THE FUNCTIONAL SIGNIFICANCE OF PLASMA-ACCESSIBLE CARBONIC ANHYDRASE FOR CARDIOVASCULAR OXYGEN TRANSPORT IN TELEOSTS

by

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Abstract

A novel mechanism has recently been discovered in rainbow trout that allows these fish to enhance the partial pressure of O_2 (PO_2) in their muscles. Teleosts have evolved highly pH-sensitive haemoglobins (Hb), where an arterial-venous pH shift (ΔpH_{a-v}) can severely reduce Hb-O_2 binding affinity. Most teleosts create large ΔpH_{a-v} by actively regulating the intracellular (pH_i) of their red blood cells (RBC) through adrenergically stimulated sodium-proton exchangers (β-NHE). This creates H^+ gradients across the RBC membrane that are short-circuited in the presence of plasma-accessible carbonic anhydrase (paCA) at the tissues, to greatly enhance O_2 unloading from pH-sensitive Hb. Thus, I hypothesised that teleosts increase the O_2 capacitance of their blood (β_b) by a mechanism of active RBC pH_i regulation that is modulated through a heterogeneous distribution of paCA, which has functional significance for O_2 transport *in vivo*. Mechanistically, I discovered that in rainbow trout, the time-course of β-NHE short-circuiting in the capillaries and the recovery of RBC pH_i during venous transit, are consistent with a system that can enhance O_2 unloading at the tissues with every pass through the circulation. Functionally, I discovered that the inhibition of paCA in Atlantic salmon swimming at a low speed or at rest, required a compensatory increase in cardiac output of ~30%, corroborating a role of paCA in O_2 transport over a broad range of conditions. Further, I discovered paCA in the heart lumen of coho salmon; thus, also cardiac O_2 supply in salmonids may rely on β-NHE short-circuiting. In teleosts, the evolution of β-NHE short-circuiting required the loss of paCA at the gills. However, in Antarctic icefish, I propose that the loss of Hb and RBCs released the functional constraint on the expression of paCA at the gills, and allowed for the enzyme to catalyse CO_2 excretion in the absence of RBC CA. Collectively, my findings indicate that an active mechanism at the level of the RBC enhances β_b and is an integral part of the salmonid mode of cardiovascular O_2 transport, and perhaps most teleosts, with important implications for the physiology, the conservation and the evolutionary history, of nearly half of all vertebrates.

Lay Summary

Teleost fish inhabit virtually every aquatic habitat and may owe this success in part to their exceptional mode of oxygen transport in the blood. By actively regulating the pH of their red blood cells, teleosts can control the binding of oxygen to their pH-sensitive haemoglobin, and the enzyme carbonic anhydrase (CA) promotes the unloading of oxygen at the tissues. I discovered that changes in red blood cell pH are fast enough to ensure oxygen uptake at the gills and to enhance its release at the tissues. By this mechanism, the hearts of Atlantic salmon need to pump less blood when the animals are swimming and this may be important in enabling successful spawning migrations of all salmon species, especially in the face of increasing river temperatures. Further, this system may be found in all teleosts, which would profoundly change our understanding of oxygen transport in nearly half of all vertebrates.

Preface

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Animal husbandry and all experiments were conducted according to the guidelines of the Canadian Council on Animal Care and approved by the UBC Animal Care Committee (Protocol no. A15-0266).

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List of Symbols and Abbreviations

AE	anion exchanger or Cl^-/HCO_3^- exchanger
AIC	Akaike information criterion
APS	aminopropylsilane
ATP	adenosine triphosphate
Az	acetazolamide
bpm	beats per minute
C18	positively charged, membrane-impermeable sulfonamide inhibitor of carbonic anhydrase
CA	carbonic anhydrase
Ca^{2+}	calcium
C_aO_2	oxygen content of the arterial blood
CDS	coding sequences
Cl^-	chloride
CO_2	carbon dioxide
CT_{max}	critical thermal maximum
C_vO_2	oxygen content of the venous blood
DAPI	4′,6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
EIPA	ethyl isopropyl amiloride
f_h	heart rate
Fulton's K	measure of condition factor
GPI	glycophosphatidylinositol
H^+	proton
Hb	haemoglobin
Hb P_{50}	partial pressure of oxygen at which haemoglobin is half-saturated
HCO_3^-	bicarbonate
Hct	haematocrit
HRP	horseradish peroxidase
I.U.	international enzyme unit
ISO	isoproterenol

K^+	potassium
k_{cat}	turnover number
k_i	inhibition constant
MCHC	mean cell haemoglobin concentration
$\dot{M}O_2$	rate of oxygen consumption
$\dot{M}O_{2max}$	maximal rate of oxygen consumption
MYA	million years ago
Na^+	sodium
n_H	Hill coefficient
NKA	sodium-potassium ATPase
O_2	oxygen
OCLTT	oxygen and capacity limited thermal tolerance
OEC	oxygen equilibrium curve
paCA	plasma-accessible carbonic anhydrase
P_aO_2	arterial partial pressure of oxygen
PBS	phosphate buffered saline
pH	$-\log[H^+]$
pH_e	extracellular pH
pH_i	intracellular pH
PI-PLC	phosphatidylinositol-specific phospholipase C
pK_a	apparent dissociation constant
PO_2	partial pressure of oxygen
P_vO_2	venous partial pressure of oxygen
$\dot{Q}$	cardiac output
RBC	red blood cell
RQ	respiratory quotient
RVM	relative ventricular mass
s.e.m.	standard error of the mean
S_aO_2	arterial haemoglobin-oxygen saturation
$S_{a\text{-}v}O_2$	difference between arterial and venous haemoglobin-oxygen saturation that represents tissue oxygen extraction

SDS	sodium dodecyl sulfate
SO_2	haemoglobin oxygen-saturation
S_vO_2	venous haemoglobin-oxygen saturation
$t_{1/2}$	halftime
TTBS	Tris buffered saline with 0.05% Tween 20
U_{crit}	critical swimming speed
V_s	stroke volume of the heart
β_b	capacitance of the blood for oxygen
β-NHE	β-adrenergically stimulated sodium-proton exchanger
β_{plasma}	plasma non-bicarbonate buffer capacity
ΔpH_{a-v}	arterial-venous pH difference
ΔPO_{2max}	magnitude of the change in the partial pressure of oxygen
ΔZ_H	fixed-acid Haldane effect
Φ	Bohr coefficient

Glossary

anadromous	adult stages live in the ocean but spawn in freshwater
anion ratio	ratio of intracellular over extracellular anions (A_i/A_o)
Bohr effect	reduction of haemoglobin oxygen-binding affinity due to a reduction in pH (reciprocal: Haldane-effect)
capacitance of the blood for oxygen (β_b)	Defined as: the change in blood oxygen content over the change in partial pressure of oxygen; includes both physical solubility and oxygen bound to respiratory pigments.
cardiac work	product of heart rate, stroke volume and ventral aortic blood pressure (typically mJ g^{-1}), whereas power output (mW g^{-1})
Donnan-like equilibrium	distribution of ions across a semi-permeable membrane, where at least one ion is not permeable through the membrane (also Gibbs-Donnan equilibrium)
erythropoiesis	process of new red blood cell formation
hypercapnia	elevated carbon dioxide concentration in the blood
hypercarbia	elevated environmental carbon dioxide concentration
$\dot{M}O_2$	rate of oxygen consumption, typically expressed as molar quantity; throughout this dissertation $\dot{M}O_2$ is expressed as mg O_2 kg^{-1} h^{-1}, for consistency with relevant previous studies
neoteny	retention of embryonic characters
oxygen carrying capacity	maximal oxygen content of the blood under physiological conditions
polycythemia	condition of elevated haematocrit
Root effect	reduction of absolute maximal haemoglobin oxygen-carrying capacity due to a reduction in pH
semelparous	reproductive strategy with a single spawning before death

Throughout this dissertation, carbonic anhydrase (CA) isoforms are described according to the ZFIN zebrafish gene nomenclature conventions, where:

ca4a	carbonic anhydrase isoform 4 gene, duplicate copy “a”
Ca4	fish carbonic anhydrase isoform 4 protein
CA4	mammalian carbonic anhydrase isoform 4 protein
Ca2-like	Ca2 homologous to mammalian, but orthology is ambiguous

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To my family
and especially Laura.

Chapter 1: General Introduction

Teleost fishes are the most diverse taxon within the vertebrates and, at over 30,000 named species (Nelson et al., 2006) they comprise 96% of all extant fishes and half of all vertebrate species. This astounding species diversity is reflected in the largest variation in body plans, trophic levels, reproductive- and feeding ecologies among fishes (Helfman et al., 2009). Teleosts occupy virtually every aquatic habitat on earth: from the frigid Southern Ocean, at -1.9°C (Eastman, 1993), to African lakes at over 43°C (Wood et al., 2016); from oceanic trenches at depths of 8000 m (Gerringer et al., 2017), to high-altitude freshwater lakes (Wood et al., 2007); from the ion-poor waters of the Amazon river (Val and Almeida-Val, 1995), to hypersaline desert lakes (Sardella et al., 2004); from seasonally anoxic ponds (Vornanen et al., 2009) to hyperoxic tide pools (Richards, 2011); and from lakes at pH 10 to Amazonian tributaries at pH 4.8 (Gonzalez et al., 2017). Teleosts are, without a doubt, the most successful aquatic vertebrates today.

Modern teleosts are the result of four major radiations that produced the Osteoglossomorpha, Elopomorpha, Otocephala and Euteleostei, the last being by far the most diverse. These radiations date back to the early- to mid-Triassic (200-250 MYA; Hughes et al., 2018) and involved major morphological changes to bones in the jaws and the tails of teleosts, the positions of the major fins, and a decrease in external armour; innovations that allowed a greater diversity in locomotion and feeding strategies (Helfman et al., 2009). In addition, it has been argued that a greater hypoxia tolerance was key to the success of teleosts during the Permian crisis (Randall et al., 2014), when atmospheric oxygen (O_2) levels decreased dramatically and remained low for another 100 million years (Clack, 2007). The Permian crisis led to the loss of 90% of all marine fish species; however, teleosts experienced an explosive adaptive radiation, perhaps the most extensive one in vertebrate evolution (Helfman et al., 2009). Not surprisingly, the unique adaptations that enable cardiovascular O_2 transport in teleosts are credited, in part, for this success; and during this transition, the physiological innovation of highly pH-sensitive haemoglobin (Hb) changed fundamentally the mode of O_2 transport in teleosts (Berenbrink et al., 2005). It set the stage for the evolution of specialised structures that exploit this pH sensitivity to generate staggering partial pressures of O_2 (PO_2) that allowed the swimbladder to be filled at depth and to supply O_2 to the avascular retina of the eyes (Wittenberg and Wittenberg, 1974);

innovations that enabled the life-styles, habitats and morphologies of modern teleost species. Recently, a novel mechanism of Hb-O_2 unloading was described in rainbow trout (*Oncorhynchus mykiss*) that likewise hinges on the pH sensitivity of Hb and that augments PO_2 in the fish muscle (Rummer and Brauner, 2011; Rummer et al., 2013). The present dissertation explores the idea that the pH sensitivity of Hb is a fundamental aspect of the teleost mode of O_2 transport, that it enhances Hb-O_2 unloading in non-specialised tissues, such as the heart and muscle, and that it is critical to enable the exercise performances of modern teleosts.

Salmonidae (within Salmoniformes) are a subfamily of Protacanthopterygii, a moderately derived clade within the Euteleostei, and comprise seven Eurasian and North American genera of significant commercial value (in the interest of readability taxonomic names will henceforth be given in their anglicised form; e.g., salmonids and teleosts). The actual number of species, and generally, the classification of the numerous salmonid subspecies and populations, has been a matter of debate, even for such important distinctions as the genera of Pacific and Atlantic salmon and trout, or the naming of the ubiquitous rainbow trout (Smith and Stearley, 1989). Salmonids have a long history in the research of exercise physiology in fishes and rainbow trout is perhaps the best-studied "lower" vertebrate in this respect. Many representatives of the salmonids display remarkable exercise performances in temperate waters. Some Pacific and Atlantic salmon species migrate thousands of kilometers during their oceanic life-stages (Hartt et al., 1966; Royce et al., 1968) and perform strenuous up-river migrations to their spawning grounds (Cooke et al., 2006). Due to their anadromous, and in some cases, semelparous life-cycles, where animals have a single opportunity to procreate in freshwater, selective pressures in salmonids may act directly on physiological mechanisms that enhance exercise performance (Eliason et al., 2011; Eliason et al., 2013). Therefore, salmonids are a well-studied group among the teleosts, where reproductive success is closely tied to their ability to increase cardiovascular O_2 transport. Thus, salmonids represent an excellent model to explore the specialised adaptations of cardiovascular O_2 transport in teleosts that likely shaped the evolution of the clade as a whole.

The following chapters of the introduction will review the characteristics of cardiovascular O_2 transport in fishes (Chapter 1.1), and discuss those properties of Hb that

determine the capacity to transport O_2 in the blood (Chapters 1.2 and 1.3). Teleosts have evolved highly pH sensitive Hbs that allow O_2 transport to be modulated *in vivo* by adjustments to the RBC intracellular environment. Specialised vascular structures in teleosts exploit the high pH sensitivity of Hb and enhance Hb-O_2 unloading to the eyes and the swimbladder through mechanisms that have been largely resolved (Chapter 1.4). Central to my dissertation, however, is a novel, largely uncharacterised mechanism that may extend the benefits of enhanced Hb-O_2 unloading to all tissues in salmonids through a mechanism of active red blood cell (RBC) intracellular pH (pH_i) regulation and a heterogeneous distribution of plasma-accessible carbonic anhydrase (paCA) in the vasculature (Chapter 1.5). Building on this recent discovery, the main objectives of this dissertation were to increase our mechanistic understanding of this novel mechanism, and to generate *in vivo* evidence that enhanced Hb-O_2 unloading is critical for sustaining exercise performance in salmonids (Chapter 1.6).

1.1 Cardiovascular oxygen transport

Vertebrate life has evolved a strong reliance on O_2, which functions as the final electron acceptor in oxidative phosphorylation, the principal pathway that generates ATP in the mitochondria. This preference of aerobic over anaerobic ATP synthesis may be rooted in yields that are ~18-fold higher for oxidative phosphorylation compared to substrate-level phosphorylation, which occurs in glycolysis (Hochachka and Somero, 2002). The consumption of O_2 in the mitochondria requires that O_2 is continuously supplied to the tissues. A shortage of O_2 at the tissues through hypoxemia will limit ATP production *via* oxidative phosphorylation, forcing the cells to recruit the less efficient anaerobic pathways in order to prevent a mismatch between ATP supply and demand. Therefore, mechanisms of O_2 transport in vertebrates must respond rapidly to changes in the rate of O_2 consumption at the tissues ($\dot{M}O_2$). With the exception of some embryonic and larval stages, where surface-area to volume ratios are such that O_2 can be supplied entirely by diffusion through the skin, vertebrates rely on their cardiovascular systems to deliver O_2 to the tissues. Thus, O_2 is taken up from the environment at the gas exchange surface, *via* diffusion down a PO_2 gradient into the blood, followed by convective transport in the circulatory system that is actively driven by the heart, and finally diffusion from the capillaries to the mitochondria (see e.g., Dejours,

1981). The convective step in this O_2 transport cascade is described by the Fick equation (Fick, 1870):

$$1)\ \dot{M}O_2 = \dot{Q} \times (C_aO_2 - C_vO_2)$$

where $\dot{Q}$ is cardiac output, and C_aO_2 and C_vO_2 are the contents of O_2 in the arterial and venous blood, respectively. The limitations of the Fick equation to model whole animal $\dot{M}O_2$ are its neglect of cutaneous respiration (Farrell et al., 2014) and the O_2 consumption of the gills (Daxboeck et al., 1982), largely for osmoregulation and acid-base regulation, which under certain conditions and for some species may be important contributors to $\dot{M}O_2$ (Farrell et al., 2014). An increase in $\dot{M}O_2$ during exercise in teleosts is supported, in about equal parts, by increases in $\dot{Q}$ and (C_aO_2-C_vO_2) that describes O_2 extraction at the tissues (Stevens and Randall, 1967; Kiceniuk and Jones, 1977; Brauner et al., 2000a).

Adjustments to $\dot{Q}$ are typically a combination of changes in heart rate (f_h) and stroke volume (V_s). However, adjustments to V_s predominate during exercise in fish, whereas adjustments to f_h modulate $\dot{Q}$ in response to temperature changes (Farrell and Jones, 1992; Farrell and Smith, 2017). Most fish species can increase $\dot{Q}$ by 2-3-fold during exercise or elevated temperature, and this limitation will, in part, determine their ability to compensate for the increase in $\dot{M}O_2$ under these conditions (Farrell and Smith, 2017). Blood flow is a function of pressure differences, generated by the heart, and resistance to flow, generated by the vasculature; in fish this is the pressure difference between the ventral aorta and the central veins and the serial resistances of the gill and the systemic vascular beds (Sandblom and Gräns, 2017). Control of blood flow is largely accomplished by changes in vascular resistance, and even small changes in the diameter of resistance vessels, typically arterioles, has a great effect on vascular resistance and blood flow (described by Poiseuille's law). Resistance vessels respond rapidly to local changes in $\dot{M}O_2$, through active hyperaemia, autocrine and paracrine pathways, signalling from the autonomous nervous system, and several vasoactive hormones (Sandblom and Gräns, 2017). The recruitment of additional capillaries in the tissues is a principal mechanism by which vertebrates increase O_2 extraction from the blood (Krogh, 1919a, b; Egginton, 2002). The result is a larger surface area for gas exchange, a shorter diffusion distance between capillaries and mitochondria (Weibel et al.,

1992) and a longer capillary transit time; typically a major limitation to O_2 extraction in exercising humans (Saltin, 1985). However, an increase in the cross-sectional area of the capillary beds reduces systemic vascular resistance (assuming a constant tortuosity), which may result in a decrease in arterial blood pressure. Mean arterial pressure in fish is controlled centrally within narrow limits, and the input comes from baroreceptors located in the gills (Wood and Shelton, 1980; Bushnell et al., 1992). Thus, an increase in capillary recruitment is typically matched by an increase in $\dot{Q}$ to maintain mean arterial pressure (Jung et al., 1999; Behnke et al., 2001; Sandblom and Gräns, 2017). Fish, like other vertebrates, cannot increase $\dot{Q}$ sufficiently to maximally perfuse all vascular beds simultaneously and thus, at values of $\dot{Q}$ that approach maximal levels, tissues must necessarily compete for the available blood flow (Randall and Daxboeck, 1982; Thorarensen et al., 1993; Thorarensen, 1994; Farrell et al., 2001).

The capacity to increase arterial O_2 transport ($\dot{Q}\times C_aO_2$) is generally a good predictor of maximal $\dot{M}O_2$ in fish (Gallaugher et al., 2001). According to Henry's law, C_aO_2 is a function of the capacitance of blood for O_2 (β_b) and the arterial partial pressure of O_2 (P_aO_2):

$$2)\ C_aO_2 = \beta_b \times P_aO_2$$

where β_b is defined as the increment in C_aO_2 per increment in P_aO_2, and includes both the physically dissolved O_2 as well as O_2 bound to respiratory pigments (Piiper et al., 1971):

$$3)\ \beta_b = \frac{\Delta C_aO_2}{\Delta P_aO_2}$$

Therefore, convective O_2 transport in the cardiovascular system can be expressed as:

$$4)\ \dot{M}O_2 = \dot{Q} \times \beta_b(P_aO_2 - P_vO_2)$$

The latter equation is central to this dissertation, as it illustrates that animals can balance O_2 supply and demand using different strategies. Changes in $\dot{Q}$ are a principal mechanism to support a higher $\dot{M}O_2$ in all vertebrates, and the response, as well as its

limitations, are well-characterised in fish (Randall and Daxboeck, 1984; Butler and Metcalfe, 1989; Farrell, 1992; Farrell and Jones, 1992; Fritsche and Nilsson, 1993; Farrell, 2007; Gamperl and Driedzic, 2009; Farrell and Smith, 2017). Adjustments to β_b are another, effective means, to modulate cardiovascular O_2 transport, and can serve to alleviate the requirements on $\dot{Q}$ proportionally. Clearly, the potential of animals to modulate β_b is intimately associated with the PO_2 of the arterial and venous blood, an aspect that will be treated in some detail. The following sections will discuss the principal mechanisms by which vertebrates can regulate β_b *in vivo* through changes in: i) O_2 carrying capacity; ii) O_2 extraction at the tissues, and iii) pH-sensitive Hb-O_2 binding. The last aspect in particular, represents a central mechanism by which teleosts modulate β_b *in vivo* that is enabled by the exceptional pH sensitivity of their Hb.

1.2 O_2 carrying capacity

Haemoglobin is the principal O_2 carrier in the blood and greatly elevates the O_2 content at physiological PO_2, and therefore increases β_b. In addition, Hb provides a large sink for O_2 within the animal that allows O_2 uptake from the water against its concentration gradient. This is possible because Hb maintains large PO_2 gradients between the venous blood and the water, which allows for an efficient diffusional uptake of O_2 within the short residence time that blood has in the gill lamellae (about 1-3 s; Cameron and Polhemus, 1974; Booth, 1978; Hughes et al., 1981; Randall, 1982b). The content of physically dissolved O_2 in the plasma is typically of minor importance (Wood et al., 1979a, b), however, its contribution increases at lower Hb concentrations, lower temperatures and higher PO_2. For example, in Hb-less icefishes that live at sub-zero temperatures, O_2 transport in the cardiovascular system relies entirely on physically dissolved O_2 in the plasma (Ruud, 1954), which is somewhat facilitated by the higher solubility of O_2 in plasma at low temperatures (Boutilier et al., 1984), but that also required other important adaptations to their cardiovascular system.

Haemoglobin is contained within the erythrocytes or RBCs, the functional unit of O_2 transport in vertebrates. Haematocrit (Hct) is the percentage of blood volume that is occupied by the RBCs. In mature RBCs, Hb concentration is close to its solubility limit (Riggs, 1976); thus, there is typically a good correlation between Hb concentration and Hct (Farrell, 1991; Fänge, 1992; Farrell, 1992). In fishes, interspecific variation in Hct ranges from 0 to > 50%

(Gallaugher and Farrell, 1998; Harter and Brauner, 2017), and *in vivo* changes in Hct may offset challenges to O_2 transport by increasing C_aO_2 and β_b. Acutely, an increase in Hct is accomplished by splenic contraction that releases RBCs into the circulation (Yamamoto et al., 1985; Vermette and Perry, 1988; Perry and Kinkead, 1989). Chronically, fish may increase Hct by producing new RBCs, a process termed erythropoiesis, in response to exercise training (Hochachka, 1961; Farlinger and Beamish, 1978; Zbanyszek and Smith, 1984; Dougan, 1993; Thorarensen et al., 1993; Gallaugher, 1994; Gallaugher et al., 2001), hypoxia acclimation (Wood and Johansen, 1972; Härdig et al., 1978; Murad et al., 1990; Frey et al., 1998; Chapman et al., 2002; Timmerman and Chapman, 2004; Lai et al., 2006; Rutjes et al., 2007; Petersen and Gamperl, 2011), or simply to balance the loss due to lysis and senescence of RBCs (Weinberg et al., 1976). However, a higher Hct will also result in a higher blood viscosity that increases cardiac work, which may limit the benefits of polycythemia on arterial O_2 transport (Graham and Fletcher, 1983; Wells and Weber, 1991; Gallaugher et al., 1995; Egginton, 1996). The optimal Hct hypothesis states that mammals balance these conflicting requirements on β_b and cardiac function, to maximise arterial O_2 transport (Richardson and Guyton, 1959; Crowell and Smith, 1967). However, in fish this framework has been criticised as it fails to explain the observed variability in Hct within and between fish species (Wells and Baldwin, 1990; Wells and Weber, 1991; Gallaugher et al., 1995; Gallaugher and Farrell, 1998). Nevertheless, increasing Hct is a costly means of sustaining a higher β_b. Therefore, many fishes store a substantial portion of their blood O_2 carrying capacity in the spleen, which is only recruited when it is needed; a strategy that may significantly reduce routine cardiac work (Farrell and Steffensen, 1987b; Franklin et al., 1993; Gallaugher and Farrell, 1998; Sandblom and Gräns, 2017). Indeed, some polar fishes can increase Hct by over 40% due to splenic contraction (Wells et al., 1989) to sustain higher C_aO_2, but may otherwise avoid the elevated cardiac work that results from a higher blood viscosity, especially at low temperatures. In icefishes that lack Hb, the relationship between blood O_2 content and PO_2 is linear and determined exclusively by the physical solubility of O_2 in the plasma. However, in most vertebrates, the O_2 carrying capacity of the blood is overwhelmingly determined by Hb, and therefore, also β_b is largely a function of the O_2-binding properties of Hb.

1.3 O_2 extraction

1.3.1 Haemoglobin structure and function

Haemoglobin is perhaps the best-studied protein to date, and excellent reviews describe in detail the structure of fish Hbs in relation to their O_2-binding properties (Riggs, 1970; Jensen et al., 1998; Weber and Fago, 2004; Brittain, 2005). Briefly, most fishes possess tetrameric Hb (agnathans have monomeric Hb and Antarctic icefishes do not possess Hb), a globular protein consisting of two α and two β chains that form two α-β dimers. Each subunit has a haem group with a central iron atom that is capable of reversibly binding one molecule of O_2. For most physiological applications it suffices to describe Hb-O_2 binding by a two-state allosteric model (Monod et al., 1965), in which Hb is in equilibrium between two alternate conformations: the low affinity (T)ense-state, and the high affinity (R)elaxed-state. The affinity of Hb-O_2 binding is described by the Hb P_{50} value (the PO_2 at which Hb is 50% saturated), where higher P_{50} values represent a lower binding affinity. The relationship between PO_2 and Hb-O_2 saturation (SO_2) is illustrated by the O_2 equilibrium curve (OEC; Fig. 1-1). In the absence of ligand binding, the Hb molecule will be present in the thermodynamically more stable T-state and a transition to the R-state occurs with the binding of O_2. This transition induces changes in the protein's tertiary and quaternary conformations that provide the molecular mechanism for cooperativity and the linkage between Hb (de)oxygenation and the binding of allosteric effectors (Perutz, 1970; Nagai et al., 1985; Perutz et al., 1987). The cooperative nature of Hb-O_2 binding in vertebrates arises from a decrease in P_{50} during the T-R transition, which is described by the Hill coefficient (n_H), and this results in the characteristic sigmoidal shape of the OEC. For a given set of environmental and metabolic conditions, the characteristics of Hb-O_2 binding and the shape of the OEC determine how much O_2 can be taken up at the gills and how much can be unloaded at the tissues, and therefore, influence β_b by setting the *in vivo*-limits of the OEC.

1.3.2 The shape of the OEC

While increasing the circulating Hb concentration will increase the maximal O_2 carrying capacity, this full potential is never realised *in vivo*. Under routine conditions rainbow trout utilise less than half of their O_2 carrying capacity; while Hb is nearly fully saturated at the gill (S_aO_2 ~100%), venous S_vO_2 remains high, ~68% (Kiceniuk and Jones, 1977). Therefore, a large amount of O_2 is circulated without being unloaded from Hb at the

tissues and this O_2 store is termed the venous reserve. During exercise, O_2 extraction at the tissues increases as additional capillaries are recruited, and the venous reserve is increasingly utilised (Gallaugher et al., 2001; Egginton, 2002). At maximal swimming speed (U_{crit}) in rainbow trout, S_vO_2 becomes just 15-20%, while S_aO_2 remains largely unchanged (Kiceniuk and Jones, 1977; Brauner et al., 2000a). Thus, at rest, ~30% of the total blood O_2 carrying capacity is utilised compared to > 80% at U_{crit}.

Consequently, *in vivo* relevant changes in β_b are best described by the slope of the OEC between the points of O_2 loading and unloading. In the upper half of the OEC an increase in O_2 extraction at the tissues and a reduction S_vO_2, will increase β_b (see change in slope from β_b' to β_b" in Fig. 1-1). As a word of caution, an increase in β_b through O_2 extraction does not increase arterial O_2 transport ($C_aO_2 \times \dot{Q}$), but increases the fraction of the total O_2 carrying capacity that is utilised by the animal. This increase in the effective (or *in vivo*) β_b results in a higher flux of O_2 from the water to the mitochondria per unit of $\dot{Q}$. In addition, by recruiting the venous reserve, animals exploit the steeper ranges of the OEC, where large β_b can be realised and may alleviate cardiovascular adjustments to exercise. While the sigmoidal shape of the OEC ensures that a large fraction of O_2 is unloaded at the tissues over a narrow range of PO_2. Still, an increase in O_2 extraction must necessarily decrease P_vO_2, the main determinant of diffusion gradients from Hb to the mitochondria in the tissues (Jones, 1986; Wagner, 1992). Therefore, the upper and lower boundaries of the OEC that can be used for O_2 transport are set by P_aO_2 that is largely a function of the prevailing environmental PO_2, and by a minimum P_vO_2 that is required to maintain O_2 diffusion gradients to the mitochondria. Within these constraints, the β_b that can be realised *in vivo* must be largely a function of Hb P_{50}.

1.3.3 Hb P_{50}

Haemoglobin P_{50} represents a trade-off between conflicting requirements on O_2 loading at the gas exchange surface and unloading at the tissues. A number of excellent studies have addressed the idea of an optimal P_{50} in mammals (Turek et al., 1973; West and Wagner, 1980; Willford et al., 1982; Tenney, 1995) and in fish (Brauner and Wang, 1997; Wang and Malte, 2011). When interpreted carefully, the concept of optimality is a powerful tool for developing hypotheses and fits well within the comparative approach, as long as the results are not over-interpreted and adaptive value is indiscriminately assigned to *in vivo*

conditions in the animal (for reviews see Wells, 1990; Dudley and Gans, 1991). An optimal Hb P_{50} that maximises O_2 delivery to the mitochondria can be defined in terms of: i) maximal tissue O_2 extraction, and ii) maximal P_vO_2.

Based on the analysis of Willford et al. (1982), Figure 1-2A shows how an optimal P_{50} maximises O_2 extraction at the tissue (shown as $S_{a-v}O_2$). For a given combination of P_aO_2 and P_vO_2, this corresponds to the largest β_b that can be realised under these conditions. The optimal P_{50} is strongly influenced by the shape of the OEC and the range over which O_2 loading and unloading occur, and therefore the maximal β_b occurs at neither the maximal S_aO_2 nor minimal S_vO_2 (Fig. 1-2B). A more pronounced sigmoidal shape (larger n_H) will lead to a more narrow window over which P_{50} is optimal, compared to an OEC with a more parabolic shape (see Kobayashi et al., 1994). In addition, as P_aO_2 decreases during hypoxia, the optimal P_{50} shifts to a lower PO_2 value, and the maximal β_b that can be realised during hypoxia is lower as well (Wang and Malte, 2011). *In vivo* studies that altered blood P_{50} in mammals are generally in line with the latter analysis. During normoxia, there was a beneficial effect of higher Hb P_{50} on O_2 delivery (Oski et al., 1971; Woodson et al., 1973; Rice et al., 1975; Bakker et al., 1976; Harken and Woods, 1976; Moores et al., 1978; Zaroulis et al., 1979), whereas in hypoxia a lower P_{50} was beneficial (Hall et al., 1936; Eaton et al., 1974; Penney and Thomas, 1975; Hebbel et al., 1978).

The theoretical importance of an optimal Hb P_{50} in maintaining P_vO_2 is illustrated schematically in Figure 1-3 for three species that largely span the spectrum of P_{50} observed in vertebrates. During normoxia a high P_{50} will sustain a high P_vO_2. Therefore, animals with a high P_{50} can exploit the venous reserve to a larger extent to increase β_b, as a critical P_vO_2 is reached at a lower S_vO_2. This situation is beneficial for active species with a larger scope to increase $\dot{M}O_2$ and tissue O_2 extraction (dashed lines indicate $S_{a-v}O_2$ = 75%). However, the benefits of a high P_{50} decrease with P_aO_2, and in hypoxia a lower P_{50} can sustain a higher P_vO_2 and β_b. This is the case in the carp (*Cyprinus carpio*), where P_vO_2 is largely independent of P_aO_2; a situation that is beneficial for an animal that frequently experiences hypoxia in its habitat. However, the maximal P_vO_2 that can be realised is low as well, which greatly reduces the PO_2 gradient from the blood to the mitochondria, and may require compensation by other physiological or morphological adaptations (Vornanen et al., 2009). In fish, maintaining P_vO_2 is especially pertinent for the heart that is situated downstream of

all other tissues. While many fish, especially more active species, have a coronary supply of arterial blood to the compact myocardium, the heart of all fishes, relies to varying degrees on the O_2 supplied by the venous return (Farrell and Jones, 1992). Therefore, the teleost heart may set the lower limits for P_vO_2 (Davie and Farrell, 1991b; Farrell and Clutterham, 2003), and thus, the lower limits for P_{50}.

Clearly, adjustments to Hb P_{50} can increase β_b by ensuring that O_2 transport occurs over an appropriate range of the OEC. The optimal P_{50} always represents a compromise between the requirements for O_2 loading and unloading, within the constraints set by the prevailing environmental and metabolic condition of the animal. However, vertebrates can modulate Hb P_{50} *in vivo*, even within a single pass through the circulatory system, and teleosts in particular take advantage of this plasticity in P_{50} to enhance β_b.

1.4 pH sensitive Hb-O_2 binding

1.4.1 Plasticity of haemoglobin P_{50}

The intrinsic O_2 binding characteristics of Hb (including P_{50}) are determined by the protein's amino acid sequence, and thus are genetically coded. Fish, like many other ectotherms, typically show Hb multiplicity, where functional heterogeneity between Hb isoforms may allow for adequate O_2 transport over a wide range of fluctuating conditions (Nikinmaa, 1990; Weber, 1990, 1996; Weber, 2000; Wells, 2009). RBCs in fish are nucleated and retain the ability to synthesise proteins, including Hb, until a late state of maturation (Speckner et al., 1989). Maturation of RBCs takes place in the circulation, and it is possible that *de novo* synthesis would favor Hb isoforms that suit the prevalent environmental conditions (Marinsky et al., 1990; Nikinmaa, 2001). On top of this genotypic variation in Hb characteristics, a higher temperature typically increases P_{50}, because Hb-O_2 binding is an exothermic reaction, and some fishes, typically regional heterotherms, have evolved mechanism to modulate this temperature sensitivity (Weber and Fago, 2004; Weber et al., 2010). In addition, the binding of allosteric effectors to the T-state of Hb stabilises this conformation and increases Hb P_{50} (Jensen and Weber, 1987; Jensen et al., 1998). Important allosteric effectors in vertebrates are organic phosphates, chloride (Cl^-), carbon dioxide (CO_2), and protons (H^+), and by regulating their concentrations within the RBC cytoplasm, vertebrates can control Hb P_{50} *in vivo* (Nikinmaa, 1990).

In teleosts, Hb-O_2 binding is especially sensitive to H^+ and a decrease in RBC pH_i can greatly increase P_{50}. The resulting right-shift of the OEC is termed the (alkaline) Bohr effect (Bohr et al., 1904) and describes H^+ binding to titratable groups on the Hb molecule above pH 6. At lower pH values, many vertebrate Hbs show a reverse, or acid Bohr effect, which decreases P_{50} when additional H^+ are bound (Kilmartin and Rossi-Bernardi, 1973; Riggs, 1988; Jensen et al., 1998; Bonaventura et al., 2004; Giardina et al., 2004; Jensen, 2004; Berenbrink, 2006). Mathematically, the Bohr coefficient of Hb (Φ) is defined as:

$$5)\ \Phi = -\frac{\Delta log\ P_{50}}{\Delta pH}$$

The physiological significance of the alkaline Bohr effect is apparent: CO_2 released into the blood by metabolically active tissues decreases blood pH at the capillaries, creating an arterial-venous pH difference ($\Delta pH_{a\text{-}v}$). This decrease in pH increases Hb P_{50}, promoting the release of O_2 to the tissues. The situation is reversed at the gill, where low CO_2 tensions and high pH decrease P_{50} and promote Hb-O_2 binding. Therefore, the Bohr effect can greatly increase β_b as it enables dynamic changes in P_{50} throughout the circulation, maintaining local conditions that benefit O_2 loading at the gills and unloading at the tissues.

