement of both O$_2$ and CO$_2$ is driven by their respective partial pressures and, in many species, is further facilitated by interactions at the level of the red blood cell (RBC). O$_2$ unloading to the tissues can be calculated as the product of cardiac output and the arterio-venous difference in O$_2$ concentration ($\Delta$a-VO$_2$). The latter can be increased in most animals due to the existence of pH sensitive hemoglobins (Hb; for example, the Bohr effect (the reduction in Hb-O$_2$ affinity due to a reduction in pH (fig. 1.3; Bohr et al., 1904)) in conjunction with metabolically produced CO$_2$ that acidifies the blood (section 1.3.4). In salmonids, and likely most teleosts (Berenbrink et al., 2005; Harter & Brauner, 2017; Shu et al., 2022), blood $\Delta$a-VO$_2$ is further increased due to the presence of Hbs that exhibit a large Root effect (the reduction in Hb carrying capacity and subunit cooperativity at low pH (Root, 1931; Nikinmaa, 1990; section 1.3.4) in addition to the Bohr effect. Increased Hb pH sensitivity compared to other vertebrates is the first component, which along with red blood cell (RBC) intracellular pH (pHi) protection by way of a beta adrenergically stimulated sodium proton exchanger ($\beta$-NHE) and heterogeneous distribution of plasma accessible carbonic anhydrase (paCA), comprise the unique teleost oxygenation system.
The unique teleost oxygenation system comprised of the abovementioned three components functions as follows: at the gills, RBC pH must be appropriate for environmental O$_2$ uptake by highly pH sensitive teleost Hbs (Baur, 1974). During a generalised acidosis (for example, due to exhaustive exercise; see section 1.3.6 (Wood et al., 1983)), RBC pH is reduced and catecholamines are released triggering removal of H$^+$ from the RBC by the β-NHE thereby elevating pH and protecting O$_2$ uptake (Nikinmaa et al., 1990). At the respiring tissues, in the presence of paCA, H$^+$ exported from the RBC by β-NHE recombine with HCO$_3^-$ to produce CO$_2$ in the plasma at the fast catalysed rate (Lessard et al., 1995; Harter & Brauner, 2017). Consequently, this CO$_2$ diffuses back into, and reacidifies, the cytoplasm of the RBCs, effectively short circuiting the β-NHE pH protection system (Lessard et al., 1995; Harter & Brauner, 2017). Due to the high pH sensitivity of teleost Hbs this greatly enhances O$_2$ unloading to the tissues (fig. 1.2E; Rummer et al., 2013; Harter & Brauner, 2017). If paCA were present in the branchial vasculature of teleosts, RBC pH protection would be short-circuited and O$_2$ uptake would be compromised due to the increased Hb pH sensitivity (Regan & Brauner, 2010). The increased pH sensitivity of teleost Hbs is associated with a reduced Hb buffer capacity (βHb; Lappenas, 1983; Berenbrink et al., 2005; Randall et al., 2014). The functional outcome of this is a switch from pH regulated to O$_2$ regulated Hb H$^+$ binding that affects Δa-vO$_2$ and post branchial pH disequilibrium state development (Gilmour, 1998). It has therefore been hypothesised that the loss of branchial paCA in teleosts is selected for in the presence of increased Hb pH sensitivity and RBC β-NHE (Rummer & Brauner, 2011; Rummer et al., 2013). For a more comprehensive explanation of this system’s function see chapter 1 and reviews by Rummer (2010) and Harter & Brauner (2017).

Of the three major components of the teleost oxygenation system, the importance of a heterogeneous distribution of paCA has received the least attention (section 1.3.7). The carbonic
anhydrases are a family of zinc metalloenzymes responsible for the reversible hydration of CO$_2$ to bicarbonate (HCO$_3^-$) and as such play crucial roles in gas exchange, acid-base balance, and ion homeostasis (Tashian, 1989; Chegwidden et al., 2000; Schwartz et al., 2003; Esbaugh & Tufts, 2006; Georgalis et al., 2006a; Purkerson & Schwartz, 2007; Gilmour, 2010; Harter & Brauner 2017). Many CA isoforms exist, including RBC and cytoplasm specific isoforms, and those bound to cell membranes (Gilmour, 2010), all of which differ in their catalytic efficiencies and abundances (Esbaugh & Tufts, 2006) with the RBC CA-II isoform representing the largest available pool in vertebrates (Maren, 1967). Despite the great degree of CA isoform specialisation and diversity observed inter- and intra-specifically, the catalytic mechanism is conserved (Silverman, 1988; Lindskog, 1997). The major membrane bound isoform, CA-IV, is bound via a glycoprophosphatidylinositol (GPI) anchor to the apical surface of plasma membranes where it is accessible to the blood plasma (Zhu & Sly, 1990). Expression of various isoforms with differing cellular localisation functions to maintain appropriate cellular compartmentalisation of CA catalysis. For example, in the freshwater crustacean gill CA associated with the basolateral membrane mobilises haemolymph HCO$_3^-$ for CO$_2$ excretion, while cytoplasmic CA in the gill catalyses the reverse reaction providing counter ions for ion uptake (Henry, 1988). Many teleosts possess a highly potent endogenous plasma inhibitor of CA (PICA; Henry et al., 1997), a trait absent from the elasmobranchs (Gilmour et al., 2002; McMillan et al, 2019) but present in many air breathing vertebrates (Booth, 1938; Hill, 1986; Henry & Heming, 1998; Heming et al., 1993). Plasma inhibitors of CA are thought to protect against potential CA release during RBC lysis and aid in zinc recycling (Henry & Heming, 1998). Additionally, teleosts lack the circulating plasma CA isoform found in elasmobranchs (Gilmour et al., 1997; Gilmour et al., 2001; Gilmour et al., 2002; Rummer et al., 2013; McMillan et al, 2019) and possess a higher activity RBC CA isoform
compared to elasmobranchs (Henry et al., 1997). Recently, a correlation between the high activity RBC CA and a lack of branchial paCA in a few teleosts has been observed, with the reverse pattern existing in elasmobranchs (McMillan et al., 2019; Dichiera et al., 2020).

The increased pH sensitivity of Hb has been associated with the loss of paCA at the gas exchange surface in teleosts. As described above, the lack of branchial paCA protects O₂ uptake at the gill while allowing short-circuiting of this protective system during a generalised acidosis at the respiring tissues, thereby enhancing O₂ unloading (Harter & Brauner, 2017). The loss of branchial paCA in teleosts appears to be a highly derived trait that is not reminiscent of the ancestral vertebrate condition, or present in extant elasmobranchs and air breathing vertebrates (Harter & Brauner, 2017; McMillan et al., 2019); in most vertebrates paCA is present at the gas exchange surface where it is thought to supply a small role in CO₂ excretion (Henry & Swenson, 2000; Gilmour & Perry, 2004; McMillan et al., 2019). CA-IV, the major paCA isoform was recently confirmed to be absent in all four gill arches of rainbow trout but present in almost all other tissues (except the stomach; fig. 2.4, table 2.2; Nelson et al. 2023 - chapter 2). In addition, functional studies have provided evidence that paCA isoforms are critical to maintaining swimming ability and hypoxia tolerance even under relatively mild exposures to stress in Atlantic salmon (Salmo salar, section 1.3.7.1; Harter et al., 2019; Carless & Brauner, unpublished observation).

Randall et al. (2014) suggest that the unique teleost oxygenation system evolved during a period of low environmental O₂ (fig. 1.1) and enabled this group’s explosive adaptive radiation in the wake of the Permian Period mass extinction event. Berenbrink et al. (2005) used ancestral state reconstruction to plot the evolution of several traits throughout the Percomorpha (fig. 1.4) illustrating that the increasing magnitude of the Root effect occurred in parallel with decreasing
βHb and preceded the evolution of the β-NHE system of RBC pH i protection. While the evolution of the Hb pH sensitivity, β-NHE, and βHb have been well established, the evolution of other features, including paCA, have received less attention (section 1.4). Nothing is known regarding the presence of branchial paCA, or other CA characteristics (RBC CA activity, plasma CA activity, PICA availability) in the basal actinopterygians and as such these species represent an important group in which to explore the transition from paCA availability at the gas exchange surface in elasmobranchs and air-breathing vertebrates to the loss of branchial paCA in the teleosts, a group encompassing half of all vertebrates.

The aim of the current study was to fill knowledge gaps regarding the evolutionary loss of branchial paCA in the teleosts. I focused on three species representative of basal actinopterygian groups that cover the major transitional period between the elasmobranch and teleost respiratory models (Berenbrink et al., 2005): alligator gar (lepisosteiformes), white sturgeon (acipensiformes), and Senegal bichir (polypteriformes). These species were chosen based on availability, but importantly represent extant groups that may reflect transitional stages in the evolution of the teleost respiratory system (fig. 1.4). I used established biochemical methods to assess each species for: 1) branchial paCA, 2) circulating plasma CA, 3) high/low activity RBC CA, and 4) endogenous PICA. I hypothesised that: 1) all groups would possess branchial paCA and circulating plasma CA due to the lack of a physiologically relevant Root effect (Regan & Brauner, 2010), 2) species with high βHb would not have high activity RBC CA, 3) species with high activity RBC CA would not have branchial paCA, and 4) only species with high activity RBC CA and no circulating plasma CA would have endogenous PICA. I expected these characteristics to exist on a spectrum as is the case with the respiratory features that have already been investigated (for example: Root effect magnitude (Berenbrink et al., 2005), Root effect pH onset (Regan & Brauner,
2010), and β-NHE activity (Berenbrink et al., 2005); fig. 1.4). For ease of visualisation, I present these data alongside representative data for a teleost (rainbow trout, *Oncorhynchus mykiss*) and an elasmobranch (blacktip reef shark, *Carcharhinus melanopterus*). Blacktip reef shark was chosen as it appeared to best represent the patterns observed in the elasmobranchs overall in the analysis by McMillan et al. (2019), which is the source of the data included here. Rainbow trout data (branchial paCA) was sourced from chapter 2 (Nelson et al., 2023) or measured as part of the current study (circulating plasma CA, RBC CA activity, endogenous PICA). Gaining understanding of how these CA characteristics change across these transitionary groups will expand our knowledge of how the unique teleost oxygenation system evolved, and how the various components may enhance and/or constraint the system as a whole. These data will help assess the principal hypothesis of my thesis that the presence of highly pH sensitive Hbs constrains the availability of branchial paCA in teleosts.