The Haldane effect is reciprocal to the Bohr effect (Christiansen et al., 1914), where the oxygenation of Hb causes conformational changes during the T-R transition that alter the molecular environment surrounding titratable Bohr groups, decreasing their pK_a and releasing bound H^+ (Perutz et al., 1960; Perutz, 1970; Perutz, 1990). These oxygenation-dependent H^+s are quantified by the fixed-acid Haldane effect (ΔZ_H in mol H^+ between oxy- and de-oxy Hb at constant pH) and their availability at the gills is important to support bicarbonate (HCO_3^-) dehydration and thus CO_2 excretion (Brauner, 1995, 1996; Brauner and Randall, 1996). Thermodynamic evidence that Bohr and Haldane effects are indeed mirror images of the same phenomenon was provided by Wyman (1964), who elegantly summarised the linkage between Bohr and Haldane coefficients for a symmetrical OEC and in the absence of additional allosteric effectors:

$$6)\ -\frac{\Delta log\ P_{50}}{\Delta pH} = \frac{1}{4}\Delta Z_H$$

The magnitude of a Bohr shift is a function of the Bohr coefficient of Hb and $\Delta pH_{a\text{-}v}$ during capillary transit. However, due to the linkage between Bohr and Haldane effects (Wyman, 1964, 1979), the magnitude of a $\Delta pH_{a\text{-}v}$ at the capillaries is reduced as Hb binds H^+ during de-oxygenation, thereby dampening the benefits of the Bohr effect for Hb-O_2 unloading. Therefore, it has been proposed that an optimal Bohr-Haldane coefficient for tissue O_2 delivery would be half of the respiratory quotient (RQ = CO_2 produced/ O_2 consumed, typically 0.7-1.0). This represents an optimal compromise between a Haldane coefficient that is small enough to allow for some $\Delta pH_{a\text{-}v}$ and a Bohr coefficient that is large enough to cause a significant Bohr shift; conditions that are met in most air-breathing vertebrates (Lapennas, 1983). However, the Bohr-Haldane effect in teleosts is non-linear over the *in vivo* range of the OEC (Brauner, 1995, 1996; Brauner and Randall, 1996). At rest many teleosts have large Bohr-Haldane coefficients that are in the range of RQ (Weber and Lykkeboe, 1978; Albers et al., 1981; Nikinmaa, 1983; Berenbrink et al., 2005), which is likely more beneficial for H^+ buffering and CO_2 transport than for O_2 delivery. But as the animal recruits the venous reserve during exercise, the Bohr-Haldane coefficient decreases to values that are close to half of RQ (Brauner and Randall, 1998) and are optimal for O_2 transport, and that notably occur when $\dot{M}O_2$ is high (Brauner, 1995; Brauner et al., 2000b; Brauner et al., 2001). These findings point towards a role of the Bohr-Haldane effect in sustaining an elevated β_b in exercising teleosts. However, modulating Hb P_{50} throughout the circulation requires rapid changes in RBC pH_i and that acid-base equivalents are effectively transferred across the RBC membrane, a process that is largely facilitated by the activity of the enzyme carbonic anhydrase (CA).

1.4.2 The physiology of carbonic anhydrase

Carbonic anhydrase is a zinc metalloenzyme that catalyses the reversible hydration/dehydration reactions of CO_2 (for general reviews see Maren, 1967; Chegwidden and Carter, 2000; Chegwidden et al., 2000; Forster and Dodgson, 2000; Hewett-Emmett, 2000; Gilmour, 2012):

$$CO_2 + H_2O \overset{i}{\leftrightarrow} H_2CO_3 \overset{ii}{\leftrightarrow} HCO_3^- + H^+$$

The second term of this equation (ii), the ionisation of carbonic acid (H_2CO_3), is extremely fast (Eigen and DeMaeyer, 1963) and can safely be considered instantaneous under physiological conditions (Forster and Dodgson, 2000). Also term (i) will proceed at an appreciable rate even without a catalyst at physiologically relevant temperatures. At 37°C the rate constants of the uncatalysed hydration and dehydration reactions are 0.18 s^{-1} and 79 s^{-1}, respectively (Itada and Forster, 1977; Wang et al., 2009). However, these reaction rates are highly temperature-dependent and a reduction in temperature from 37°C to 0°C decreases the uncatalysed rates 16-fold (Forster, 1991). Thus, in rainbow trout blood at 10°C, the halftime ($t_{1/2}$) for the dehydration reaction of HCO_3^- is ~90 s (Kern, 1960; Heming, 1984), and thus exceeds by two orders of magnitude the residence time of blood at the gills of fish ~1-3 s (Cameron and Polhemus, 1974; Hughes et al., 1981). Clearly, the uncatalysed reaction is not rapid enough to support significant CO_2 excretion in any vertebrate, but this is especially problematic for poikilothermic ectotherms. These limitations are overcome by CA that is abundantly present in all vertebrate RBCs (Meldrum and Roughton, 1933; Maren, 1967) and accelerates the hydration and dehydration reactions by up to 17,000-fold such as in human (*Homo sapiens*) RBCs at 37°C (human CAII $k_{cat} = 10^6$ s^{-1}; Khalifah and Silverman, 1991; Forster and Dodgson, 2000).

Sixteen α-CAs (the vertebrate gene family of CA isozymes) have been identified in mammals (Chegwidden and Carter, 2000; Hewett-Emmett, 2000), of which only 13 isoforms possess catalytic activity. There are five membrane-bound CA isoforms in mammals (Sly, 2000; Hilvo et al., 2005), three with trans-membrane domains (CA9, 12 and 14) and two (CA4 and 15) which are anchored to the membrane by the common linkage molecule glycophosphatidylinositol (GPI; Cross, 1987; Low et al., 1988). It appears that all membrane-bound CAs have a high catalytic activity, perhaps with the exception of CA15 (see Esbaugh and Tufts, 2006). Cytosolic CA in teleosts differ from those in mammals and it appears that teleosts have retained an ancestral state of single isoform with a high catalytic activity that has recently been renamed into Ca17 (previously referred to as Ca2-like; Ferreira-Martins et al., 2016). Ca17 has two distinct isozymes in teleosts of which Ca17b is mainly expressed in RBCs and Ca17a is widely expressed across tissue types and particularly abundant in gills, kidney and RBCs (Esbaugh et al., 2004; Esbaugh et al., 2005; Lin et al., 2008).

The molecular diversity and near ubiquity of CA in vertebrate tissues, including fishes, reflects its involvement in a wide range of physiological processes: CO_2 excretion at the cellular and systemic levels (Randall and Val, 1995; Henry and Heming, 1998; Tufts and Perry, 1998; Henry and Swenson, 2000; Tufts et al., 2003; Evans et al., 2005; Esbaugh and Tufts, 2006), acid-base regulation (Haswell et al., 1980; Perry and Laurent, 1990; Marshall and Grosell, 2006; Perry and Gilmour, 2006; Gilmour and Perry, 2009; Gilmour, 2011) and ion regulation (Maetz, 1971; Maetz and Bornancin, 1975; Haswell et al., 1980; Pelis and Renfro, 2004; Evans et al., 2005; Tresguerres et al., 2006; Gilmour and Perry, 2009). More generally, however, the role of CA in physiological processes can be described as facilitated membrane transport (Forster and Dodgson, 2000). Lipid membranes are relatively impermeable to charged ions, such as HCO_3^- and H^+, while CO_2, a small uncharged molecule, is highly lipid soluble and will rapidly diffuse across plasma membranes (Forster and Steen, 1969). In the presence of CA, CO_2-HCO_3^--H^+ species will rapidly equilibrate, both within a compartment and across lipid membranes, *via* the highly diffusible CO_2 (Forster and Dodgson, 2000). Further, the diffusion of CO_2 across RBC membranes is facilitated to a great extent by aquaporins (Endeward et al., 2006) and rhesus proteins (Endeward et al., 2008). Therefore, the rate at which acid-base equivalents passively equilibrate across a membrane is a function of CA activity on either side of the membrane and the membrane permeability for the involved reactive species; in RBCs this process is termed the Jacobs-Stewart cycle (Jacobs and Stewart, 1942).

1.4.3 The Jacobs-Stewart cycle

Under steady-state conditions, when secondarily-active transporters are silent, the RBC can be considered functionally impermeable to cations (Nikinmaa, 1990; Nikinmaa, 1992). Exchangeable ions, such as HCO_3^- and Cl^-, are passively distributed across the RBC membrane and their concentration is determined by the overall net charge of the impermeable polyions within the cell, mainly Hb and organic phosphates, according to a Donnan-like equilibrium (Hladky and Rink, 1977; Heinz, 1981). The net charge of the intracellular polyions is negative under physiological conditions, resulting in a small anion ratio (intracellular over extracellular anions; A_i/A_o) and in a lower pH_i compared to extracellular pH (pH_e; Hladky and Rink, 1977; Heming et al., 1986; Jensen, 2004). This trans-membrane pH gradient is ~0.2 in human RBCs (Hladky and Rink, 1977) and typically

~0.7 pH units in teleosts due to a due to their higher pH_e and similar pH_i (Jensen and Weber, 1982). However, despite this pH gradient under steady-state conditions, H^+s are in equilibrium across the RBC membrane because the concentrations of HCO_3^- and H^+ are linked *via* the Jacobs-Stewart cycle (Funder and Wieth, 1966; Hladky and Rink, 1977; Heming et al., 1986).

The Jacobs-Stewart cycle describes the passive equilibration of H^+s across the RBC membrane (Jacobs and Stewart, 1942). When an acid load is added to a RBC suspension this creates a CO_2^--HCO_3^--H^+ disequilibrium in the plasma and across the RBC membrane (Forster and Dodgson, 2000). The Cl^-/HCO_3^- exchanger (or anion exchanger; AE) on the RBC membrane that quickly corrects pH disequilibria across the membrane can only respond to changes in Cl^- or HCO_3^- gradients, but not to H^+s, and thus, immediately following H^+ addition the transporter will remain silent. In the plasma, H^+ and HCO_3^- react to form CO_2 and water, notably at the slow, uncatalysed rate. Once formed, CO_2 rapidly equilibrates across the cell membrane ($t_{1/2}$ ~1 ms in human RBCs at 37°C; Forster, 1969; Wagner, 1977; Swenson and Maren, 1978), and within the RBC, CO_2 is re-hydrated into HCO_3^- and H^+s, a reaction that is catalysed by the abundant intracellular CA pool (Maren, 1967). The result is the transfer of H^+s into the RBC that is buffered by organic phosphates and Hb. Consequently, the charge on intracellular polyions becomes less negative, causing intracellular HCO_3^- and Cl^- concentrations to increase, which results in osmotic swelling of the RBCs (Van Slyke et al., 1923; Nikinmaa, 1990). Finally, because the anion-ratio increases, the difference in pH_i-pH_e is lower at the new equilibrium (Duhm, 1972; Duhm, 1976). The formation of HCO_3^- within the RBC will activate the AE, exporting HCO_3^- and taking up additional Cl^-. Importantly, the rate at which H^+s are transferred into the RBC is limited by the slow uncatalysed rate of CO_2 formation in the plasma, a limitation that is overcome in the presence of extracellular CA (Jacobs and Stewart, 1942; Motais et al., 1989; Nikinmaa, 1990; Nikinmaa et al., 1990; Nikinmaa, 1992). By this mechanism, an extracellular acidosis can be rapidly transferred into the RBC at the tissue capillaries to enhance β_b *via* the Bohr effect.

1.4.4 Magnitude of the Bohr effect

At the tissue capillaries, the magnitude of a Bohr shift is a function of the Bohr coefficient of Hb (Φ) and $\Delta pH_{a\text{-}v}$ during capillary transit. Based on these parameters the change in P_{50} that will result from a Bohr shift can be calculated as:

$$7)\ P_{v50} = P_{a50} \times \left(10^{(\Phi \times \Delta pH_{a-v})}\right)$$

where P_{v50} is the venous P_{50} after the Bohr shift and P_{a50} is the original arterial P_{50}. In rainbow trout with a P_{a50} = 24.1 mmHg (Tetens and Lykkeboe, 1981), Φ = -0.91 (Rummer and Brauner, 2015) and a $\Delta pH_{a\text{-}v}$ = -0.2 (Kiceniuk and Jones, 1977), P_{v50} becomes 37 mmHg (Fig. 1-1). As the OEC is right-shifted at the tissues, O_2 extraction can be increased substantially, and importantly, without compromising P_vO_2, a situation that greatly increases β_b (see β_b' to β_b'"). In many vertebrates, the benefits of the Bohr effect are limited by the low pH sensitivity of Hb, however teleosts have evolved Hbs with an exceptionally large Bohr coefficient (Berenbrink et al., 2005). Rummer and Brauner (2015) calculated that with a $\Delta pH_{a\text{-}v}$ of -0.2, the pH sensitivity of Hb in rainbow trout may enhance Hb-O_2 unloading by over 70% relative to the situation in mammals with a moderate Bohr coefficient.

In addition to the Bohr effect, many teleosts have a Root effect that at low pH prevents Hb from becoming fully O_2 saturated, even at above atmospheric PO_2 (Root, 1931; Scholander and Van Dam, 1954), due to a negative cooperativity of Hb-O_2 binding (Yokoyama et al., 2004). Root effect Hbs do not exhibit an acid Bohr effect, which contributes to the continued increase in P_{50} with decreasing pH (Brittain, 2005). The Root effect may have evolved as a by-product of the mechanism by which actinopterygians increased the magnitude of their Bohr effect; however, the molecular mechanisms underlying the two phenomena are different (Perutz and Brunori, 1982; Brittain, 1987; Berenbrink et al., 2005). A Root effect is first observed in the basal actinopterygians, the polypteriforms and acipenseriforms (Berenbrink et al., 2005), but in these species is only expressed at pH values that are never encountered in the general circulation (Regan and Brauner, 2010b). However, with the evolution of specialised vascular structures, pH in the blood could be decreased sufficiently to trigger the Root effect, with tremendous benefit to β_b in select organs.

1.4.5 Specialised structures in teleosts that increase β_b

The *retia mirabilia* (singular *rete mirabile*) are dense vascular counter-current exchangers that create a large $\Delta pH_{a\text{-}v}$ locally (Scholander and Van Dam, 1954; Wittenberg and Haedrich, 1974; Wittenberg and Wittenberg, 1974; Pelster, 1997; Pelster and Randall, 1998; Pelster, 2004). The *choroid rete* evolved first, in the common ancestor of *Amia* and teleosts, and thereafter, swimbladder *retes* evolved independently, at least five times (Berenbrink et al., 2005). The acidification of blood in the *choroid rete* is likely induced by the retina itself whereas the *rete* in the swimbladder is closely associated with an acid producing gas gland. These structures produce large quantities of CO_2 from glucose *via* the pentose-phosphate shunt and of lactate and H^+ by glycolysis of glucose (Hoffert and Fromm, 1970; Baeyens et al., 1971; Pelster, 1995; Pelster, 2004). In the presence of RBC CA and a membrane-bound CA isoform the acidosis is rapidly transferred into the RBC *via* the Jacobs-Stewart cycle (Pelster, 1995; Gervais and Tufts, 1998; Würtz et al., 1999). Since the divergence of the polypteriforms, the teleost lineage has experienced a marked reduction in Hb and plasma buffer capacity compared to more basal jawed vertebrates, and as a consequence the amount of acid required to acidify the blood in the *retes* is greatly reduced (Berenbrink et al., 2005). The evolution of a Root effect likely released a functional limitation on the evolution of *retes*, because without Root effect Hbs a high arterial PO_2 would result in O_2 simply being shunted into venous vessels (Berenbrink et al., 2005). Instead, the high PO_2 and PCO_2 in venous vessels of the *retes* diffuse into nearby arterial vessels that run in parallel, magnifying and "recycling" the acidosis, and multiplying PO_2 with every pass through the system (Kuhn et al., 1963). The result is a mechanism that can generate large $\Delta pH_{a\text{-}v}$, which increases Hb P_{50} *via* the Bohr effect and reduces the O_2-carrying capacity *via* the Root effect, resulting in an extremely high PO_2.

The *choroid rete* generates PO_2 values greatly in excess of 1 atm and provides the necessary diffusion gradient to deliver O_2 across a large diffusion distances to the avascular retina (Wittenberg and Wittenberg, 1974). This, likely lifted the constraints on higher metabolic rates in the eye and increased visual acuity in teleosts (Barnett, 1951; Wittenberg and Haedrich, 1974; Wittenberg and Wittenberg, 1974), which is a beneficial trait for eye-sight predators and for the exploration of deeper and darker habitats (Pelster and Weber, 1991). Thus, it has been suggested that with the evolution of the *choroid rete* the Root effect

came under positive selection and increased in magnitude, such as observed in *Amia* and the osteoglossomorpha (Berenbrink et al., 2005). Remarkably, the swimbladder *retes* of many teleosts can generate PO_2 values of several hundred atm (Scholander and Van Dam, 1954; Pelster, 1997) that are adequate to explain the presence of fish with a gas filled swimbladder at depths of several thousand meters (Nielsen and Munk, 1964). Clearly, the evolution of Root effect Hbs and the *retia mirabilia* were key innovations that allowed the life-styles, habitats and morphologies of modern teleosts (Randall et al., 2014). In the counter-current systems of the *retes*, large ΔpH_{a-v} are effectively localised, preventing an expression of the Root effect in the general circulation; however, the beneficial increase in β_b is restricted to these morphological structures and is not available to other tissues without *retes*.

1.5 A novel mechanism for enhancing β_b in all tissues

Recently, a novel mechanism has been proposed that localises large ΔpH_{a-v} to sites of plasma-accessible carbonic anhydrase (paCA) activity in the circulation, thus potentially enhancing β_b in all tissues (Rummer and Brauner, 2011; Rummer et al., 2013). As in the *retes*, this system hinges on the high pH sensitivity of teleost Hb. In addition, two other requirements must be met that will be explored in detail in the following sections: i) active RBC pH_i regulation, and ii) a heterogeneous distribution of paCA in the vasculature.

1.5.1 Active RBC pH_i regulation

In the basal teleosts the Root effect continued to increase in magnitude and its expression approached the range of pH values normally encountered in the general circulation (Berenbrink et al., 2005). However, an expression of the Root effect in the general circulation would severely decrease the O_2 carrying capacity of the blood, and would impair arterial O_2 transport, leading to hypoxemia. This functional constraint was released by the evolution of active RBC pH_i regulation that is first observed in the elopomorphs, and that allowed a further increase in the onset pH of the Root effect. Upon a reduction in arterial PO_2 or pH, catecholamines (chiefly adrenaline and noradrenaline) are released into the blood and bind to β-adrenergic receptors on the RBC membrane (Tetens and Christensen, 1987; Nikinmaa, 1992; Randall and Perry, 1992; Guizouarn et al., 1993; Weaver et al., 1999). In rainbow trout, the RBC adrenergic receptor is more sensitive to noradrenaline than adrenaline (Tetens and Lykkeboe, 1988), however, sensitivity varies between teleost species (Salama and Nikinmaa, 1990). The stimulated receptor activates an excitation cascade that

involves the intracellular accumulation of cyclic adenosine monophosphate (cAMP; Mahe et al., 1985), which in turn activates β-adrenergically stimulated sodium-proton exchangers (β-NHE). As Na^+ moves down its concentration gradient (established by the Na^+/K^+-ATPase, NKA) H^+s are displaced from equilibrium and pH_i increases. RBC CA will rapidly correct the intracellular disequilibrium by hydrating CO_2 to HCO_3^- and H^+. As HCO_3^- accumulates within the RBC it is exported into the plasma in exchange for Cl^- and the intracellular accumulation of Na^+ and Cl^- causes osmotic swelling (Motais and Garcia-Romeu, 1988). The increase in RBC pH_i is related to the slower AE compared to NHE activity. This may seem surprising as the maximal rate of AE (5 mol kg cell dry weight^{-1} min^{-1}, in rainbow trout at 15°C; Romano and Passow, 1984; Nikinmaa and Boutilier, 1995) is two orders of magnitude faster, compared to the initial rate of the β-NHE (30 mmol kg cell dry weight^{-1} min^{-1}, in rainbow trout at 20°C; Nikinmaa et al., 1990). However, the rate of passive H^+ equilibration across the RBC membrane is not limited by the capacity of the AE, but by the uncatalysed dehydration rate of HCO_3^- in the plasma (Jacobs and Stewart, 1942; Motais et al., 1989). This aspect is of pivotal importance for RBC pH_i regulation. It allows for a large H^+ extrusion, and thus a large increase in pH_i, before HCO_3^- dehydration in the plasma can produce a significant amount of CO_2 that will re-acidify the RBC *via* the Jacobs-Stewart cycle. As extracellular H^+ concentration increases, the uncatalysed dehydration reaction accelerates, because the plasma H_2CO_3 pool increases. At the same time β-NHE activity slows as extracellular H^+ and intracellular Na^+ accumulate (Garcia-Romeu et al., 1988), despite an increase in NKA activity during the β-adrenergic response (Palfrey and Greengard, 1981; Bourne and Cossins, 1982; Ferguson and Boutilier, 1989). At this point, the apparent H^+ fluxes of the β-NHE and the Jacobs-Stewart cycle become equal and there is no net movement of H^+s. In this dynamic equilibrium, a H^+ gradient is actively maintained across the RBC membrane by NKA activity, and RBC pH_i is uncoupled from pH_e. The increase in pH_i will depend on the buffer capacity of Hb and organic phosphates within the RBC, where the low buffer capacity of teleost Hb drastically reduces the amount of H^+s that need to be extruded for a given change in pH_i (Nikinmaa, 1997). An elevated pH_i decreases Hb P_{50} *via* the Bohr effect and protects the O_2 carrying capacity of blood during an acidosis (Nikinmaa, 1983; Cossins and Richardson, 1985). In fact, it has been shown that C_aO_2 in teleosts is compromised when the adrenergic response is inhibited during a blood acidosis

(Nikinmaa et al., 1984; Primmett et al., 1986; Tetens and Christensen, 1987; Vermette and Perry, 1988; Perry and Kinkead, 1989).

1.5.2 β-NHE short-circuiting

Creating H^+ gradients across the RBC membrane hinges on a Jacobs-Stewart cycle that is limited by the uncatalysed production of CO_2 in the plasma and therefore an absence of extracellular CA activity. However, if circulating RBCs encounter a site where paCA is anchored to the endothelium, the Jacobs-Stewart cycle accelerates and the H^+s extruded by β-NHE will immediately combine with HCO_3^- in the plasma to form CO_2 that re-acidifies the RBC, importantly, at a rate that exceeds the H^+ efflux *via* β-NHE. Under these conditions, H^+ extrusion becomes futile, and pH_i and pH_e once again become coupled *via* the Jacobs-Stewart cycle, effectively "short-circuiting" β-NHE activity (Motais et al., 1989). The result is a rapid transfer of H^+s from the plasma into the RBC, *via* diffusion of CO_2, which decreases pH_i and increases Hb P_{50} *via* the Bohr effect. Therefore, short-circuiting RBC pH_i regulation will create a large $\Delta pH_{a\text{-}v}$ within the RBC and increase β_b in every tissue that has paCA at its capillaries.

Accumulating evidence indicates that paCA is present at the tissue capillaries of teleosts, whereas it is likely absent at the gills and in the venous system. If this were true, the heterogeneous distribution of paCA would modulate the ability of β-NHE to regulate RBC pH_i and selectively couple, and uncouple pH_e and pH_i, throughout the circulation (see Fig. 1-4). At the gills and in the venous system, where paCA is absent, pH_e and pH_i are uncoupled, thus preventing an expression of the Root effect and safeguarding O_2 uptake during an acidosis. However, it is presently unknown whether β-NHE activity is sufficiently rapid to restore RBC pH_i during venous transit; a knowledge gap that is directly addressed in Chapter 2 of this dissertation. In contrast, paCA at the tissue capillaries, would couple pH_e and pH_i creating a large $\Delta pH_{a\text{-}v}$ that short-circuits β-NHE activity and may greatly enhance β_b. The parallels to the situation in the *retes* are evident: Large $\Delta pH_{a\text{-}v}$ are created by actively pumping H^+s across the RBC membrane, instead of acid secretion from a gas gland, and the $\Delta pH_{a\text{-}v}$ is localised to sites of paCA activity, instead of vascular counter-current exchangers. However, β-NHE short-circuiting is not restricted to these specialised morphological structures and may enhance β_b in all tissues, and thus, increase the efficiency of cardiovascular O_2 transport on a systemic level.

1.5.3 Evidence that paCA is absent at the teleost gill

The evolution of β-NHE activity in teleosts must have required a functional absence of extracellular CA activity in the plasma, at the very least at the gills, where an elevated RBC pH_i is critical to protect O_2 loading in species with a Root effect. Despite the potential implications that the distribution of paCA may have for our understanding of O_2 transport in teleosts, this aspect has not been studied systematically. Data are available for only a few model species and the fact that studies have used a variety of different methods to measure paCA activity complicates the issue; in most cases, data from different studies are only conclusive if taken together. An exhaustive summary of these findings was recently published and allows drawing some tentative conclusions (Harter and Brauner, 2017).

Among teleosts the most compelling case for an absence of branchial paCA can be made for rainbow trout: this is supported by biochemical characterisation of the branchial CA isoforms (Henry et al., 1993; Henry et al., 1997b; Gilmour et al., 2001; Stabenau and Heming, 2003), functional measurements of CO_2 excretion in combination with specific inhibitors or injection of soluble CA (Perry et al., 1982; Wood and Munger, 1994; Currie et al., 1995; Julio et al., 2000; Desforges et al., 2001; Desforges et al., 2002; for review see Perry and Gilmour, 2002), measurements of post-branchial pH disequilibrium states (Gilmour and Perry, 1994; Gilmour et al., 1994; Gilmour et al., 1997; Perry et al., 1997), immunohistochemical localisation of CA (Rahim et al., 1988) and mRNA expression (Georgalis et al., 2006). Limited data on other teleosts is generally in line with the findings on rainbow trout, and paCA seems to be absent at the gills of other salmonids, such as coho salmon (*Oncorhynchus kisutch*; Perry et al., 1982), steelhead trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*; Stabenau and Heming, 2003) and six other teleost species (Henry et al., 1988; Rahim et al., 1988; Sender et al., 1999; Gilmour et al., 2002; Stabenau and Heming, 2003). The only exceptions among teleosts are found within the Antarctic notothenioids where paCA may be present at the gills of three Hb-less icefish species (Maffia et al., 2001; Tufts et al., 2002; Harter et al., unpublished data), but data on three red-blooded species of the clade remain conflicting; a knowledge gap that is addressed in detail in Chapter 6 of this dissertation.

Stabenau and Heming (2003) provide, perhaps, the broadest survey of paCA across vertebrate gas exchange organs, including mammals, birds, reptiles, amphibians and fishes;

importantly using a comparable methodology (some of the original data were collected by Heming et al., 1994; Stabenau et al., 1996; Stabenau and Heming, 1999; Stabenau and Vietti, 2002). It appears that among vertebrates, teleost fishes stand out in their absence of paCA at the gas exchange surface, a trait that is otherwise highly conserved across vertebrate phyla. This is perhaps no surprise given the potential benefit of paCA for CO_2 excretion. In air-breathers, paCA at the lung endothelium will also eliminate CO_2-HCO_3^--H^+ disequilibrium states in the plasma that may confound the sensing of respiratory set-points (Swenson et al., 1995b; Swenson et al., 1998; Swenson, 2000); a functional constraint that may not apply to fishes. However, dogfish (*Squalus acanthias*), which have been extensively studied in this respect, have a paCA isoform at the gills (Henry et al., 1997b; Wilson et al., 2000; Gilmour et al., 2001; Gilmour et al., 2007) that plays a role in CO_2 excretion (Swenson et al., 1995a; Swenson et al., 1996; Gilmour et al., 1997; Patel et al., 1997; Wilson et al., 2000; Gilmour et al., 2001; Gilmour and Perry, 2004). Further, evidence on other chondrichthyans (Gilmour et al., 2002; McMillan et al., unpublished data) and on the basal Pacific hagfish (*Eptatretus stoutii*; Esbaugh et al., 2009) also support a role of paCA in CO_2 excretion, and in addition indicate the presence of soluble CA isoforms in the plasma, that may originate from RBC lysis (Wood et al., 1994; Gilmour et al., 1997; Henry et al., 1997b; Gilmour et al., 2001; Gilmour et al., 2002; Esbaugh and Tufts, 2004; Esbaugh et al., 2009). In contrast, most euteleostomes have potent inhibitors of CA in the plasma that are highly specific to cytosolic CA isoforms and ineffective against membrane-bound isoforms (Hill, 1986; Roush and Fierke, 1992; Heming et al., 1993; Gervais and Tufts, 1998), which may indicate a function in inactivating or scavenging CA released from RBC or tissue lysis (Booth, 1938; Henry et al., 1997b; Henry and Heming, 1998). Also, teleosts have plasma CA inhibitors (Maetz, 1956; Haswell et al., 1983; Heming, 1984; Heming and Watson, 1986; Dimberg, 1994; Gervais and Tufts, 1998; Peters et al., 2000) and the potency of these inhibitors may correlate with the pH sensitivity of Hb (Henry et al., 1997b). In combination, the available data indicate a successive loss of CA activity in the blood compartments in vertebrates, from the basal condition in hagfishes, where CA is ubiquitously present, to the condition in teleosts, where CA is retained only within the RBCs. The loss of paCA at the gills of teleosts and the complete absence of soluble CA in the plasma, likely released the functional constraint on

the evolution of active RBC pH_i regulation and enabled the active generation of H^+ gradients across the RBC membrane to protect O_2 loading at the gills during an acidosis.

1.5.4 Evidence for paCA activity at the tissues

In mammals, a number of studies provide strong evidence for the presence of paCA activity in the capillaries of skeletal muscle (Zborowska-Sluis et al., 1974; Effros and Weissman, 1979; Ridderstrale, 1979; Lönnerholm, 1980; Riley et al., 1982; Geers and Gros, 1984; Dermietzel et al., 1985; Geers et al., 1985; Bruns et al., 1986; Hemptinne et al., 1987; Gros and Dodgson, 1988; Wetzel and Gros, 1990; Wetzel et al., 1990; Waheed et al., 1992; Wetzel and Gros, 1998), brain (Ghandour et al., 1992) and the heart (Sender et al., 1994; Decker et al., 1996; Vandenberg et al., 1996; Sender et al., 1998; Knüppel-Ruppert et al., 2000) that is located on the luminal surface of the capillary endothelium (Sender et al., 1994; Decker et al., 1996). The physiological functions of membrane-bound extracellular CA at the tissues are diverse, and a role has been shown in cellular CO_2 (Geers and Gros, 1988), ammonia (Henry et al., 1997a; Wang et al., 1998) and lactate excretion (Wetzel et al., 2001), and in facilitating rapid calcium (Ca^{2+}) movements across the sarcoplasmic reticulum during muscle contraction (Wetzel et al., 1990; Geers and Gros, 2000; Sly, 2000).

While much less work has been done on fish tissues, it appears that white muscle in rainbow trout has a CA distribution similar to that in mammals, including a membrane-bound Ca4 isoform (Sanyal et al., 1982; Siffert and Gros, 1982; Sanyal et al., 1984) with some functional evidence for a plasma accessible orientation (Henry et al., 1997a; Henry and Heming, 1998; Wang et al., 1998). In contrast, pH disequilibrium states in the venous system of rainbow trout, which reach as far as the bulbus arteriosus, indicate that paCA is functionally absent in the venous circulation (Perry et al., 1997). These results are in line with the immuno-histochemical localisation of paCA in the dorsal aorta of glass catfish (*Kryptopterus bicirrhis*), but its absence in the dorsal vein (Randall et al., 2014). Further, *ca4* mRNA and protein were detected in the brains and the heart of rainbow trout (Georgalis et al., 2006), which is in line with the results of mammalian studies where paCA was reported in the capillaries of these tissues. However, the cellular orientation of these putatively paCA isoforms remains to be confirmed; a knowledge gap that is directly clarified for the hearts of coho salmon in Chapter 5 of this dissertation.

Based on the combined evidence it appears that paCA activity is present in all parts of the circulation in hagfish, chondrichthyans and tetrapods; however, the tetrapod kidney may be a notable exception (Effros and Nioka, 1983). Therefore, most vertebrates may have a largely homogenous presence of paCA throughout the vasculature, which is also the situation in many invertebrates and may represent the ancestral state (Henry, 1984, 1988b, a; Stabenau and Heming, 2003). However, it appears that teleosts have selectively lost paCA at the gills, and in the venous system, while paCA is present at the tissue capillaries and perhaps in the arterial system in general. Therefore, unlike any other vertebrate studied to date, teleosts appear to have a heterogeneous distribution of paCA in the vasculature (Harter and Brauner, 2017).

1.5.5 Evidence for β-NHE short-circuiting

In vitro experimental evidence supports a mechanism of RBC β-NHE short-circuiting in rainbow trout. Motais et al. (1989) and Nikinmaa et al. (1990) showed that β-NHE stimulation in RBC suspensions will cause a reduction in pH_e due to H^+ extrusion; a response that is abolished when RBCs are incubated in the presence of extracellular CA. In addition, Nikinmaa et al. (1990) also showed that the increase in RBC pH_i due to β-NHE stimulation was inhibited in the presence of CA. Rummer et al. (2011) effectively avoided the challenges of measuring RBC pH_i, by loading blood into a closed vial and measuring PO_2 instead. In rainbow trout, that have pH-sensitive Hb, closed-system changes in PO_2 can be used as a proxy for changes in RBC pH_i and give direct insight into the effects of short-circuiting on Hb-O_2 binding. When extracellular CA was added to adrenergically stimulated RBCs, PO_2 increased by up to 25 mmHg. This result provided the first *in vitro* evidence that short-circuiting β-NHE activity decreases RBC pH_i and can greatly enhance O_2 unloading from pH-sensitive Hb. The effect of CA on PO_2 was completely abolished in the presence of the NHE inhibitor EIPA (ethyl isopropyl amiloride), thus substantiating the involvement of NHE activity in the response. Interestingly, when CA was added to RBCs without β-NHE stimulation a small increase in PO_2 (up to 6 mmHg) was still observed. It was concluded that another "housekeeping" NHE isoform may generate a H^+ gradient that can be short-circuited by CA. Evidence for an involvement of non-adrenergic NHE isoforms in hypoxic and hypercapnic RBC volume and pH_i regulation has been reported in sablefish (Rummer et al., 2010). However, the data of Tetens et al. (1988), who measured a high sensitivity of rainbow

trout RBC adrenoreceptors for noradrenaline, indicate that some stimulation of β-NHE is likely, even at the low catecholamine concentrations typical of resting fish, creating a background level of β-NHE activity. Whether background β-NHE activity or other putative RBC NHE isoforms may create H^+ gradients across the membrane in resting teleosts deserves further investigation. If substantiated these H^+ gradients may be short-circuited in the presence of paCA, increasing β_b even under routine conditions; a knowledge gap that is directly addressed in Chapters 2 and 3 of this dissertation.

In vivo studies support a mechanism in teleosts that enhances β_b, however, typically without direct evidence for β-NHE short-circuiting. Nikinmaa et al. (1984) measured arterial PO_2 in exercised stressed striped bass that were higher than water and atmospheric PO_2. Haematological parameters clearly indicated β-NHE activity and these findings are in line with β-NHE short-circuiting by paCA in the post-branchial circulation. McKenzie et al. (2004) measured red muscle PO_2 values in rainbow trout that were consistently higher than those reported in mammalian studies, and unlike the mammalian measurements, were maintained (at > 40 mmHg) even during spontaneous struggling, exercise or hypoxia. In light of previous *in vitro* data, these results are consistent with β-NHE short-circuiting that facilitates unloading of O_2 at a higher PO_2. However, measurements of blood flow and systemic vascular resistance are important to discount alternative hypothesis. Higher Mass-specific blood flow rates in rainbow trout may be double those in mammals (Egginton, 1987; Taylor et al., 1996; Egginton, 2002), and diffusion distances in rainbow trout and striped bass are about 20% shorter than those in rat and hamster *tibialis anterior* (Egginton, 2002). In addition, rainbow trout have a higher P_{50} of myoglobin, an intracellular haem molecule, compared to that of mammals (Helbo and Fago, 2011); conditions that favor a higher muscle PO_2 in the first place.

The first study to specifically address a role of paCA *in vivo*, measured red muscle PO_2 in tubocurarine paralysed rainbow trout that were force ventilated (Rummer et al., 2013). Exposure to hypercarbia (1.5% CO_2) caused a reduction in pH_e and RBC pH_i and increased red muscle PO_2 by 30 mmHg (~65%), and this response was abolished by injection of the membrane-impermeable CA inhibitor C18 (Scozzafava et al., 2000). The specificity of this CA inhibitor supports an involvement of paCA in enhancing red muscle PO_2 during a respiratory acidosis. The injection of C18 into normocapnic trout (in normoxia) had no effect

on red muscle PO_2, indicating that the enhancement of β_b may not occur under routine conditions, in the absence of an acidosis. Catecholamine levels were not elevated above resting levels throughout the entire experiment, but this may not preclude some low β-NHE activity (Tetens and Lykkeboe, 1988), especially in combination with a reduction in blood pH (Nikinmaa, 1992; Guizouarn et al., 1993; Weaver et al., 1999). The conditions of an acidosis induced by hypercarbia, and the fact that the animals were paralysed, and thus red muscle $\dot{M}O_2$ was likely low, are noteworthy and raise some concern about the relevance of these results for unrestrained fish.

Petersen and Gamperl (2010a) found that hypoxia-acclimated Atlantic cod (*Gadus morhua*) had a lower $\dot{Q}$ during rest or exercise, compared to normoxia-acclimated fish. However, both groups reached similar $\dot{M}O_{2max}$ and U_{crit} values, resulting in a higher $\dot{M}O_2/\dot{Q}$ in hypoxia-acclimated animals. Blood O_2 carrying capacity was only slightly elevated in hypoxia-acclimated fish, and P_{50} and n_H were unchanged, indicating that these were not primary mechanisms that explain a higher β_b. Similar results were observed in hypoxia-acclimated steelhead trout that were unable to match the increase in $\dot{Q}$ that was observed in normoxia-acclimated fish during a temperature challenge, whereas $\dot{M}O_2$ increased to the same degree in both groups and consequently, their aerobic scope did not differ (Motyka et al., 2017). An increase in $\dot{M}O_2/\dot{Q}$ during different aerobic challenges is in line with a system that increases β_b in teleosts by short-circuiting RBC β-NHE in the presence of paCA. This reduces the requirements on $\dot{Q}$ to support a given $\dot{M}O_2$ and thus, cardiovascular O_2 transport becomes more efficient with respect to $\dot{Q}$. If hypoxia-acclimated fish are unable to increase $\dot{Q}$ to the same degree as normoxia-acclimated conspecifics (Petersen and Gamperl, 2010b) they may be more reliant on a higher β_b to maintain aerobic performance. Thus, Chapter 3 of this dissertation examined the role of paCA in sustaining an elevated $\dot{M}O_2/\dot{Q}$ in normoxia- and hypoxia-acclimated Atlantic salmon (*Salmo salar*) during a routine exercise challenge.

1.6 Thesis objectives and structure

The present dissertation builds on the recent discovery of a novel mechanism of enhanced Hb-O_2 unloading in salmonids (Rummer, 2010; Rummer and Brauner, 2011; Rummer et al., 2013). By selectively generating and eliminating H^+ gradients across the RBC membrane, teleost may create large $\Delta pH_{a\text{-}v}$ that are localised to the tissue capillaries and greatly enhance β_b. The primary objective of the present dissertation was to increase our

mechanistic understanding of this system by addressing the previously identified gaps in knowledge, and to generate *in vivo* evidence for its importance for cardiovascular O_2 transport and to sustain exercise performance in teleosts. Thus, the over-arching hypothesis of this dissertation is:

Teleosts increase the O_2 capacitance of their blood (β_b) by a mechanism of active RBC pH_i regulation that is modulated through a heterogeneous distribution of paCA, which has functional significance for O_2 transport *in vivo*.

Previous work has focused on rainbow trout, a well-studied teleost species, with a history as a model in the exercise physiology of fish. Conceptually, three basic requirements must be met for a species to enhance β_b *via* this mechanism: i) a pH-sensitive Hb, ii) RBC pH_i regulation, and iii) a heterogeneous distribution of paCA. The present work will focus on several salmonid species where it has been shown, specifically, that rainbow trout, Atlantic salmon, and coho salmon largely meet these requirements (Berenbrink et al., 2005; Harter and Brauner, 2017; Shu et al., 2017), and that RBC β-NHE can be short-circuited *in vitro* to increase closed-system PO_2 (Shu, Harter and Brauner., unpublished data). However, these three requirements may be met by most teleost species and therefore, the present results will be discussed in light of the potential implications for the clade as a whole. Salmonids represent an excellent group to study a mechanism that may enhance β_b and thus alleviate the requirements on $\dot{Q}$ in a group where reproductive success is closely tied to their ability to increase cardiovascular O_2 transport (Eliason et al., 2011; Eliason et al., 2013). The remaining dissertation is structured into four data chapters, followed by a General Discussion and Conclusion section (Chapter 6):

1.6.1 The time-course of RBC pH_i recovery

Chapter 2 explores the time-courses of RBC β-NHE short-circuiting and the subsequent recovery of pH_i in relation to venous transit times in rainbow trout. Short-circuiting of β-NHE activity will only increase β_b on a systemic level if RBC pH_i is restored during venous transit, enabling O_2 loading at the gills, and the $\Delta pH_{a\text{-}v}$ experienced by Hb is effectively localised to the tissues.

Chapter 2 tested the hypothesis that the time-course of RBC pH_i recovery after short-circuiting must concur with the estimated time of venous transit.

To this end, the time-courses of β-NHE activation, short-circuiting and RBC pH_i recovery were assessed *in vitro*, with a closed-system preparation that has been validated previously (Rummer and Brauner, 2011). To confirm that the observed changes in closed-system PO_2 are, in fact, due to β-NHE short-circuiting, experiments were performed on rainbow trout and on white sturgeon (*Acipenser transmontanus*) that do not possess RBC β-NHE (Berenbrink et al., 2005). While this closed-system preparation can quickly generate qualitative data that is comparable to previous work, RBC pH_i recovery was also measured in a continuous-flow apparatus that more closely resembles the *in vivo* conditions that RBCs experience in the circulation. Results from the combined approach provide strong evidence that RBC β-NHE activity and tissue paCA can create and localise large $\Delta pH_{a\text{-}v}$ at the tissues and greatly enhance the β_b in teleosts.

1.6.2 The in vivo relevance of paCA for O_2 transport

Chapter 3 examined the role of paCA in sustaining an elevated β_b in normoxia- and hypoxia-acclimated Atlantic salmon during routine swimming and at rest. Conceptually, a system of β-NHE short-circuiting will increase β_b, allowing teleosts to transport more O_2 per unit of blood flow. This is consistent with what has been observed in hypoxia-acclimated teleosts that were unable to increase $\dot{Q}$ to the same extent as normoxia-acclimated conspecifics (Petersen and Gamperl, 2010b), but attained the same aerobic performance, in terms of $\dot{M}O_{2max}$, U_{crit}, or critical thermal maximum (CT_{max}; Petersen and Gamperl, 2010b; Petersen and Gamperl, 2011; Motyka et al., 2017).

Chapter 3 tested the hypothesis that teleosts enhance β_b, at least in part, by short-circuiting β-NHE and that this response is increased after hypoxia-acclimation.

To this end, Atlantic salmon were acclimated to normoxia or hypoxia (~40% air saturation for > 6 weeks) and exercised in a swim tunnel at a constant speed and $\dot{M}O_2$. If paCA is part of the mechanism that allows hypoxia-acclimated fish to sustain a higher $\dot{M}O_2/\dot{Q}$, we predicted that an inhibition of paCA would result either in a decrease in $\dot{M}O_2$ and thus a failure to sustain swimming, or in a compensatory increase in $\dot{Q}$, to maintain $\dot{M}O_2$ at the respective swimming velocity. In fact, results show conclusively that paCA plays a role in O_2 transport in teleosts; and for the first time this phenomenon has been demonstrated *in vivo*, and in a situation with functional and ecological relevance.

1.6.3 The presence of paCA in the salmon heart

Chapter 4 explored the putative presence of paCA in the lumen of the coho salmon heart. While experimental evidence in rainbow trout supports this mechanism for enhanced oxygen delivery to red muscle (Rummer et al., 2013), its involvement in oxygen delivery to other metabolically active tissues is unknown.

Chapter 4 tested the hypothesis that the salmon heart contains a membrane-bound CA isoform that is accessible to blood in the lumen.

To this end, the CA isoforms were localised in coho salmon hearts using histological and molecular techniques. Further, the plasma-accessible orientation of CA in the atrium was demonstrated using a novel assay that measured the catalytic rate of these isoforms in the lumen, *in situ*. The presence of paCA in the contractile elements of the salmon heart may enhance β_b during critical periods of low P_vO_2 and blood acidosis, which salmon must experience during their exhausting spawning migrations.

1.6.4 Functional constraints on branchial paCA in teleosts

Chapter 5 examined the functional relationship between teleost Hb characteristics and the presence of branchial paCA. This complex question was conveniently addressed in two species of Antarctic notothenioids, one of which displays typical teleost Hb characteristics, whereas the other has lost Hb and RBCs from the circulation; a fantastic model system to study the cardio-respiratory physiology of vertebrates. Teleost fishes have lost paCA activity at the gills (reviewed by Harter and Brauner, 2017; and see above), and thus HCO_3^- dehydration is shifted entirely into the RBC, creating a strong coupling between O_2 and CO_2 transport; this strategy is clearly not available to icefishes, which are teleosts, but lack RBCs. Previous studies found biochemical markers for the presence of a membrane-bound Ca4 isoform in the gills of icefish (Feller et al., 1981; Maffia et al., 2001; Tufts et al., 2002). However, the cellular orientation of putatively paCA isoforms was not assessed, and a potential involvement of a Ca4 isoform in CO_2 excretion remained unresolved for icefishes.

Chapter 5 tested the hypothesis that icefishes express a membrane-bound CA isoform at the gill that is accessible to the blood plasma where it would catalyse CO_2 excretion in the absence of RBC CA.

To this end, the CA isoform distribution in the gills of the icefish *Champsocephalus gunnari* and the red-blooded *Notothenia rossii* were compared by biochemical, molecular

and immunohistochemical techniques. The obtained results shed new light on a divergent strategy of CO_2 excretion in icefishes that is unlike that found in any other adult vertebrate.

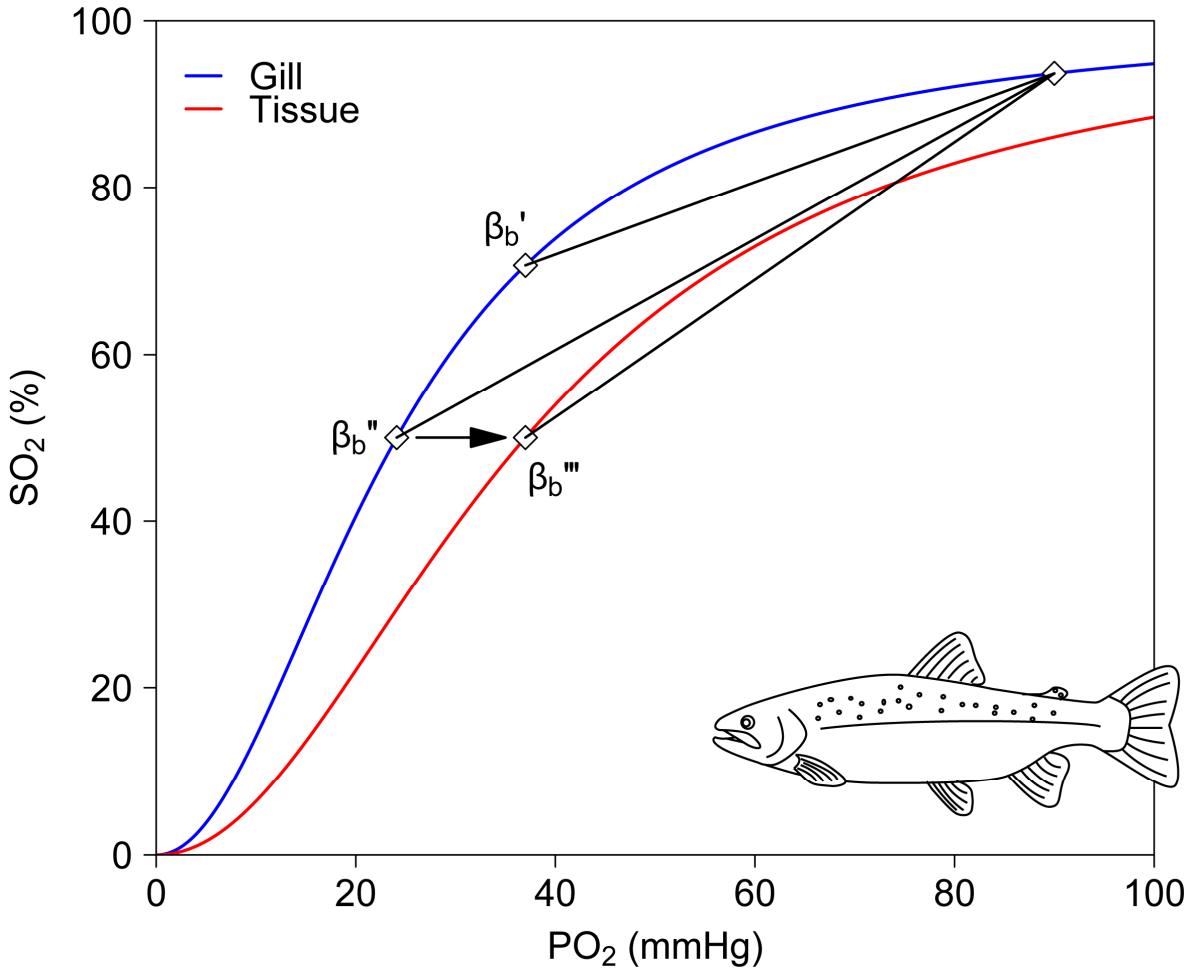


Figure 1-1 Oxygen equilibrium curves (OEC) for rainbow trout blood (*Oncorhynchus mykiss*) calculated before (Gill) and after (Tissue) a Bohr shift during capillary transit. This analysis uses the Hill equation (Hill, 1910) and previously reported blood parameters for rainbow trout (Tetens and Lykkeboe, 1981), and assumes an arterial partial pressure of O_2 (P_aO_2) of 100 mmHg, a Bohr coefficient (Φ) of -0.91 (Rummer and Brauner, 2015) and an arterial-venous pH difference ($\Delta pH_{a\text{-}v}$) at the capillaries, of -0.2 (Kiceniuk and Jones, 1977). The change in Hb P_{50} due to a Bohr shift is indicated by the arrow. The capacitance of the blood for O_2 (β_b) is the slope of the oxygen OEC between the points of O_2 loading at the gas exchange surface and unloading at the tissues. β_b' represent a low tissue O_2 extraction where arterial SO_2 (S_aO_2) is 94% and venous SO_2 (S_vO_2) is 70%, such as may be observed at rest. During routine exercise β_b'' increases due to an increase in O_2 extraction. And a decrease in blood pH at the tissue capillaries will further increase β_b''' *via* a Bohr shift.

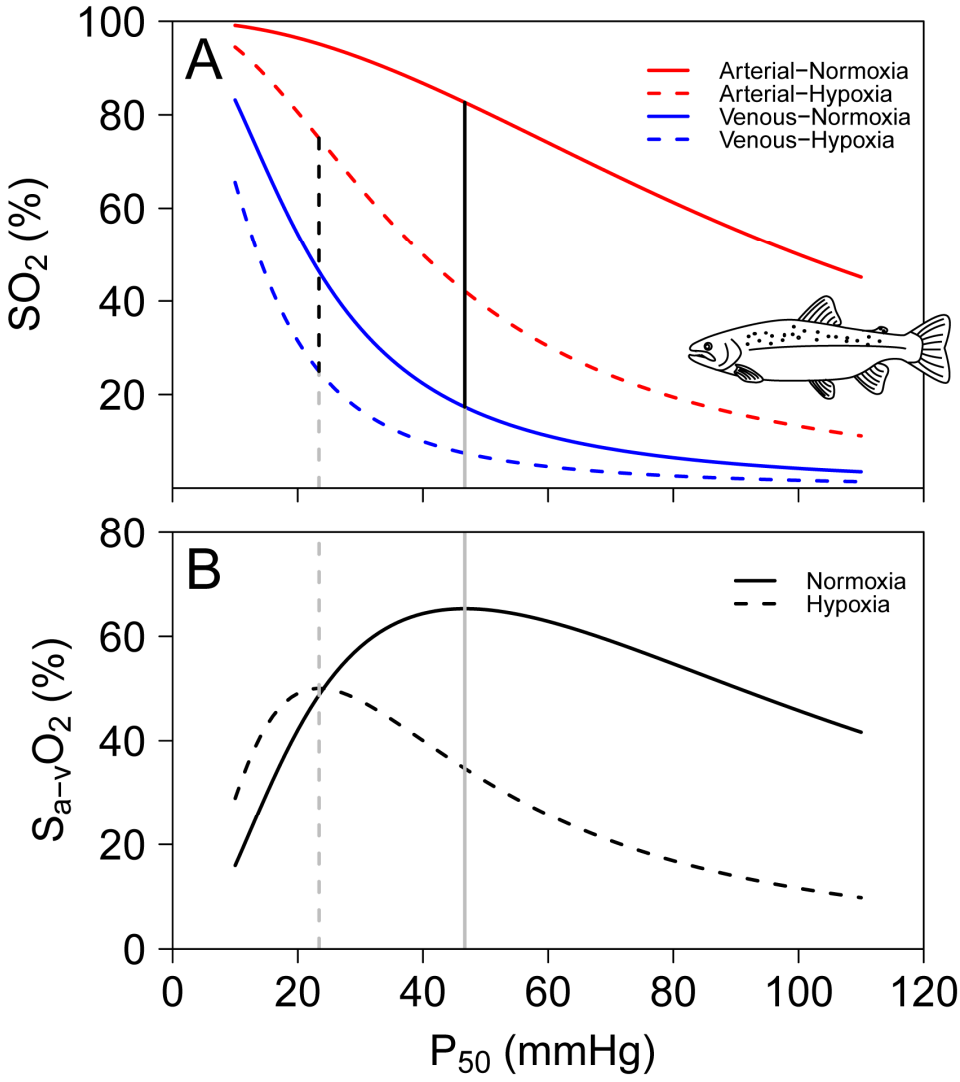


Figure 1-2 A) Haemoglobin (Hb) oxygen saturation (SO_2) as a function of Hb P_{50} in rainbow trout (*Oncorhynchus mykiss*). Solid lines are normoxia (arterial partial pressure of O_2, P_aO_2 = 100 mmHg) and dashed lines are hypoxia (P_aO_2 = 40 mmHg). P_{50} is the PO_2 at which Hb is 50% saturated. The oxygen equilibrium curves for arterial (red) and venous blood (blue) were calculated according to Willford et al. (1982), using the Hill equation (Hill, 1910) and assuming a constant venous P_vO_2 (22 and 14 mmHg for the normoxic and hypoxic scenarios). Tissue O_2 extraction was calculated as the difference between S_aO_2 and S_vO_2 ($S_{a\text{-}v}O_2$) and is represented by the vertical distance between the two curves. B) $S_{a\text{-}v}O_2$ as a function of P_{50} during normoxia and hypoxia at constant P_aO_2 and P_vO_2. $S_{a\text{-}v}O_2$ is maximal at the theoretical optimal P_{50}, which is 47 mmHg in normoxia and 23 mmHg in hypoxia.

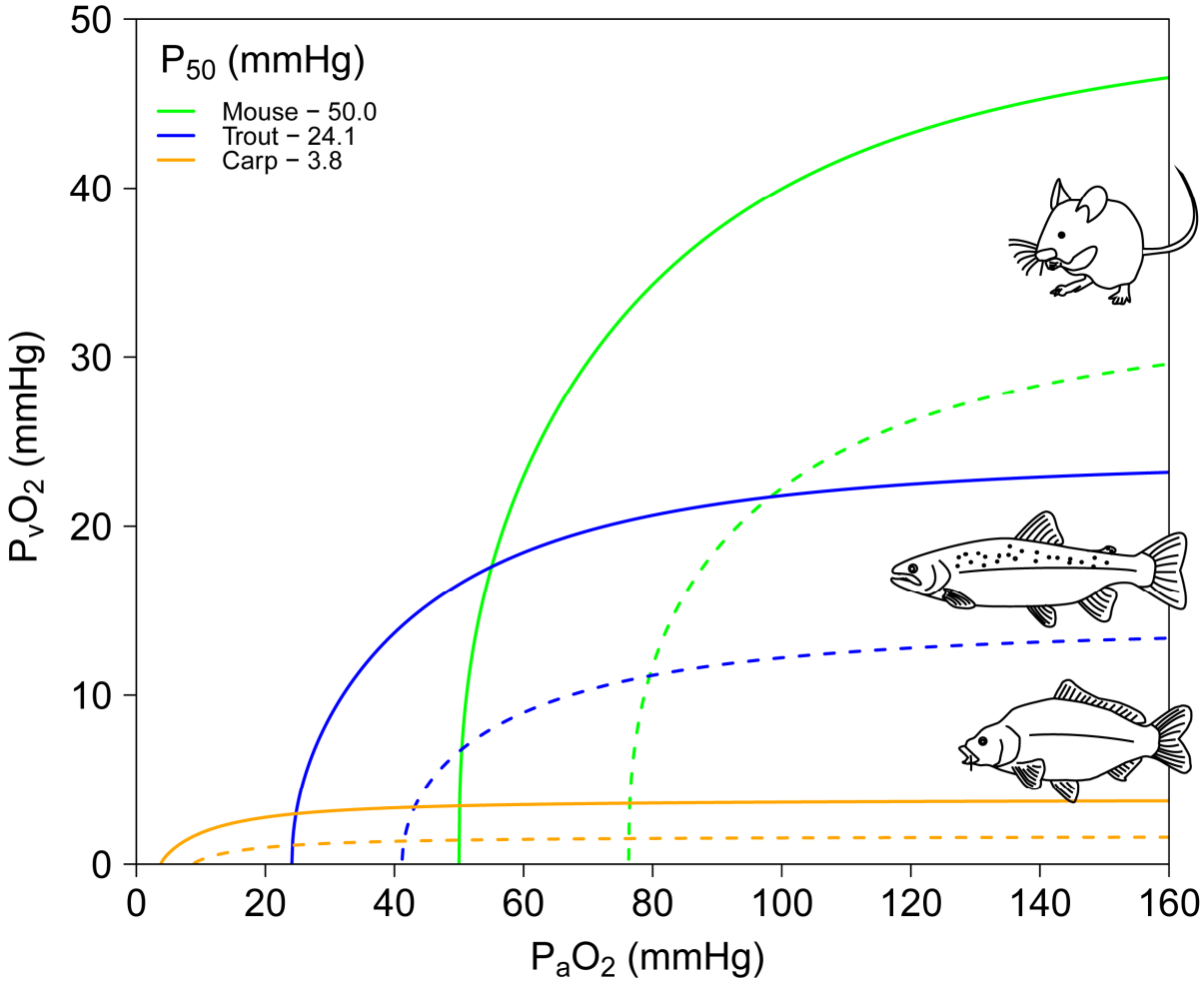


Figure 1-3 Venous partial pressure of oxygen (P_vO_2), as a function of arterial PO_2 (P_aO_2) in three species with different Hb P_{50}. Mouse (*Mus musculus*, green lines; P_{50} = 50 mmHg, n_H = 2.6; Newton and Peters, 1983), rainbow trout (*Oncorhynchus mykiss*, blue lines; P_{50} = 24.1 mmHg, n_H = 2.05; Tetens and Lykkeboe, 1981) and carp (*Cyprinus carpio*, orange lines; P_{50} = 3.8 mmHg, n_H = 1.3; Brauner et al., 2001), calculated using the equations described in Willford et al. (1982). P_{50} is the PO_2 at which haemoglobin (Hb) is 50% saturated, and n_H is the Hill coefficient. Solid lines are calculated at a constant Hb-O_2 unloading, expressed as the difference between arterial and venous Hb-O_2 saturations, $S_{a\text{-}v}O_2$ = 50%, and dashed lines at $S_{a\text{-}v}O_2$ = 75%.

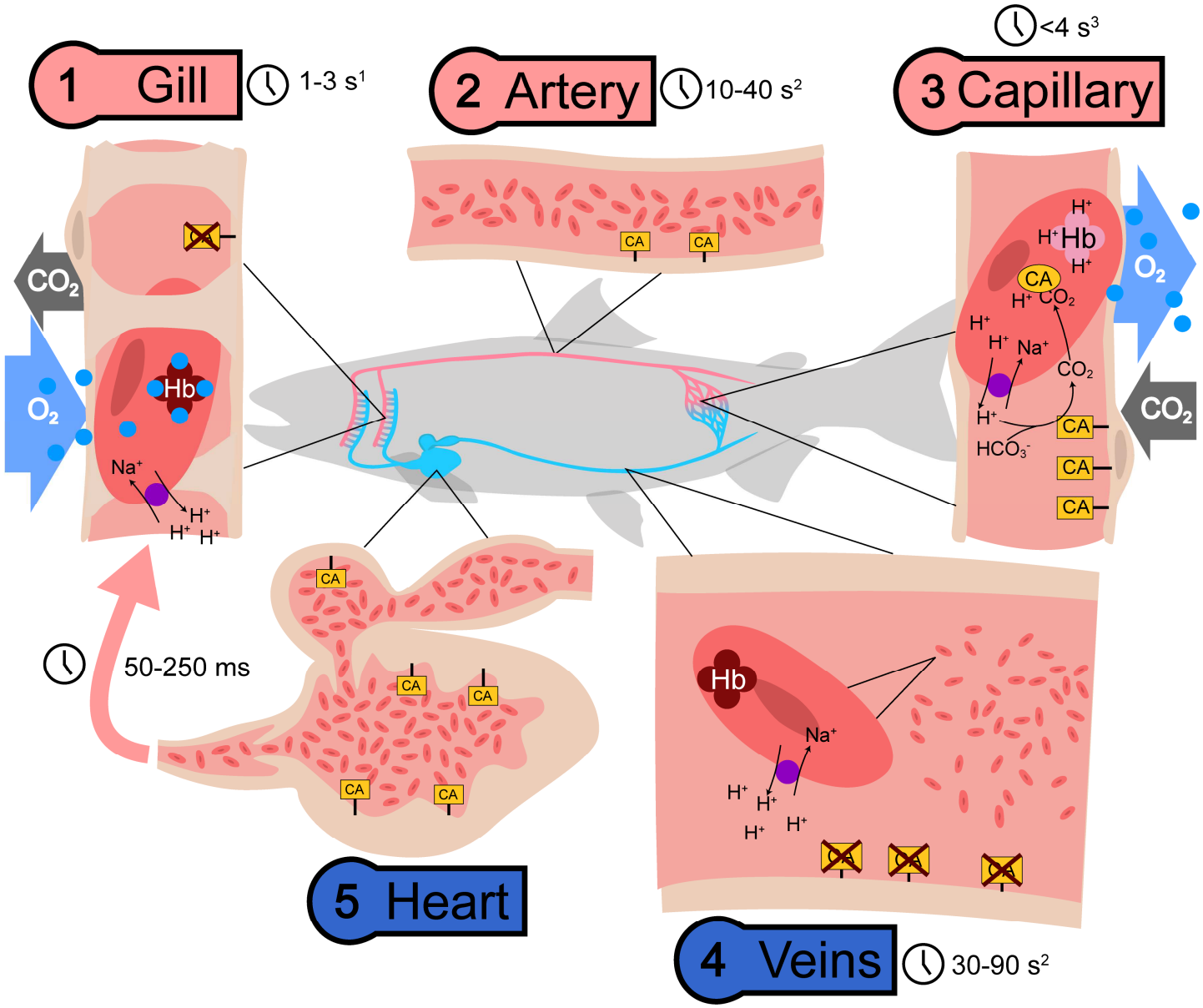

1 Gill
1-3 s1
2 Artery
10-40 s2
3 Capillary
<4 s3
CO2
O2
Hb
Na+
H+
CA
HCO3-
50-250 ms
5 Heart
4 Veins
30-90 s2

Figure 1-4 Schematic and description of the teleost mechanism of enhanced haemoglobin-oxygen (Hb-O_2) unloading. 1) During a blood acidosis, catecholamines are released into the blood and activate β-adrenergic sodium-proton exchangers (β-NHE) on the red blood cell (RBC) membrane. By actively extruding H^+ into the plasma, β-NHEs protect RBC intracellular pH (pH_i) and O_2 loading to pH sensitive haemoglobin (Hb). 2-3) At the tissue capillaries (and perhaps in the arterial system in general) plasma accessible carbonic anhydrase (paCA) is anchored to the endothelium and catalyses CO_2-HCO_3^--reactions in the blood plasma (Henry et al., 1997a; Wang et al., 1998; Randall et al., 2014). Thus, H^+ that are extruded by β-NHE, immediately combine with HCO_3^- to form CO_2, a small lipid soluble molecule that quickly diffuses across RBC membranes ($t_{1/2}$ ~1 ms; Forster, 1969). Within the RBCs CO_2 is re-hydrated to form H^+ and HCO_3^-, a reaction that is catalysed by the abundant CA pool within the RBC (Maren, 1967). The result is a rapid transfer of H^+ into the RBC that effectively short-circuits β-NHE activity; plasma pH (pH_e) and pH_i become coupled, and this increases tissue PO_2 *via* the Bohr effect. 4) In the venous system paCA is absent and the transfer of H^+ across the RBC membrane is rate-limited by the uncatalysed production of CO_2 in the plasma ($t_{1/2}$ ~90 s at 10°C; Heming, 1984). Thus when RBCs leave the capillaries, β-NHE activity will recover pHi during venous transit and protect renewed O_2 uptake at the gills (Harter et al., In Press). 5) In the teleost heart paCA is anchored to walls and trabeculae of the atrium and ventricle (Alderman et al., 2016), and β-NHE short-circuiting may secure the O_2 supply to the avascular spongy myocardium (Farrell and Jones, 1992). The large volume of the lumen precludes an effective exposure of RBCs to paCA and the bulk blood flow (~90%, based on ventricular ejected fraction (Franklin and Davie, 1992)) will retain its high O_2 affinity; this is critical as transit times in the ventral aorta are too short to allow for a recovery of RBC pH_i before the gill. Additional references: [1](Cameron and Polhemus, 1974; Hughes et al., 1981); [2]Chapter 2 and (Randall et al., 2014); and [3](Honig et al., 1977; Maren and Swenson, 1980). The graphical design in this figure is the work of Jacelyn Shu.

Chapter 2: The time-course of red blood cell intracellular pH recovery following short-circuiting in relation to venous transit times in rainbow trout, *Oncorhynchus mykiss*[1]

2.1 Synopsis

Accumulating evidence is highlighting the importance of a system of enhanced haemoglobin-oxygen (Hb-O_2) unloading for cardiovascular O_2 transport in teleosts. Adrenergically stimulated sodium-proton exchangers (β-NHE) create H^+ gradients across the red blood cell (RBC) membrane that are short-circuited in the presence of plasma-accessible carbonic anhydrase (paCA) at the tissues; the result is a large arterial-venous pH shift (ΔpH_{a-v}) that greatly enhances O_2 unloading from pH-sensitive Hb. However, RBC intracellular pH (pH_i) must recover during venous transit (30-90 s), to enable O_2-loading at the gills. The halftimes ($t_{1/2}$) and magnitudes of RBC β-adrenergic stimulation, short-circuiting with paCA and recovery of RBC pH_i were assessed *in vitro*, on rainbow trout whole blood, and using changes in closed-system PO_2 as a sensitive indicator for changes in RBC pH_i. In addition, the recovery rate of RBC pH_i was assessed in a continuous-flow apparatus that more closely mimics RBC transit though the circulation. Results indicate that: i) the $t_{1/2}$ of β-NHE short-circuiting is likely within the residence time of blood in the capillaries; ii) the $t_{1/2}$ of RBC pH_i recovery is 17 s and within the time of RBC venous transit; and iii) after short-circuiting RBCs re-establish the initial H^+ gradient across the membrane and can potentially undergo repeated cycles of short-circuiting and recovery. Thus, teleosts have evolved a system that greatly enhances O_2 unloading from pH-sensitive Hb at the tissues, while protecting O_2 loading at the gills; the resulting system increases the flux of O_2 from the water to the mitochondria per unit of blood flow and may enable the tremendous athletic ability of salmonids.

[1]A version of this chapter has been published as: **Harter, T. S., May, A. G., Federspiel, W. J., Supuran, C. T. and Brauner, C. J.** (In Press). The time-course of red blood cell intracellular pH recovery following short-circuiting in relation to venous transit times in rainbow trout, *Oncorhynchus mykiss*. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*

2.2 Introduction

The haemoglobins (Hb) of most vertebrates exhibit some pH sensitivity and typically Hb-oxygen (O_2) binding affinity decreases with a reduction in pH, termed the Bohr effect (Bohr et al., 1904). The Bohr effect permits a high Hb-O_2 affinity at the gas-exchange surface, where pH is high, and a low affinity at the tissue capillaries, where metabolically produced CO_2 diffuses into the blood and pH is low. In this way the Bohr effect can increase the difference between arterial and venous Hb-O_2 saturation and elevate the capacitance of the blood for O_2 (β_b), thus allowing more O_2 to be unloaded per unit of blood flow. The magnitude of the Bohr effect is a function of the pH sensitivity of Hb (expressed as the Bohr coefficient) and the pH change that Hb, within the red blood cell (RBC), experiences at the tissue capillaries (ΔpH_{a-v}). Teleost Hb typically has a large Bohr coefficient (Berenbrink et al., 2005) and in addition many species exhibit a Root effect, where a reduction in pH will prevent Hb from becoming fully O_2 saturated even at O_2 tensions above atmospheric (Root, 1931; Scholander and Van Dam, 1954). To exploit this extreme pH sensitivity, teleosts have evolved dedicated morphological structures (*retia mirabilia*) that create large ΔpH_{a-v} locally and can generate the high partial pressures of O_2 (PO_2) that are necessary to fill the swimbladder at depth and to deliver O_2 to the avascular retina in the eye (Wittenberg and Wittenberg, 1974).

However, during a blood acidosis, pH-sensitive Hb-O_2 binding may compromise O_2 loading at the gills and reduce the O_2 carrying capacity of teleost blood (Nikinmaa et al., 1984). Enhanced O_2 unloading by acidifying Hb at the tissues, can only increase β_b on a systemic level, if O_2 loading at the gas exchange surface is not compromised; consequently large ΔpH_{a-v} must be localised to the tissues. At the swimbladder and the eyes of teleosts this is enabled by the *retes*, and thus, it was predicted that enhanced Hb-O_2 unloading was restricted to these specialised structures. More recently however, a novel mechanism has been proposed that localises large ΔpH_{a-v} to sites of plasma-accessible carbonic anhydrase (paCA) in the circulation, thus potentially enhancing Hb-O_2 unloading to all tissues that possess paCA (Rummer et al., 2013; Randall et al., 2014).

Many teleost species that have a Root effect can actively regulate RBC intracellular pH (pH_i) and thus protect Hb-O_2 binding at the gills during an acidosis. In stressful situations, such as exercise or hypoxia, that may generate an acidosis, catecholamines are

released into the blood that bind to β-adrenergic receptors on the RBC membrane and activate sodium-proton exchangers (β-NHE). These β-NHEs actively pump H^+s from the RBC cytosol into the plasma and thus increase RBC pH_i. In the plasma these H^+s will combine with HCO_3^- to form CO_2 that can freely diffuse into the RBC where it is rapidly converted back into H^+s and HCO_3^-, a reaction that is catalysed by the abundant CA pool within RBCs (Maren, 1967); this passive transfer of H^+s across the RBC membrane *via* CO_2 is termed the Jacobs-Stewart cycle (Jacobs and Stewart, 1942). In the absence of extracellular CA activity, the Jacobs-Stewart cycle is limited by the uncatalysed rate of CO_2 production in the plasma, which allows β-NHE activity to create a H^+ gradient across the RBC membrane. However, the addition of extracellular CA activity removes this limitation and H^+s extruded by β-NHE will immediately combine with HCO_3^- in the plasma to form CO_2 that re-acidifies the RBC. Thus, in the presence of extracellular CA, H^+ extrusion is futile, and β-NHE activity is effectively short-circuited (Motais et al., 1989). Teleosts lack soluble CA in the plasma and paCA at the gills (reviewed by Harter and Brauner, 2017), enabling β-NHE activity and active RBC pH_i regulation. At the tissue capillaries of teleosts, where paCA is likely present (Henry et al., 1997a), β-NHE activity will be short-circuited, rapidly transferring H^+s into the RBC and creating a large $\Delta pH_{a\text{-}v}$ that enhances the unloading of O_2 from Hb. However, short-circuiting of β-NHE activity will only increase β_b on a systemic level if RBC pH_i is restored during venous transit, enabling O_2 loading at the gills; thus the recovery of RBC pH_i will effectively localise the $\Delta pH_{a\text{-}v}$ experienced by Hb, to the tissues.