4.3 Materials and Methods

4.3.1 Experimental animals

Rainbow trout (mean size: 401 g, 34.3 cm) and white sturgeon (*Acipenser transmontanus*, mean size: 2.5 kg, 71.1 cm) were obtained from Miracle Springs Hatchery, Mission, BC, Canada and Vancouver Island University, Nanaimo, BC, Canada, respectively, before transfer to The University of British Columbia (UBC) where they were held in recirculating freshwater tanks (rainbow trout: 2,100 L, white sturgeon: 15,000 L) supplied with dechlorinated Vancouver tap water (*Na^+* = 0.09, *Cl^−* = 0.10, *Ca^{2+}* = 0.10, *Mg^{2+}* = 0.011, *K^+* = 0.004 mmol l⁻¹, hardness as CaCO₃ = 3.3 mg l⁻¹, pH = 7.0) at 10 °C. Fish were fed three times a week to satiation with commercial dry
pellets (Skretting, Stavanger, Norway). All procedures were conducted according to the policies and guidelines of The Canadian Council on Animal Care and approved by the UBC Animal Care Committee (protocol no.: A19-0284-A009).

Alligator gar (*Atractosteus spatula*, mean size: 1.2 kg, 62.4 cm) were obtained from The University of Mississippi, USA in collaboration with Dr. Peter Allen. Prior to sampling, they were reared in 3,500 L recirculating tanks fed by local well water at ~3.8 °C. Fish were fed with commercial warm water feed containing 32% protein, 5 times per week to satiation. All sampling procedures were conducted according to guidelines approved by the Institutional Animal Care and Use Committee at Mississippi State University (protocol no.: 21-161).

Senegal bichir (*Polypterus senegalus*, mean size: 191 mm, 48 g) were obtained from the pet trade (Mirdo Importations Canada, Inc) and held individually in 1 L tanks as part of a 300 L recirculating rack system at The University of Ottawa, ON, Canada in collaboration with Dr. Emily Standen. Fish were fed five days per week on an alternating diet of high protein fish pellets and beef heart and all procedures were conducted in accordance with the policies and guidelines of The Canadian Council on Animal Care and approved by the University of Ottawa Animal Care Committee (protocol no.: BL-3625 and BL3671).

### 4.3.2 Tissue collection

For all species, individual fish were euthanized using Aqualife TMS (50-100 mg/L; Syndel) buffered with NaHCO₃. Upon cessation of opercular beating, rainbow trout, white sturgeon, and alligator gar were injected with heparin (1000 U; Sigma, H3393, St Louis, MO, USA) into the caudal vein to prevent clotting. This was impossible in Senegal bichir due to their small size. Blood samples were collected from rainbow trout, white sturgeon, and alligator gar
using a heparinized syringe from the caudal vein. Packed RBCs and plasma were separated using centrifugation (3000 g, 2 min) after which plasma was transferred into a separate tube and both were flash frozen using liquid N\(_2\). Blood from Senegal bichir was collected using heparinized microhematocrit tube from severed tail, separated by centrifugation and frozen directly. Gill samples were collected (second gill arch only from white sturgeon and alligator gar; whole gill baskets from Senegal bichir) rinsed with saline to remove blood clots and flash frozen in liquid N\(_2\). Alligator gar and Senegal bichir samples were shipped to UBC in liquid N\(_2\) and all samples were stored at \(-80\) °C until further use. Sample sizes for all species/measurements are available in table A1.

### 4.3.3 Tissue preparation

All tissue sample processing and experimental procedures were conducted at UBC. Thawed gill samples were dissected using scissors and scalpel to allow for easy homogenisation. 1-2 g of gill filaments with lamellae tissue (mean ± SEM: 1.66 ± 0.13 g) was added to 2 mL of ice-cold ΔpH assay buffer (in mmol l\(^{-1}\): 225 mannitol, 75 sucrose and 10 Tris base, adjusted to pH 7.3 with 10% phosphoric acid) and homogenised (Polytron PT1200; Lucern, Switzerland) on ice. Crude homogenate underwent differential centrifugation at 4 °C following the protocol previously described (800 G for 20 min, 8000 G for 20 min – centrifuge: Beckman Coulter Allegra X-22R, 100,000 G for 90 min – centrifuge: Beckman Coulter Optima L-100XP; see Henry et al. (1991) and Harter et al. (2018a) for further detail). For Senegal bichir, gill samples were pooled due to their small size and each n contains gills from many fish (total number of individual fish contained in end sample size of 3 = 91). At each stage aliquots were flash frozen in liquid N\(_2\) and retained at -80 °C for further analysis. This additional freeze-thaw cycle has been shown to have
no significant effect on activity (T. S. Harter, unpublished observation). Aliquots frozen from the supernatant and pellet after spin three are referred to as cytoplasm and pre-wash microsomes respectively.

4.3.4 Branchial carbonic anhydrase activity measurement

Carbonic anhydrase activity was measured using the electrometric ΔpH assay (Henry, 1991). The reaction medium consisted of 6 mL ΔpH assay buffer in a 10 mL glass reaction vessel submerged in water in a 35 mL thermostatted vessel at 4 °C. After the addition of enzyme source (100 μL tissue fraction) the reaction was initiated via the addition of 200 μL of 4 °C CO₂ saturated water from a 250 μL gas tight Hamilton syringe. The reaction velocity was continuously measured over a pH change of 0.15 pH units. To obtain the true rate of reaction, the uncatalyzed rate (measured separately in the absence of enzyme source) was subtracted from the observed catalysed rate, and the absolute buffer capacity (measured in separate titrations and calculated from the buffer curve of the assay buffer over the tested pH range) was taken into account to convert from pH units min⁻¹ to μmol H⁺ min⁻¹. Protein content was measured spectrophotometrically at 595 nm using the Bradford assay (Sigma, B6916, St Louis, MO, USA) and bovine serum albumin standards (Sigma, A4612) to give final CA activity in μmol H⁺ min⁻¹ mg⁻¹. pH was measured using a Mettler Toledo InLab cool combined electrode (Mettler Toledo, 51343174, Columbus, OH, USA) with Mettler Toledo pH amp (ADInstruments, FE165) and PowerLab (ADInstruments, PL2602), and data were visualised in LabChart (version 8, ADInstruments). Microsomes were washed using an additional round of ultracentrifugation to remove any persistent non-membrane bound CA (100 000 G, 90 minutes, 4 °C – centrifuge: Beckman Coulter Optima L-100XP). Pellets were resuspended in 2 mL ΔpH assay buffer using mild sonication. Pre-wash refers to CA activity
data collected before the ultracentrifugal washing step, and post-wash refers to that measured after ultracentrifugal washing. The effect of washing was quantified by comparing CA activity between pre- and post-wash microsomes where no significant change in activity is indicative of the measured CA activity resulting from a membrane bound isoform.

4.3.5 Red blood cell and plasma carbonic anhydrase measurement and analysis of plasma inhibitors of carbonic anhydrase

Blood was collected as described in section 4.3.2 to measure RBC and plasma CA activities and to test for the presence of endogenous PICA. These inhibitors are extremely potent in salmonids and are thought to protect against unwanted CA release during RBC lysis as well as aiding in zinc recycling (Dimberg, 1994; Henry et al., 1997). The RBC lysates were produced from 50 µl packed RBCs that were diluted 100-fold in ice cold distilled water to ensure cell lysis. CA activity and protein concentration was measured separately in 40 µl of lysate and 100 µl plasma following the ΔpH assay described above. Additionally, lysate and plasma were assayed together to assess inhibitory effect of plasma on RBC lysate. Percentage inhibition was calculated by comparing activity of the assay run with only the RBC lysate to that also containing plasma.

4.3.6 Statistical analyses

All analyses and figure production were conducted in R Studio (version 1.2.5042). Carbonic anhydrase activity data are presented in μmol H⁺ min⁻¹ mg⁻¹. Inhibition data are presented as % inhibition compared to control values. Data were assessed for normality using the Shapiro-Wilk test. Paired t-tests were used to analyse the effects of ultracentrifugal washing and plasma inhibition within individual species. Comparisons between species (RBC CA activity, plasma CA
activity, PICA) were conducted using two-way ANOVA with Tukey pairwise comparisons used to test for differences where required. Statistically significant differences within species are indicated on figures by asterisks where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, or letters (between species analyses). Raw CA activity data for washed microsomes and the proportion of activity lost with washing can be found in table A4.1.

4.4 Results

In the current study I assessed species from three transitionary groups (as identified from Berenbrink et al., 2005) for respiratory characteristics relevant to the evolutionary development of the unique teleost oxygenation system and compared them with previously published data for a representative elasmobranch and teleost. Data for blacktip reef shark (from McMillan et al., 2019) are shown in figures 4.1, 4.2 and 4.3 to enable visual comparison with the elasmobranch respiratory model. In figure 4.4, blacktip reef shark data were calculated from the data included in figures 4.2 and 4.3 from McMillan et al. (2019). Rainbow trout was included in the current study for comparison of transitionary groups to a model species for which most research on the unique teleost oxygenation system has been conducted; branchial paCA data (fig. 4.1) was sourced from chapter 2 of my thesis (Nelson et al., 2023) and all other rainbow trout data (figs. 4.2, 4.3, 4.4) were collected as part of the current study and confirm previous findings. Raw CA activity data for washed microsomes and the proportion of activity lost with washing varied between 27% and 124% (table A4.1).
4.4.1 Alligator gar

Ultracentrifugal washing provided evidence for a lack of branchial paCA in alligator gar as a significant drop in CA activity was detected between pre- and post-wash microsomes (fig. 4.1; p < 0.001). Alligator gar possessed low activity RBC CA that had significantly reduced activity compared to the high activity rainbow trout RBC CA (fig. 4.2; p < 0.001) and was similar to data for blacktip reef shark (McMillan et al., 2019). Alligator gar plasma contained circulating CA (fig. 4.3) for which activity was significantly greater than rainbow trout (p < 0.001). Alligator gar plasma also contained highly potent PICA that was not significantly different from that found in rainbow trout (p > 0.05; fig. 4.4).

4.4.2 White sturgeon

Ultracentrifugal washing provided no evidence for branchial paCA in white sturgeon as a significant drop in CA activity was detected between pre- and post-wash microsomes (fig. 4.1; p < 0.01). White sturgeon possessed low activity RBC CA that had significantly reduced activity compared to the rainbow trout RBC CA (fig. 4.2; p < 0.001) and was similar to values for blacktip reef shark (McMillan et al., 2019). White sturgeon plasma contained circulating CA (fig. 4.3) for which activity was significantly higher than all other species investigated (p < 0.001) and similar to data for blacktip reef shark (McMillan et al., 2019). White sturgeon plasma also contained endogenous PICA that were significantly less potent that those found in all other species investigated (p < 0.001; fig. 4.4) but still highly effective (92% inhibition) compared to blacktip reef shark.
4.4.3 Senegal bichir

Ultracentrifugal washing provided evidence for branchial paCA in this species as no significant drop in CA activity was detected between pre- and post-wash microsomes (fig. 4.1; p>0.05). Senegal bichir possessed low activity RBC CA that had significantly reduced activity compared the rainbow trout RBC CA (fig. 4.2; p <0.001) and was similar to data for blacktip reef shark (McMillan et al., 2019). Senegal bichir plasma contained low levels of circulating CA (fig. 4.3) for which activity was not significantly different from rainbow trout (p >0.05). Senegal bichir plasma also contained endogenous PICA that were significantly less potent than those found in rainbow trout (p <0.001; fig. 4.4) but still highly effective compared to blacktip reef shark.

4.5 Discussion

In the current study I present an analysis of several respiratory characteristics in species representative of transitionary groups basal to the teleost lineage. I initially made four hypotheses: 1) all groups would possess branchial paCA and circulating plasma CA, 2) species with high βHb would not have high activity RBC CA, 3) species with high activity RBC CA would not have branchial paCA, and 4) only species with high activity RBC CA and no circulating plasma CA would have endogenous PICA. My data show that 1) Senegal bichir possesses branchial paCA, a trait that is absent in any of the more derived species (fig. 4.1), 2) all basal actinopterygian species investigated possessed low activity RBC CA (fig. 4.2), 3) all basal actinopterygian species investigated had circulating plasma CA activity, although likely at a physiologically irrelevant level (fig. 4.3), and 4) all species investigated possessed endogenous PICA (fig. 4.4), with potency appearing to have increased through evolutionary time. In general, these data do not support my hypotheses, and raise many further questions regarding the potential evolutionary trajectory of the
disappearance of branchial paCA in teleosts which I explore below. These data fill important gaps in our knowledge and complement previous findings on the evolution of βHb, Root effect magnitude and β-NHE activity (Berenbrink et al., 2005; figs. 1.4, 4.5). The following discussion will centre the current results within species specific respiratory strategies before speculating on potential evolutionary trajectories of these characteristics within the development of the unique teleost oxygenation system.

4.5.1 Alligator gar

Of the four species investigated in this study, the alligator gar is the most closely related to the teleosts (fig. 4.5) and possessed the following characteristics: no branchial paCA (fig. 4.1), low activity RBC CA (fig. 4.2), minimal circulating plasma CA activity (fig. 4.3), and highly potent PICA (fig. 4.4). The lack of paCA in the branchial vasculature of gar contrasts with my hypothesis and is noteworthy considering this species also lacks a physiologically relevant Root effect and RBC β-NHE (Berenbrink et al., 2005; Regan & Brauner, 2010). The respiratory strategy shown here in gar also fails to support the hypothesis of Dichiera et al. (2020) who suggested that the presence of a high activity RBC CA may be linked to the loss of branchial paCA. Instead, my data suggest that the high activity RBC CA found in rainbow trout may be a more recently derived characteristic, restricted to the teleosts or even salmonids. While I did not conduct genetic analysis, and so cannot confirm that the low RBC CA activity shown here is the same isoform expressed in elasmobranchs, the RBC CA activity measured for alligator gar was almost two orders of magnitude lower than that measured for rainbow trout (fig. 4.2) and very similar to activity found in blacktip reef shark by McMillan et al. (2019).
The alligator gar therefore possesses an interesting combination of respiratory characteristics, unlike either the teleost or elasmobranch respiratory models. Without highly pH sensitive Hbs or RBC β-NHE, gar would be unable to enhance O\textsubscript{2} unloading during stress as is seen in teleosts (fig. 1.2E; Berenbrink et al., 2005; Regan & Brauner, 2010; Harter & Brauner, 2017). Consequently, there would not be strong coupling of O\textsubscript{2} and CO\textsubscript{2} through the RBC (Brauner & Randall, 1998; Jensen, 2009), which may explain the presence of a low activity RBC CA isoform in this species. Support for this comes from the greater βHb in alligator gar compared to teleosts (Berenbrink et al., 2005), which is associated with a smaller Bohr/Haldane coefficient (Brauner & Randall, 1998). Coupling of O\textsubscript{2}/CO\textsubscript{2} through the RBC is affected by availability of CA to the plasma, a trait that teleosts lack (Harter & Brauner, 2017). While I detected significantly higher levels of CA activity in the plasma of alligator gar compared to rainbow trout (fig. 4.3), the activity detected was minimal and is unlikely to be biologically relevant (Gilmour et al., 2001). Together with the lack of branchial paCA, the lack of circulating plasma CA suggests that CO\textsubscript{2} excretion across the gills in alligator gar requires transport through the RBC due to the almost complete lack of plasma catalytic activity for HCO\textsubscript{3}\textsuperscript{-} dehydration (Jensen, 2004). Therefore, the respiratory strategy of alligator gar appears to be a scenario where, like in teleosts, both O\textsubscript{2} and CO\textsubscript{2} require transport through the RBC possibly due to buffer availability and lack of plasma CA activity, but is intermediate between elasmobranchs and teleosts due to the reduced pH sensitivity of alligator gar Hbs compared to teleosts.

4.5.2 White sturgeon

White sturgeon was included in this study as representative of the acipenseriformes and therefore represent the next most basal phylogenetic branch after alligator gar (Hughes et al., 2018;
White sturgeon possessed a generally similar set of respiratory characteristics to the alligator gar (fig. 4.5) with no branchial paCA (fig. 4.1), low activity RBC CA (fig. 4.2), and endogenous PICA (fig. 4.4). White sturgeon possessed greater circulating plasma CA activity compared to all other actinopterygian species studied (fig. 4.3), a characteristic that would affect the relative buffering capacities of the two-compartment blood system (Desforges et al., 2001). Elasmobranchs possess circulating plasma CA activity, which was originally thought to contribute greatly to CO₂ excretion (along with paCA isoforms; Wood et al., 1994). The elasmobranchs are not thought to have the high plasma buffer capacities needed to utilise such a pool of CA (Heisler, 1986; Henry et al., 1997; McMillan et al., 2019), however, which is also likely true of white sturgeon, suggesting a role for circulating plasma CA in direct CO₂ excretion in white sturgeon is likely minimal. The reduced potency of PICA in white sturgeon compared to all other species measured is interesting but also likely lacks physiological relevance; addition of plasma still reduced RBC CA activity by almost 92 % (compared to >96 % in all other species; fig. 4.4), suggesting white sturgeon PICA are still highly effective against RBC CA. Despite these differences white sturgeon appear to employ a generally similar respiratory strategy to alligator gar and are thus intermediate between elasmobranchs and teleosts for the same reasons.

4.5.3 Senegal bichir

Senegal bichir were included in this study as representative of the polypteriformes and represent the most basal phylogenetic branch investigated by the current study (Hughes et al., 2018; fig. 4.5). While the sample size (n=3) for this species was less than desired, I remain confident in these results due to the number of individuals pooled in each measurement (see section 4.3.3 for further detail). This species displayed an apparent shift in respiratory strategy compared
to alligator gar and white sturgeon. They were the only species investigated to possess branchial paCA (fig. 4.1) and as such represent the most derived basal actinopterygian group shown to possess this characteristic. Senegal bichir also possessed low activity RBC CA (fig. 4.2), minimal circulating plasma CA (fig. 4.3), and potent PICA (fig. 4.4). The discovery of branchial paCA in the Senegal bichir represents the first evidence for the availability of paCA in the branchial capillaries of a basal actinopterygian and suggests this species employs a respiratory strategy more similar to that of elasmobranchs. Thus, I propose that in the Senegal bichir both the RBC and plasma compartments contribute to HCO₃⁻ dehydration at the gills, similar to elasmobranchs. Two key differences between the respiratory strategies of the Senegal bichir and the elasmobranchs, however, are 1) the presence of endogenous PICA that are absent in elasmobranchs (McMillan et al., 2019), and 2) the lack of circulating plasma CA in Senegal bichir, which is present to various degrees in elasmobranchs (McMillan et al., 2019).

A possible explanation for the apparent disappearance of branchial paCA after the divergence of the polypteriformes is related to βHb. The polypteriformes are the most derived actinopterygian group to maintain high βHb (Berenbrink et al., 2005), which is reminiscent of the assumed ancestral vertebrate condition. Such correlation between high βHb and presence of branchial paCA can be used to inform further species of interest; several teleost species have secondarily lost the Root effect (including Anguilla, Siluriformes and Monopterus) and possess greater βHb that is comparable to values seen in non-teleost species (Berenbrink et al., 2005). If retention of high βHb is linked to retention of branchial paCA in basal actinopterygians, I may expect teleosts that have secondarily lost the Root effect, and regained βHb strength to also regain branchial paCA as observed in the species described above.
4.5.4 Evolutionary perspectives

It is useful to position the findings of the current study within the previous knowledge of the evolution of the teleost respiratory system (fig. 1.4; Berenbrink et al., 2005) and speculate how the traits investigated here (branchial paCA, circulating plasma CA, RBC CA activity, PICA) may have evolved over this teleostean lineage. Previously work has demonstrated that βHb first decreased, before a large increase in the magnitude of the Root effect and its physiological relevance may have selected for RBC pHi protection by way of the β-NHE exchanger that preceded the evolution of the choroid and swimbladder retia (fig. 1.4; section 1.4; Berenbrink et al., 2005). My work suggests that: 1) branchial paCA was lost earlier than initially hypothesised, 2) high activity RBC CA may be restricted to derived teleostean species and not linked to branchial paCA availability, 3) endogenous PICA may be present in all non-elasmobranch fishes, and 4) circulating plasma CA may be an evolutionary relic that is incompatible with potent PICA.