Although only investigated in a few studies, it appears that paCA activity is absent in the venous circulation of teleosts (Perry et al., 1997; Randall et al., 2014), a requirement for RBC pH_i recovery in this compartment. The transit time of RBCs through the venous circulation has not been measured in fish (Olson, 1992; Sandblom and Axelsson, 2007) and best estimates are based on cardiac output and the volume of the primary circulation. Cardiac output in rainbow trout ranges from 19 ± 4 mL kg^{-1} min^{-1} at rest to 56 ± 6 mL kg^{-1} min^{-1} during maximal exercise (mean ± s.e.m. based on data from Kiceniuk and Jones, 1977; Thorarensen et al., 1996b; Brauner et al., 2000b). Total blood volume in rainbow trout has been assessed by a variety of methods and on average is 41 ± 3 mL kg^{-1} (mean ± s.e.m. from 13 studies using a variety of methods; reviewed by Olson, 1992). Consequently, average

blood transit times through the entire circulation will vary between 130-43 s, depending on the level of activity. If the venous blood volume in fish, like in mammals, is ~70% of total blood volume (Rothe, 1993; Pang, 2001), then venous transit time at rest is ~91 s, and ~30 s during maximal exercise.

Therefore, we **hypothesised that the time-course of RBC pH_i recovery after short-circuiting must concur with the estimated time of venous transit (30-91 s).** To test this hypothesis we used a two-pronged approach. The time-courses of β-NHE activation, short-circuiting and RBC pH_i recovery were assessed in a closed-system preparation that has been validated previously (Rummer and Brauner, 2011), and that elegantly avoids the difficulty of measuring RBC pH_i, by using changes in closed-system PO_2 as a sensitive proxy measurement. To validate that the observed changes in closed-system PO_2 are, in fact, due to β-NHE short-circuiting, experiments were performed on rainbow trout and on white sturgeon that do not possess RBC β-NHE (Berenbrink et al., 2005). While this closed-system preparation can quickly generate qualitative data that are comparable to previous work, RBC pH_i recovery was also measured in a continuous-flow apparatus that more closely resembles the *in vivo* conditions that RBCs experience in the circulation. Results from the combined approach provide strong evidence that RBC β-NHE activity and tissue paCA can create and localise large $\Delta pH_{a\text{-}v}$ at the tissues and greatly enhance β_b.

2.3 Materials and Methods

2.3.1 Animals and housing

Rainbow trout, *Oncorhynchus mykiss* Walbaum (Average body mass, Series 1: 2,266 ± 207 g; Series 2: 2,943 ± 188 g), were obtained from Miracle Springs Inc. (Mission, BC, Canada) and white sturgeon, *Acipenser transmontanus* Richardson (761 ± 34 g) were obtained from Vancouver Island University (Nanaimo, BC, Canada). All animals were maintained at The University of British Columbia (UBC) aquatic facility in 4000 L tanks, supplied with flow-through dechlorinated municipal tap water (Vancouver, BC, Canada) at 12°C and under a 12:12 h photoperiod. Fish were fed to satiation every second day using commercial trout pellets (Skretting, Orient 4-0, Vancouver, BC, Canada) and feeding was suspended the day before surgeries. Animal husbandry and all experiments were conducted according to the guidelines of the Canadian Council on Animal Care and approved by the UBC Animal Care Committee (Protocol no. A15-0266).

2.3.2 *Blood sampling and preparation*

Fish were anaesthetised in 0.2 g L^{-1} tricaine methanesulfonate (MS222, Argent, Redmond, WA) buffered with $NaHCO_3^-$. After loss of equilibrium animals were placed on a surgery table, where oxygenated and cooled (12°C) water, with a maintenance dose of anesthetic (0.1 g L^{-1} buffered MS222), was irrigated over the gills. Fish were chronically cannulated with a catheter (PE50, BD Intramedic, Franklin Lakes, NJ) into the dorsal aorta according to Soivio et al. (1975). Thereafter, fish were recovered by irrigating the gills with freshwater and then transferred to individual holding tanks, supplied with flow-through water. Fish were allowed to recover for 48 h after surgery and before blood sampling, and cannulae were flushed with heparinised Cortland's saline (in mM: 124.1 NaCl; 5.1 KCl; 1.6 $CaCl_2$; 0.9 $MgS0_4$; 11.9 $NaHCO_3$; 3.0 NaH_2PO_4, at pH 7.4; Wolf 1963; 50 I.U. Na-Heparin, Sigma H3149) twice per day to prevent blood clotting.

Blood sampling was conducted in the mornings, before the lights were turned on. Blood was drawn slowly from the cannula into a heparinised syringe and care was taken not to disturb the animal; sampling was suspended if the fish responded to the procedure. After sampling, animals were euthanised in 0.2 g L^{-1} buffered MS222 and blood was stored on ice for processing. Haematocrit (Hct) was measured in triplicate, in 15 µL capillary tubes that were centrifuged at 10,000 g for 3 min. Hct of the samples was then adjusted to 25% by adding Cortland's saline or by gently centrifuging the blood (500 g for 3 min) and removing plasma. Thereafter, 3 mL of blood was loaded into Eschweiler tonometers and was equilibrated (1 h) to a humidified, custom gas mixture generated by a Woesthoff pump (Bochum, Germany). Gas tensions were chosen to produce ~75% Hb-O_2 saturation, and based on preliminary trials and previous work, gases were mixed at 3.8 mmHg CO_2 (0.5 kPa), 76 mmHg O_2 (10 kPa) in N_2 for rainbow trout (Rummer, 2010; Rummer and Brauner, 2011), and 3.8 mmHg CO_2, 38 mmHg O_2 in N_2 for white sturgeon (Burggren and Randall, 1978).

2.3.3 *Series 1: Closed-system experiment*

In order to determine the recovery rate of RBC pH_i after short-circuiting, we used a two-pronged approach. First, recovery rates were determined using a closed-system preparation that has been the standard method to characterise RBC β-NHE short-circuiting in fishes (Rummer and Brauner, 2011). After equilibration of rainbow trout or white sturgeon

blood in tonometers, a subsample (150 µL) was removed to measure initial Hct (as described above) and blood pH with a thermostatted (12°C) microelectrode (16-705 and 16-702; Microelectrodes Inc., Bedford, NH). Thereafter, 2 mL of blood was loaded into a glass vial that was sealed with a septum, carefully excluding any air. This vial was thermostatted at 12°C and fitted with a magnetic stir bar that kept the sample well mixed. PO_2 within the vial was measured continuously with a fiberoptic O_2 microsensor (Loligo, Viborg, Denmark; and PreSens MicroTX3 meter, Regensburg, Germany) that was pierced through the septum. Due to the pH sensitivity of Hb, changes in RBC pH_i are reflected in qualitative changes in closed-system PO_2 (ΔPO_2). This allowed changes in RBC pH_i to be monitored in real-time, which is typically not possible with other available techniques (Baker et al., 2009). Furthermore, the use of ratio-metric fluorophores (e.g., SNARF-1) in RBCs is complicated by the high concentration of Hb and its fluorescent properties (Swietach et al., 2010).

According to previous work (Rummer and Brauner, 2011), blood within the closed system was acidified by injecting HCl, β-adrenergically stimulated with isoproterenol (ISO, a synthetic β-agonist), and short-circuited with soluble CA; treatments that have large effects on RBC pH_i and thus closed-system PO_2 (Rummer and Brauner, 2011; Shu et al., 2017). We extended this protocol by a final injection of C18, a membrane-impermeable CA inhibitor (Scozzafava et al., 2000) that inhibits the soluble CA injected previously, but that has no significant effect on RBC intracellular CA within 1 h of injection (Rummer et al., 2013). The inhibition of the extracellular CA pool with C18, allows for a renewed recovery of RBC pH_i by β-NHE activity. The following solutions were made up in Cortland's saline and were injected into the vial (total injection volume was 3% of vial volume) in 5 minute intervals (as determined in preliminary trials): i) 20 µl of 500 mM HCl, to acidify the blood by ~0.3 pH units according to (Wood et al., 1982); ii) 20 µl of 2 mM ISO (Sigma I-5627), for a final concentration of 0.01 mM; iii) 10 µl of 0.2 mM CA (CA2 from bovine erythrocytes, Sigma C3934) for a final concentration of 10^{-3} mM; and iv) 10 µl of 40 mM C18 (in 20% DMSO), for a final concentration of 0.2 mM C18 (0.1% DMSO). At the end of the trial, a subsample of blood was removed from the vial to measure final Hct and pH. In a separate trial, solvent controls were run to assess the effect of 0.1% DMSO on ΔPO_2, however no effects were observed (ΔPO_2 was 0.0 ± 2.6 mmHg, $N = 3$).

2.3.4 Series 2: Continuous-flow apparatus

While the results obtained in the closed-system preparation were qualitatively informative, it became apparent that RBC pH_i recovery occurred at a rate that was likely to be confounded by: i) the rate of mixing within the closed system that delayed the delivery of drugs (ISO, CA or C18) and the measurement of PO_2; and ii) the response time of the fiberoptic sensors that was in the order of several seconds for the observed ΔPO_2; these constraints have been identified previously (Rummer and Brauner, 2011). Therefore, to supplement the closed-system experiment, RBC pH_i recovery was additionally measured in a continuous-flow apparatus that is robust against these confounding factors (see below), and that replicates more closely the dynamic conditions that RBCs experience in the circulation. A similar approach has been used previously, to measure the rate of rapid reactions that exceed the response time of standard measuring techniques, such as Bohr and Root shifts in blood and CO_2 diffusion across RBC membranes (Forster and Steen, 1968; Forster, 1969; Forster and Steen, 1969).

After equilibration in tonometers, 3 mL of blood was loaded into a 10 mL, gas-tight, Hamilton syringe at 12°C, excluding any air. As in the closed system, blood in the syringe was acidified with HCl (by ~0.3 pH units) and β-adrenergically stimulated with ISO (at 0.01 mM). The sample was carefully mixed by inverting the syringe and this process was aided by a small stir bar within the syringe that moved through the sample with every tilt. Thereafter, the syringe was connected to the system (see Fig. 2-1A for system schematic) *via* a blunted 18 gauge needle, fitted to gas-impermeable tubing (PTFE #20 AWG Cole-Parmer, Vernon Hills, IL). Blood was drawn out of the syringe by a peristaltic pump and as the volume of blood decreased, this retracted the syringe plunger (ensuring no air exposure). The flow rate of the peristaltic pump was set to 0.2 mL min^{-1} as determined in preliminary trials. Downstream of the peristaltic pump, blood was perfused through a section of glass-capillary tubing (L = 10 mm, ID = 0.15 mm) that was fitted with ten polymethyl-pentene (PMP) fibers (Oxyplus, Membrana GmbH, Wuppertal, Germany). These fibers were coated with CA (CA2 from bovine erythrocytes, Sigma C3934) according to previously described methods (Kaar et al., 2007; Arazawa et al., 2012), creating a site of localised CA activity, such as blood may experience during capillary transit in fish. Thus, during the residence time of blood within this section (~8 s) plasma CO_2-HCO_3^- reactions were catalysed and β-NHE activity of

stimulated RBCs was short-circuited. When blood left this site, plasma CO_2-HCO_3^- reactions became, once again, uncatalysed and RBC β-NHE activity restored H^+ gradients across the membrane. Therefore, whether plasma CO_2-HCO_3^- reactions were catalysed depended only on the position of the RBC within the system and, because no CA inhibitor was required, the confounding effects of mixing and inhibition kinetics were avoided.

At a continuous blood-flow rate and a constant diameter of the PTFE tubing (ID = 0.81 mm), the distance that blood has travelled after leaving the site of CA will correspond to the time of RBC pH_i recovery. PO_2 in the blood was measured at 5, 15, 30, 60 and 120 s after short-circuiting, by placing fiberoptic sensors at fixed distanced from the site of CA (32, 96, 193, 385, 771 mm). Therefore, each PO_2 sensor performed measurements at a constant PO_2 that corresponded to one time-point in the RBC pH_i recovery reaction. The system was run continuously for up to 15 min, which allowed sufficient time for complete equilibration of the PO_2 sensors and avoided the issue of slow sensor response times. Finally, after blood had passed through the system, a blood sample was collected at the outlet for measurements of final Hct and pH (as described above).

2.3.5 Validation of the continuous-flow apparatus

New PMP fibers were coated with CA every day and were fitted into the glass capillary before experiments. The presence of CA activity within the capillary was confirmed before running trials, by using a buffer in pH disequilibrium and stop-flow measurements of pH according to (Gilmour, 1998). Briefly, a continuous flow of assay buffer (in mM: 225 mannitol, 75 sucrose, 10 TRIS base, and pH adjusted to 7.4 with 10% phosphoric acid) was rapidly loaded with CO_2 in a hollow-fiber membrane gas exchanger (see Fig. 2-1B), by equilibrating the buffer to a humidified, high-CO_2 gas (38 mmHg CO_2 in N_2, from a Woesthoff gas mixing pump). Thereafter, the buffer was immediately perfused over the CA-coated fibers (or non-coated fibers as a Ctrl) and pH was measured downstream using a microelectrode (Microelectrodes Inc., Bedford, NH). After equilibration of the pH readings the flow was stopped and the magnitude and direction of the pH change (ΔpH) was measured.

To confirm that CA activity was not washed out from the fibers, perfusate buffer (1 mL) was collected from the previous CA-validation trial and analysed for CA activity using the electrometric ΔpH assay (Henry, 1991). Reactions were in 6 mL of assay buffer

(described above) at 4°C using 100 µL CO_2 saturated water as a substrate and in the presence or absence of 100 µL of perfusate sample. The reaction kinetics were assessed as the time for a 0.15 unit pH change, with a GK2401C electrode and PHM84 meter (Radiometer, Copenhagen, Denmark), and absolute rates were calculated from the buffer curve of the assay buffer over the tested pH range.

2.3.6 Data analysis and statistics

All data are reported as mean ± s.e.m. and were analysed in R studio v1.0.153 (R v3.4.1) and figures were generated with the ggplot2 v.2.2.1 package (Wickham, 2009). Normality of the data was tested with the Shapiro-Wilk test and homogeneity of variances with the Levene's test ($P < 0.05$). Parametric tests typically have a higher statistical power compared to non-parametric alternatives and thus, the former were used whenever the underlying assumptions of the data distribution were met. However, in situations where data transformation could not correct issues with the parametric assumptions, statistical analysis was carried out with non-parametric tests (here and throughout the dissertation). The rate of RBC pH_i recovery was assessed by measuring changes in closed-system PO_2 and taking advantage of the pH sensitivity of Hb. PO_2 data were normalised to the initial PO_2 values within each run and expressed as a change in PO_2 over time (ΔPO_2). To ensure that the RBC pH_i recovery between the two systems were comparable, the continuous PO_2 traces from the closed system were subsampled at those times corresponding to measurements in the continuous-flow apparatus (i.e. 5, 15, 30, 60 and 120 s after short-circuiting) using LabChart v8.1.5 (ADInstruments, Dunedin, New Zealand). In the closed system, the rate of O_2 consumption ($\dot{M}O_2$) of the RBCs was determined as the average slope (mmHg s^{-1}) of the PO_2 trace (over a 40 s interval) immediately before and after treatments (injections of ISO, CA or C18). In the continuous-flow apparatus, RBC $\dot{M}O_2$ was determined as the slope of the PO_2 trace for each PO_2 sensor; all ΔPO_2 data were corrected for the average RBC $\dot{M}O_2$ over the tested period. However, no significant differences (Paired-samples t-test; $P > 0.05$) were observed between the $\dot{M}O_2$ corrected and the uncorrected data set; the $\dot{M}O_2$ corrected data are presented here. ΔPO_2 data for all treatments were analysed by fitting two non-linear models to each data set:

1) Michaelis-Menten enzyme kinetics model:

$$\Delta PO_2 = \frac{at}{(b + t)}$$

2) Hill-model:

$$\Delta PO_2 = \frac{at^b}{(c^b + t^b)}$$

Where *t* is the time after treatment, *a* represents the magnitude of the response (ΔPO_{2max}) and *b* (eqn. 1) and *c* (eqn. 2) represent the $t_{1/2}$, respectively. The fit of both models was compared with the Akaike information criterion (AIC; Akaike, 1974) and the model with the lower AIC was used for analysis. Representative models were run on the pooled data sets for each treatment and system, and these are depicted in the figures. However, for statistical analysis each individual trace was analysed as described above yielding parameter estimates $t_{1/2}$ and ΔPO_{2max} that are reported as means ± s.e.m. ($N = 15$, unless otherwise indicated). Due to the very rapid and variable ΔPO_2 after acidification (see Fig. 2-2), HCl data were not analysed by fitting a model. Therefore, $t_{1/2}$ was not determined for this treatment and ΔPO_{2max} was calculated as the difference between PO_2 at HCl injection and the maximal PO_2 after acidification. Differences in ΔPO_{2max} between treatments (HCl, ISO, CA and C18) and $t_{1/2}$ between treatments (ISO, CA and C18) in the closed system, were tested with Kruskal-Wallis one-way analysis of variance ($P < 0.05$, $N = 15$, unless otherwise indicated) and the kruskalmc function (R pgirmess package) for post-hoc analysis. Differences between initial and final blood pH and Hct ($N = 15$ in rainbow trout and $N = 6$ in white sturgeon), and differences between ΔPO_{2max} and $t_{1/2}$ between the closed and continuous-flow systems ($N = 15$) were tested with the Wilcoxon rank-sum test ($P < 0.05$). Finally, differences between Ctrl and CA treatments in the validation of the continuous-flow apparatus were tested with independent samples t-tests ($P < 0.05$, $N = 11$).

2.4 Results

2.4.1 Series 1: Closed-system experiment

In the closed-system experiment, the initial Hct of rainbow trout blood was 25.1 ± 0.7% and the experimental protocol increased Hct to 29.9 ± 0.8% ($P < 0.001$). Initial blood pH was 7.62 ± 0.02 and decreased to 7.34 ± 0.02 by the end of the experimental protocol ($P < 0.001$); a pH reduction of 0.28 ± 0.03 that was in line with the planned degree of acidification with HCl. In white sturgeon blood, Hct was 23.1 ± 0.4% and did not significantly increase due to the experimental protocol ($P = 0.750$). Blood pH in white

sturgeon decreased significantly ($P = 0.005$) from 7.63 ± 0.01 to 7.47 ± 0.02, a pH reduction of 0.15 ± 0.02.

Figure 2-2 shows representative traces from two closed-system trials on rainbow trout and white sturgeon blood respectively. In rainbow trout, the ΔPO_2 due to ISO, CA and C18 injections were analysed on 15 individual traces by fitting non-linear models to the data and representative models were fitted to the pooled data sets shown in Figure 2-3; the parameter estimates for all representative models are summarised in Table 2-1. The pooled datasets were best described by a sigmoidal Hill curve for the ISO response (Fig. 2-3A) and a Michaelis-Menten curve for the CA (Fig. 2-3B) and C18 responses (Fig. 2-3C).

Based on the analysis of individual traces, the stimulation of RBC β-NHE with ISO had a ΔPO_{2max} of -27.5 ± 3.2 mmHg with a $t_{1/2}$ of 63.9 ± 2.3 s. The addition of extracellular CA had a ΔPO_{2max} of 29.5 ± 2.0 mmHg with a $t_{1/2}$ of 12.8 ± 1.3 s. And, the inhibition of extracellular CA with C18 had a ΔPO_{2max} of -27.4 ± 2.6 mmHg with a $t_{1/2}$ of 53.5 ± 5.2 s. Significant differences were detected between the average $t_{1/2}$ for ISO, CA and C18 responses in rainbow trout (Fig. 2-4A). The ΔPO_{2max} after HCl addition was on average 46.0 ± 4.0 mmHg and significantly larger compared to the responses after ISO, CA and C18 injections, which were not significantly different from one another (Fig. 2-4B). In white sturgeon, the ΔPO_2 due to acidification was significantly lower compared to rainbow trout ($P < 0.001$) and on average 10.8 ± 1.1 mmHg; subsequent injections of ISO, CA or C18 had no effect on ΔPO_2 in white sturgeon (Fig. 2-5).

2.4.2 Series 2: Continuous-flow apparatus

In the continuous-flow apparatus, the experimental protocol increased Hct of rainbow trout blood from 23.6 ± 0.4% to 32.1 ± 0.6% ($P < 0.001$), and decreased blood pH from 7.65 ± 0.02 to 7.39 ± 0.02 ($P < 0.001$); no significant differences in pH ($P = 0.309$) or Hct ($P = 0.833$) were observed between the closed-system and the continuous-flow experiments. RBC pH_i recovery in the continuous-flow apparatus was analysed on 15 individual traces by fitting non-linear models to the data. The pooled dataset was best described by a Michaelis-Menten curve, which is depicted in Figure 2-6, with the previous closed-system RBC pH_i recovery data (C18 treatment) for comparison. RBC pH_i recovery in the continuous-flow apparatus had a $t_{1/2}$ of 17.2 ± 2.5 s and was significantly faster compared to the closed system (Fig. 2-

7A). The ΔPO_{2max} in the continuous-flow apparatus was -34.4 ± 3.0 mmHg and not significantly different from that in the closed system (Fig. 2-7B).

2.4.3 Validation of the continuous-flow apparatus

Buffer that was perfused over control fibers (Ctrl) showed a large negative ΔpH during stop-flow, indicative of an ongoing hydration of CO_2 and production of H^+s, and thus an absence of CA activity (Fig. 2-8A). However, in CA-coated fibers ΔpH was positive and significantly different from Ctrl. This is indicative of a complete hydration of CO_2 within the time the buffer was in contact with the coated fibers and a clear sign of CA catalytic activity within the glass capillary. Buffer without CO_2 loading was run as an additional control and also showed a positive ΔpH. No significant difference in CA activity was detected in perfusates from Ctrl or CA-coated fibers (Fig. 2-8B), indicating that the enzyme was appropriately bound to the fibers and did not wash out, which is an important pre-requisite for the recovery of RBC pH_i after short-circuiting. In addition, the ΔpH assay was validated by measuring a sample of the CA solution that was used to coat fibers (a positive Ctrl), which confirmed the sensitivity of the assay.

2.5 Discussion

It is critical that teleost recover RBC pH_i during venous transit. Only then, can β-NHE short-circuiting enhance Hb-O_2 unloading at the tissues during a blood acidosis, without compromising O_2 uptake at the gill; a system that increases the difference between arterial and venous Hb-O_2 saturations and therefore β_b. The transit time of blood through the venous circulation in rainbow trout may vary between 30-91 s in exercise and resting scenarios, respectively (as described above). The results of the present work place the $t_{1/2}$ of RBC pH_i recovery, after short-circuiting in a continuous-flow apparatus, at 17 s, thus providing strong evidence that β-NHE activity is rapid enough to largely recover Hb-O_2 affinity during venous transit, even under conditions of maximal exercise and a severe acidosis.

Importantly, the present study used two independent experimental systems to measure the rate of RBC pH_i recovery. The closed-system preparation has been the standard method for studying β-NHE short-circuiting in teleost RBC (Rummer and Brauner, 2011); the $t_{1/2}$ and PO_{2max} values presented here, for the activation (ISO) and the short-circuiting (CA) of β-NHE, resemble closely those of previous work. However, the slow mixing kinetics

and sensor response times in the closed system may be inadequate to accurately assess the $t_{1/2}$ of fast changes in PO_2. And in fact, the measured $t_{1/2}$ of RBC pH_i recovery was >3-fold slower in the closed system compared to a continuous-flow apparatus (Fig. 2-7A) that is robust to these confounding factors. These results lend support to the idea that the closed system overestimates $t_{1/2}$ measurements for all treatments and therefore these values will not be discussed in detail. The $t_{1/2}$ of 17 s, obtained in the continuous-flow apparatus, is considered a more accurate measure of the rate of RBC pH_i recovery after short-circuiting. All the same, closed-system results provide important insight into the magnitude of HCl, ISO, CA and C18 responses. Therefore, only combined data from the two systems can reveal both the temporal kinetics and response magnitudes that characterise the teleost system of enhanced Hb-O_2 unloading.

ΔPO_{2max} did not differ between the additions of CA and C18, indicating that the original H^+ gradient was re-established across the RBC membrane and that full recovery of pH_i was achieved after short-circuiting. Thus, based on the model of best fit (Table 2-1), it can be estimated that ΔPO_2 recovered by 64% within the venous transit time available during exercise, and by 84% at rest. ΔPO_2 is a useful proxy for qualitative changes in RBC pH_i; however, due to the non-linear relationship between ΔPO_2 and RBC pH_i the models presented here have limited predictive value to assess quantitative RBC pH_i recovery. The activity of β-NHE increases RBC pH_i and therefore shifts the oxygen equilibrium curve towards a higher Hb-O_2 affinity (left shift). Consequently, Hb binds additional O_2, Hb-O_2 saturation increases and PO_2 in the closed system decreases. However, as Hb-O_2 saturation increases, changes in Hb-O_2 affinity will have an increasingly larger effect on closed-system PO_2, but a decreasing effect on Hb-O_2 saturation. Consequently, the $t_{1/2}$ estimated based on ΔPO_2 underestimates the recovery of Hb-O_2 saturation that will be largely complete during venous transit in rainbow trout, indicating that Hb can be fully oxygenated by the time RBCs return to the gills.

Rainbow trout blood was equilibrated to gas tensions that represent venous conditions during aerobic exercise (3.8 mmHg CO_2 and 76 mmHg O_2) resulting in an extracellular pH (pH_e) of 7.63 ± 0.01 (averaged data from both systems) that corresponds closely to *in vivo* measurements (Kiceniuk and Jones, 1977; Brauner et al., 2000a). The addition of HCl further lowered blood pH_e to 7.34 ± 0.01, a value consistent with a severe metabolic acidosis that *in*

vivo may occur due to glycolytic ATP production at the exercising muscle. Final pH_e was measured at the end of the trial and after the activation of β-NHE whereby H^+ extrusion from the RBCs into the plasma will have contributed to the low pH_e measured. The initial acidification of blood by injection of HCl occurred in the absence of β-NHE activity, thus pH_e and pH_i were coupled (Heming et al., 1986) and the acidosis was transferred into the intracellular compartment by the Jacobs-Stewart cycle. The reduction in pH_i due to HCl addition caused a ΔPO_2 of 46 mmHg, a result of the high pH sensitivity of rainbow trout Hb (Bohr coefficient of -0.91; Rummer and Brauner, 2015).

The large ΔPO_2 observed after acidification of rainbow trout blood is the result of a large reduction in Hb-O_2 affinity and illustrates the dangers of a pH-sensitive Hb that, in teleosts, may fail to become fully oxygenated at the gas exchange surface. Therefore, during a blood acidosis *in vivo*, catecholamines released into the blood will activate RBC β-NHE and restore Hb-O_2 affinity; *in vitro* RBCs were stimulated with ISO at concentrations that induce maximal β-NHE activity in rainbow trout (Rummer and Brauner, 2011). The addition of ISO caused a ΔPO_{2max} of 28 mmHg (Fig. 2-4B) that was significantly smaller than the ΔPO_{2max} observed after acidification with HCl. Clearly, more H^+s entered the RBC during acidification than were subsequently removed by β-NHE activity and thus RBC pH_i was not recovered to its original set point. As secondarily-active transporters, β-NHE build up H^+ gradients across the RBC membrane that are ultimately driven by the activity of the Na^+-K^+-ATPase (NKA). As extracellular H^+s and intracellular Na^+ accumulate (Garcia-Romeu et al., 1988), β-NHE activity slows, despite an increase in NKA activity during the β-adrenergic response (Palfrey and Greengard, 1981; Bourne and Cossins, 1982; Ferguson and Boutilier, 1989). The acidification of the plasma increases the H_2CO_3 pool and thus the rate of uncatalysed HCO_3^- dehydration, accelerating the Jacobs-Steward cycle and the passive transfer of H^+s into the RBC. Eventually the apparent H^+ fluxes of the β-NHE and the Jacobs-Stewart cycle become equal, and net H^+ extrusion comes to a halt. Thus, the degree of pH_i recovery will depend on trans-membrane ion gradients and NKA activity, as well as the buffer capacities of the intra- and extracellular media. Teleost Hb, the primary buffer within RBCs, typically has a low buffer capacity thus minimising the amount of H^+s that need to be extruded for a given change in pH_i (Nikinmaa, 1997). Under severely acidotic conditions, such as those induced here, high extracellular H^+ concentrations may set thermodynamic

limits upon β-NHE activity (Parks et al., 2008) limiting complete recovery of pH_i. The maximal H^+ gradient that can be produced by β-NHE activity may be rather constant for a given species' blood characteristics. And this H^+ gradient will dictate the maximal ΔpH_{a-v} that can be created upon β-NHE short-circuiting to enhance Hb-O_2 unloading.

A critical finding of the present work was that the stimulation of β-NHE with ISO, the short-circuiting with CA and RBC pH_i recovery after addition of C18, produced similar ΔPO_{2max} (Fig. 2-4B). This indicates that the entire H^+ gradient across the RBC membrane that was initially established by β-NHE activity is available to enhance ΔpH_{a-v} and thus Hb-O_2 unloading at the tissues of teleosts, where paCA is present. In addition, when RBCs leave the site of paCA activity, β-NHE activity can re-establish the original H^+ gradient that was present before short-circuiting. Importantly, this indicates that Hb-O_2 loading at the gill is protected, but also that the same H^+ gradient is available for renewed short-circuiting, when RBCs reach the tissues once again. Thus, potentially, RBCs in rainbow trout, can undergo repeated cycles of short-circuiting and recovery with every pass through the circulation, and thus maintain an elevated β_b during the adrenergic response.

Maximally stimulating the RBC β-NHEs with ISO resulted in a time lag before changes in PO_2 were observed; thus, these data were best described by a sigmoidal Hill model that can accommodate a delayed response onset (Fig. 2-3A). The mechanism underlying the delayed ISO response is likely found in the excitation cascade that translates the stimulation of the RBC β-adrenergic receptor by catecholamines into an activation of the transporter, which involves adenylate cyclase and the accumulation of cAMP as a secondary messenger (Mahe et al., 1985; Nikinmaa, 1990). In contrast, the inhibition of CA by C18 was best described by a Michaelis-Menten curve indicating the absence of a time lag (Fig. 2-3C). Conceptually, the addition of CA to stimulated RBCs will accelerate the Jacobs-Steward cycle and abolish the H^+ gradient across the RBC membrane, despite ongoing β-NHE activity. And clearly, the large decrease in closed-system PO_2, caused by C18 addition, is evidence that β-NHE activity is ongoing but futile, in the presence of CA. Therefore, β-NHEs are already activated when blood leaves the capillaries after short-circuiting and this may be of physiological significance, as it will allow for a rapid onset of pH_i recovery.

In the continuous-flow apparatus, the recovery of RBC pH_i was about 3-fold faster compared to the closed system, which likely overestimated the $t_{1/2}$ of all treatments. If CA

short-circuiting was confounded to a similar degree, then the $t_{1/2}$ was likely in the order of 4 s (3-fold faster than the measured $t_{1/2}$ = 12.8 s). This is perhaps a conservative estimate, as confounding factors may have a relatively larger contribution to the measured $t_{1/2}$ of fast responses (CA) compared to slower ones (ISO, C18); and evidence from other studies indicates that the short-circuiting of β-NHE activity by CA is likely an extremely rapid process. The addition of extracellular CA to blood catalyses the CO_2-HCO_3^- reactions in the plasma, which due to the extremely high turnover rate of the enzyme (human CA2 at 25°C, $k_{cat} = 10^6$ s^{-1}) can be considered instantaneous in physiologically relevant time-scales (Khalifah and Silverman, 1991; Forster and Dodgson, 2000), and the same applies to intracellular CO_2-HCO_3^- reactions that are catalysed by the RBC CA pool (Maren, 1967). Therefore, the intracellular acidification of RBCs in the presence of paCA is likely rate limited by the diffusion of CO_2 across the RBC membrane, a rapid process with a $t_{1/2}$ of <1 ms (at 37°C in human RBCs; Forster, 1969; Swenson and Maren, 1978). In fact, when extracellular CA (0.02 g L^{-1}) was added to eel RBC suspended in a solution that was in pH disequilibrium the resulting Bohr shift and release of O_2 from Hb (ΔPO_2 19 mmHg), had a $t_{1/2}$ of ~0.5 s (Forster and Steen, 1969). Thus, without venturing into an exact estimate of the $t_{1/2}$ of CA short-circuiting in rainbow trout, the combined data from both systems, and data from previous work, indicate that it may be sufficiently rapid to enhance Hb-O_2 unloading during capillary transit (<4 s; Honig et al., 1977; Maren and Swenson, 1980), which is in line with the results from *in vivo* work on rainbow trout (Rummer et al., 2013).

To demonstrate that the large changes in PO_2 observed here were, in fact, a direct consequence of the H^+ gradients established by β-NHE activity, a second set of experiments were run on white sturgeon, a species that lacks a RBC β-NHE (Berenbrink et al., 2005). The activation of β-NHE leads to the intracellular accumulation of Na^+ and Cl^- that causes osmotic swelling of RBCs and thus an increase in Hct. In rainbow trout blood, the addition of ISO increased Hct from 24.4 ± 0.4 to 31.0 ± 0.5%, which is in line with results from previous work (Caldwell et al., 2006; Rummer and Brauner, 2011; Shu et al., 2017). As expected, white sturgeon RBC showed no β-adrenergic swelling and Hct was unaffected by ISO addition. The evolution of β-NHE activity in teleosts correlates with the advent of highly pH-sensitive Hbs (Berenbrink et al., 2005). White sturgeon have a moderate Root effect, however, that is only expressed at low pH values that are likely never encountered in the

circulation *in vivo* (Regan and Brauner, 2010b); thus in white sturgeon, β-NHE activity is not vital to protect Hb-O_2 loading at the gills. Acidification of white sturgeon blood, to a similar degree as in rainbow trout, resulted in a ΔPO_2 of only 11 mmHg (compared to 46 mmHg in rainbow trout); a results of the lower pH sensitivity of white sturgeon Hb (Bohr coefficient -0.4; Burggren and Randall, 1978; Crocker and Cech, 1998). Thus, in white sturgeon, the potential benefits of the Bohr effect for Hb-O_2 unloading are limited by the low pH sensitivity of Hb, and by the absence of RBC β-NHE that, when short-circuited at the tissues, create large ΔpH_{a-v} that magnify the Bohr effect in teleosts.

It has been proposed that other transporters on the RBC membrane may create H^+ gradients that can be short-circuited in the presence of CA (Rummer et al., 2010; Rummer and Brauner, 2011). The idea that other taxa, which lack β-NHE, may benefit from a similar mechanism of enhanced Hb-O_2 unloading is intriguing, and may bear consequences for our understanding of O_2 transport in all vertebrates (Brauner and Harter, 2017). However, in white sturgeon, the absence of a ΔPO_2 after CA addition (Fig. 2-2 and 2-5) indicates that H^+s were distributed passively across the RBC membrane and refutes the idea that other, non-adrenergic, transporters may create H^+ gradients that are available to enhance Hb-O_2 unloading. Likewise, in rainbow trout, the addition of a specific NHE inhibitor (EIPA) abolished the ΔPO_2 that is otherwise observed after the addition of CA to stimulated RBCs, thus corroborating an involvement of an NHE in this response (Rummer and Brauner, 2011). Based on these results, it appears that short-circuiting of H^+ gradients across the RBC membrane is a mechanism that is exclusive to the teleost clade, through a mechanism that requires β-NHE activity and a heterogeneous distribution of paCA in the circulation; conditions that are not met by most vertebrates. An acidosis, such as that induced by the injection of HCl, or a moderate pH sensitivity of Hb, such as observed in white sturgeon, do not appear to be sufficient to enhance Hb-O_2 unloading in the presence of paCA.

In the closed system, the addition of CA to stimulated RBCs caused an increase in PO_2 of 30 mmHg, which is in line with a previous study that found a ΔPO_2 of 25 mmHg under similar conditions (Rummer and Brauner, 2011). These reproducible findings add to an increasing body of literature that illustrates the great potential for β-NHE short-circuiting to enhance Hb-O_2 unloading in teleosts. This mechanism may explain why rainbow trout can sustain higher red-muscle PO_2 compared to mammals, even during spontaneous struggling,

exercise or hypoxia (McKenzie et al., 2004); or why stressed striped bass (*Morone saxatilis*) have arterial PO_2 that are higher than those in water (Nikinmaa et al., 1984). When paCA in the circulation was inhibited with C18 *in vivo*, red-muscle PO_2 in hypercapnic rainbow trout decreased significantly, indicating a role of paCA in maintaining elevated tissue PO_2 (Rummer et al., 2013). Similarly, the injection of C18 into the circulation of swimming Atlantic salmon either resulted in collapse, or at lower speeds, in a large compensatory increase in $\dot{Q}$, indicating that enhanced Hb-O_2 unloading, mediated by paCA, facilitates O_2 transport during exercise (Chapter 3). In the heart of coho salmon, paCA activity may enhance Hb-O_2 unloading to the spongy myocardium that relies exclusively on the O_2-depleted venous return (Alderman et al., 2016). And more broadly, recent work indicates that β-NHE short-circuiting may be important for migratory salmonids and perhaps teleosts in general (Shu et al., 2017), potentially enhancing Hb-O_2 unloading by over 70% compared to what mammals can achieve with their moderate Bohr effect alone (Rummer and Brauner, 2015).