I suggest that my data may fit into the evolutionary trajectory proposed by Berenbrink et al. (2005) that is illustrated in figure 4.5, as follows: the increasing magnitude of the Bohr effect, and associated transition from pH regulated to O₂ regulated Hb H⁺ binding, affected post branchial pH disequilibrium state development (Gilmour, 1998). A large Bohr effect couples O₂/CO₂ transport through the RBC (Nikinmaa, 1997) and resulted in the reduced effectiveness of Hb as a buffer, ensuring that H⁺ were available for HCO₃⁻ dehydration (Randall & Brauner, 1996). This reduced βHb may therefore have favoured the loss of branchial paCA after the divergence of the polypteriformes due to the increased magnitude of the Bohr effect. The presence of circulating plasma CA would also affect post-branchial pH disequilibrium state development (Nikinmaa, 1997) and may have been selected against early in the lineage leading to derived teleosts (prior to the divergence of the polypteriformes), a hypothesis that is supported by the presence of highly
potent PICA in all species investigated. The continued decrease in βHb thereby allowed for the increasing magnitude and physiological relevance of the Bohr/Root effect in the absence of branchial paCA and circulating plasma CA. Selective loss of branchial paCA in such a scenario may have functioned to maintain higher RBC pHi and protect branchial O₂ uptake. Larger disequilibrium states may develop in the absence of branchial paCA, alongside increasing Bohr magnitude and decreasing βHb, due to the catalysis of CO₂ hydration reactions being confined to the RBC (Perry et al., 1997; Gilmour, 1998). Consequently, RBC pHi would be higher during gill transit, thereby aiding O₂ uptake by Hb with an increasing Bohr magnitude. I can then speculate that the loss of branchial paCA after the divergence of the polypteriformes may have acted as a primitive RBC pHi protection mechanism, which was beneficial due to low atmospheric O₂ during this period (Randall et al., 2014) and allowed exploitation of the increasing Bohr magnitude at the respiring tissues without compromising gill O₂ uptake. Finally, with the evolution of a large, physiologically relevant Root effect, RBC pHi protection by β-NHE and retinal and swimbladder retia (Berenbrink et al., 2005), a high-activity RBC CA activity evolved only in the teleosts, further enhancing disequilibrium state development.

It is important to mention that these findings are based on a limited number of species chosen as representatives for their respective groups. Furthermore, two of the species studied (alligator gar and white sturgeon) are water breathers while the other (Senegal bichir) is a facultative air breather (Graham et al., 2014). The impact of life history traits such as respiratory medium on the respiratory characteristics investigated in the current study is unknown and would be an interesting direction for future research.

My study indicates that paCA was likely lost from the branchial vasculature in the basal actinopterygians much earlier than originally expected and highlights the need for future work in
this area. An important next step in this work is to broaden the number of species investigated from these basal actinopterygian groups to determine if the pattern remains. In general, it appears that the respiratory strategies of elasmobranchs and teleosts, which are often held as divergent solutions to the same environmental challenge, might be more accurately thought of as opposite ends to a diverse spectrum, with various intermediary phenotypes.
Species

CA activity (μmol H⁺ min⁻¹ mg⁻¹)

Fraction
- Post-wash
- Pre-wash

Blacktip reef shark
Senegal Bichir
White Sturgeon
Alligator Gar
Rainbow Trout
Figure 4.1: Carbonic anhydrase (CA) activity of gill tissue homogenates prior to (pre-wash - green) and following (post-wash - purple) high speed ultracentrifugal washing (100 000 G, 4 °C, 90 minutes) for blacktip reef shark (data reproduced from McMillan et al., 2019), Senegal bichir, white sturgeon, alligator gar and rainbow trout (rainbow trout data reproduced from chapter 2 (Nelson et al., 2023); from left (most basal) to right (most derived)). Solid points with bars represent mean ± SEM, translucent points represent individual data values. A significant decrease in activity (as indicated by asterisks: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$) between pre- (CA activity measured prior to an additional ultracentrifugal spin) and post-wash (CA activity measured after an additional ultracentrifugal spin) samples indicates a lack of paCA as CA activity in the respective species was reduced by ultracentrifugal washing, suggesting the CA was not truly membrane bound. Conversely, the lack of a significant change between pre- and post-wash activity indicates the presence of paCA.
Figure 4.2: Red blood cell (RBC) carbonic anhydrase (CA) activity for blacktip reef shark (data reproduced from McMillan et al., 2019), Senegal bichir, white sturgeon, alligator gar, and rainbow trout (from left (most basal) to right (most derived)). Solid points with bars represent mean ± SEM, translucent points represent individual data values. Different letters represent significant differences between species analysed in the current study.
Figure 4.3: Plasma carbonic anhydrase (CA) activity for blacktip reef shark (data reproduced from McMillan et al., 2019), Senegal bichir, white sturgeon, alligator gar, and rainbow trout (from left (most basal) to right (most derived)). Solid points with bars represent mean ± SEM, translucent points represent individual data values. Different letters represent significant differences between species analysed in the current study.
Figure 4.4: Presence of plasma inhibitors for carbonic anhydrase (PICA) in blacktip reef shark (data calculated from McMillan et al., 2019), Senegal bichir, white sturgeon, alligator gar, and rainbow trout (from left (most basal) to right (most derived)). Solid points with bars represent mean ± SEM, translucent points represent individual data values. Dashed line through zero indicates lack of significant inhibition in any species with data crossing the line. Different letters represent significant differences between species analysed in the current study.
Elevated Bohr effect
Decreasing \( \beta \)Hb
Physiologically relevant Root effect
Choroid rete
RBC \( \beta \)-NHE
High activity
RBC CA
Loss of branchial paCA
Decreasing \( \beta \)Hb
Elevated Bohr effect
PICA
Loss of plasma CA

Blacktip reef shark
Senegal Bichir
White Sturgeon
Alligator Gar
Rainbow Trout

tetrameric Hb, no RBC \( \beta \)-NHE, no retia, no Root effect, high \( \beta \)Hb, plasma CA, RBC AE-II, small Bohr effect, branchial paCA, low RBC CA, no PICA
Figure 4.5: Summary figure for basal fishes study. Phylogenetic relationships of species investigated illustrating assumed ancestral condition (traits contained in box at base of tree), suggested evolutionary trajectory including data from the literature in open, black ovals (Berenbrink et al., 2005; McMillan et al., 2019), and data from the current study in filled, coloured ovals. Hb: hemoglobin, RBC: red blood cell, β-NHE: beta adrenergically stimulated sodium proton exchanger, βHb: Hb buffer value, CA: carbonic anhydrase, AE-II: type II anion (Cl⁻/HCO₃⁻) exchanger, paCA: plasma accessible CA, PICA: endogenous plasma inhibitor of CA.
Chapter 5: General Discussion and Conclusions

The principal goal of my thesis was to assess the hypothesis: **hemoglobins with a large, physiologically relevant Root effect have imposed constraints on branchial plasma accessible carbonic anhydrase availability within teleost fishes.** I used three observational studies to approach my principal hypothesis from a variety of angles. The motivation for these studies came directly from curiosity driven questions arising in the wake of my principal hypothesis that addresses key gaps in our current understanding of the unique teleost oxygenation system. Teleosts have a highly derived, unique system to enhance O₂ unloading to the tissues that is comprised of three major components: highly pH sensitive hemoglobins (large Bohr/Root effect), RBC β-NHE pHi protection, and a heterogeneous distribution of paCA (absence in the gills, and presence in other tissues; Harter & Brauner, 2017; Nelson et al., 2023). While the first two components have received much research interest, the distribution of paCA within the teleost vasculature has been subject to many assumptions and less rigorous scientific investigation. Through my thesis I hoped to fill some of these knowledge gaps and advance our understanding of a system that is likely present in half of all vertebrates.

One assumption that has persisted in this field, and is directly addressed by my thesis, is the notion that the lack of branchial paCA in gill arch 2 is reminiscent of the pattern of paCA localisation in all four gill arches. Prior to my thesis, availability of paCA had only been investigated in gill arch 2 (Henry et al., 1988; Henry, 1991; Henry et al., 1993; Gilmour et al., 2002; Harter et al., 2018a) and the remaining three arches have been assumed to match this pattern. This assumption represents the focus of chapter 2 of my thesis where I used a suite of biochemical and molecular methods to assess 14 different rainbow trout tissues for paCA availability to test the hypotheses that paCA is absent from all four gill arches but present in all other tissues. These data
are summarised in table 2.2 and figure 2.4 and collectively provide strong support for the first part of my hypothesis: paCA was found to be absent from all four gill arches, but only partially supports the second, as I found paCA to be absent from the stomach but present in all other tissues analysed.

The remaining two studies in my thesis focussed on utilising natural systems where Hb pH sensitivity is diminished or absent to look for patterns of branchial paCA availability. These systems comprise developing rainbow trout (chapter 3) and basal actinopterygians (chapter 4). Until now these groups represented significant gaps in our knowledge. In developing rainbow trout I found a strong correlation between HbE expression (that lacks a large Bohr/Root effect), and branchial paCA availability in pre-hatch animals. I also found that the RBC β-NHE pHi protective mechanism was not available pre-hatch suggesting that embryonic rainbow trout use a strikingly different respiratory strategy compared to their adult counterparts (fig. 3.4). This apparent embryonic strategy changed through development, as paCA was lost at hatch in conjunction with the increased magnitude of the Bohr/Root effect and appearance of RBC β-NHE expression. These data provide strong support for my thesis’ principal hypothesis; however, I was unable to replicate such support in my investigation of basal actinopterygians. I predicted that the evolutionary trajectory of branchial paCA in basal actinopterygians would follow a similar pattern to the developmental trajectory of branchial paCA found in rainbow trout; however, branchial paCA appears to have been lost much earlier in the evolutionary trajectory than expected based on this prediction and appears to correlate with the increasing magnitude of the Bohr coefficient and the concomitant decrease in βHb in the teleostean lineage (fig. 4.5).

Overall, my thesis provides persuasive evidence that the presence of a large, physiologically relevant Root effect in teleost Hbs is not the single constraining factor on the presence of paCA in the branchial vasculature (fig. 5.1). While I confirmed the assumption that
paCA is absent from all four gill arches in adult rainbow trout (table 2.2, fig. 2.4) and found a correlation between availability of branchial paCA and highly pH sensitive Hbs in developing rainbow trout (fig. 3.4), this pattern was not replicated in the basal actinopterygians (fig. 4.5). If branchial paCA is constrained by an alternative factor, our understanding of how the unique teleost oxygenation system functions, develops, and evolved would change fundamentally. The remainder of this chapter will focus on the most interesting implications of my findings (section 5.1) before discussing suggestions for future directions (section 5.2) and overarching conclusions (section 5.3).

5.1 Thesis highlights, major contributions, and implications

The following sections discuss the most exciting implications of the findings from all studies of my thesis and contains abundant speculation regarding the mechanisms, functional importance, and correlations involved.

5.1.1 paCA is absent from all four gill arches in rainbow trout

In chapter 2 of my thesis, I confirmed the long-standing assumption that paCA is absent from all four gill arches of rainbow trout (table 2.2, fig. 2.4). While these data may not appear as immediately exciting as other results of my thesis, it represents an important confirmation for our understanding of how the respiratory system of half of all vertebrates operates (Helfman et al., 2009). Henry et al. (1988) originally demonstrated that rainbow trout lack paCA in gill arch 2, and it has since been assumed to be absent from all four gill arches due to the associated risks to O$_2$ uptake (Randall & Brauner, 1991; Rummer & Brauner, 2011; Harter & Brauner, 2017). Considering that the lack of paCA in the gills of teleosts appears to be an essential component of
their unique system of O₂ unloading, it is surprising that this assumption has not been scientifically confirmed for more than 35 years. Confirming this long-standing assumption is a component of my thesis that represents a particularly important basic advancement of our knowledge.

5.1.2 paCA is not present in all non-branchial tissues of rainbow trout

A second interesting outcome of chapter 2 of my thesis is the presence of paCA in all tissues I investigated, except for the stomach tissue that lacked paCA (table 2.2, fig. 2.4). I originally hypothesised that paCA would be present in all non-branchial tissues of rainbow trout, a hypothesis that I rejected due to the data for the stomach. A possible explanation for the absence of paCA in the stomach is linked to the requirement to excrete acid and thus remove CO₂, apparently at the expense of O₂ unloading during times of stress. After feeding, an alkaline tide develops (Niv & Fraser, 2002; Cooper & Wilson, 2008) where a relative increase in plasma HCO₃⁻ results from the secretion of H⁺ from the blood into the stomach lumen. I speculate that if paCA were present in the vasculature of the stomach, the PCO₂ would be reduced due to elevated HCO₃⁻ and consequently could impair H⁺ excretion from the blood. Support for this speculation comes from the observation that fish possess a much higher PCO₂ in the stomach lumen compared to the blood (Wood & Eom, 2019; Jung et al., 2022) and interestingly, paCA is also absent from the stomach in several mammals (Lönnerholm, 1983; Fleming et al, 1995, Purkerson & Swartz, 2005). Therefore, it is plausible that the absence of paCA in the stomach is a result of prioritisation of luminal acid excretion and a broadly vertebrate trait (Fleming et al, 1995; Kivelä et al, 2005), independent of the large Bohr/Root effect in teleosts. This would be a deserving area for further study.
In addition, we know that in many animals, including fishes, blood flow to the digestive tract is modulated by various conditions including feeding status, infection, temperature, and stress (Matheson et al., 2000; Seth et al., 2010; Brijs et al., 2018). Under stressful conditions, blood flow to the digestive tract is highly reduced, and so paCA would likely play no significant role in enhancing O\textsubscript{2} unloading even if it were present. A logical hypothesis is that paCA in the stomach would serve no adaptive role, due to the risks described above relating to acid secretion and lack of benefit to O\textsubscript{2} delivery during stress due to diverted blood flow. However, the presence of paCA in all other digestive tract tissues suggests the restriction of blood flow alone may not be a strong selective force causing the loss of paCA in the stomach tissues. Selective inhibition of paCA at a tissue level (section 5.2.6) under a variety of conditions (for example: feeding status and/or stress) would provide interesting insight.

The absence of paCA in the stomach (in addition to the gills) allows speculation that paCA may have undergone secondary losses (compared to the assumed ancestral condition of homogenous expression) in specific tissues to protect other mechanisms vital to the function of the tissue in question. In the stomach, the lack of paCA may act to protect luminal acid secretion as discussed above, whereas in the gills O\textsubscript{2} uptake by highly pH sensitive Hbs is safeguarded due to the loss of paCA. This acts as an important reminder that CAs are involved in a myriad of physiological processes (Chegwidden et al., 2000; Georgalis et al., 2006a; Gilmour, 2010) and it is exceptionally difficult to tease apart a primary role for a given enzyme at any location. The variety of roles in which CAs are involved also adds difficulty to the principal aim of my thesis that was to assess the hypothesis that Hbs with a large, physiologically relevant Root effect have imposed constraints on branchial paCA availability within teleost fishes. With so many different roles for CA enzymes, it is almost impossible to say with certainty that any given factor is the
proximate cause of a change in availability, activity, or expression, and more likely represents a compromise among competing physiological processes.

5.1.3 Potential plasticity in paCA expression

An unexpected implication of my thesis was the indication that paCA availability may be plastic within individuals both due to changing environmental conditions and throughout development. Georgalis et al. (2006a) previously found evidence for CA-IV in the posterior kidney, a conclusion I was unable to fully support in chapter 2 of my thesis (table 2.2). It is possible to speculate that this divergence may be due to environmental water parameters. Fish from my study were held in dechlorinated Vancouver tap water, which is soft and so low in calcium carbonate, compared to the relatively hard water that supplies Ottawa and was used in the previous study (Georgalis et al., 2006a; Kumai et al., 2014). Extracellular CA isoforms have been shown to play an important role in lactate transport (Svichar & Chesler, 2003) with fish subjected to exhaustive exercise in soft water displaying elevated blood lactate (Kieffer et al., 2002; Dussault et al., 2008). It is therefore possible that availability of paCA isoforms in the kidney may be a plastic trait associated with environmental conditions. Interestingly, Dichiera et al. (2022) found CA-IV expression did not change significantly in red muscle or heart of red drum exposed to hypoxia. In a companion study to that of Dichiera et al. (2022), Negrete et al. (2022) showed that the same fish used changes in Hb isoform expression to modulate tissue O₂ delivery rather than changes in paCA catalytic availability. It is possible that due to the number of processes in which CA catalysis is important, adjustments in expression of different CA isoforms may not be the leading means of adjustment. Whether other environmental variables affect CA-IV availability is
experimentally testable and would reveal interesting insights into the scope of plasticity of CA-IV in different tissues.

In chapter 3 I showed that paCA expression through development in rainbow trout is plastic; pre-hatch animals express branchial CA-IV that is abruptly lost with hatch and correlates with the transition to HbA isoform expression (Bianchini & Wright, 2013). It would be of great interest to know if what appears to be the retention of the ancestral condition (i.e., presence of paCA at the gas exchange surface), and the transition from branchial paCA expression with HbE to loss of branchial paCA with HbA occurs in other teleosts, and if this apparent plasticity in branchial paCA availability can be exploited under similar conditions during adult life stages. Fishes that inhabit extremely hypoxic or anoxic environments may be interesting subjects for future study in this regard. The ability to modulate paCA availability with changing internal conditions is of great interest and sparks many questions relating to whether fishes can increase paCA concentrations in certain metabolically active tissues during times of stress, for example during strenuous migrations in salmon, or extended hypoxic exposures in species that inhabit winter frozen lakes.

5.1.4 Pre-hatch rainbow trout are more like an elasmobranch than a salmonid

Perhaps one of the most exiting findings of my thesis comes from chapter 3 and pertains to the respiratory characteristics of developing rainbow trout. I showed, for the first time, that a teleost, which has highly pH sensitive Hbs and RBC β-NHE activity as an adult, can exhibit branchial paCA in another life stage. By illustrating significant expression of CA-IVa (and a lack of expression of β-NHE) in pre-hatch rainbow trout I present the first evidence for a switch from a respiratory strategy similar to that seen in adult elasmobranchs, to the classic teleost suite of
respiratory characteristics (fig. 3.2). These data lend support to the hypothesis of Harter et al. (2018a) who suggested that the presence of branchial paCA in the Antarctic icefishes may be a paedomorphic trait retained into adulthood due to their loss of RBCs and Hb (Kock, 2005). It also supports the notion that a homogenous distribution of paCA represents the ancestral vertebrate condition that is lost in the highly derived adult teleost due to the increased pH sensitivity of their Hb (Harter & Brauner, 2017). I therefore hypothesise that the respiratory strategy observed in pre-hatch rainbow trout in chapter 3 of my thesis is reminiscent of the ancestral vertebrate condition and likely exists in pre-hatch animals of other species. Expanding upon my work to address this hypothesis directly would be of great interest.

5.1.5 Developmental and evolutionary trajectories in the loss of branchial paCA

The studies conducted in chapters 3 and 4 of my thesis aimed to investigate scenarios where the Root effect is absent to investigate whether the constraint on branchial paCA availability is released, and therefore address my principal hypothesis. I originally predicted that I would see the same pattern in the developing rainbow trout and in the basal actinopterygians. This prediction was accurate at the broadest level – pre-hatch rainbow trout did possess a respiratory strategy reminiscent of that seen in elasmobranchs and thought to be the ancestral vertebrate condition, but differences emerge when considering the details. In the developing rainbow trout, I observed an abrupt change in respiratory strategy from HbE without a large Bohr/Root effect, no β-NHE expression, and the presence of branchial paCA (significant expression of CA-IVa) pre-hatch, to HbA with highly pH sensitive Hbs, RBC β-NHE pHi protection (significant expression of β-NHE) and lack of branchial paCA (no significant mRNA expression of either CA-IV isoform) post-hatch (fig. 3.4). Across the basal actinopterygians I investigated, these changes appear sequentially rather
than abruptly, and in a different order, with the loss of branchial paCA apparently occurring prior to the evolution of a large, physiologically relevant Root effect (figs. 1.4, 4.5). While Ernst Haeckel’s (1866) concept of ontogeny recapitulating phylogeny is rejected today (Olsson et al., 2017), data from chapters 3 and 4 of my thesis do support this notion at the broadest level. The hourglass model, proposed by Duboule, (1994) describes the similarities seen between diverse animals at specific developmental stages and suggests that these similarities arise not due to recapitulation of prior adult stages, but conservation of molecular mechanisms (for example: signalling pathways or transcription factors). It is possible to speculate that the highly derived condition in teleosts has itself impacted the development of these characteristics during the early life stages investigated here. The Root effect of adult teleost Hbs is large in magnitude and physiologically relevant in the general circulation (Berenbrink et al., 2005; Verde et al., 2007; Regan & Brauner, 2010). If the principal hypothesis of my thesis is correct, then this large, physiologically relevant Root effect likely acts as a strong selective force to ensure a lack of branchial paCA due to the associated risks to O₂ loading (as discussed in chapter 1). Therefore, in developing rainbow trout, the transition from HbE to HbA, and the concurrent appearance of increased Hb pH sensitivity may represent an inflection point in which branchial paCA goes from being adaptive to highly problematic (jeopardising O₂ uptake across the gills in already hypoxic conditions; fig. 1.1; Randall et al., 2014) and could explain the abrupt switch in branchial paCA availability that I observed in chapter 3 (fig.3.2). To then consider my results for the basal actinopterygians (chapter 4) in this light, I can speculate that because the Hb pH sensitivity slowly increased over time (a pattern that is also reflected in β-NHE activity; fig. 1.4; Berenbrink et al., 2005; Regan & Brauner, 2010), it is logical that there may not be an abrupt inflection point at which the loss of branchial paCA was absolutely necessary but more likely a sequential series of
changes in the various respiratory characteristics involved (figs. 1.4, 4.5). Therefore, the loss of branchial paCA after the divergence of the polypteriformes may be viewed as the point at which branchial paCA was no longer conducive to the changing suite of respiratory characteristics.