In conclusion, the present study provides strong evidence that in rainbow trout: i) the rate of RBC pH_i recovery after short-circuiting is sufficiently rapid to largely restore Hb-O_2 affinity during venous transit; ii) the short-circuiting of β-NHE activity by paCA may be sufficiently rapid to enhance Hb-O_2 unloading during capillary transit; and iii) after short-circuiting, RBCs re-establish the initial H^+ gradient across the membrane, which protects O_2 loading at the gills, and sets the system up for renewed short-circuiting. Thus, potentially, β-NHE short-circuiting may enhance Hb-O_2 unloading with every pass through the circulation and maintain an elevated β_b during an adrenergic response. In addition, based on the results on white sturgeon, we found no indications that other, non-adrenergic transporters create H^+ gradients across the RBC membrane that can be short-circuited to enhance Hb-O_2 unloading. The temporal kinetics of β-NHE short-circuiting and RBC pH_i recovery corroborate that a system of enhanced Hb-O_2 unloading is not only feasible, but likely a major pillar of the teleost O_2-transport system. Thus, approximately half of all vertebrates may enhance β_b by selectively creating and eliminating H^+ gradients across the RBC membrane.

Table 2-1 Non-linear models of best fit and parameter estimates for changes in closed-system PO_2 of rainbow trout whole blood. Treatments were stimulation with isoproterenol (ISO), short-circuiting with extracellular carbonic anhydrase (CA) and recovery of red blood cell intracellular pH after inhibition of CA with C18 in a closed system, or absence of CA in a continuous-flow apparatus.

Treatment	Parameter estimates			Model
Closed system	a	b	c	
ISO	-27.64 ± 1.71***	2.50 ± 0.392***	63.65 ± 4.74***	Hill-model
CA	31.84 ± 3.48***	13.02 ± 3.88**		Michaelis-Menten
C18	-33.12 ± 6.23***	71.84 ± 26.75**		Michaelis-Menten
Continuous-flow apparatus	-36.13 ± 2.89***	16.97 ± 4.53***		Michaelis-Menten

Significance level of parameter estimates indicated as $P <$ ***0.001, **0.01

Michaelis-Menten enzyme kinetics model: $\Delta PO_2 = \frac{at}{(b+t)}$; Hill-model: $\Delta PO_2 = \frac{at^b}{(c^b+t^b)}$; where t is the time after treatment, a represents the magnitude of the response (ΔPO_{2max}) and b (Michaelis-Menten) and c (Hill-model) represent the $t_{1/2}$, respectively.

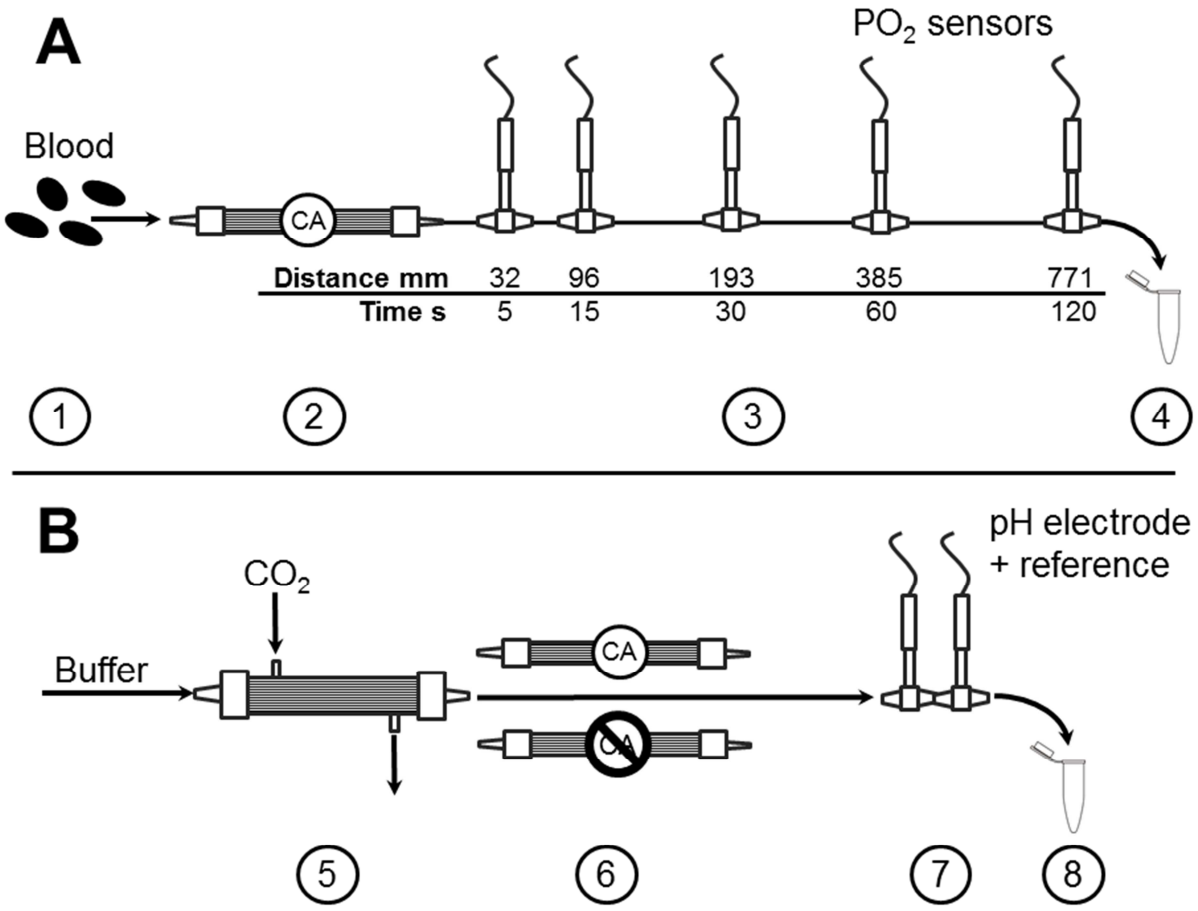

A
PO2 sensors
Blood
CA
Distance mm 32 96 193 385 771
Time s 5 15 30 60 120
1
2
3
4
B
pH electrode
+ reference
CO2
Buffer
CA
CA
5
6
7
8

Figure 2-1 Schematic and detailed description of the continuous-flow apparatus. A) 1) Blood from cannulated rainbow trout was equilibrated in tonometers, acidified and β-adrenergically stimulated with isoproterenol, a β-agonist. 2) Blood was perfused (0.2 mL min^{-1}) through a glass capillary containing carbonic anhydrase (CA)-coated polymethyl-pentene (PMP) fibers (Kaar et al., 2007; Arazawa et al., 2012). In the presence of CA, red blood cell (RBC) sodium-proton exchangers (β-NHE) are short-circuited, leading to a decrease in RBC intracellular pH (pH_i). 3) When blood leaves the site of CA, β-NHE activity recovers RBC pH_i which is reflected in a change in the partial pressure of O_2 (ΔPO_2), due to the pH sensitivity of haemoglobin-O_2 binding in rainbow trout. By placing fiberoptic PO_2 sensors at fixed distances from the site of short-circuiting, the ΔPO_2 response was re-constructed using the readings from all sensors, each corresponding to a time-point in the response. 4) A blood sample was collected for measurements of final Hct and pH. B) Setup for validation of the continuous-flow apparatus. 5) Buffer was continuously (1 mL min^{-1}) equilibrated to 38 mmHg CO_2 in N_2 in a hollow-fiber gas exchanger, to create a pH disequilibrium (at 12°C). 6) The buffer in disequilibrium was immediately perfused through a glass capillary containing CA-coated or un-coated (Ctrl) fibers. 7) Buffer pH was measured downstream with a flow-through pH microelectrode after stopping the flow. The change in pH (ΔpH) during stop-flow is indicative of the residual pH disequilibrium in the buffer; an absence of a ΔpH indicates the presence of CA catalytic activity in the system. 8) Perfusate buffer was collected for the analysis of CA activity that was washed-out from the coated fibers, using the electrometric ΔpH assay (Henry, 1991).

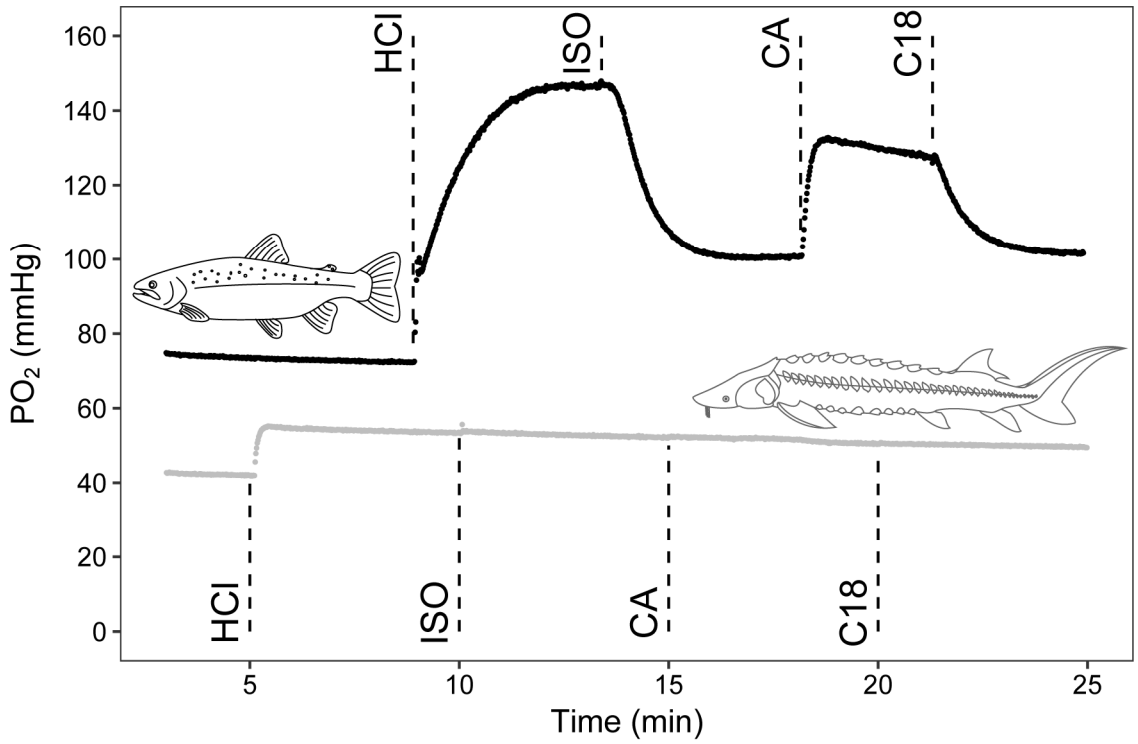


Figure 2-2 Representative trace of changes in the closed-system partial pressure of oxygen (PO_2; mmHg) in blood from rainbow trout and white sturgeon. Rainbow trout are indicated in black and white sturgeon in grey. Whole blood (Hct = 25%) was sampled from cannulated animals at rest and equilibrated in tonometers to conditions that mimic venous blood: 76 mmHg (rainbow trout) or 38 mmHg O_2 (white sturgeon), 3.8 mmHg CO_2 in N_2 at 12°C. Blood was loaded into a 2 mL vial as described previously (Rummer and Brauner, 2011). Dashed lines indicate injections (through a sealed septum) of: i) HCl to acidify the sample by ~0.3 pH units; ii) isoproterenol (ISO), a synthetic β-agonist at 0.01 mM; iii) carbonic anhydrase (CA) at 10^{-3} mM; and iv) a membrane-impermeable CA inhibitor (C18) at 0.2 mM.

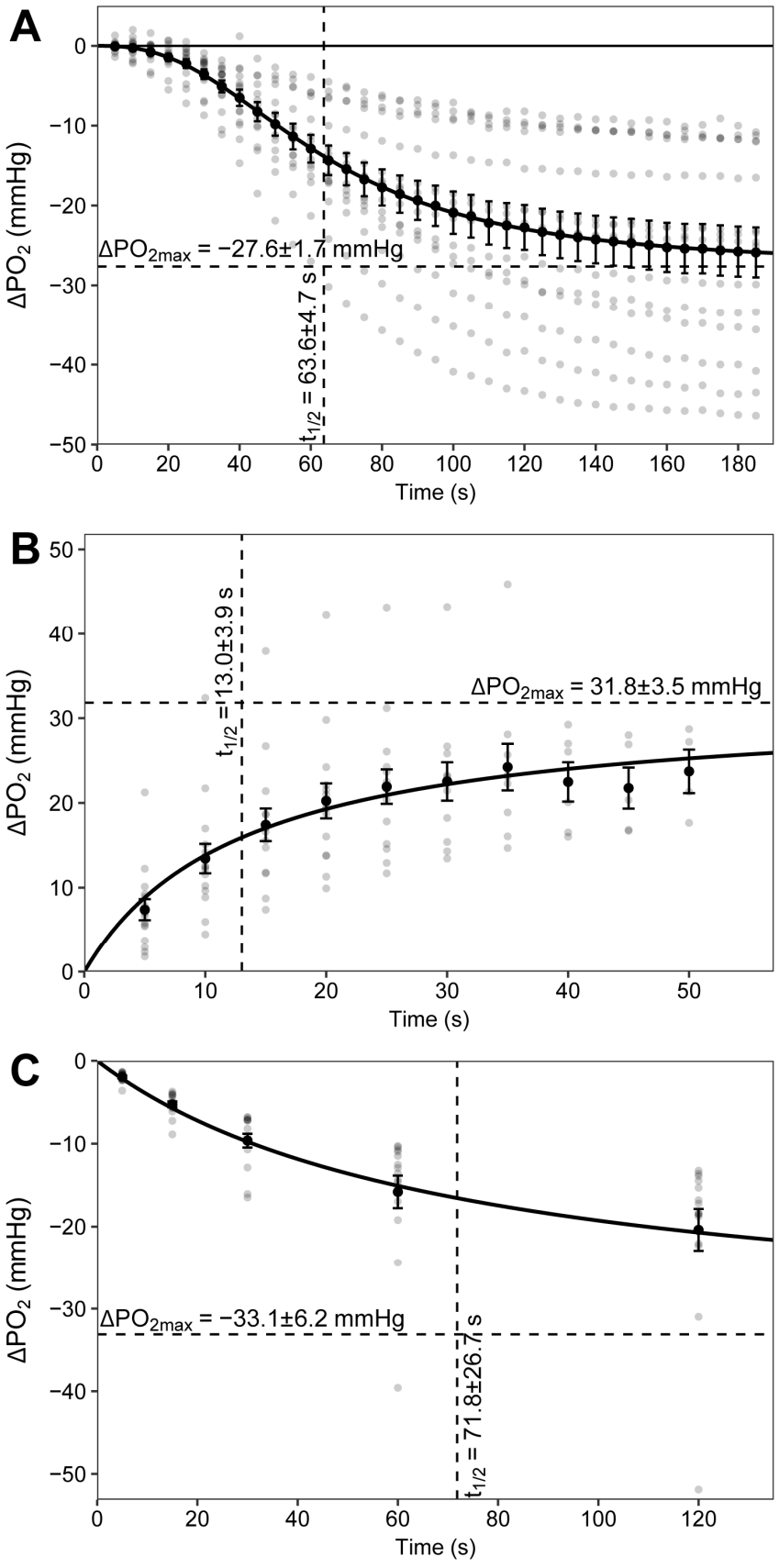

A
ΔPO2 (mmHg)
ΔPO2max = −27.6±1.7 mmHg
t1/2 = 63.6±4.7 s
Time (s)
B
ΔPO2 (mmHg)
t1/2 =13.0±3.9 s
ΔPO2max = 31.8±3.5 mmHg
Time (s)
C
ΔPO2 (mmHg)
ΔPO2max = −33.1±6.2 mmHg
t1/2 = 71.8±26.7 s
Time (s)

Figure 2-3 Closed-system change in the partial pressure of oxygen (ΔPO_2; mmHg) in rainbow trout blood. Measurements were in whole blood (see Fig. 2-2 caption for blood preparation) after injections of: A) isoproterenol (ISO, 0.01 mM), a synthetic β-agonist; B) carbonic anhydrase (CA, 10^{-3} mM); or C) a membrane-impermeable CA inhibitor (C18, 0.2 mM). Individual data points are indicated by light circles and means ± s.e.m. (N = 15) by dark circles, and the models of best fit are indicated by continuous lines (Hill-model for ISO, Michealis-Menten models for CA and C18). The response halftime ($t_{1/2}$) and magnitudes (ΔPO_{2max}) are indicated by vertical and horizontal dashed lines as means ± s.e.m., and were determined from the models fitted to the pooled data (these do not exactly resemble the parameter estimates used for statistical analysis that represent averages of individually analysed traces).

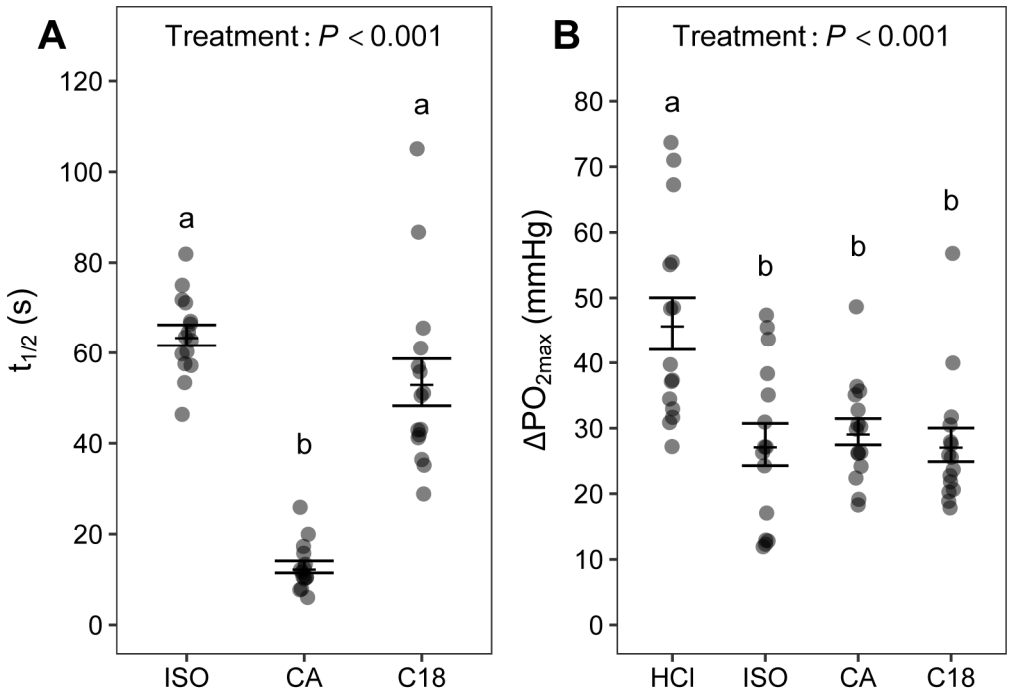


Figure 2-4 A) Halftimes ($t_{1/2}$; s) and B) magnitudes (ΔPO_{2max}; mmHg) of a closed-system change in the partial pressure of oxygen (ΔPO_2) in rainbow trout blood. Measurements were in whole blood (see Fig. 2-2 caption for blood preparation), after the addition of isoproterenol (ISO, 0.01 mM), carbonic anhydrase (CA, 10^{-3} mM) and a membrane-impermeable CA inhibitor (C18, 0.2 mM). Individual data points are indicated by circles with means ± s.e.m.. The $t_{1/2}$ and ΔPO_{2max} were determined by fitting non-linear models to individual traces. Significant main effects of treatment were detected for $t_{1/2}$, but not for ΔPO_{2max} as determined with a non-parametric Kruskal-Wallis test ($P < 0.05$, $N = 15$), significant differences between treatments are indicated by different superscript letters.

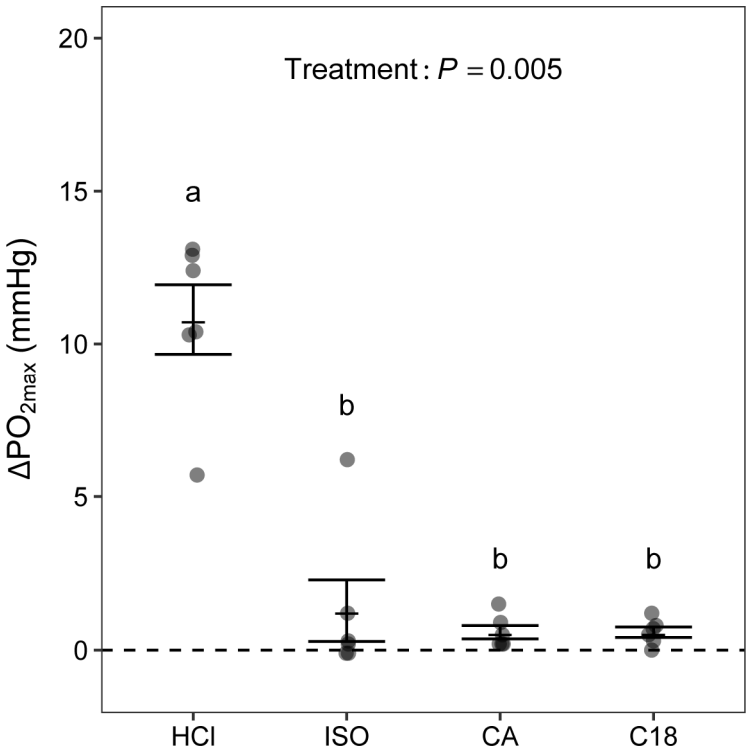


Figure 2-5 Magnitude (ΔPO_{2max}; mmHg) of a closed-system change in the partial pressure of oxygen (ΔPO_2) in white sturgeon blood. Measurements were in whole blood (see Fig. 2-2 caption for blood preparation), after the addition of HCl to acidify the sample by ~0.3 pH units, isoproterenol (ISO, 0.01 mM), carbonic anhydrase (CA, 10^{-3} mM) and a membrane-impermeable CA inhibitor (C18, 0.2 mM). Individual data points are indicated by circles with means ± s.e.m.. A significant main effect of treatment was detected with a non-parametric Kruskal-Wallis test ($P < 0.05$, $N = 15$), significant differences between treatments are indicated by different superscript letters.

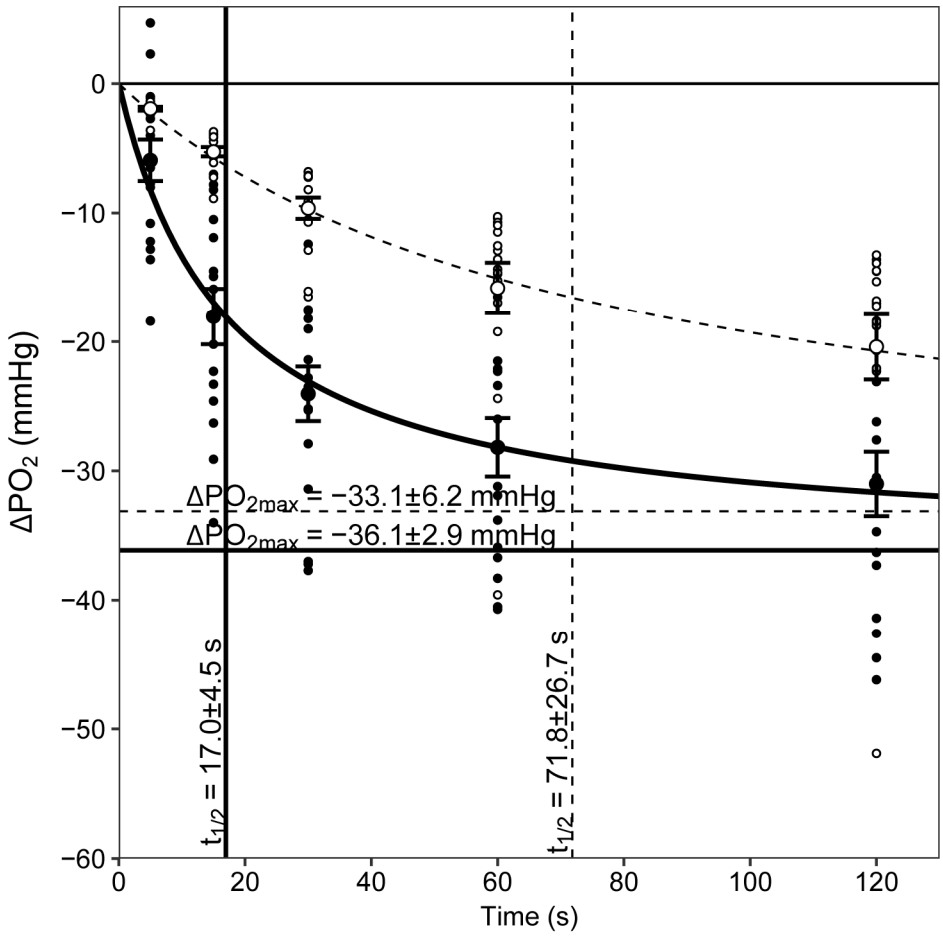


Figure 2-6 The rate of red blood cell (RBC) intracellular pH (pH_i) recovery in rainbow trout blood. The partial pressure of oxygen (ΔPO_2; mmHg) was used to indicate changes in RBC pH_i, in whole blood (see Fig. 2-2 caption for blood preparation), using two different methods. Results from the continuous-flow apparatus, after blood has left the site of CA activity (see Fig. 2-1A), are shown as solid points and lines. The previous results from the closed system (see Fig. 2-3C, C18 treatment) are shown as open circles and dashed lines, allowing for a direct comparison. Individual data points and means ± s.e.m. are indicated by solid and open circles for the two methods respectively. The response halftime ($t_{1/2}$) and magnitudes (ΔPO_{2max}) are indicated by vertical and horizontal lines as means ± s.e.m., and were determined by fitting a non-linear model (Michaelis-Menten) to the pooled data sets (*N* = 15).

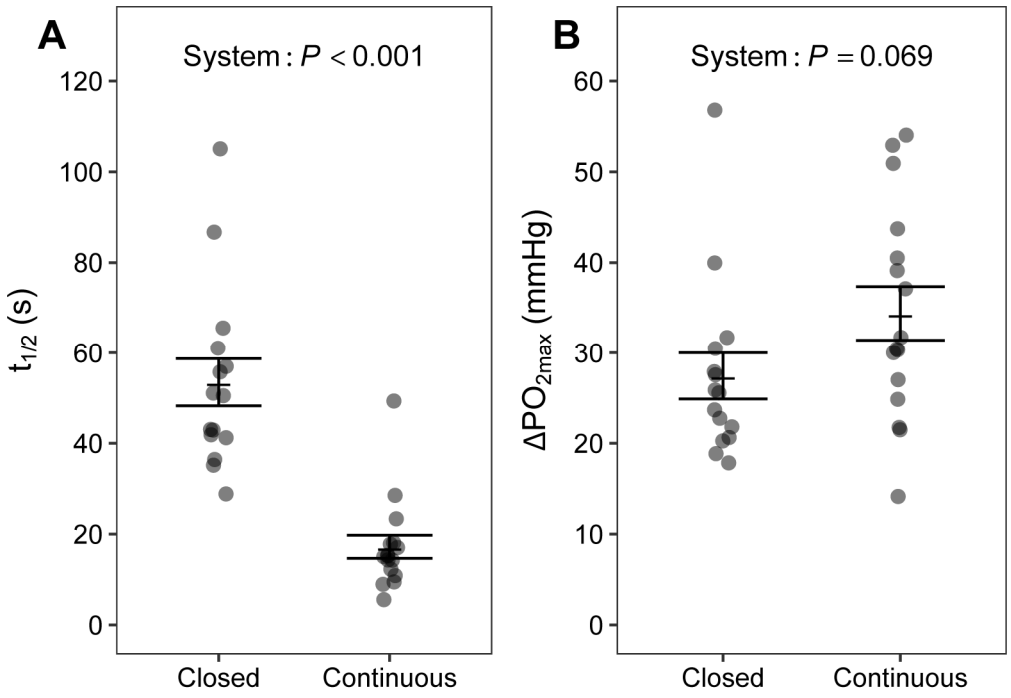


Figure 2-7 A) Halftimes ($t_{1/2}$; s) and B) magnitudes (ΔPO_{2max}; mmHg) of red blood cell (RBC) intracellular pH (pH_i) recovery in rainbow trout blood. The partial pressure of oxygen (ΔPO_2, mmHg) was used to indicate changes in RBC pH_i. Measurements were on whole blood (see Fig. 2-2 caption for blood preparation) in a closed-system preparation after addition of a membrane-impermeable CA inhibitor (C18, 0.2 mM), and in a continuous-flow apparatus after blood left the site of CA activity. Individual data points are indicated by circles with means ± s.e.m.. The $t_{1/2}$ and ΔPO_{2max} were determined by fitting non-linear models to individual traces ($N = 15$). Significant main effects of system were detected for $t_{1/2}$, but not for ΔPO_{2max} with a non-parametric Kruskal-Wallis test ($P < 0.05$, $N = 15$).

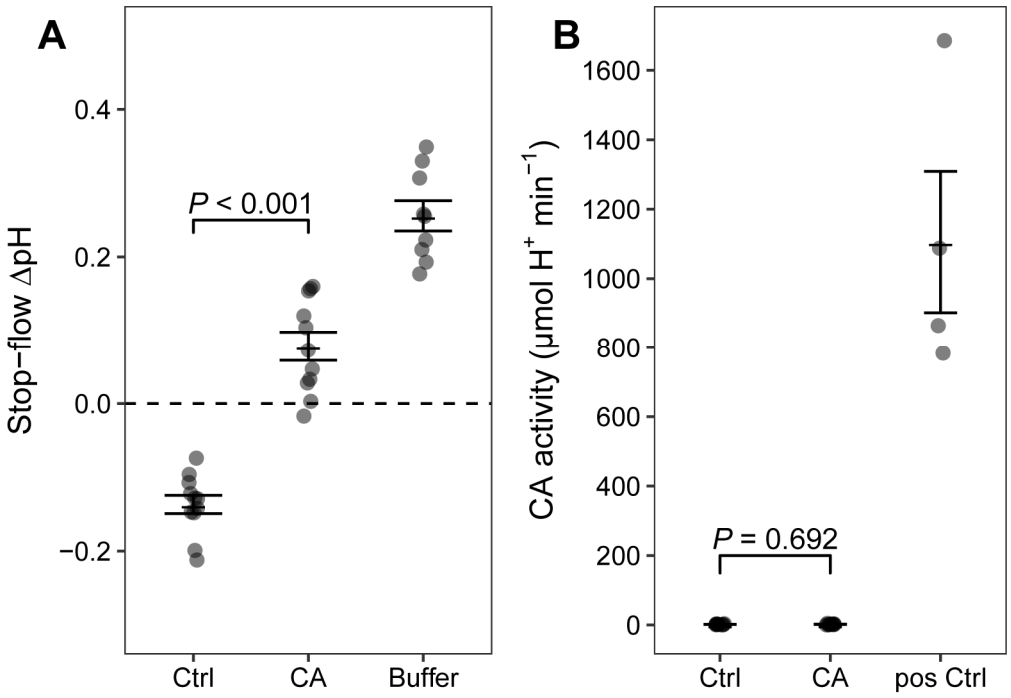


Figure 2-8 Validation of the continuous-flow apparatus and the coating of polymethylpentene (PMP) fibers with carbonic anhydrase (CA). A) Validation of CA activity in uncoated (Ctrl) and CA-coated fibers fitted into a glass capillary that was perfused (1 mL min^{-1}) with a buffer in pH disequilibrium; changes in pH (ΔpH) were measured during periods of stopped flow with a pH microelectrode (see Fig. 2-1B for description of the setup). The system was also perfused with buffer alone (Buffer) to account for the effects of flow on pH measurements. Individual data points are indicated by circles with means ± s.e.m.. Differences between treatments were tested with an independent samples t-test ($P < 0.05$, $N = 11$). B) CA activity (µmol H^+ min^{-1}) measured with the electrometric ΔpH assay (Henry, 1991) on perfusate buffers collected from the previous validation experiment. Differences in CA activity between treatments were tested with an independent samples t-test ($P < 0.05$, $N = 11$). No significant difference in CA activity was detected between perfusates from CA and Ctrl fibers, thus indicating that no CA wash-out occurred from coated fibers. The assay was validated by running positive controls (pos Ctrl) on the CA solution used for coating the fibers (1 mg mL^{-1} bovine CA2).

Chapter 3: The importance of plasma-accessible carbonic anhydrase for enhancing tissue oxygen extraction during exercise in Atlantic salmon

3.1 Synopsis

A successful spawning migration in salmon depends on their amazing athletic ability and thus, on their specialised mode of cardiovascular oxygen (O_2) transport. Teleost fishes may enhance oxygen (O_2) unloading at their tissues by an active cellular mechanism at the level of the red blood cell (RBC) that is modulated by a heterogeneous distribution plasma-accessible carbonic anhydrase (paCA) in the vasculature. By actively coupling and uncoupling RBC intracellular pH (pH_i) from the pH in the blood plasma (pH_e), teleosts can greatly increase the partial pressure at which O_2 is unloaded at the tissues, allowing for a greater extraction of O_2 from the blood. Thus, we hypothesised that paCA allows teleosts to sustain a higher rate of O_2 consumption relative to cardiac output ($\dot{M}O_2/\dot{Q}$). Elevated $\dot{M}O_2/\dot{Q}$ values have been reported previously, after hypoxia acclimation in several teleosts species. Therefore, to assess the role of paCA in O_2 transport, Atlantic salmon were acclimated to normoxia or hypoxia (40% air saturation for > 6 weeks) and injected with a membrane-impermeable CA inhibitor (C18) at rest and during moderate exercise in a swim tunnel. Results show that: 1) paCA plays a role in enhancing tissue O_2 extraction *in vivo* and this reduces cardiac work by a third; 2) the recruitment of paCA is plastic and increases following hypoxia acclimation; and 3) maximal exercise performance in Atlantic salmon is not possible without paCA. All salmonids and most teleost species may rely on paCA to enhance tissue O_2 extraction, a finding that would profoundly change our understanding of O_2 transport in half of all vertebrates.

3.2 Introduction

The Bohr effect of vertebrate haemoglobins (Hb) can enhance the capacitance of blood for O_2 (β_b) by right-shifting the oxygen equilibrium curve (OEC) at the tissues (Bohr et al., 1904), which increases the difference in O_2 content between arterial and venous blood. An increase in β_b enables a higher flux of O_2 from the water to the mitochondria per unit of blood flow, because the available O_2 carrying capacity is utilised to a larger extent. However, in most vertebrates, the Bohr effect does not enhance Hb-O_2 unloading by more than ~2% (Rummer and Brauner, 2015), and this limitation is related to the moderate pH sensitivity of Hb and the small arterial-venous pH shift (ΔpH_{a-v}) that can be realised at the tissues. In contrast, teleosts Hbs typically have large Bohr coefficients, and often a Root effect that prevents Hb from becoming fully saturated at low pH, even when the partial pressure of O_2 (PO_2) is above atmospheric levels (Root, 1931; Scholander and Van Dam, 1954). In addition, the *retia mirabilia*, specialised vascular structures in teleosts (Steen, 1963; Kobayashi et al., 1990), create large ΔpH_{a-v} by acidifying the blood, and generate the high PO_2 required to fill the swimbladder at depth and to supply the avascular retina with O_2 (Wittenberg and Wittenberg, 1974). By recycling the acidosis in counter-current vascular systems, teleosts *retes* ensure that blood pH does not decrease on a systemic level. This is important, as an expression of the Root effect at the gills would compromise O_2 loading into the blood and rapidly lead to hypoxemia; therefore, the benefits of the Root effect are restricted to those few organs that have *retes*.