5.1.6 A spectrum of respiratory characteristics

In much of the previous literature on this topic, the elasmobranchs (namely dogfishes) and teleosts (namely rainbow trout) are held as examples of divergent respiratory strategies to a common challenge: ensuring adequate respiratory gas exchange during changing environmental conditions. However, recently it has been suggested that this may be inaccurate, and that dogfishes and rainbow trout may instead be more accurately viewed as opposite ends of a respiratory spectrum, and not necessarily representative of the elasmobranch and teleost groups respectively (McMillan et al., 2019). McMillan et al. (2019) showed through analysis of 13 elasmobranch species, that dogfishes may actually represent an extreme of the elasmobranch group, rather than an adequate model. Similarly, rainbow trout have been shown to have exaggerated characteristics compared to many other teleosts (Esbaugh et al., 2004) suggesting that they too may be unlikely to be an adequate representative of the teleostean respiratory pattern. The teleosts comprise half of all vertebrates (Nelson et al., 2006; Helfman et al., 2009, Ravi & Venkatesh, 2018) and exhibit vast variation in many aspects of physiology and so the notion that any one individual species may be representative of a group so large and diverse is unrealistic. Chapter 4 of my thesis adds breadth to this topic by presenting data for several species that appear intermediary between the elasmobranch and teleost respiratory phenotypes (fig. 4.5). By broadening our knowledge across a variety of species, we can start to assess the relevance of extrapolating patterns from a small number of species to whole groups. Adding to our knowledge the respiratory characteristics of
numerous, phylogenetically divergent teleosts, in a similar way to that of McMillan (2018) for elasmobranchs, would be highly worthwhile. While Shu et al. (2022) recently provided evidence for the enhanced $O_2$ unloading system in two non-salmonid teleosts (cobia and mahi mahi), further expansion of this work is greatly needed.

The suggested respiratory spectrum with dogfishes and rainbow trout representing opposite ends may be fundamentally routed in $CO_2$ excretion method and post-branchial acid-base disequilibrium state development. In adult rainbow trout that lack branchial paCA, plasma pH slowly rises in the post-branchial circulation (Gilmour et al., 1994) due to rebalancing of the $HCO_3^-$ buffer system reactions at the slow, uncatalyzed rate. In contrast, dogfish plasma pH decreases in the post branchial circulation (Gilmour, 1998) due to the availability of paCA in the gills of elasmobranchs. The increased portion of $HCO_3^-$ dehydration that occurs in the plasma of dogfish compared to rainbow trout (due to the availability of branchial paCA), coupled with the low buffering capacity of plasma results in relatively greater depletion of protons in this compartment (and causes the disequilibrium in the opposing direction to that seen in rainbow trout). In both groups, this disequilibrium is then re-equilibrated via the Jacobs-Stewart cycle (see: Nikinmaa et al., 1990; Gilmour, 1998) in the post-branchial blood. Hypoxia has been shown to exacerbate disequilibrium states in both species (Gilmour & Perry, 1994), reflecting greater $H^+$ imbalance across the RBC membrane, and highlights the importance of relative RBC and plasma buffer capacities (Gilmour, 1998). The direction of the disequilibrium observed in these species is associated with the presence of pH sensitive Hb in teleosts (large Bohr/Root effect) and absence in elasmobranchs, with the opposite pattern of branchial paCA availability in these species (Gilmour, 1998).
5.1.7 Branchial paCA is likely constrained by something other than the Root effect

In chapter 4, I found that branchial paCA appears to have been lost after the divergence of the polypteriformes (fig. 4.5), which is much earlier than predicted by my principal hypothesis and the previous literature. Despite the persuasive support for my principal hypothesis found in chapter 3 (in pre-hatch rainbow trout, branchial paCA is correlated with the absence of highly pH sensitive Hbs), this result led me to reject the hypothesis that the lack of a large, physiologically relevant Root effect would permit the presence of paCA in all basal groups investigated. In rejecting my principal hypothesis, I am then able to speculate on alternative reasons for the loss of branchial paCA after the divergence of the polypteriformes. When framing our new knowledge within the extensive analysis of Berenbrink et al. (2005), it becomes apparent that the loss of branchial paCA after the divergence of the polypteriformes correlates with decreasing βHb (fig. 1.4). The decreased βHb in teleosts is associated with increased magnitude of the Bohr coefficient (Jensen, 1986; Jensen, 1989) and the transition from pH mediated, to O₂ mediated H⁺ binding (Gilmour, 1998). The large Bohr coefficient observed in teleosts effectively couples O₂/CO₂ transport through the RBC (Nikinmaa, 1997) and results in the reduced effectiveness of Hb as a buffer, ensuring that H⁺ are available for HCO₃⁻ dehydration (Randall & Brauner, 1996; Brauner and Randall, 1998). Reduced βHb may therefore have favoured the loss of branchial paCA due to increased magnitude of the Bohr coefficient. The correlation between reduced βHb and the loss of branchial paCA is also found in various species of teleosts that have secondarily lost the Root effect (including Anguilla, Siluriformes and Monopterus) and possess greater βHb, comparable to that seen in non-teleost species (Berenbrink et al., 2005). If retention of high βHb is linked to retention of branchial paCA in basal actinopterygians, I would expect teleosts that have
secondarily lost the Root effect, and regained βHb capacity to also recover branchial paCA availability.

The increased magnitude of the Bohr coefficient in the teleosts is linked to the evolution of the Root effect exclusively in this group and may provide another explanation for the loss of branchial paCA after the divergence of the polypteriformes (fig. 1.4). The increased magnitude of the Bohr coefficient is believed to have occurred after the divergence of the lobe-finned fishes (Berenbrink et al., 2005; fig. 1.4) and is linked to the reduced βHb in more derived actinopterygian fishes (Berenbrink, 2006). Reduction in the βHb increases efficiency of a given acid load to cause changes in Hb-O2 affinity via the Bohr coefficient (Berenbrink, 2006), especially under hypoxic conditions. Therefore, the evolution of a larger Bohr coefficient, and its concomitant decrease in βHb could have provided the selective drive for the loss of branchial paCA after the divergence of the polypteriformes. In the presence of increasing Bohr coefficient magnitude and reduced βHb, branchial paCA may have been selectively lost to maintain higher RBC pH and protect O2 uptake at the gills. In the presence of branchial paCA (alongside increasing Bohr magnitude and decreasing βHb), disequilibrium state development would likely be small due to the availability of CA to catalyse reactions in both the RBC and the plasma (Gilmour et al., 1997; Gilmour, 1998). Therefore, acid loads would more easily be transferred to the RBC and, due to the increasing magnitude of the Bohr coefficient, this would increase P50 resulting in challenges to O2 uptake (fig. 1.3). In the absence of branchial paCA larger disequilibrium states could develop due to the catalysis of the CO2 hydration reaction only occurring inside the RBC (Perry et al., 1997; Gilmour, 1998). Under such conditions, RBC pH would be higher during gill transit, thereby aiding O2 uptake by Hb with increasing pH sensitivity. I hypothesise that the loss of branchial paCA may have acted as a primitive RBC pH protective mechanism in these basal actinopterygians, which
was beneficial due to the low environmental O\textsubscript{2} concentration during their evolution (fig. 1.1; Randall et al., 2014). This would have allowed them to exploit their increasing Bohr coefficient magnitude at the respiring tissues without risking branchial O\textsubscript{2} uptake. It would be of great interest to conduct extra-corporeal loop experiments (Gilmour et al., 1994; Gilmour et al., 1997; Gilmour, 1998) to investigate post-branchial disequilibrium state development in basal actinopterygians. Such studies would be a useful first step in testing the pertinence of the hypothesis that branchial paCA is constrained by a large Bohr coefficient and the concomitant reduction in \( \beta \text{Hb} \).

If the increasing magnitude of the Bohr coefficient is what constrains branchial paCA availability, then I predict, based on the data from chapter 3 of my thesis, that the Bohr coefficient (\( \Phi \)) at which branchial paCA becomes a detriment to O\textsubscript{2} uptake is around \( \Phi = -0.5 \) (\( \Phi = \Delta \log P_{50}/\Delta \text{pH} \); Bohr, 1904). In chapter 3, developing rainbow trout exhibited the loss of branchial paCA at hatch, which coincided with the increasing magnitude of the Bohr coefficient surpassing -0.5 (fig. 3.4; Bianchini & Wright, 2013). Senegal bichir have been shown to possess a small Bohr coefficient (\( \Phi = -0.43 \); Vokac et al., 1972) whereas white sturgeon and alligator gar possess larger Bohr coefficients (\( \Phi = -0.5 \); Regan & Brauner, 2010) similar to the magnitude shown for hatching rainbow trout (Bianchini & Wright, 2013). Interestingly, a Bohr coefficient of -0.5 also corresponds to the optimal Bohr coefficient for O\textsubscript{2} uptake and delivery, as proposed by Lapennas (1983). While this calculation was based on steady state conditions and demonstrated only to exist in air breathing vertebrates, the hypothesis that branchial paCA is constrained by Bohr coefficient magnitude and its role in O\textsubscript{2} uptake is an intriguing possibility. Senegal bichir are facultative air breathers, which may explain the correlation between their Bohr coefficient and Lapennas’ (1983) proposed optimum, however this does not apply to the developing rainbow trout that uptake O\textsubscript{2} from the surrounding water, predominantly via the skin. Investigating those species that have
retained a large Bohr coefficient, but secondarily lost the Root effect and β-NHE, for example: the Asian swamp eel (Monopterus albus; Φ = -0.79; Damsgaard et al., 2014; Berenbrink et al., 2005) would be an interesting way to test this hypothesis and dissect apart the impacts of the Bohr and Root effects on branchial paCA availability.

5.2 Limitations and future directions

The following sections will explain some limitations of the methodologies used in my thesis and make suggestions for future directions in this field of research.

5.2.1 Development of a diagnostic test for CA-IV

A fundamental challenge of my thesis surrounds the methodologies commonly used to assess paCA availability and their application across a variety of species and life stages. The modified electrometric ΔpH assay, which is commonly used to test for paCA availability (Henry, 1991), is a biochemical assay and as such requires a relatively large volume of tissue (1-2 g). This requirement conferred a significant challenge in chapter 4 of my thesis as Senegal bichir have reduced gills and facultatively utilise a modified swimbladder for environmental O₂ uptake (Magid, 1967), and in chapter 3 due to the small size of developing rainbow trout. In chapter 4, I pooled individuals to achieve tissue weight requirements, however this then resulted in low n numbers (total fish required to achieve n=3 was 91). Unfortunately, in chapter 3 pooling of individuals to achieve sufficient tissue was impossible due to the minute size of the developing fish (10-21 mm total embryo length; Vernier, 1969) and therefore I used qPCR to measure CA-IV mRNA expression and assess developing rainbow trout gills for paCA availability. While the use of molecular methods was effective in chapter 3, and arguably provides greater clarity of
information, it could not easily be applied in chapter 4 due to the requirement to design and validate new primer pairs for each species. An additional challenge surrounding the use of molecular methods is the assumption that mRNA expression implies protein production and activity. In chapter 2, I used biochemical methods to measure paCA activity and confirmed that mRNA expression was correlated to biochemical activity data, providing confidence in my data from chapter 3. Immunohistochemical methods provide an alternate method for such validation which was attempted as part of chapter 2; however, staining proved challenging and I was unfortunately unsuccessful.

Due to the lack of a diagnostic test, it is common in the literature to use a suite of methods to provide evidence for paCA availability (Gilmour et al., 2002; Gilmour et al., 2006; Esbaugh & Tufts, 2004; Georgalis et al., 2006a; Harter et al., 2018a), which I did in chapter 2 (Nelson et al., 2023). Conducting a suite of analyses such as this is both time and resource consuming, and I showed that the probability of any one test matching the result of the entire suite was around 80% (see chapter 2; table 2.2). This consistency is encouraging and supported my decision to utilise only one biochemical test (ultracentrifugal washing) to assess paCA availability in the species investigated in chapter 4.

Despite the techniques I used in my thesis, the lack of a simple diagnostic test for CA-IV is a significant barrier to research, and indeed is somewhat surprising considering the implications of this isoform in human medicine (Supuran, 2011; Swenson, 2014; Nocentini & Supuran, 2019; Provensi et al., 2019). The development of a diagnostic test for CA-IV would vastly improve the ease of these kinds of analyses and undoubtably lead to great advances in our understanding as a result.
5.2.2 Investigating the functional difference between CA-IVa and CA-IVb

An additional complication involved in the development of a diagnostic test for CA-IV, is the fact that many species possess several copies. In chapters 2 and 3 I incorporated molecular methods to distinguish CA-IV availability between two isoforms: CA-IVa and CA-IVb. I used non-specific biochemical methods to measure pCaA activity in microsomal tissue fractions and related this to molecular data for CA-IV isoform specific mRNA expression. Multiple CA-IV isoforms exist in teleosts due to an additional whole genome duplication event, but their retention suggests they may have since evolved divergent roles (Lin et al., 2008; Dichiera et al., 2023). Dichiera et al. (2023) found rainbow trout to possess as least three copies of the CA-IV isoform (CA-IVa, b, and c), however, were only able to measure expression of CA-IVa and CA-IVb in adult fish. In chapter 2 I confirmed the results of Dichiera et al. (2023) by reporting almost complete divergence of CA-IV tissue expression patterns (fig. 2.3); the anterior intestine was the only tissue measured to have significant expression of both CA-IVa and CA-IVb genes. In consensus with what has already been suggested in the literature (Dichiera et al., 2023), I found that CA-IVa was generally expressed in tissues associated with major roles in gas exchange (for example in the heart) and that CA-IVb was generally expressed in tissues associated with major roles in ion, and acid-base balance (for example in the intestines, pyloric caeca, and kidney). While such differential expression data are an important first step in outlining potentially divergent roles for multiple isoforms of a gene, it does not provide any functional evidence. Specific inhibition of CA-IVa and CA-IVb separately would provide great insight into whether these two CA-IV isoforms do indeed have divergent roles. The generation of knockout animals with non-functional CA-IVa or CA-IVb, respectively would be a powerful direction from which to assess this hypothesis. I would predict that if these two isoforms serve divergent functions that are not
achievable by the other version (for example: CA-IVa expressed in the kidney does not lead to the same functional outcome as CA-IVb expression) then these animals would not survive or would be compromised, however if these CA-IV isoforms are similar enough one may be able to be expressed in the absence of the other (for example: CA-IVa is expressed in kidney due to knockout of CA-IVb) with minimal physiological implications. Since the catalytic mechanism of CA is highly conserved across all isoforms (Chegwidden et al., 2000), I would expect that knockout of one CA-IV isoform would lead to increased expression of the other and not present fatal consequences. Under such a scenario, we are left with the question of why we do see divergent tissue expression of different CA-IV isoforms. A second interesting avenue of investigation would be to use immunohistochemical techniques to investigate potential differences in cellular localisation between CA-IVa and CA-IVb. If differences in localisation are exhibited e.g. CA-IVa facing into capillaries and CA-IVb facing into intestinal lumen or interstitial spaces, this may explain the divergent expression patterns. Currently available immunohistochemical probes are not able to distinguish between copies of the CA-IV isoform but the development of such probes would be of great interest to the advancement of this field.

5.2.3 How else do pre-hatch rainbow trout differ from adults?

In chapter 3 I predicted that developing rainbow trout would possess branchial paCA while they express HbE, due the lack of a large, physiologically relevant Root effect. Fig. 3.3 indicates that my prediction was correct and lends support to the hypothesis that branchial paCA is an embryonic trait that is lost in adult teleosts due to the presence of highly pH sensitive Hb (Harter et al., 2018a), as well as the principal hypothesis of my thesis. My data suggest that pre-hatch
rainbow trout use a rather different respiratory strategy compared to their adult counterparts, which is more similar to the elasmobranch than teleost respiratory model (section 5.1.4).

While this study was the first to show that embryonic individuals of a species that possess the teleost suite of characteristics as adults (highly pH sensitive Hb, RBC β-NHE, and a lack of branchial paCA) can possess branchial paCA and lack RBC β-NHE during development, many questions remain unanswered regarding the suite of respiratory characteristics during this time. It is unknown whether pre-hatch rainbow trout possess the high activity RBC CA isoform found in adult animals, or if they have circulating plasma CA activity or PICA. These three characteristics were investigated in basal actinopterygians in chapter 4 and may provide some insight for what we may expect in embryonic rainbow trout. Although ontogeny does not strictly recapitulate phylogeny (section 5.1.5), some parallels can be drawn between my work on developing rainbow trout and basal actinopterygians. In Senegal bichir I found branchial paCA, low activity RBC CA, almost no plasma CA activity, and potent PICA, whereas in adult rainbow trout I found no evidence for branchial paCA, high activity RBC CA, no plasma CA activity and highly potent PICA (fig. 4.5). In addition, Berenbrink et al. (2005) showed that β-NHE is not present in the polypteriformes, but activity is known to be high in many teleosts (Berenbrink et al., 2005; Shu et al., 2022). It is possible to speculate that pre-hatch rainbow trout, along with possession of branchial paCA and no β-NHE activity would likely possess no plasma CA activity and have potent PICA. This speculation is based on collections of characteristics observed in the basal actinopterygians I investigated in chapter 4 and does not take into account any potential constraints on embryonic rainbow trout characteristics resulting from the highly derived condition they exhibit as adults.
It is more difficult to speculate on whether pre-hatch rainbow trout would possess a high or low activity RBC CA isoform. The high activity RBC CA found in adult rainbow trout appears to be specific to teleosts (all non-teleost species investigated in chapter 4 possessed low activity RBC CA; fig. 4.5). Dichiera et al. (2020) suggested that the presence of branchial paCA may relieve the requirement for high activity RBC CA in basal ray-finned fishes; however, I showed in chapter 4 that both alligator gar and white sturgeon possess low activity RBC CA and lack branchial paCA, suggesting that this hypothesis may be incorrect. The logistics of executing a study to investigate these characteristics would be difficult due to the reasons explored above (section 5.2.1). The minute size of these developing animals makes blood collection extremely challenging, and although others have had success with a microhematocrit method (Bianchini & Wright, 2013), I was unable to achieve successful blood collection. Measurement of CA activity in RBCs and plasma, as well as PICA validation requires relatively large volumes of sample, compared to the size of these animals and so pooling of samples from many animals would be required, as well as modification of the ΔpH assay used for measuring CA activity (Henry, 1991). Despite the challenges associated with conducting such a study, analysing these respiratory characteristics through development in rainbow trout would be of great interest to see how their suite of respiratory characteristics compares to those found in adult fishes as well as basal actinopterygians.

5.2.4 Divergent life histories

One challenge in assessing the relevance of my data for basal actinopterygians from chapter 4 of my thesis concerns life history traits. Of the three species investigated, two are water breathers (alligator gar and white sturgeon) and the other is a facultative air breather (Senegal bichir). In
vertebrates generally, respiratory medium has a large impact on various respiratory characteristics, including blood CO₂ content, regulation of breathing, post-branchial acid-base disequilibria, hematocrit, and hemoglobin O₂ affinity (Morris & Bridges, 1994; Smatresk, 1994; Gilmour, 1998; Bayley et al., 2018). Differences in life history characteristics may be important in determining the availability of branchial paCA, which is a potentially confounding factor of my work in chapter 4. The fact that the availability of branchial paCA was only detected in Senegal bichir, which is a facultative air breather, may be noteworthy but does not denote causation. Broad analysis of further species of both air and water breathers from across the piscine phylogeny would be informative to assess if any link between breathing mode and branchial paCA availability exists. Indeed, if such a link was found I may speculate that branchial paCA is primarily involved in ion or acid-base balance due to the primary role for the gills in air breathing fishes (Cameron & Wood, 1978; Gilmour et al., 2007; Bayley et al., 2018). To this end, it would be useful to conduct molecular analyses (as used in chapters 2 and 3) to investigate if branchial paCA activity in Senegal bichir can be distinguished between different isoforms expressed.

There are several other life history characteristics that may be interesting in the assessment of branchial paCA availability. In developing rainbow trout, branchial paCA may be essential to gas exchange in the hypoxic egg (chapter 3). It is conceivable that reverting to the condition observed in pre-hatch rainbow trout (branchial paCA availability) may be beneficial to species that inhabit hypoxic conditions for example: winter frozen lakes (Nilsson & Renshaw, 2004) or hydrothermal vents (Weber et al., 2003). Additionally, in adult salmonids, it appears that paCA at the respiring tissues enables them to cope with such strenuous migrations (Harter et al., 2019); other species that have particularly active life histories (for example, tuna; Lowe et al., 1998) may also benefit greatly from this enhanced O₂ unloading. Species that exhibit extreme life history
traits such as those mentioned above may be interesting candidates for further study. It is evident from the data in my thesis that broader investigation is required to assess the potential links between paCA localisation and various life history traits.