Recently, however, a novel mechanism has been described (Rummer et al., 2013) that may extend the benefits of pH-sensitive Hb-O_2 unloading to all tissues in teleosts (see detailed description in Fig. 1-4). In brief, during a blood acidosis, such as may be experienced during exercise or hypoxia, teleosts protect RBC intracellular pH (pH_i) by activating adrenergically stimulated sodium-proton exchangers (β-NHE) on the RBC membrane that generate a H^+ gradient across the RBC membrane. However, in the presence of plasma-accessible carbonic anhydrase (paCA), the enzyme that catalyses CO_2-HCO_3^- reactions, the H^+ gradient collapses and β-NHE activity is effectively short-circuited (Motais et al., 1989; Rummer and Brauner, 2011). Thus, at the gills of teleosts where paCA is absent (reviewed by Harter and Brauner, 2017), RBC β-NHE activity will uncouple pH_i from pH_e and Hb-O_2 affinity is protected during an acidosis.Whereas at the tissue capillaries, where paCA is present (Henry et al., 1997a; Wang et al., 1998; Randall et al., 2014), pH_e and pH_i become coupled and the extracellular acidosis is

rapidly transferred into the RBC, releasing O_2 from Hb. Subsequently, as RBCs leave the capillaries and the site of paCA, β-NHE activity will recover pH_i in the venous system and enable renewed O_2 loading at the gills (Chapter 2). Therefore, analogous to the situation in the teleost *retes*, β-NHE activity and the heterogeneous distribution of paCA can create large ΔpH_{a-v} that are localised to the tissue capillaries, and greatly enhance tissue O_2 extraction via the Bohr effect.

Conceptually, a system of β-NHE short-circuiting will increase β_b, allowing teleosts to transport more O_2 per unit of blood flow, and thus to sustain a higher rate of O_2 consumption ($\dot{M}O_2$) per unit of cardiac output ($\dot{Q}$). This observation has been made in hypoxia-acclimated teleosts that were unable to increase $\dot{Q}$ to the same extent as normoxia-acclimated conspecifics (Petersen and Gamperl, 2010b), but attained the same aerobic performance, in terms of maximal $\dot{M}O_2$ ($\dot{M}O_{2max}$), critical swimming speed (U_{crit}) and critical thermal maximum (CT_{max}; Petersen and Gamperl, 2010b; Petersen and Gamperl, 2011; Motyka et al., 2017). Therefore, we **tested the hypothesis that teleosts enhance β_b, at least in part, by short-circuiting β-NHE and that this response is increased after hypoxia-acclimation.** To this end, Atlantic salmon were acclimated to normoxia or hypoxia (40% air saturation for > 6 weeks), and then exercised in a swim tunnel at a constant speed and $\dot{M}O_2$. If paCA is part of the mechanism that allows hypoxia-acclimated fish to sustain a higher $\dot{M}O_2/\dot{Q}$, we predicted that an inhibition of this CA pool would result either in a decrease in $\dot{M}O_2$ and thus a failure to sustain swimming, or in a compensatory increase in $\dot{Q}$, to maintain $\dot{M}O_2$ at the respective swimming velocity. In fact, our results show conclusively that paCA plays a role in O_2 transport in teleosts; and for the first time this phenomenon is demonstrated *in vivo*, and in a situation with functional and ecological relevance.

3.3 Material and Methods

3.3.1 Animals and holding conditions

Atlantic salmon (*Salmo salar*, Linnaeus 1758) were obtained from Northern Harvest Sea Farms (Saint George, NB, Canada), PIT-tagged, and kept at the Ocean Science Centre (Memorial University of Newfoundland; MUN) for 6 months before experiments. Seventy animals were weighed (average initial mass 654 ± 10 g) before being transferred into one of two 1.2 m^2 tanks that were supplied with flow-through seawater at 12°C. All animals were held for two weeks at a water PO_2 corresponding to air-saturation (~160 mmHg or 21 kPa O_2) and were fed commercial trout pellets to satiation. Thereafter, the PO_2 in one of the two tanks was lowered

to 40% air saturation, over the course of three weeks. Water PO_2 was controlled by decreasing the water flow into the hypoxic tank and by automatically bubbling N_2 into the tank when PO_2 increased over a pre-determined threshold. Despite the lower water flow, there were no evident differences in water quality between the normoxia and hypoxia tanks (assessed as water [ammonia], [nitrite] and pH). Once the target PO_2 was reached, animals were acclimated to 40% air saturation for six weeks before experiments; therefore, depending on when fish were used, acclimation time ranged from 6-10 weeks. Over this period, average water PO_2 and temperature were 41.6 ± 0.2 and 102.8 ± 0.3% air saturation, and 11.5 ± 0.1 and 11.6 ± 0.1°C, for the hypoxia- and normoxia-acclimated groups respectively. Feeding levels in both groups were matched by first feeding animals in the hypoxic group, recording their feed intake, and then feeding the same amount to the normoxic group. Fish holding and all experimental procedures were in strict compliance with the guidelines of the Canadian Council on Animal Care (CCAC) and were approved by the MUN Institutional Animal Care Committee (Protocol 17-94-KG).

3.3.2 Surgery and recovery

Atlantic salmon were anaesthetised in seawater containing 0.10 g L^{-1} tricaine methanesulfonate (MS-222). Once unresponsive, animals were measured for final weight and length and transferred to a surgery table where their gills were continuously irrigated with oxygenated and chilled water (12°C) containing a maintenance dose of 0.05 g L^{-1} MS-222. A catheter (PE 50; BD Intramedic, Franklin Lakes, NJ) was chronically implanted into the dorsal aorta according to Soivio et al. (1975). Thereafter, a Doppler flow probe (Cuff-type transducer model ES, ID = 1.3-1.8 mm, frequency = 20 MHz; Iowa Doppler Products, Iowa City, IA, USA) was fitted around the ventral aorta just outside of the pericardium (Motyka et al., 2017), and the DA cannula and Doppler flow-probe lead were sutured to the fish just anterior to the dorsal fin.

After surgery, fish were quickly recovered in aerated seawater without anesthetic. Once equilibrium was re-gained, they were placed individually in respirometry chambers that were connected to a recirculation system that was continuously flushed with aerated seawater at 12°C. Fish were allowed to recover from surgery for 48 h, and all cannulas were flushed twice per day with heparinised saline (0.9% NaCl and 50 I.U. Na-Heparin, Sigma H3149) to prevent clotting. In the evening before the swim trials, fish were lightly anaesthetised (in 0.05 g L^{-1} MS-222) and transferred to a 81 L Blazka-type swim tunnel (University of Waterloo, Biotelemetry Institute, Waterloo, ON, Canada) that is described in detail by (Petersen and Gamperl, 2010a). The tunnel

was supplied with recirculating seawater from a temperature-controlled and aerated reservoir at 10 L min^{-1}, which kept water in the tunnel at 95-100% air-saturation and at 12°C. After a short recovery period, water flow in the swim tunnel was gradually increased to 1 body length per second (bl s^{-1}) and the fish were "conditioned" to swim in the mid-front section of the tunnel for 10 min; if the salmon rested against the back end of the tunnel, an electrified grid was engaged very briefly (5 V, 0.2 A) to entice the fish to continue swimming. In addition, the front section of the tunnel was shaded and provided a refuge that the salmon generally preferred over the exposed back half of the tunnel. Preliminary experiments showed that "conditioned" fish swam more readily during the experiments on the next day. After this short episode of mild exercise, water velocity in the swim tunnel was decreased to 10 cm s^{-1}, which allowed the salmon to rest overnight at the bottom of the tunnel without swimming. The surgeries, recovery procedures, and all experiments were carried out in normoxia for both acclimation groups.

3.3.3 Experimental design

To examine the effect of paCA inhibition on cardiovascular O_2 transport, Atlantic salmon were swum at a constant swimming speed of 1 bl s^{-1}. Swimming fish were first injected with a sham (a solvent control consisting of saline; 0.9% NaCl with 0.1% DMSO), and then injected with saline containing C18 (0.2 mM final concentration or 10 µmol kg^{-1}; based on animal mass, assuming a density equal to water and a blood volume of 5%), a membrane-impermeable CA inhibitor (Scozzafava et al., 2000). The sequential injections of sham and C18 (~15 min apart) significantly reduced the amount of experimental animals required. This protocol was applied to salmon that had been acclimated to normoxia or hypoxia; thus, resulting in a mixed-model design, that compared the effect of C18 injection against the injection of the sham (solvent control), and the effect hypoxia against normoxia acclimation.

In preliminary trials it was determined that the U_{crit} of these Atlantic salmon was ~2 bl s^{-1}, which is in line with previous measurements (Deitch et al., 2006), and that the optimal swimming speed for these experiments was 1 bl s^{-1}; when swimming at 1.5 bl s^{-1} the fish would collapse and stop swimming after injection of C18 ($N = 2$). Measurements of $\dot{M}O_2$ and cardiovascular parameters ($\dot{Q}$; stroke volume, V_s; and heart rate, f_h) were performed at 5 points during the experimental protocol: i) at rest (water velocity of 10 cm s^{-1}); ii) after gradually increasing swimming speed to 1 bl s^{-1} and 10 min of steady swimming (swim); iii) 5 min after a saline injection (sham); iv) 5 min after C18 injection (C18); and v) after decreasing water

velocity to 10 cm s^{-1} and allowing the fish to recover from exercise for 10 minutes (recov). Each step in the protocol lasted ~30 min, with a total duration of ~3 h, during which the fish swam for ~2 h. In a separate trial the effect of paCA inhibition was assessed on resting Atlantic salmon. After surgery animals were transferred into individual respirometry chambers, that were continuously supplied with aerated seawater from a reservoir at 12°C; described in detail by (Motyka et al., 2017). Measurements of $\dot{M}O_2$ and cardiovascular parameters were: i) initial (rest); ii) 5 min after a saline injection (sham); and iv) 5 min after C18 injection.

A previous study on rainbow trout determined that C18 has no effect after 30 min, but significantly inhibits RBC CA by ~30% after 1 h of injection, by slowly diffusing across the RBC membrane (Rummer et al., 2013). To avoid a confounding effect of RBC CA inhibition, salmon were swum for 30 min after the injection of C18 and final measurements (recov) were performed < 50 min after C18 injection. In addition, a separate control experiment was performed to test whether $\dot{M}O_2$ and cardiovascular parameters were affected by the swimming protocol itself, the injections of saline, or the repeated sampling of blood. Therefore, normoxia-acclimated fish (N = 3) were subjected to the above swimming protocol, however, the C18 injection was replaced with a second saline injection.

3.3.4 Measurements of $\dot{M}O_2$ and cardiovascular parameters

The Doppler lead of the animal was connected to a pulsed Doppler flow meter (Model 545C-4; Department of Bioengineering, University of Iowa, Ames, IA), and the crystal's focus was adjusted so that a clean signal of the greatest magnitude was obtained before each trial. The analog signal from the Doppler flow meter was recorded continuously using a MP100 data acquisition system and Acqknowledge software v3.7.3 (BIOPAC Systems, Santa Barbara, CA). $\dot{Q}$ was assessed as the average voltage reading over a selected period, and all values were expressed relative to the average voltage measured at rest. Average f_h was measured using the Acqknowledge heart rate function, and these measurements were validated by manually measuring the time required for 20 heartbeats; if there was a discrepancy between the two measurements of f_h, the manually calculated values were used. Stroke volume, V_s, was calculated as $\dot{Q}/f_h$, and thus, was relative to average values at rest (Motyka et al., 2017).

PO_2 in the swim tunnel was measured continuously using a fiber-optic sensor (Dipping probe mini sensor, Loligo Systems, Viborg, Denmark) connected to a Witrox 1 O_2 meter (Loligo Systems). $\dot{M}O_2$ was measured using an intermittent respirometry approach, and by manually

stopping the flow of water into the swim tunnel. The duration of stop-flow at rest and during recovery was ~15 min, over which PO_2 decreased by ~3% air saturation. During swimming, stop-flow periods were shorter (~5 min) due to the higher $\dot{M}O_2$ of the animal, where PO_2 was prevented from decreasing by more than ~5% air saturation; therefore, all experiments were well within a range of PO_2 values that can be considered normoxic. $\dot{M}O_2$ was calculated in LabChart v8.1.5 (ADInstruments, Dunedin, New Zealand) from the slope of the decrease in PO_2 over the final 8 min of the stop-flow period at rest, and over the final 3 min of the stop-flow period during exercise, according to:

$$\dot{M}O_2 = -\frac{\delta PO_2}{\delta t} \times (V_c - V_a) \times \alpha_{O_2} \times \frac{3600}{m_a}$$

where $-\frac{\delta PO_2}{\delta t}$ (% air-saturation s^{-1}) is the slope of the PO_2 curve during stop-flow, V_c and V_a (L) are the volumes of the chamber and the animal (based on animal wet mass and assuming a density equal to water), α_{O_2} (mg O_2 L^{-1} % air-saturation^{-1}) is the solubility of O_2 in water (Boutilier et al., 1984) and m_a is the mass of the animal (kg). Thus, $\dot{M}O_2$ was expressed as mg O_2 kg^{-1} h^{-1} where 1 mg O_2 is ~32 µmol, a unit that is also commonly used to describe $\dot{M}O_2$.

$\dot{M}O_2$ in the resting trial was assessed using an automated intermittent respirometry system that controlled the flush pumps to individual respirometry chambers as described previously (Motyka et al., 2017). PO_2 was measured with fiber-optic sensors (Dipping probe mini sensor, Loligo systems, Viborg, Denmark) connected to an Oxy-4 mini meter (PreSens, Regensburg, Germany) that was interfaced with a DAQ-4 module (Loligo systems). $\dot{M}O_2$ was calculated automatically by the AutoResp software v2.2.2 (Loligo systems) and the average values over two respirometry cycles are reported for each measurement period.

3.3.5 Blood sampling and analysis

To account for potentially confounding changes in arterial O_2 transport due to the experimental treatments (Steinhausen et al., 2008) or hypoxia acclimation (Greaney et al., 1980; Petersen and Gamperl, 2010a) that may affect β_b, arterial blood was sampled from the catheter at four time-points during the experiment: i) a resting sample was taken between the two resting $\dot{M}O_2$ measurements; ii) a swimming/sham sample was taken after the $\dot{M}O_2$ measurement; iii) a sample was taken after the C18 $\dot{M}O_2$ measurement; and iv) a final sample was taken after the

recovery $\dot{M}O_2$ measurement and before removing the fish from the swim tunnel. A blood volume of 600 µL was removed from the animal at each sampling time-point and this volume was replaced by saline. To assess the stress response of Atlantic salmon during the swim trials and to validate an involvement of β-NHE activity in modulating β_b, plasma catecholamine concentrations were measured. Immediately after sampling, 400 µL of blood was centrifuged for 20 s with a Mini-Centrifuge 05-090-128 (Fisher Scientific, Hampton, NH) and 200 µL of plasma was immediately pipetted into a bullet tube containing 10 µL of 0.2 M EDTA and glutathione, and then rapidly frozen in liquid N_2 (within ~1 min from sampling). Plasma adrenaline and noradrenaline concentrations were measured using an ELISA kit (ABNova KA1877, Taipei City, Taiwan), following the manufacturer's instructions.

The remainder of the plasma was frozen in liquid N_2 and plasma lactate concentration was measured spectrophotometrically using an extraction kit (Sigma 826). Haematocrit (Hct) was measured in triplicate on 15 µL of blood in capillary tubes that were centrifuged at 10,000 g for 3 min. Blood pH was measured with a thermostatted (12°C) microelectrode (16-705 and 16-702; Microelectrodes Inc., Bedford, NH). Arterial O_2 content of the blood (C_aO_2) was measured on 10 µL, in triplicate, using a Tucker chamber (Tucker, 1967) and by lysing the RBCs and converting all Hb into methaemoglobin in a ferricyanide solution at 40°C. The change in PO_2 in the Tucker chamber was measured with a Clark-type electrode (E101, Radiometer, Copenhagen, Denmark) and OM200 meter (Cameron Instruments, Guelph, ON, Canada). Hb concentration was measured in triplicate on 10 µL of blood using the cyanomethaemoglobin method (Drabkin's reagent, Sigma D5941) and measuring absorbance at 540 nm using a plate reader (SpectraMax 5, Molecular Devices, Sunnyvale, CA). Hb concentration was calculated in reference to standard curves with bovine Hb (Sigma, H-2500) that were run on the same plates as the samples. Hb-O_2 saturation (SO_2) was calculated from C_aO_2 and Hb concentration after subtracting physically dissolved O_2 as previously described (Tucker, 1967; Boutilier et al., 1984). At the end of the experiment, the salmon were removed from the swim tunnel and euthanised in 0.15 g L^{-1} MS-222. Finally, the ventricle was dissected out, blotted dry and weighed on a scale, for the calculation of relative ventricular mass (RVM).

3.3.6 Data analysis and statistics

All data were analysed in R studio v1.0.153 (R v3.4.1) and figures were generated with the ggplot2 v.2.2.1 package (Wickham, 2009). The mixed-design model (lme function in R)

included a random effect of individual, a fixed effect of treatment that was nested within individual, and a fixed effect of acclimation that was independent of individual. This model tested the main effects of treatment (treat) and acclimation (accl), and their interaction (treat×accl), and these results are reported in all figures. In addition, differences between treatments were analysed with a Tukey post-hoc test ($N = 9$ for the swimming trial, $N = 5$ for the resting trial and $N = 3$ for control fish, unless indicated otherwise; $P < 0.05$). If the main model indicated a significant effect of acclimation or an interaction effect, the post-hoc analysis was carried out separately for both acclimation groups. All data are shown as means ± s.e.m..

3.4 Results

During the entire 10-week acclimation period, only one mortality was observed in the hypoxia-acclimated group, and there were no significant effects of acclimation on final animal mass (774 ± 194 g; $P = 0.618$; $N = 69$) or condition factor (Fulton's K; 1.04 ± 0.01 g cm^{-3}; $P = 0.111$). However, in those Atlantic salmon that were used in swim trials, a significant acclimation effect ($P = 0.046$) indicated that normoxia-acclimated fish were larger (868 ± 41 g; $N = 12$) compared to hypoxia-acclimated fish (768 ± 27 g; $N = 14$) and had a significantly higher RVM (0.075 ± 0.003 vs. 0.068 ± 0.002%: $P = 0.042$).

Figure 3-1 summarises the metabolic and cardiovascular response of normoxia- and hypoxia-acclimated Atlantic salmon during swim trials. A significant main effect of treatment (see figures for main effect P values) indicated that the experimental protocol affected $\dot{M}O_2$; however, there were no significant effects of hypoxia acclimation or interaction effects on $\dot{M}O_2$ (Fig. 3-1A). Average $\dot{M}O_2$ at rest was 81.6 ± 2.6 mg O_2 kg^{-1} h^{-1}, and swimming at 1 bl s^{-1} increased $\dot{M}O_2$ two-fold, to 187.8 ± 5.8 mg O_2 kg^{-1} h^{-1} ($P < 0.001$). The injections of saline ($P = 0.943$) or C18 ($P < 0.283$) into swimming Atlantic salmon, had no significant effects on $\dot{M}O_2$ and values were 180.2 ± 5.2 and 200.1 ± 8.6 mg O_2 kg^{-1} h^{-1}, respectively. During recovery from exercise $\dot{M}O_2$ decreased significantly to 103.0.0 ± 8.4 mg O_2 kg^{-1} h^{-1} ($P < 0.001$), but it remained elevated over resting values ($P = 0.001$). In normoxia-acclimated control fish that were injected with saline twice, swimming at 1 bl s^{-1} had a comparable effect on $\dot{M}O_2$, and there was no significant ($P = 1.000$) difference in $\dot{M}O_2$ between the first and second sham injection (Fig. 3-2A).

Significant treatment effects were also detected on cardiovascular parameters, $\dot{Q}$, f_h and V_s (Fig. 3-1B-D), and significant interaction effects on $\dot{Q}$ and V_s indicated that normoxia- and

hypoxia-acclimated fish responded differently to treatments. Swimming increased $\dot{Q}$ to 174.8 ± 11.6% relative to resting values (100%; Fig. 3-1B). This was due to a large increase in V_s to 157.4 ± 9.7% relative to resting values (Fig. 3-1D) and a significant ($P < 0.001$) increase in f_h (8 bpm; Fig. 3-1C). Injection of C18 into swimming Atlantic salmon significantly increased $\dot{Q}$ by 27.0% relative to sham injections (20.2% in normoxia-acclimated fish, $P = 0.002$; and 34.4% in hypoxia-acclimated fish, $P = 0.001$). This was associated with an increase in f_h by 2 bpm in both acclimation groups ($P = 0.036$), and an increase in V_s by 23.6% relative to sham injections (16.3% in normoxia-acclimated fish, $P = 0.032$; and 31.8%, in hypoxia-acclimated fish, $P = 0.004$). During recovery from exercise, $\dot{Q}$ decreased significantly ($P < 0.001$) in normoxia-acclimated fish to 138.0 ± 11.3% relative to resting values. However, in hypoxia-acclimated fish values remained elevated at 198.5 ± 17.2% during recovery ($P = 0.305$). Likewise, V_s in normoxia-acclimated fish decreased significantly ($P < 0.001$) to 128.9 ± 10.5% relative to resting values. However, in hypoxia-acclimated fish values remained elevated at 178.3 ± 13.5% during recovery ($P = 0.493$). In control fish that were injected with saline twice (Fig. 3-2B-D) there was no significant difference between the first and second sham injection on $\dot{Q}$ ($P = 0.855$), f_h ($P = 0.690$) and V_s ($P = 0.701$). During recovery in control fish, f_h remained significantly elevated above resting values ($P < 0.001$). In contrast, $\dot{Q}$ ($P < 0.001$) and V_s ($P < 0.001$) decreased significantly relative to values during exercise, to 84.6 ± 5.8 and 77.1 ± 4.3%, respectively, and thus were lower than resting values (100%).

Figure 3-3 shows markers of anaerobic metabolism in normoxia- and hypoxia-acclimated Atlantic salmon during the swim trial. Plasma lactate concentration was significantly affected by the experimental protocol as indicated by a treatment effect (Fig. 3-3A), and a non-significant trend ($P = 0.072$) may point towards a higher lactate concentration in normoxia- compared to hypoxia-acclimated fish. Plasma lactate concentration at rest was on average 0.66 ± 0.09 mM and was unaffected by swimming and sham injections ($P = 0.998$). However, the injection of C18 significantly increased ($P = 0.001$) plasma lactate concentration, in both acclimation groups, to 1.05 ± 0.11 mM, and values remained elevated above resting during recovery ($P = 0.023$). In control fish that were injected with saline twice, there was no significant difference between the first and second sham injection ($P = 0.987$), but plasma lactate concentration significantly increased ($P < 0.001$) due to exercise and the first sham injection (Fig. 3-4A).

A significant treatment effect was also detected on arterial blood pH and an interaction effect indicated that blood pH in the two acclimation groups responded differently to the experimental protocol (Fig. 3-3B). In normoxia-acclimated fish blood pH decreased significantly ($P = 0.007$) after C18 injection, compared to a sham injection. However, in hypoxia-acclimated fish blood pH was unaffected by sham ($P = 0.130$) or C18 injections ($P = 0.373$). A significant interaction effect indicated that blood pH after sham injections was significantly higher in normoxia- compared to hypoxia-acclimated fish ($P = 0.019$). During recovery, blood pH was not different compared to resting values in normoxia- ($P = 0.713$) or hypoxia acclimated fish ($P = 0.873$). In control fish that were injected with saline twice, there was no significant ($P = 0.999$) difference in blood pH between the first and second sham injection (Fig. 3-4B).

Figure 3-5 shows plasma catecholamine concentrations in normoxia- and hypoxia-acclimated Atlantic salmon during the swim trial. Significant treatment effects were detected on the concentrations of adrenaline and noradrenaline in the plasma of Atlantic salmon. On average, adrenaline concentration increased from 2.0 ± 0.4 nmol L^{-1} at rest, to 3.1 ± 0.4 nmol L^{-1} after a sham injection ($P = 0.008$) and to 5.7 ± 0.6 nmol L^{-1} after C18 injection ($P < 0.001$). During recovery, adrenaline concentration was 3.3 ± 0.5 nmol L^{-1} and significantly lower ($P < 0.001$) compared to maximal levels after C18 injection, but values remained elevated ($P = 0.003$) over resting (Fig. 3-5A). The changes in noradrenaline concentration, due to the experimental protocol, were similar to those observed for adrenaline (2 – 6 nmol L^{-1}). However, a significant effect of acclimation indicated that, on average, plasma noradrenaline concentrations were higher in normoxia- compared to hypoxia-acclimated fish (Fig. 3-5B). Injection of C18 had no effect on noradrenaline concentrations compared to sham injections in normoxia- ($P = 0.202$) or hypoxia-acclimated fish ($P = 0.973$), but values increase significantly relative to those at rest, in both normoxia- ($P < 0.001$) and hypoxia-acclimated fish ($P = 0.005$). During recovery, noradrenaline concentration was not different from resting values in normoxia- ($P = 0.476$) or hypoxia-acclimated fish ($P = 0.746$). In control fish that were injected with saline twice, adrenaline concentration increased due to exercise and the first sham injection ($P = 0.003$). However, there was no significant difference between the first and second sham injection on adrenaline ($P = 0.161$) or noradrenaline ($P = 0.988$) concentrations (Fig. 3-6A and B).

Figure 3-7 summarises the metabolic and cardiovascular responses of normoxia-acclimated Atlantic salmon during the resting trial. Resting $\dot{M}O_2$ was unaffected by the injection

of a sham ($P = 0.173$) or C18 ($P = 0.535$), and on average 74.2 ± 2.1 mg O_2 kg^{-1} h^{-1} (Fig. 3-7A). In contrast, significant treatment effects indicated that injection of C18 increased $\dot{Q}$ ($P = 0.002$), f_h ($P = 0.015$) and $\dot{V}_s$ ($P = 0.019$) compared to sham injections (Fig. 3-7B-D). The increase in $\dot{Q}$ of 27.4% relative to sham injections was associated with an increase in f_h of 5 bpm and an increase in $\dot{V}_s$ of 19.6% relative to sham injections.

Figure 3-8 shows the calculated parameter of $\Delta\dot{M}O_2/\Delta\dot{Q}$ for the swim trial (Fig. 3-8A) and the resting trial (Fig. 3-8B). A significant treatment effect indicated that $\Delta\dot{M}O_2/\Delta\dot{Q}$ was affected by the swimming protocol, but no effects of acclimation or interactions were detected. During swimming, average $\Delta\dot{M}O_2/\Delta\dot{Q}$ was 1.4 ± 0.1 and was unaffected by a sham injection ($P = 0.282$). C18 injection did not decrease $\Delta\dot{M}O_2/\Delta\dot{Q}$ relative to sham injections ($P = 0.323$); however, $\Delta\dot{M}O_2/\Delta\dot{Q}$ after C18 injection was lower compared to initial swimming values ($P = 0.003$). $\Delta\dot{M}O_2/\Delta\dot{Q}$ during recovery decreased significantly ($P < 0.001$) in both acclimation groups, to values below parity (a value of 1, where $\dot{M}O_2$ and $\dot{Q}$ change at the same rate) and on average 0.7 ± 0.1. In control fish that were injected with saline twice, there was no significant main effect of treatment on $\Delta\dot{M}O_2/\Delta\dot{Q}$ and all values remained above parity, and on average were 1.7 ± 0.1 (Fig. 3-9). As in the swim trial, the injection of C18 into normoxia-acclimated Atlantic salmon at rest significantly ($P = 0.002$) decreased $\Delta\dot{M}O_2/\Delta\dot{Q}$ relative to a sham injection, from 0.9 ± 0.0 to 0.7 ± 0.0 (Fig. 3-8B).

A significant treatment effect, but no acclimation or interaction effects were detected on blood Hct (Fig. 3-10A). In both acclimation groups, Hct decreased throughout the experiment, from 26.6 ± 1.0% at rest to 23.5 ± 0.8% during recovery, but the injection of C18 had no significant effect on Hct ($P = 0.621$). In addition, no effects of treatment, acclimation or interactions were detected on Hb concentration (Fig. 3-10B), MCHC or Hb-O_2 saturation (Fig. 3-10C), and average values were 1.0 ± 0.0 mM, 4.1 ± 0.2 mM and 95.2 ± 2.8%, respectively. However, a treatment effect was detected on C_aO_2, which decreased from 3.8 ± 0.1 mM at rest to 3.2 ± 0.2 mM during recovery (Fig. 3-10D). Injection of C18 into swimming Atlantic salmon significantly ($P = 0.020$) decreased C_aO_2 compared to sham injections (from 3.8 ± 0.1 to 3.5 ± 0.2 mM). In control fish that were injected with saline twice (Fig. 3-11A-D), there were no significant treatment effects or differences between the first and second sham injection on Hct ($P = 0.402$), Hb concentration ($P = 0.745$), Hb-O_2 saturation ($P = 0.207$) or C_aO_2 ($P = 0.465$), but Hb concentration increased transiently due to exercise and the first sham injection ($P = 0.035$).

3.5 Discussion

The present results are in line with our initial hypothesis and as such are the first functional data to support a role of paCA in cardiovascular O_2 transport in a teleost. The inhibition of paCA with C18 in Atlantic salmon had no effect on $\dot{M}O_2$, yet $\dot{Q}$ increased significantly by 27.4% at rest, by 27.0% in fish swimming at 1 bl s^{-1}, and salmon collapsed when C18 was injected at higher swimming speeds. The corresponding decrease in $\Delta\dot{M}O_2/\Delta\dot{Q}$ during exercise following C18 injection, and to a value below parity during recovery from exercise and at rest, is consistent with a reduction in β_b. The increase in $\dot{Q}$ may represent a compensatory response that allowed the animals to sustain resting $\dot{M}O_2$ and exercise performance in the absence of paCA activity. Both, normoxia- and hypoxia-acclimated Atlantic salmon used paCA to enhance tissue O_2 extraction during exercise and recovery. However, the reliance on paCA was greater in hypoxia-acclimated fish. These results indicate that paCA is an integral part of the O_2 transport system of Atlantic salmon that may reduce the requirement for cardiac work by up to a third, is recruited over a wide range of conditions including rest, exercise and recovery from exercise, and may respond plastically to hypoxia acclimation.

Animals in the present study were allowed to recover from surgery for 48 h in normoxia. This timeframe represented a trade-off between a full recovery and the risk of reversing the effects of hypoxia acclimation. Nevertheless, in Atlantic salmon that had undergone a similarly invasive hepatic portal vein cannulation, blood parameters had already recovered 24 h after surgery (Eliason et al., 2007). Furthermore, previous work on Atlantic cod (Petersen and Gamperl, 2010a; Petersen and Gamperl, 2011) and steelhead trout (Motyka et al., 2017) that used a similar protocol of acclimation, surgery and recovery, detected large effects of hypoxia acclimation, indicating that normoxic recovery from surgery for 48 h did not fully reverse acclimation effects in these fish. In Atlantic salmon, hypoxia acclimation had no effect on O_2 carrying capacity of the blood (Hct, Hb concentration, or pH; Fig. 3-10A and B; Fig. 3-3B). Likewise, there was no acclimation effect on $\Delta\dot{M}O_2/\Delta\dot{Q}$ indicating that β_b was not affected by potential differences in capillary density (Sänger et al., 1990; Egginton, 2002), myoglobin concentration (Wittenberg and Wittenberg, 2003) or mitochondrial volume (Johnston and Bernard, 1984), between acclimation groups. Whether acclimation to hypoxia changed Hb-O_2 affinity was not tested here. However, all experiments were performed in normoxia, where minor changes in Hb-O_2 affinity are not expected to affect O_2 uptake from the water, and this is

corroborated by the absence of acclimation or interaction effects on arterial O_2 transport parameters (Hb-O_2 saturation or C_aO_2; Fig. 3-10C and D).

$\dot{M}O_2$ in resting Atlantic salmon was unaffected by hypoxia acclimation (Fig. 3-1A), which is in line with previous findings in this species (Bushnell et al., 1984; Motyka et al., 2017). Atlantic salmon of the size range studied here typically attain U_{crit} of 2.0-2.2 bl s^{-1} where $\dot{M}O_2$ increases to 550% relative to resting values (McKenzie et al., 1998; Deitch et al., 2006). In the present study, fish were swum at 1 bl s^{-1}, a moderate level of exercise that represents ~50% of U_{crit}. At this swimming speed $\dot{M}O_2$ increased to 230% (Fig. 3-1A), whereas $\dot{Q}$ increased to 174.8 ± 11.6% relative to resting values (Fig. 3-1B). These results correspond well with data on other instrumented salmonids swimming at 50% U_{crit}, where $\dot{Q}$ increased to ~175% in Chinook salmon (*Oncorhynchus tshawytscha*; Gallaugher et al., 2001), ~171% in sockeye salmon (*Oncorhynchus nerka*; Eliason et al., 2013) and ~162% in rainbow trout (Kiceniuk and Jones, 1977). In teleosts, an increase in $\dot{M}O_2$ during exercise is supported, to about equal parts, by increases in $\dot{Q}$ and tissue O_2 extraction (Stevens and Randall, 1967; Kiceniuk and Jones, 1977; Brauner et al., 2000a). During moderate exercise in Atlantic salmon, $\Delta\dot{M}O_2/\Delta\dot{Q}$ increased above parity (Fig. 3-8A) and, in the absence of changes in O_2 carrying capacity of the blood (constant Hct and Hb concentration; Fig. 3-10A and B), this increase in β_b is consistent with an increase in tissue O_2 extraction.

The injection of C18 into swimming Atlantic salmon caused a 27% increase in $\dot{Q}$ relative to sham injections, and this response resulted from increases in both f_h and V_s. Adjustments to V_s are the principal mechanism by which fish increase $\dot{Q}$ during exercise (Farrell and Jones, 1992), and here the increase in V_s accounted for ~88% of the increase in $\dot{Q}$ (Fig. 3-1B-D). The high specificity of C18 for membrane-bound CA isoforms (Scozzafava et al., 2000; Supuran, 2008) and the negligible membrane permeability over the experimental time-period (Rummer et al., 2013), allow to exclude a confounding effect of RBC CA inhibition and thus systemic CO_2 excretion. On the other hand, the inhibition of paCA at the capillaries may impair the transfer of CO_2, lactate or ammonia into the blood (Geers and Gros, 2000) and thus lead to changes in PCO_2 and pH that may change vascular resistance. Previous studies indicate that resistance vessels in rainbow trout do not respond to internal changes in PCO_2 and pH (Perry et al., 1999; McKendry and Perry, 2001) and that receptors must be oriented externally at the gills (Reid et al., 2000; Sundin et al., 2000). However, some systemic vasodilation due to a reduction in blood pH has

been described in the ocean pout (Canty and Farrell, 1985) and these conflicting findings remain to be revisited. Based on the available data in salmonids the inhibition of paCA is not expected to alter systemic vascular resistance *via* internal changes in PCO_2 or pH. Therefore, the observed cardiovascular adjustments in response to C18 were likely required to offset changes in tissue PO_2, resulting from a reduction in β_b. Hypoxic systemic vasodilation is a well-described mechanism in mammals and modulated by ATP-sensitive K^+-channels that hyperpolarise vascular smooth muscle cells during hypoxia (Kozlowski, 1995; Weir and Archer, 1995). Likewise, it has been shown in rainbow trout that isolated arterial vessels dilate upon exposure to hypoxia, whereas venous vessels contract, and a role of K^+-signalling was substantiated in both responses (Smith et al., 2001). An increase in venous tone is in line with the observed increase in $\dot{Q}$ through V_s (Farrell and Smith, 2017). Combined, these results may point towards a paracrine regulation of systemic vascular resistance in response to changes in PO_2, in the tissues and in the venous blood, that result from the inhibition of paCA. However, the specific mechanism underlying this response remains to be substantiated by future experiments.

Both plasma adrenaline and noradrenaline levels were significantly elevated during exercise (Fig. 3-5), a pre-requisite for β-NHE activity. However, the plasma catecholamine concentrations reported here were low compared to maximal values in Atlantic salmon that were induced by netting and air-exposure (Deitch et al., 2006). Also in rainbow trout that were exhaustively exercised, catecholamine levels increased to values that exceed by three-fold those reported here (Gamperl et al., 1994). This, and the increase in plasma adrenaline concentration after C18 injection may indicate some capacity for a further up-regulation of the adrenergic response. In addition, Atlantic salmon in both acclimation groups experienced a mild blood acidosis during exercise and after C18 injection (Fig. 3-3B), conditions that promote β-NHE activity in teleosts (Borgese et al., 1987; Salama and Nikinmaa, 1988, 1989). By substantiating the involvement of an adrenergic response, our study is the first to provide *in vivo* evidence that a combined mechanism of paCA and β-NHE activity plays a role in cardiovascular O_2 transport in teleosts, and that the inhibition of the system must be compensated by drastic cardiovascular adjustments. Importantly, changes in cardiovascular parameters that were observed after C18 injection were absent in control fish that were injected with saline twice (Fig. 3-2B-D). The results from this validation are critical, as they confirm that: i) the effects of C18 injection in the main experiment can be attributed to the action of C18 and are not confounded by the fact that

the drug always had to be injected after the sham; and ii) there were no confounding changes in the parameters of interest over the course of the 2 h swimming trial, such as may be associated with fatigue or repeated blood sampling.