5.2.5 **Secondary losses of the Root effect**

Another area that represents an interesting avenue for future research would be to study those species that have secondarily lost the Root effect and/or β-NHE. Five teleostean groups have been identified as having independently, secondarily lost the large, physiologically relevant Root effect characteristic of the teleosts; *Gnathonemus, Anguilla, Misgurnus* and *Pangio, Silurus* and *Pelteobagrus,* and *Monopterus* (Berenbrink et al., 2005). In addition, β-NHE has been lost four times independently in the same groups (except *Gnathonemus*; Berenbrink et al., 2005) with a fifth loss reported in the sablefish (Rummer et al., 2010). If the principal hypothesis of my thesis is correct then I would expect branchial paCA to be available in these groups, in the absence of a large, physiologically relevant Root effect and RBC pHi protection. Based on the results of chapter 4 of my thesis, I predict that this may be unlikely. In chapter 4 I found that alligator gar and white sturgeon lacked branchial paCA (fig. 4.5), despite also lacking a large, physiologically relevant Root effect (Regan & Brauner, 2010) and β-NHE (Berenbrink et al., 2005). Based on the pattern of branchial paCA shown across the basal actinopterygians I investigated in chapter 4, I hypothesised that the loss of branchial paCA in teleosts may be correlated with the drop in βHb associated with the increased Bohr effect magnitude. It has been shown that several of these groups that have secondarily lost the Root effect and β-NHE have also regained increased βHb (Berenbrink et al., 2005) and so the prediction that they will possess branchial paCA may still hold (section 5.1.7). These groups would be of interest for future study, irrespective of the status of my
principal hypothesis, as they represent examples of teleosts reverting to respiratory strategies reminiscent of more basal groups.

5.2.6 paCA knockout in teleosts, and in elasmobranchs

Molecular methods are an important approach in studies of animal physiology and give us tools with which we may ask questions that would otherwise be impossible to investigate using more traditional, biochemical based methodologies. One avenue where the use of molecular methods would be particularly exciting would be in creating paCA knockout animals. It would be of great interest to know if paCA knockout fishes can survive routine conditions, or if paCA knockout only affects survival during extreme environmental exposures (for example, hypoxia, hypercapnia, exhaustive exercise, thermal extremes). A paCA knockout of rainbow trout would be a great tool for studying the importance of the heterogeneous distribution of paCA in the teleosts, while paCA knockout elasmobranchs would allow us to assess the magnitude of the role of paCA in CO$_2$ excretion in this group more accurately. Work by Harter et al. (2019) and Carless & Brauner (unpublished observation) suggest that paCA isoforms are critical to maintaining swimming ability and hypoxia tolerance even under relatively mild exposures to stress in salmonids. It is therefore possible to predict that an animal with complete paCA knockout would exhibit compromised function, even under routine conditions. A strain of CA-IV knockout mice have been created and shown to be viable with no obvious mutant phenotype (Waheed & Sly, 2013). Although mice do not employ the system of enhanced O$_2$ unloading seen in teleosts, this work suggests that other CA isoforms are able to act as substitutes. Indeed, due to the many roles CA isoforms play (Chegwidden et al., 2000), such knockout animals would likely also experience disturbances to
acid-base or ion regulation. The multiple roles of CA is a pervasive caveat of all work involving inhibition of CA isoforms and is a vast area deserving of further research.

Chemical inhibition of paCA isoforms has been used in various studies to assess the role of paCA under exhaustive exercise (Harter et al., 2019) and hypoxia (Carless & Brauner, unpublished observation), however this inhibition is temporary; C18 (paCA inhibitor used in the aforementioned studies) can cross cell membranes after approximately 60 minutes (Supuran, 2008b; Rummer et al., 2013; Harter et al., 2019), therefore limiting the analyses that can be conducted. Additionally, due to the method of delivery (injection of C18 into the bloodstream), it is impossible to selectively inhibit certain tissues. The relative mRNA expression of CA-IV in rainbow trout tissues (fig. 2.3) suggests different tissues may rely on paCA to differing extents; expression in the heart was significantly higher than expression in any other tissue investigated. This indicates that knocking out paCA may have a greater effect in some tissues than others. It is believed that paCA in the heart may be essential for gas exchange during stressful conditions (Alderman et al., 2016). A tissue with presumed high reliance on the teleost system of enhanced O₂ unloading would be a particularly interesting place to target such knockout experiments. The ability to knockout paCA from just one tissue, for example in the heart of rainbow trout, or the gills of dogfish, would enable assessment of the importance of paCA expression in the given tissue and would be possible using isolated preparation methods similar to those used by Alderman et al. (2016). Whilst studies using isolated tissues may be a useful place to start, they cannot inform us on the whole animal implications of such treatments.
5.3 Conclusion

In my thesis I set out to test the principal hypothesis that hemoglobins with a large, physiologically relevant Root effect have imposed constraints on branchial paCA availability in teleost fishes (fig. 5.1). I sought to do this via three independent observational studies that each add important basic knowledge to our understanding of a respiratory system that is present in half of all vertebrates (Nelson et al., 2006; Helfman et al., 2009; Ravi & Venkatesh, 2018). In chapter 2 I confirmed the long-held assumption that paCA is absent from the branchial vasculature of all four gill arches in a model teleost, the rainbow trout (table 2.2, figs. 2.4, 5.1) but present in most other tissues (except the stomach). In chapters 3 and 4 I used natural examples of the absence of the Root effect to see if paCA would be present under such conditions. I focussed on developing rainbow trout in chapter 3 and found that pre-hatch fish did possess branchial CA-IV, and lack RBC β-NHE, suggesting these pre-hatch animals utilise a respiratory strategy more similar to elasmobranchs than to their adult counterparts (figs. 3.4, 5.1), and reminiscent of the ancestral vertebrate condition. Phylogenetic analysis comprised chapter 4 of my thesis where I found that paCA disappears much earlier than predicted in the basal actinopterygians and appears to be correlated with the increasing magnitude of the Bohr coefficient and concomitantly decreasing βHb (figs. 1.4, 4.5, 5.1). I also found that PICA may be more widespread than previously thought (Henry et al., 1997), and that the high activity RBC CA found in rainbow trout is likely confined to teleosts and possibly even salmonids (figs. 4.5, 5.1). The findings of these studies add important knowledge to our understanding of how the unique teleost oxygenation system functions in different tissues (chapter 2), develops through ontogeny (chapter 3), and has evolved through basal actinopterygian groups (chapter 4). There are various challenges and limitations to this work, which I have endeavoured to explore in the
preceding sections of chapter 5. Most importantly to any scientific venture, the studies comprising my thesis have raised many additional questions and provide ample stimulating avenues for future research. I am excited to see where the future will lead this field of animal physiology.
**Figure 5.1: Graphical summary of the findings of my thesis.** The top panel illustrates the findings of chapter 2 investigating the distribution of plasma accessible carbonic anhydrase (paCA) in 14 tissues in adult rainbow trout. Tissues in grey-scale boxes on upper row do not have paCA and those in red and blue boxes in lower row showed evidence for CA-IVa and CA-IVb respectively (two isoforms of the major plasma accessible CA: CA-IV). The middle panel illustrates the findings of chapter 3 investigating the system of enhanced oxygen unloading found in some teleosts through development in rainbow trout. Left group of fish/characteristics are pre-hatch (indicated by egg below) and right group of fish/characteristics are post-hatch. From left to right characteristics are as follows: large Bohr/Root effect, red blood cell (RBC) intracellular pH (pHi) protection by way of a beta adrenergically stimulated sodium proton exchanger (β-NHE), and presence of paCA. Grey scale diagrams indicate an absence of the given characteristic, and full colour diagrams indicate the presence of the given characteristic. The bottom panel illustrates the findings of chapter 4 investigating various CA characteristics in basal actinopterygians. Fish silhouettes on left hand side are ordered from most basal (lowest) to most derived (highest). The presence of a box in each column to the right of the silhouettes indicates the presence of that characteristic in the species on that row. Coloured outline boxes indicate data comes from my thesis and black outlined boxes indicate data comes from the previous literature and is included for clarity (exception: all elasmobranch data sourced from the literature; McMillan et al., 2019). From left to right the characteristics are as follows: blue: free circulating plasma CA, purple: branchial paCA, green: plasma inhibitors of CA (PICA), black: large Bohr effect, black: low hemoglobin buffer capacity (βHb), black: large Root effect, black: choroid rete mirabile, black: RBC β-NHE, red: high activity RBC CA.
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Kieffer, J. D., Rossiter, A. M., Kieffer, C. M., Davidson, K., & Tufts, B. L. (2002). Physiology and survival of Atlantic salmon following exhaustive exercise in hard and softer water:


Nikinmaa, M. (1982). Effects of adrenaline on red cell volume and concentration gradient of


Rombough, P. J. (2002). Gills are needed for ionoregulation before they are needed for O$_2$ uptake in developing zebrafish, *Danio rerio*. *Journal of Experimental Biology* **205**, 1787-1794.


Appendix

Table A1: Sample sizes for all assays conducted as part of chapters 2, 3, and 4.

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Table A2.1: Raw microsomal carbonic anhydrase activity values and washed microsome activity presented as a percentage of unwashed microsome activity, for all 14 tissues analysed.

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<th>% of unwashed microsomal activity</th>
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Figure A2.1: Validation of the presence of plasma inhibitors of carbonic anhydrase (PICA) in rainbow trout plasma. A) PICA presence was confirmed by measurement of CA activity in RBC lysates (black), plasma (blue), and RBC lysates + plasma (PICA; orange) to determine B) the inhibitory effect (%) of PICA on cytoplasmic CA isoforms.
Table A3.1: mRNA expression and respective sampling data collected and analysed as part of chapter 3 and displayed in figures 3.2 and 3.3. dpf: days post fertilisation, n: sample size, stage: developmental stage (Vernier, 1969), STD: standard deviation, SEM: standard error of the mean, mean LN expression: mean natural log transformed expression data used for statistical analyses. Mean relative expression was standardised to CA-IVa stage 27 for both CA-IVa and CA-IVb, and to β-NHE adult for β-NHE.

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Figure A3.1: Comparison of ct values for reference gene (b-actin) used in chapter 3. ANOVA was used to compare ct values between stages. No significant differences were detected ($p = 0.6$) and so b-actin was deemed to be an acceptable reference gene for this study.
Figure A3.2: Comparison of two adult groups (20 g and 100 g fish) for A - CA-IVa, B - CA-IVb, and C - β-NHE analysed as part of chapter 3. No significant differences were detected between the two groups for any of the three genes (p > 0.05).
Table A4.1: Raw microsomal carbonic anhydrase activity values and washed microsome activity presented as a percentage of unwashed microsome activity, for three basal actinopterygian species analysed (alligator gar, white sturgeon, Senegal bichir) as well as rainbow trout and blacktip reef shark as representatives of the teleosts and the elasmobranchs respectively.

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