The increase in $\dot{Q}$ after C18 injection occurred rapidly, in some cases within seconds. Several animals showed burst-and-glide swimming that lasted minutes after C18 injection, and 3 out of 26 animals (2 hypoxia- and 1 normoxia-acclimated) were unable to sustain swimming in the presence of C18. While these incomplete trials were excluded from the analysis, individual variation in the inability to sustain a given level of exercise in the presence of C18 is also consistent with a role of paCA in sustaining O_2 transport in teleosts. The injection of C18 caused a significant, albeit numerically small, increase in plasma lactate concentration in swimming Atlantic salmon in both acclimation groups (Fig. 3-3A), which was not observed in control fish after the second saline injection (Fig. 3-4A). Consequently, the observed increase in $\dot{Q}$ may not have fully compensated for a reduction in β_b during exercise, and thus, was complemented by some anaerobic ATP production. These results are in line with the observation of burst-and-glide swimming after C18 injection and indicate a transient recruitment of glycolytic white muscle (Bone, 1978). A non-significant trend ($P = 0.072$) may point towards a lower plasma lactate concentration in hypoxia-acclimated fish. Whether this was related to a lower reliance on anaerobic metabolism or a higher capacity to metabolise produced lactate, remains to be investigated. However, these results are in remarkable agreement with the "lactate paradox" observed in high-altitude native humans (Hochachka et al., 2002).

A striking result was that injection of C18 into resting Atlantic salmon also increased $\dot{Q}$ by 27.4% relative to sham injections, while $\dot{M}O_2$ was unaffected (Fig. 3-7A and B). As in swimming fish, this was associated with some increase in f_h (by 5 bpm) and a large increase in $\dot{V}_s$ (by 19.6%). The similarity of this response to that observed in swimming fish is remarkable, and thus, it appears that paCA played a comparable role in supporting O_2 transport at rest and during moderate exercise. Even at rest, some background activity of β-NHE is likely in salmonids (Tetens and Lykkeboe, 1988). Shu et al. (2017) established a dose-response relationship between isoproterenol concentration and the increase in closed-system PO_2 that can be achieved by β-NHE short-circuiting in rainbow trout. If a similar relationship holds for RBCs of Atlantic salmon stimulated with noradrenaline, the resting values observed here (3.4 ± 0.2 nmol L^{-1}) would allow for a 6 mmHg (~0.8 kPa) increase in PO_2, when β-NHE are short-

circuited at the tissues. Clearly, this dose-response relationship needs to be validated in Atlantic salmon and using the typically lower-potency natural catecholamine, noradrenaline (Tetens and Lykkeboe, 1988). Interestingly, however, the calculated increase in PO_2 is in line with that observed when "non-stimulated" rainbow trout RBC were short-circuited *in vitro* (Rummer and Brauner, 2011), results that may also be explained by a low level of background β-NHE activity. At rest, presumably all vascular beds are perfused at a low blood-flow rate and thus, even small changes in tissue PO_2 that affect all tissues may require a significant change in $\dot{Q}$ to compensate. In combination, these results indicate that β-NHE short-circuiting is recruited to elevate β_b over a broad range of conditions in Atlantic salmon, and thus, that the system may represent a fundamental part of the teleost mode of O_2 transport.

If teleost routinely maintain an elevated β_b that allows for a reduction in cardiac function by 30%, then inhibiting paCA must set limits to maximal aerobic performance. After C18 injection at 50% U_{crit}, $\dot{Q}$ in Atlantic salmon was elevated to 225.7 ± 16.5% relative to resting values. In teleosts, interspecific variation in the scope for $\dot{Q}$ typically ranges from 200-300% relative to rest (Farrell and Smith, 2017). Swimming Chinook salmon maximally increased $\dot{Q}$ to 194% at U_{crit} (Gallaugher et al., 2001), Atlantic cod to 193% (Petersen and Gamperl, 2010a), sockeye salmon to ~290% (Eliason et al., 2013) and rainbow trout to values from 183% (Thorarensen et al., 1996b) to 298% (Kiceniuk and Jones, 1977). Thus in the present study, C18 injected Atlantic salmon may have largely utilised their scope to increase $\dot{Q}$, especially considering that these were hatchery fish that typically do not match the exercise performance of wild conspecifics (Eliason et al., 2013). In preliminary trials, normoxia-acclimated fish that were swum at 1.5 bl s^{-1} were unable to sustain swimming after C18 injection (N = 2). Although $\dot{Q}$ data are not available for these runs, this may be a powerful indication that animals swimming at the higher speed did not have enough scope to increase $\dot{Q}$ by the additional ~30% that is required to compensate for the inhibition of paCA. Thus, it appears that in the absence of paCA activity and the related enhancement of β_b, Atlantic salmon are unable to attain levels of aerobic performance that even remotely resemble U_{crit}.

The effect of C18 injection on $\Delta\dot{M}O_2/\Delta\dot{Q}$ was less pronounced than initially hypothesised and no significant difference was detected relative to sham injections. However, $\Delta\dot{M}O_2/\Delta\dot{Q}$ did decrease relative to initial values in swimming fish (Fig. 3-8A). The observation of burst-and-glide swimming and an increase in plasma lactate concentration point towards a transient

recruitment of anaerobic ATP production that may have masked an immediate decrease in $\Delta\dot{M}O_2/\Delta\dot{Q}$. In fact, the effects of C18 injection become clear during recovery from exercise, where $\Delta\dot{M}O_2/\Delta\dot{Q}$ decreased significantly to values below parity (on average 0.7 ± 0.1). Importantly, this was not observed in control fish that were injected with saline twice, and that maintained a constant $\Delta\dot{M}O_2/\Delta\dot{Q}$ of 1.7 ± 0.1 (Fig. 3-9). In control fish $\dot{Q}$ and V_s during recovery decreased below resting levels (84.6 ± 5.8 and 77.1 ± 4.3%, respectively), a response that is typically observed after exercise in teleosts (Thorarensen et al., 1996b; Petersen and Gamperl, 2010a), whereas $\dot{Q}$ and V_s in C18 injected fish remained elevated during recovery. These results indicate that the recovery from aerobic exercise in Atlantic salmon is also supported by an enhancement in β_b that is mediated by paCA.

Unlike previous results on Atlantic cod and steelhead trout (Petersen and Gamperl, 2010a; Petersen and Gamperl, 2011; Motyka et al., 2017), hypoxia-acclimated Atlantic salmon swimming at a moderate speed were able to increase $\dot{Q}$ and V_s to the same extent as normoxia-acclimated fish, despite their lower RVM. These results do not necessarily stand in conflict with previous data, as the discrepancy in $\dot{Q}$ between acclimation groups was typically observed at higher $\dot{Q}$ and $\dot{M}O_2$ values than those tested here. However, differences in cardiac function between the acclimation groups became evident during recovery from exercise. After C18 injection, $\dot{Q}$ and V_s during recovery from exercise were significantly higher in hypoxia- compared to normoxia-acclimated fish, while $\dot{M}O_2$ was not different between the two groups (Fig. 3-1). As a result $\Delta\dot{M}O_2/\Delta\dot{Q}$ was numerically lower in hypoxia-acclimated fish (Fig. 3-8A), indicating a greater reliance of these fish on β-NHE short-circuiting and that there is some phenotypic plasticity in the recruitment of this mechanism that responds to hypoxia acclimation.

An increase in O_2 extraction during exercise can be accomplished entirely by recruiting additional capillaries in the tissues (Krogh, 1919a, b). However, at a higher tissue O_2 extraction and as blood becomes increasingly desaturated, the PO_2 at which O_2 is unloaded from Hb decreases, thus reducing the diffusion gradient from Hb to the mitochondria (Jones, 1986; Lau et al., 2017). And herein may lie the inherent advantage of β-NHE short-circuiting, as the mechanism right-shifts the OEC and thus, may allow for a higher tissue O_2 extraction without compromising diffusion gradients. The magnitude of the induced Bohr shift at the tissues will depend on the H^+ gradient across the RBC membrane. These H^+ gradients are actively generated by RBC β-NHE activity (driven by Na^+ gradients that are set up by the Na^+-K^+-ATPase) that is

modulated by the circulating catecholamine concentration (Shu et al., 2017). Therefore, in addition to recruiting more capillaries, teleosts may enhance tissue O_2 extraction by an active cellular mechanism at the level of the RBC that is under adrenergic control, and critically, without compromising tissue PO_2.

Maintaining venous PO_2 may be especially pertinent for teleosts, where the heart is situated downstream of all other tissues, and the avascular spongy myocardium must extract its O_2 entirely from the low-PO_2 venous return (Farrell, 1991; Farrell and Jones, 1992). In fact, previous studies indicate that rainbow trout strictly maintain a minimum venous PO_2 of 15 mmHg (~2 kPa) that decreases only at swimming speeds approaching U_{crit} (Farrell and Clutterham, 2003). Thus, at high exercise intensities, the PO_2 of the venous return may limit O_2 extraction at the tissues upstream. However, paCA has recently been detected in the heart lumen of coho salmon, and therefore, the teleost heart too, may rely on β-NHE short-circuiting to enhance O_2 extraction (Alderman et al., 2016). Of course cardiac paCA may be important for other physiological functions besides O_2 unloading to the myocardium, such as cellular CO_2 (Geers and Gros, 1988), ammonia (Henry et al., 1997a; Wang et al., 1998) and lactate excretion (Wetzel et al., 2001), and generally the maintenance of tissue and interstitial pH homeostasis (Geers and Gros, 2000). The inhibition of these functions by C18 may have deleterious effects on cardiac performance and thus, cardiovascular O_2 transport. However, in the present study, $\dot{Q}$, f_h and V_s increased in the presence of C18 and clearly, any adverse effects on myocardial waste removal or acid-base regulation, were permissive of an increase in cardiac work. If the O_2 supply to the spongy myocardium sets limits to exercise performance and thermal tolerance in teleosts (Farrell, 2002; Farrell and Clutterham, 2003), a potential role of cardiac paCA in alleviating these limitations is clearly worthwhile investigating.

The results from our study provide the first *in vivo* evidence that β-NHE short-circuiting is a fundamental aspect in the mode of cardiovascular O_2 transport in Atlantic salmon. The mechanistic requirements for β-NHE short-circuiting appear to be met in most teleost species (Berenbrink et al., 2005; Harter and Brauner, 2017) and certainly in all salmonids (Shu et al., 2017), further extending the significance of these findings. During their yearly up-river migrations, Pacific- and Atlantic salmon species display some of the most astounding exercise performances among fishes. Previous work on sockeye salmon, has identified cardiovascular O_2 transport as a major limiting factor during spawning migrations, especially in the face of

increasing river temperatures (Eliason et al., 2011; Eliason et al., 2013). Clearly, a mechanism that enhances β_b, and thus alleviates the requirements on $\dot{Q}$, may be critical in a clade where reproductive success is so closely tied to the ability to increase cardiovascular O_2 transport. It appears that paCA in teleosts may accomplish this by selectively creating and abolishing H^+ gradients across the RBC membrane that are actively generated by RBC β-NHE. The result is a large $\Delta pH_{a\text{-}v}$ that right-shifts the OEC at the tissues and enables a larger O_2 extraction from the blood; a system with obvious similarities to the *retes* in the eyes and swimbladder of teleosts. Thus, potentially half of all vertebrates may enhance O_2 unloading from Hb by an active cellular mechanism at the level of the RBC that responds to adrenergic control.

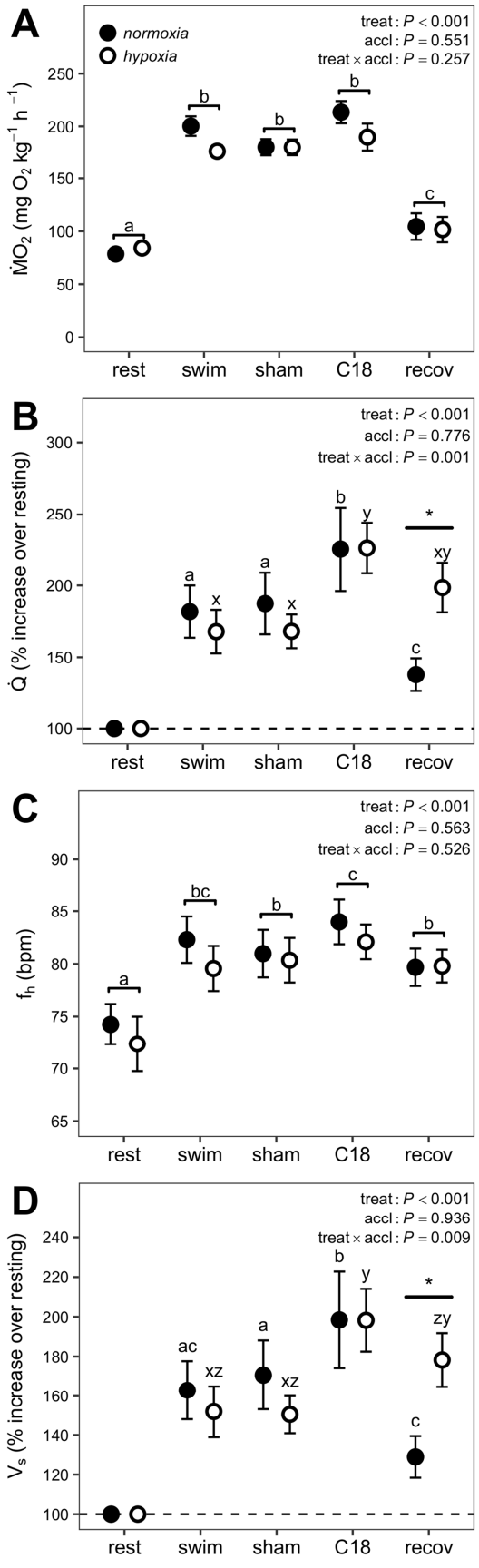

A
normoxia
hypoxia
treat : P < 0.001
accl : P = 0.551
treat × accl : P = 0.257
ṀO2 (mg O2 kg−1 h−1)
250
200
150
100
50
0
a
b
b
b
c
rest
swim
sham
C18
recov
B
treat : P < 0.001
accl : P = 0.776
treat × accl : P = 0.001
Q̇ (% increase over resting)
300
250
200
150
100
a
x
a
x
b
y
*
xy
c
rest
swim
sham
C18
recov
C
treat : P < 0.001
accl : P = 0.563
treat × accl : P = 0.526
fh (bpm)
90
85
80
75
70
65
a
bc
b
c
b
rest
swim
sham
C18
recov
D
treat : P < 0.001
accl : P = 0.936
treat × accl : P = 0.009
Vs (% increase over resting)
240
220
200
180
160
140
120
100
ac
xz
a
xz
b
y
*
zy
c
rest
swim
sham
C18
recov

Figure 3-1 A) Rate of oxygen consumption ($\dot{M}O_2$; mg O_2 kg^{-1} h^{-1}), B) Cardiac output ($\dot{Q}$; % increase over resting), C) heart rate (f_h; bpm) and D) stroke volume (V_s; % increase over resting), of normoxia- (black) and hypoxia- (white) acclimated Atlantic salmon.
Measurements were taken at rest, during moderate exercise at 1 bl s^{-1} (swim), after injection with saline (sham), after injection with a membrane-impermeable CA inhibitor (C18; 0.2 mM), and after the swim trial, 10 min into recovery (recov). All trials were performed in normoxia for both acclimation groups. Data were analysed with a linear mixed model and the main effects of treatment (treat), acclimation (accl) and their interaction (treat×accl) are shown for the full model. Differences between treatments were analysed with a Tukey post-hoc test (N = 9-14; P < 0.05) and are indicated by different superscript letters. If the main model detected significant acclimation effects, post-hoc analysis was carried out separately for both acclimation groups, and significant interaction effects are labelled with "*". All data are means ± s.e.m..

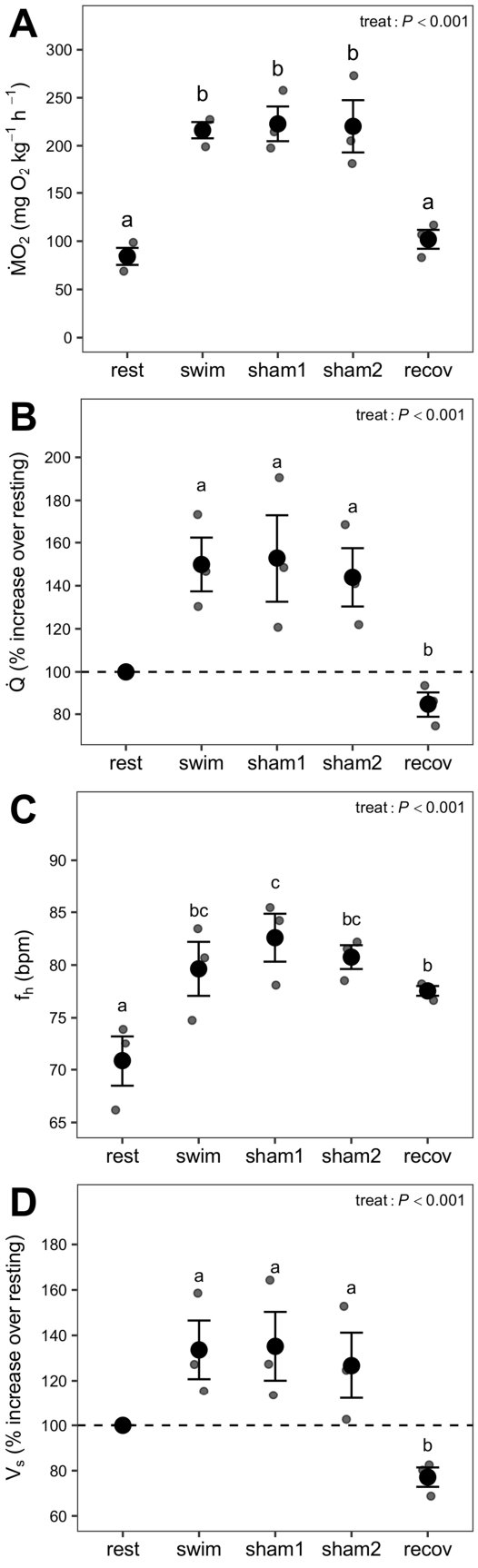

A
treat : P < 0.001
$\dot{M}O_2$ (mg O_2 kg^{-1} h^{-1})
300
250
200
150
100
50
0
a
b
b
b
a
rest
swim
sham1
sham2
recov
B
treat : P < 0.001
$\dot{Q}$ (% increase over resting)
200
180
160
140
120
100
80
a
a
a
b
rest
swim
sham1
sham2
recov
C
treat : P < 0.001
f_h (bpm)
90
85
80
75
70
65
a
bc
c
bc
b
rest
swim
sham1
sham2
recov
D
treat : P < 0.001
V_s (% increase over resting)
180
160
140
120
100
80
60
a
a
a
b
rest
swim
sham1
sham2
recov

Figure 3-2 Rate of oxygen consumption ($\dot{M}O_2$; mg O_2 kg^{-1} h^{-1}), B) Cardiac output ($\dot{Q}$; % increase over resting), C) heart rate (f_h; bpm) and D) stroke volume (V_s; % increase over resting), of normoxia-acclimated Atlantic salmon. Measurements were taken at rest, during moderate exercise at 1 bl s^{-1} (swim), after duplicate saline injections (sham1 and sham2) that corresponded to the time points of saline and C18 injection in the main experiment, and after the swim trial, 10 min into recovery (recov). Data were analysed with a linear mixed model and the main effect of treatment (treat) is shown for the full model. Differences between treatments were analysed with a Tukey post-hoc test ($N = 3$; $P < 0.05$) and are indicated by different superscript letters. Raw values are plotted as small points and means ± s.e.m. are shown as large circles with error bars.

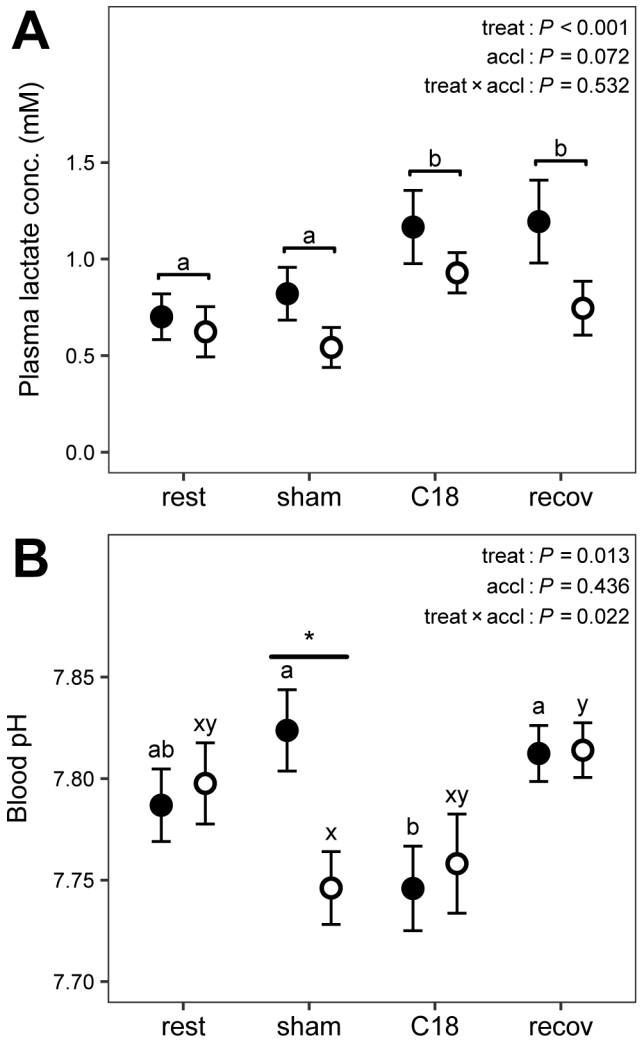


Figure 3-3 A) Plasma lactate concentration (mM) and B) blood pH, of normoxia- (black) and hypoxia- (white) acclimated Atlantic salmon. Blood was sampled from the arterial catheter at four time-points during the experiment: at rest, during moderate exercise at 1 bl s^{-1} and after injection with saline (sham), after injection with a membrane-impermeable CA inhibitor (C18; 0.2 mM), and after the swim trial, 10 min into recovery (recov). All trials were performed in normoxia for both acclimation groups. Data were analysed with a linear mixed model and the main effects of treatment (treat), acclimation (accl) and their interaction (treat×accl) are shown for the full model. Differences between treatments were analysed with a Tukey post-hoc test ($N = 9$; $P < 0.05$) and are indicated by different superscript letters. If the main model detected significant acclimation effects, post-hoc analysis was carried out separately for both acclimation groups, and significant interaction effects are labelled with "*". All data are means ± s.e.m..

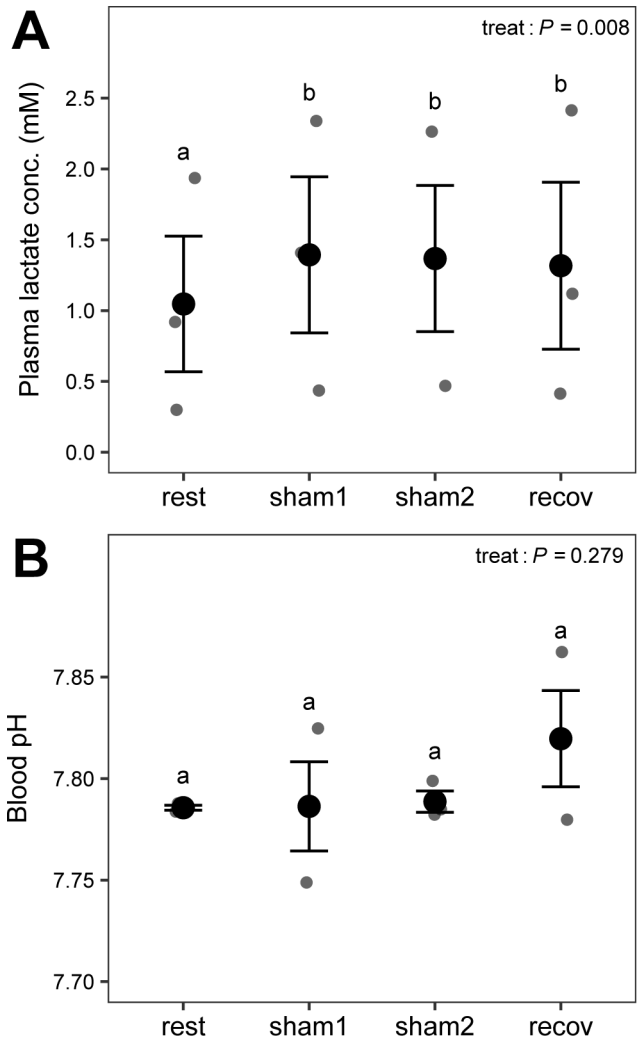


Figure 3-4 A) Plasma lactate concentration (mM) and B) blood pH, of normoxia-acclimated Atlantic salmon. Blood was sampled from the arterial catheter at four time-points during the experiment: at rest, during moderate exercise at 1 bl s^{-1} and after injection with saline (sham1), after a second saline injection (sham2) that corresponded to the time-point of C18 injection in the main experiment, and after the swim trial, 10 min into recovery (recov). Data were analysed with a linear mixed model and the main effect of treatment (treat) is shown for the full model. Differences between treatments were analysed with a Tukey post-hoc test ($N = 3$; $P < 0.05$) and are indicated by different superscript letters. Raw values are plotted as small points and means ± s.e.m. are shown as large circles with error bars.

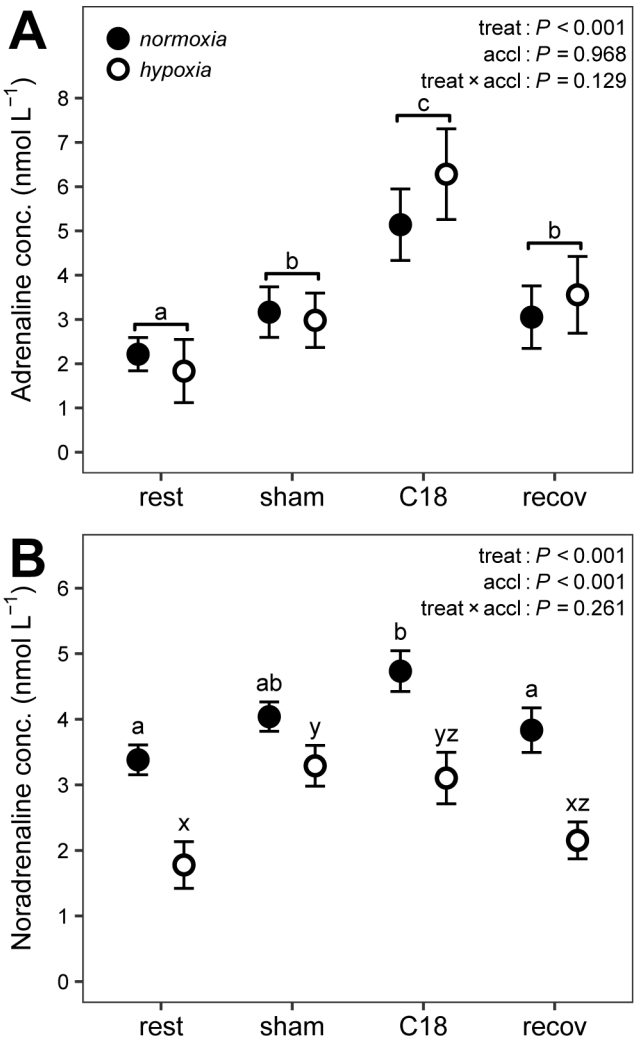


Figure 3-5 A) Plasma adrenaline and B) noradrenaline concentrations (nmol L^{-1}), of normoxia- (black) and hypoxia- (white) acclimated Atlantic salmon. Blood was sampled from the arterial catheter at four time-points during the experiment: at rest, during moderate exercise at 1 bl s^{-1} and after injection with saline (sham), after injection with a membrane-impermeable CA inhibitor (C18; 0.2 mM), and after the swim trial, 10 min into recovery (recov). All trials were performed in normoxia for both acclimation groups. Data were analysed with a linear mixed model and the main effects of treatment (treat), acclimation (accl) and their interaction (treat×accl) are shown for the full model. Differences between treatments were analysed with a Tukey post-hoc test ($N = 9$; $P < 0.05$) and are indicated by different superscript letters. If the main model detected significant acclimation effects, post-hoc analysis was carried out separately for both acclimation groups. All data are means ± s.e.m..

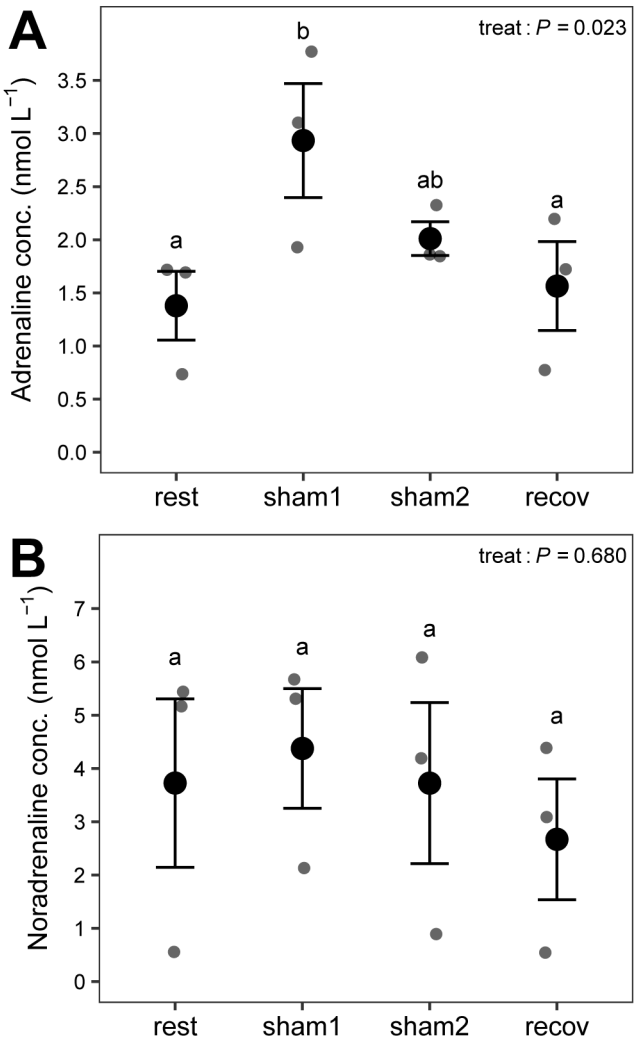


Figure 3-6 A) Plasma adrenaline and B) noradrenaline concentrations (nmol L^{-1}), of normoxia-acclimated Atlantic salmon. Blood was sampled from the arterial catheter at four time-points during the experiment: at rest, during moderate exercise at 1 bl s^{-1} and after injection with saline (sham1), after a second saline injection (sham2) that corresponded to the time-point of C18 injection in the main experiment, and after the swim trial, 10 min into recovery (recov). Data were analysed with a linear mixed model and the main effect of treatment (treat) is shown for the full model. Differences between treatments were analysed with a Tukey post-hoc test (N = 3; $P < 0.05$) and are indicated by different superscript letters. Raw values are plotted as small points and means ± s.e.m. are shown as large circles with error bars.

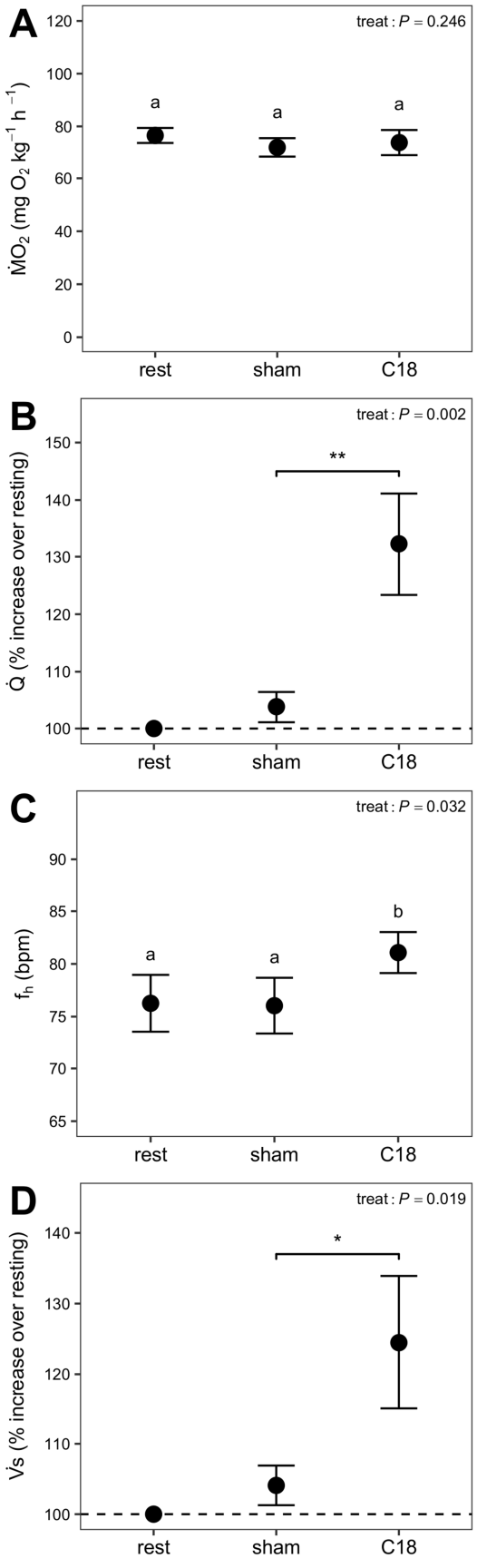

A
treat : P = 0.246
$\dot{M}O_2$ (mg O_2 kg^{-1} h^{-1})
120
100
80
60
40
20
0
a
a
a
rest
sham
C18
B
treat : P = 0.002
$\dot{Q}$ (% increase over resting)
150
140
130
120
110
100
**
rest
sham
C18
C
treat : P = 0.032
f_h (bpm)
90
85
80
75
70
65
a
a
b
rest
sham
C18
D
treat : P = 0.019
$\dot{V}s$ (% increase over resting)
140
130
120
110
100
*
rest
sham
C18

Figure 3-7 A) Rate of oxygen consumption ($\dot{M}O_2$; mg O_2 kg^{-1} h^{-1}), B) Cardiac output ($\dot{Q}$; % increase over resting), C) heart rate (f_h; bpm) and D) stroke volume (V_s; % increase over resting), of normoxia-acclimated Atlantic salmon at rest. Measurements were taken at rest ($\dot{M}O_2$ and f_h), after injection with saline (sham) and after injection with a membrane-impermeable CA inhibitor (C18; 0.2 mM). Data were analysed with a linear mixed model and the main effect of treatment (treat) is shown for the full model. Differences between treatments were analysed with a Tukey post-hoc test ($N = 5$; $P < 0.05$) and are indicated by different superscript letters (in A and C) and significant differences between sham and C18 are indicated as $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ (in B and D). All data are means ± s.e.m..

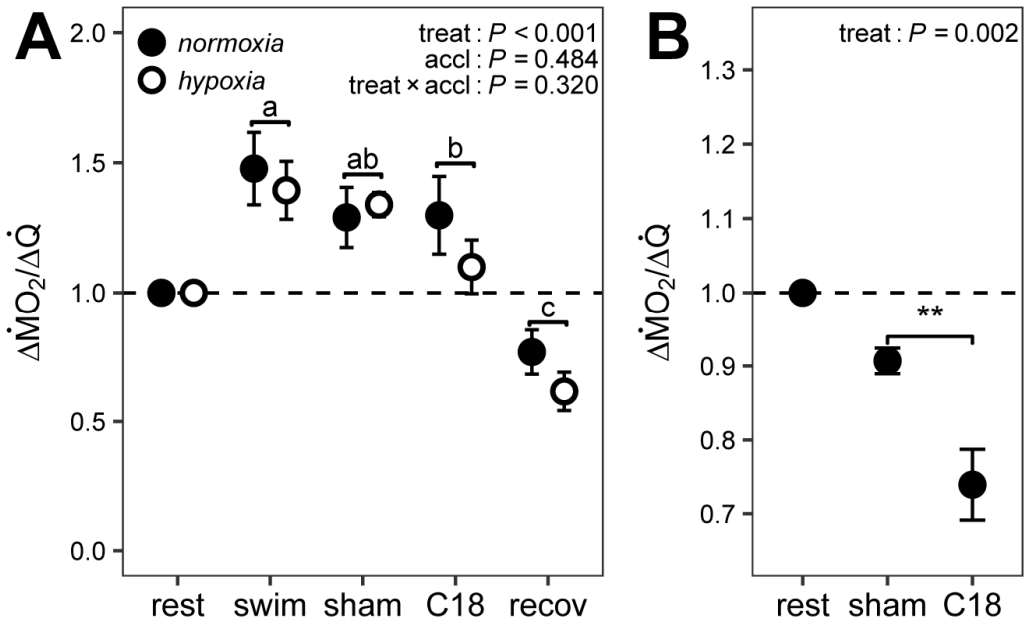


Figure 3-8 Ratio between the change in oxygen consumption rate and the change in cardiac output ($\Delta\dot{M}O_2/\Delta\dot{Q}$), relative to resting values. A) Normoxia- (black) and hypoxia- (white) acclimated Atlantic salmon, were measured at rest, during moderate exercise at 1 bl s^{-1} (swim), after injection with saline (sham), after injection with a membrane-impermeable CA inhibitor (C18; 0.2 mM), and after the swim trial, 10 min into recovery (recov). All trials were performed in normoxia for both acclimation groups and significant differences between treatments are indicated by different superscript letters. B) Resting, normoxia-acclimated Atlantic salmon, injected with saline (sham) and C18 (0.2 mM). A resting value of 1, where $\dot{M}O_2$ and $\dot{Q}$ change at the same rate, is indicated by the dashed line. Data were analysed with a linear mixed model and the main effects of treatment (treat), acclimation (accl) and their interaction (treat×accl) are shown for the full model. Differences between treatments were analysed with a Tukey post-hoc test (A, $N = 9$ and B, $N = 5$; $P < 0.05$) and are indicated by different superscript letters (in A) and significant differences between sham and C18 are indicated as $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (in B). All data are means ± s.e.m..

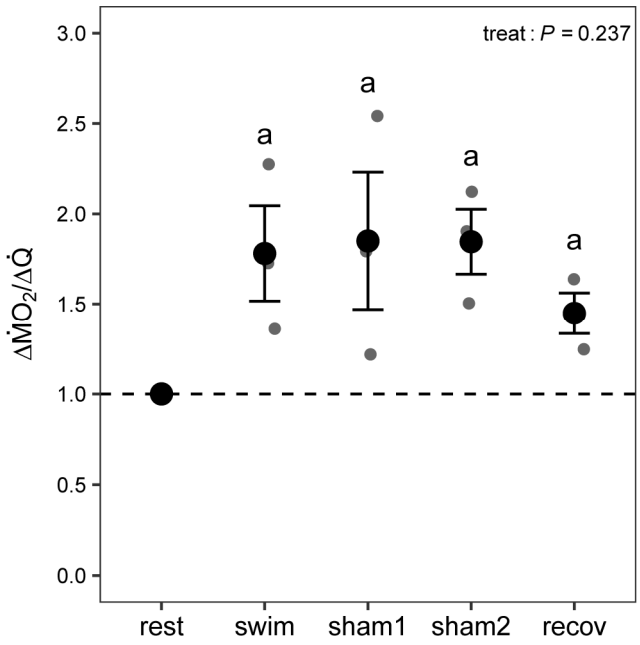


Figure 3-9 Ratio between the change in oxygen consumption rate and the change in cardiac output ($\Delta\dot{M}O_2/\Delta\dot{Q}$), relative to resting values, of normoxia-acclimated Atlantic salmon. Measurements were taken during moderate exercise at 1 bl s^{-1} (swim), after duplicate saline injections (sham1 and sham2) that corresponded to the time points of saline and C18 injection in the main experiment, and after the swim trial, 10 min into recovery (recov). A resting value of 1, where $\dot{M}O_2$ and $\dot{Q}$ change at the same rate, is indicated by the dashed line. Data were analysed with a linear mixed model and the main effect of treatment (treat) is shown for the full model. Differences between treatments were analysed with a Tukey post-hoc test ($N = 3$; $P < 0.05$) and are indicated by different superscript letters. Raw values are plotted as small points and means ± s.e.m. are shown as large circles with error bars.

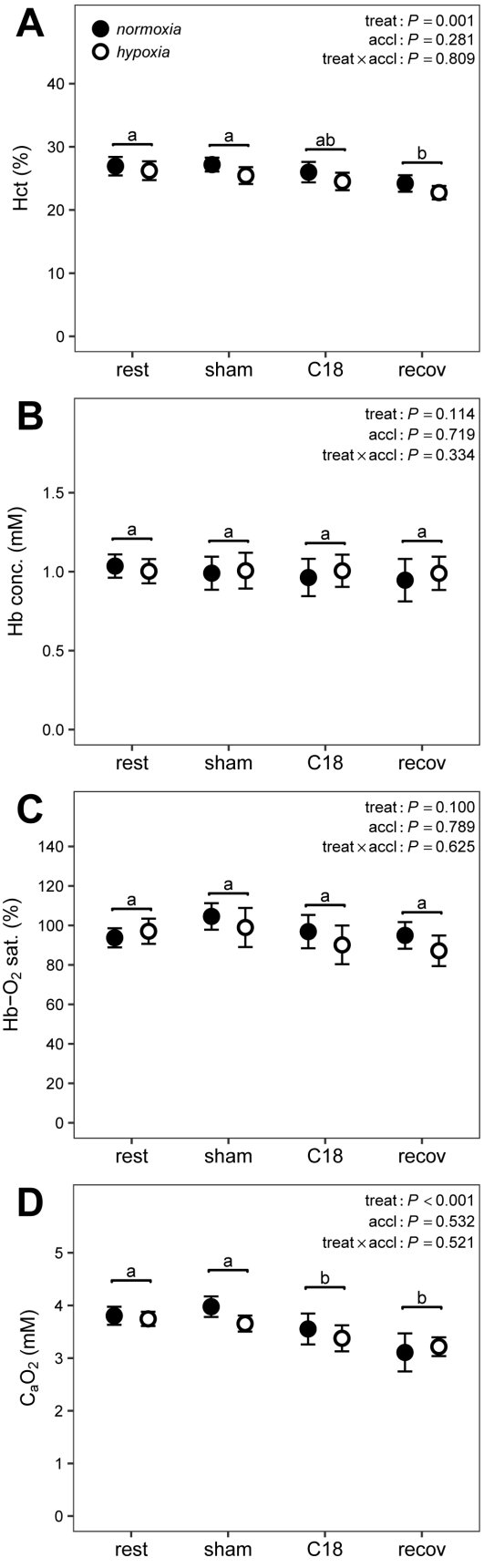

A
normoxia
hypoxia
treat : P = 0.001
accl : P = 0.281
treat × accl : P = 0.809
Hct (%)
0
10
20
30
40
a
a
ab
b
rest
sham
C18
recov
B
treat : P = 0.114
accl : P = 0.719
treat × accl : P = 0.334
Hb conc. (mM)
0.0
0.5
1.0
1.5
a
a
a
a
rest
sham
C18
recov
C
treat : P = 0.100
accl : P = 0.789
treat × accl : P = 0.625
Hb–O2 sat. (%)
0
20
40
60
80
100
120
140
a
a
a
a
rest
sham
C18
recov
D
treat : P < 0.001
accl : P = 0.532
treat × accl : P = 0.521
CaO2 (mM)
0
1
2
3
4
5
a
a
b
b
rest
sham
C18
recov

Figure 3-10 A) Haematocrit (Hct; %), B) haemoglobin concentration (mM), C) haemoglobin oxygen saturation (Hb-O_2 sat.; %) and D) arterial O_2 content of the blood (C_aO_2; mM), of normoxia- (black) and hypoxia- (white) acclimated Atlantic salmon. Blood was sampled from the arterial catheter at four time-points during the experiment: at rest, during moderate exercise at 1 bl s^{-1} and after injection with saline (sham), after injection with a membrane-impermeable CA inhibitor (C18; 0.2 mM), and after the swim trial, 10 min into recovery (recov). All trials were performed in normoxia for both acclimation groups. Data were analysed with a linear mixed model and the main effects of treatment (treat), acclimation (accl) and their interaction (treat×accl) are shown for the full model. Differences between treatments were analysed with a Tukey post-hoc test ($N = 9$; $P < 0.05$) and are indicated by different superscript letters. Mean cell Hb concentration (MCHC) was also calculated and there were no significant effects main effects or interactions on this parameter. All data are means ± s.e.m..

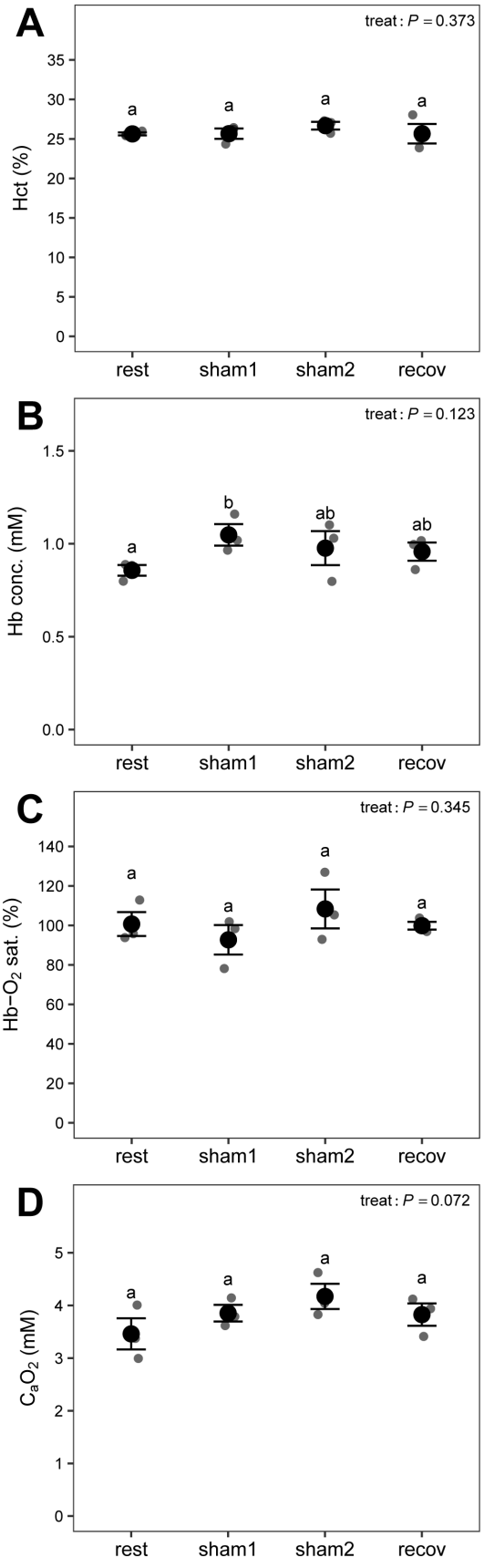

A
treat : P = 0.373
Hct (%)
35
30
25
20
15
10
5
0
a
a
a
a
rest
sham1
sham2
recov
B
treat : P = 0.123
Hb conc. (mM)
1.5
1.0
0.5
0.0
a
b
ab
ab
rest
sham1
sham2
recov
C
treat : P = 0.345
Hb−O2 sat. (%)
140
120
100
80
60
40
20
0
a
a
a
a
rest
sham1
sham2
recov
D
treat : P = 0.072
CaO2 (mM)
5
4
3
2
1
0
a
a
a
a
rest
sham1
sham2
recov

Figure 3-11 A) Haematocrit (Hct; %), B) haemoglobin concentration (mM), C) haemoglobin oxygen saturation (Hb-O_2 sat.; %) and D) arterial O_2 content of the blood (C_aO_2; mM), of normoxia-acclimated Atlantic salmon. Blood was sampled from the arterial catheter at four time-points during the experiment: at rest, during moderate exercise at 1 bl s^{-1} and after injection with saline (sham1), after a second saline injection (sham2) that corresponded to the time-point of C18 injection in the main experiment, and after the swim trial, 10 min into recovery (recov). Data were analysed with a linear mixed model and the main effect of treatment (treat) is shown for the full model. Differences between treatments were analysed with a Tukey post-hoc test ($N = 3$; $P < 0.05$) and are indicated by different superscript letters. Raw values are plotted as small points and means ± s.e.m. are shown as large circles with error bars. Mean cell Hb concentration (MCHC) was also calculated and there were no significant effects main effects or interactions on this parameter.

Chapter 4: Evidence for a plasma-accessible carbonic anhydrase in the lumen of the salmon heart that may secure myocardial oxygen supply[2]

4.1 Synopsis

Oxygen (O_2) supply to the heart of many teleosts, including salmonids, relies in part or in whole on the venous return, which typically has a low partial pressure of O_2 (PO_2). Given that plasma-accessible carbonic anhydrase (paCA) in rainbow trout has recently been shown to play a role in maintaining the high red muscle PO_2 in this species, we tested the hypothesis that paCA is present in the lumen of the coho salmon heart (*Oncorhynchus kisutch*), and may therefore assist in the luminal O_2 supply to the spongy myocardium which has no coronary circulation. Here, we demonstrate a widespread distribution of CA throughout the heart chambers, including lumen-facing cells in the atrium, and confirm that the membrane-bound isoform *ca4* is expressed in the atrium and ventricle of the heart. Further, we confirm that CA catalytic activity is available to blood in the atrial lumen using a modified electrometric ΔpH assay in intact atria in combination with either a membrane-impermeable CA inhibitor, or specific cleavage of the Ca4 membrane anchor. Combined, these results support our hypothesis that paCA is present in the salmon heart, where it may enhance the O_2 supply to the avascular spongy myocardium. This condition may be important to secure cardiac function and thus whole-animal performance when PO_2 in the venous blood becomes greatly reduced, such as after repeated burst exercise that is common during spawning migrations in salmon.

[2]A version of this chapter has been published as: **Alderman*, S. L., Harter*, T. S., Wilson, J. M., Supuran, C. T., Farrell, A. P. and Brauner, C. J.** (2016). Evidence for a plasma-accessible carbonic anhydrase in the lumen of salmon heart that may enhance oxygen delivery to the myocardium. *J. Exp. Biol.* **219**, 719-724.

*indicates equal contribution by the authors

4.2 Introduction

Swimming performance in teleosts is dependent on adequate cardiac function, which in turn requires sufficient oxygen (O_2) supply to the cardiac muscle. This O_2 supply is met by a variable combination of a coronary circulation and the cardiac circulation (i.e., venous return contained in the lumen of the cardiac chambers). Very few teleost fish have a coronary supply to the entire myocardium, and the hearts of at least a third of teleosts are completely devoid of coronary vessels (Davie and Farrell, 1991; Tota et al., 1983; Farrell et al., 2012). O_2 diffusion from the cardiac circulation into the myocardium depends on the partial pressure of O_2 in the venous blood (P_vO_2) that can decrease substantially after burst exercise (Farrell and Clutterham, 2003) and during environmental hypoxia (Holeton and Randall, 1967). Therefore, the heart appears precariously placed in the teleost circulation by having to derive the majority, if not all, of its O_2 supply from the venous blood. Thus, burst exercise, environmental hypoxia and perhaps supra-optimal water temperature may be situations where P_vO_2 is too low to fully support the heart's O_2 demand (Farrell, 2009). Indeed, numerous studies report threshold P_vO_2 values below which teleost hearts fail (see Davie and Farrell, 1991). For example, a minimum P_vO_2 of 7-10 mmHg is required for Chinook salmon (*Oncorhynchus tshawytscha*) exercising at elevated temperature (Clark et al., 2008), and in rainbow trout (*O. mykiss*) exercising in hypoxic water (Steffensen and Farrell, 1998).

Given that the fish heart is poised as a potential weak link in the O_2 transport cascade, adaptations to support O_2 delivery to the myocardium should be of selective advantage. A recent theory (Randall et al., 2014) suggests that the evolutionary success of the teleost lineage owes to a series of physiological adaptations that enhance the capacitance of blood for O_2 (β_b), potentially to all tissues: (i) highly pH sensitive haemoglobins (Hb); (ii) red blood cell (RBC) intracellular pH (pH_i) regulation; and (iii) a heterogeneous expression of plasma-accessible carbonic anhydrase (CA), the enzyme that catalyses CO_2-HCO_3^- reactions. In most vertebrates, a decrease in RBC pH_i will reduce Hb-O_2 affinity (Bohr effect), but in fishes excess H^+s will also reduce O_2 carrying capacity of the blood (Root effect). The Root effect is well-known for its critical role in oxygenating the poorly vascularised retina and pressurising gases in the swimbladder in actinopterygians (Berenbrink, 2011). During a general blood acidosis, such as that occurring with intense exercise or stress, highly pH-sensitive Hb-O_2 binding can be a

liability if Hb re-oxygenation is hindered at the gills. Thus, teleosts with highly pH-sensitive Hbs have evolved a mechanism to protect RBC pH_i whereby activation of adrenergically stimulated sodium-proton exchangers (β-NHE) maintains RBC pH_i by extruding H^+s in exchange for Na^+ (Nikinmaa et al., 1990). In the absence of CA activity in the plasma, H^+s extruded from the RBC will combine with HCO_3^- to form CO_2 at the slow, uncatalysed rate, enabling β-NHEs to maintain a H^+ gradient across the plasma membrane, thereby effectively protecting Hb-O_2 affinity. In the presence of plasma-accessible CA (paCA), however, the extruded H^+s very rapidly dehydrate plasma HCO_3^-, and the resulting CO_2 readily diffuses back into the RBC where it is quickly rehydrated by intracellular CA (Rummer and Brauner, 2011). H^+ production in the RBC out-paces its extrusion by β-NHE due to the high catalytic activity of CA within and outside the RBC, and the net acidification of the RBC reduces Hb-O_2 affinity, unloading O_2 and raising the local PO_2. While experimental evidence in rainbow trout supports this mechanism for enhanced Hb-O_2 unloading at the red muscle (Rummer et al., 2013), its involvement in enhancing O_2 unloading to other metabolically active tissues is unknown. Given the vital importance of maintaining O_2 supply to the myocardium, we **tested the hypothesis that the salmon heart contains a membrane-bound CA isoform that is accessible to blood in the lumen.** We provide histological and molecular evidence for CA in the atrium and ventricle of coho salmon hearts, and demonstrate, using a novel *in vitro* assay, that membrane-bound CA is available to blood in the avascular atrial lumen. This localisation of CA could facilitate O_2 delivery during critical periods of low P_vO_2 and blood acidosis, which salmon must experience during their exhausting spawning migrations.

4.3 Materials and Methods

4.3.1 Experimental animals and heart collection

Adult female coho salmon (*O. kisutch*) obtained as smolts from Target Marine Hatcheries (Sechelt, BC, Canada) were housed in 10 000 L, flow-through, outdoor tanks supplied with dechlorinated Vancouver city water (~12^{o}C) at The University of British Columbia (UBC), Vancouver. Fish were held under a natural photoperiod and fed every-other day with commercial salmon chow. Prior to use, individual fish were terminally anesthetised in MS-222, weighed, and injected with 100 I.U. heparin into the caudal vein. The whole heart was then removed and perfused with 100 I.U. mL^{-1} heparinised saline (in mM: 124.1 NaCl, 2.50 KCl, 0.93 $MgSO_4 \cdot 7H_2O$, 2.52 $CaCl_2 \cdot 2H_2O$, 5.55 glucose, 3.87 TES acid and 6.13 TES salt, pH 7.4) to

remove blood from the lumen prior to further processing, as described below. Care and use of the animals was approved by the UBC Animal Care Committee according to the principles of the Canadian Council for Animal Care.

4.3.2 Enzyme histochemistry

Following saline perfusion, hearts were immersed in phosphate buffered saline (PBS; pH 7.4) containing 4% paraformaldehyde and 1.5% glutaraldehyde for 4 h at room temperature, then cryoprotected in a 30% sucrose solution in PBS prior to embedding in Cryomatrix (Thermo Shandon, Pittsburgh, PA). Cryosections (16 µm) were thaw-mounted onto filter paper discs (0.45 µm pore size; Millipore Corporation, Bedford, MA), air-dried, then processed immediately or temporarily stored at -20^{o}C. CA enzymatic activity was confirmed using Hansson's histochemical method of cobalt sulphide precipitation (Hansson, 1967). Briefly, individual filter paper discs containing heart sections were floated on the surface of a small weigh boat filled with freshly prepared reaction solution (in mM: 1.75 $CoSO_4$, 53 H_2SO_4, 11.7 KH_2PO_4, 15.7 $NaHCO_3$; with or without 10^{-5} M acetazolamide; Az) for 30 min at room temperature. After rinsing briefly in 0.67 mM phosphate buffer (pH 5.9), sections were immersed in 0.5% $(NH_4)_2S$ in MilliQ water for 3 min then rinsed several times in water. In order to ensure that the integrity of the tissue slice was maintained for imaging, filter papers were mounted directly onto glass slides then photomicrographed with a 20x objective on a Nikon Eclipse 90i (Tokyo, Japan).

4.3.3 Cloning and expression of ca4 in the heart

Freshly dissected tissues (gill, bulbus arteriosus, atrium, ventricle, isolated spongy and compact layers of ventricle) were placed in RNAlater then total RNA was extracted using Trizol (Life Technologies, Carlsbad CA, USA). Following DNase I treatment, 500 ng RNA was reverse transcribed to cDNA using the High Capacity Reverse Transcription kit following manufacturer's instructions throughout (Life Technologies). Duplicate cDNA reactions lacking the Multiscribe RT enzyme were included for each sample to control for genomic DNA contamination. Degenerate primers were designed for *ca4* (f: 5'-CAGTCCCCATYAACATTGT-3', r: 5'-GAGCCMTGRTAGCGGWAGTA-3') based on a homology alignment of *O. mykiss* and *Squalus acanthias* (Accession numbers AY514871 and DQ092628, respectively), and used in standard polymerase chain reactions on atrium cDNA with One*Taq* DNA Polymerase (New England Biolabs, Whitby, ON, Canada). A single product of the expected size was purified, subcloned (PGEM-T Easy Vector and JM109 competent cells, Promega, Madison, WI), and

sequence-verified. RT-PCR was repeated as above on cDNAs from all isolated tissues using nested primers specific to this sequence (f: 5' – GATTGGTGGAGACCTGGAGA – 3'; r: 5' – GTCTTTCAGGGCATCTTCCA – 3'). Cytoplasmic *β-actin* was amplified in separate reactions for quality control (primers from Veldhoen et al., 2010). All non-RT control templates failed to amplify.

4.3.4 Carbonic anhydrase assays in atrial chambers

The atrium was isolated intact and fitted with afferent and efferent cannulae (flared PE60 tubing secured with 4-0 silk sutures). The afferent cannula was modified to include a short nested cannula of PE10 tubing glued in place. In *Experiment 1*, cannulated atria ($N = 6$) were connected to a stop-flow apparatus that allowed the atrial chamber to be continuously perfused at 1 mL min^{-1} with assay buffer (in mM: 225 mannitol, 75 sucrose, 10 Tris base, adjusted to pH 7.4 at 4°C with 10% phosphoric acid; Henry, 1991) from an aerated, constant-pressure reservoir maintained at 4°C. A needle-type pH microsensor (PreSense, Regensburg, Germany) was inserted through the myocardium and positioned in the approximate center of the atrial lumen. Chamber pH was continuously recorded to the nearest 0.01 pH unit, in real-time at a frequency of once per second using the pH1 View software package (Presense) while assay buffer continued to be perfused. CA activity in the atrial lumen was measured using the electrometric ΔpH method (Henry, 1991) where perfusion was stopped and the CO_2 hydration reaction was initiated by adding 20-40 µl (~10% luminal volume) CO_2-saturated MilliQ water through the nested cannula. After the reaction had proceeded to completion (stable pH), perfusion was re-initiated and the procedure was repeated twice. Next, the chamber was perfused with assay buffer containing 0.2 mM of C18, a membrane impermeable CA inhibitor (Supuran et al., 2000; Rummer et al., 2013), and the CO_2 hydration rate was measured in triplicate as above. Finally, the chamber was perfused again with assay buffer in the absence of C18 and the CO_2 hydration rate was re-measured as above to confirm that the inhibition was reversible. Atria were then blotted dry and weighed.

In *Experiment 2*, atria were isolated and cannulated as above, and then filled with 400 µl of either saline alone, or saline containing 1 I.U. phosphatidylinositol-specific phospholipase C (PI-PLC; Life Technologies) and incubated at room temperature for 90 min ($N = 6$). PI-PLC is known to release GPI-anchored proteins, such as Ca4, from cell surfaces (Cross 1987; Low et al. 1988; Zhu and Sly, 1990; Bottcher et al., 1994; Gilmour et al., 2002; Esbaugh and Tufts, 2004). Immediately following this pre-treatment, individual atria were perfused with assay buffer and

the CO_2 hydration rate was measured in triplicate as described above. Atria were blotted dry after the assay and weighed.

4.3.5 Data analysis

Initial pH (pH_1) was calaculated as the average pH recording for the 30 s after the flow was stopped and prior to CO_2 injection, and final pH (pH_2) was calculated as the average pH recording for the final 15 s of the assay prior to re-initiating flow. Triplicate recordings were analysed to determine the average reaction velocity (V, in pH units·s^{-1}) for each atrial chamber as follows:

$$V = \frac{\Delta pH}{\Delta t}$$

where

$$\Delta pH = pH_2 - pH_1$$

and Δt is the time (s) between the pH_1 and pH_2 measurements. Despite large differences in Δt between treatments for both experiments, ΔpH varied on average less than 0.05 pH units with intra-assay coefficients of variation of 36.9% and 25.9% for *Experiments 1* and *2*, respectively. For *Experiment 1*, a one-way Repeated Measures Analysis of Variance (RM ANOVA) and Holm-Sidak post-hoc test for multiple comparisons were used to determine differences in V between control, C18 inhibited, and recovery traces ($N = 6$, $P < 0.05$). For *Experiment 2*, a Student's two-tailed t-test was used to compare the V of control and PI-PLC treated atria ($N = 6$, $P < 0.05$). A one-way ANOVA was used to compare atrial mass among assay groups. All data are expressed as mean ± s.e.m..

4.4 Results

4.4.1 Distribution of CA in the heart

CA activity and distribution in the coho heart was visualised by enzyme histochemistry using cobalt sulphide precipitation (Hansson, 1967). In this technique, cobalt precipitates at sites of CA activity as the enzyme catalyses the alkalisation of the reaction solution (Maren, 1980), and the black deposits are readily visualised in tissue sections. Multiple cell types were stained

with this technique, as distinguished by size, shape and location (Fig. 4-1). In the atrium, squamous cells lining the lumen were frequently seen (Fig. 4-1A). In the ventricle, myocytes in the compact layer were abundantly stained and clearly demarcated the spongy layer boundary (Fig. 4-1B). Small round cells in the spongy layer of the ventricle (Fig. 4-1B) were readily distinguished from the few large, plump, and deeply stained RBCs scattered in the lumens of the heart and blood vessels (Fig. 4-1A-C). No staining was observed in the bulbus arteriosus (not shown). The specificity of the staining protocol was confirmed by inhibiting CA activity with the addition of Az to the reaction solution, which completely abolished cobalt precipitation (Fig. 4-1D).

4.4.2 Cloning and expression of ca4

Homology cloning using atrium cDNA yielded a 472 bp fragment of a carbonic anhydrase sequence that shared 69-83% identity with other known teleost *ca4* sequences (GenBank Accession number KT362379). The coho *ca4* transcript was expressed in all of the contractile regions of the heart (atrium, ventricle, isolated spongy and compact layers of the ventricle) but was not detected in the bulbus arteriosus or gills (Fig. 4-2).

4.4.3 CA activity in the atrial lumen

To functionally demonstrate that CA localised in endocardial cells is oriented towards the plasma, we modified the electrometric ΔpH assay (Henry, 1991) to measure CA activity in the lumen of intact atria. The atrium was chosen for this assay for two reasons: i) it is avascular and therefore wholly dependent on luminal O_2 supply, and ii) the relatively larger atrial lumen that is free of trabeculae, as compared to the ventricle, allowed consistent placement of the delicate pH microsensor into its lumen. In *Experiment 1*, individual atria were assayed sequentially with and without the membrane-impermeable CA inhibitor, C18. In all atria, addition of C18 to the reaction buffer reduced the reaction velocity by 3-fold from the initial uninhibited control reaction ($-5.2\times10^{-4} \pm 6.6\times10^{-5}$ vs. $-1.5\times10^{-3} \pm 3.5\times10^{-4}$ pH units s^{-1}, respectively; $N = 6$, $P < 0.001$; Fig. 4-3A). This inhibition by C18 of CA in the atrial chambers was reversible, with reaction velocities returning to control values upon removal of the inhibitor (128.7 ± 35.7% recovery; $P > 0.05$; Fig. 4-3A). As further confirmation for the orientation of CA on atrial endothelial cells, and to help elucidate which CA isozymes may be present, *Experiment 2* began by pre-incubating the cannulated atria with either saline alone or in combination with PI-PLC. The PI-PLC treated atria had an average reaction velocity of $-1.0\times10^{-3} \pm 5.6\times10^{-5}$ pH units$\cdot s^{-1}$,

which was over 2-fold slower than the reaction velocity of control atria pre-treated with saline alone ($-2.3\times10^{-3} \pm 4.6\times10^{-4}$ pH units$\cdot s^{-1}$; $N = 6$; $P = 0.02$; Fig. 4-3B). In both *Experiments 1* and *2*, the triplicate assays yielded highly reproducible traces of the decrease in pH associated with CO_2 hydration, although the rate of acidification varied with treatment (Fig. 4-3C). There was no significant difference in the mass of atria used in these assays (*Experiment 1*: 84.9 ± 2.5 mg; *Experiment 2*: Control 83.8 ± 13.9 mg, PI-PLC 68.8 ± 7.8 mg; $P = 0.42$).

4.5 Discussion

The results of this study show that CA, including the membrane-bound isoform *ca4*, is broadly expressed in the salmonid heart and that either inhibiting or removing CA from the blood-facing membrane of endocardial cells within the atrium slowed the CO_2 hydration rate in the lumen. This study, therefore, is the first to demonstrate a functional membrane-bound paCA in the salmonid heart lumen. Given that paCA can facilitate Hb-O_2 unloading from the highly pH-sensitive teleost Hb during a blood acidosis (Rummer and Brauner, 2011; Rummer and Brauner, 2015), our findings support the hypothesis that also the salmonid heart may benefit from this mechanism (Rummer et al., 2013). Importantly, such a system would increase the PO_2 at which O_2 is unloaded from Hb and thus overcome the major limitation of O_2 diffusion during periods of low P_vO_2. But, as discussed below, there could also be other functional advantages to having paCA in the heart lumen.

We demonstrated the widespread distribution of CA in the coho heart by using Hansson's enzyme histochemical method (Hansson, 1967), which relies on the precipitation of cobalt to visualise sites of CA activity in the tissue sections (Maren, 1980). The multiple cell types and unique staining patterns of the different heart regions observed using this technique suggests an array of functions for CA in the heart. Important to the present study, numerous squamous cells lining the lumen of the atrium were stained, supporting CA activity in the endocardium of the heart. To determine if the CA expressed in the atrium included a membrane-bound isoform, we cloned a *ca4* fragment from the coho atrium and demonstrated its expression in the contractile regions of the heart. This extends earlier findings of Ca4 protein expression in rainbow trout hearts and absence in gills (Georgalis et al., 2006), and of membrane-associated CA in the hearts of cyclostomes (Esbaugh and Tufts, 2004; Esbaugh et al., 2009) that are completely avascular (Farrell et al., 2012).

There are 16 CA isozymes characterised in mammalian systems and many have also been identified in other vertebrate species. The CA4 isozyme has a high catalytic activity and is anchored to the plasma membrane by a GPI residue (Esbaugh and Tufts, 2006; Hilvo et al., 2005). In mammalian hearts, CA4 is localised to the endothelium of blood vessels, the sarcolemma and the sarcoplasmic reticulum (Sender et al., 1998), and CA4 functions in facilitating the efflux of metabolic wastes into the blood and in Ca^{2+} cycling during muscle contraction and relaxation (Geers and Gros, 2000). Similarly, in teleosts, CA in skeletal muscle is also important for facilitating the diffusion of metabolic wastes into the blood. Perfusion of isolated rainbow trout trunk muscle with various CA inhibitors demonstrated a role for both intra- and extracellular CA in CO_2 and NH_3 excretion, although the relative contribution of these CAs varied depending on the extent of CO_2 production in the muscle (Henry et al., 1997; Wang et al., 1998). More recently, paCA in rainbow trout red muscle was shown to increase PO_2 during a hypercarbia-induced blood acidosis (Rummer et al., 2013), and we propose that the *ca4* expressed in the coho heart may enhance PO_2 and thus diffusion gradients to the myocardium in a similar manner. While paCA surely plays additional functional roles in heart, including associated gas exchange and metabolic waste removal, we focus most of the following discussion on how this localisation may enhance Hb-O_2 unloading.

The model for enhanced Hb-O_2 unloading in teleosts requires localised catalytic activity of CA in the plasma to short circuit RBC pH_i regulation during an acidosis in order to acidify the RBC and reduce Hb-O_2 affinity (Randall et al., 2014). Thus, CA would need to be located on the luminal wall of the coho atrium to participate in enhancing Hb-O_2 unloading to the myocardium. To functionally demonstrate this distribution, we measured CA activity in the lumen of intact atria using a modification of the electrometric ΔpH assay that quantifies the rate of CO_2 hydration. Repeating the assay in the same atria in the presence of the membrane-impermeable CA inhibitor, C18, caused a reversible reduction in the measured reaction velocity. Similarly, pre-treating the atria with PI-PLC to cleave the CA-membrane anchor also significantly decreased the reaction rate compared to saline-treated control atria. Together, these results confirm the presence of a paCA in the salmonid atrium that is likely the GPI-linked, membrane-bound isozyme, Ca4. Given that paCA in rainbow trout can more than double tissue PO_2 under relevant physiological conditions (Rummer and Brauner, 2015; Rummer et al., 2013), our demonstration of a similar CA distribution in the heart of a closely related species may have

significant bearing on our current understanding of myocardial oxygenation in salmonids and other teleosts.

The modified electrometric ΔpH assay used in this study, in combination with the CA inhibitor C18, provides functional evidence for the presence and luminal orientation of membrane-bound CA. These insights can not be obtained by studying isolated plasma membranes, due to the high CA activity in the microsomal fraction (Bruns and Gros, 1992; Geers et al., 1992), as CA4 is present on both endothelial cell and cardiomyocyte membranes (Knüppel-Ruppert et al., 2000; Sender et al., 1998), as well as potential contamination from intracellular CA4 activity (Schneider et al., 2013). Nonetheless, limitations to our method include the inability to measure the true uncatalysed reaction rate and a lack of mixing of the reaction volume, which prevent quantification of absolute CA activity.

We have now added a novel discovery to illustrate how well the salmonid heart is adapted to extract O_2 from the O_2-depleted venous blood. In addition to paCA on the luminal wall that may increase the local PO_2 gradient, other adaptations include a high surface area of the spongy ventricular myocardium, the arrangement of trabeculae into thin sheets of muscle to reduce the diffusion distance (Pieperhoff et al., 2009), the narrow spindle-shaped myocytes that minimise intracellular diffusion distances, and a high capillary density in the compact ventricular myocardium (Egginton and Cordiner, 1997; Cox, G.K., personal communication). Under routine conditions this morphology is suggested to be more than sufficient for meeting myocardial O_2 demand (Farrell, 2002). Similarly, these morphological characteristics would aid the diffusion of metabolically produced CO_2 from cardiomyocytes, a process that is facilitated by paCA in striated muscle capillaries of fish (Henry et al., 1997a) and other vertebrates (Geers and Gros, 2000). Plasma-accessible CA may become critical for cardiac function during stressful situations in which normal P_vO_2 might be insufficient to sustain cardiac function, such as during high levels of aerobic exercise, recovery from burst exercise, environmental hypoxia, and supra-optimal temperature. All of these situations are stressful to the animal, can lower blood pH as a result of metabolic or respiratory acidosis, and will trigger catecholamine release into the venous blood. They also raise CO_2 and other waste production by the heart, which may be working harder. Circulating catecholamines will activate RBC β-NHEs and create a H^+ disequilibrium across the RBC membrane which will be short-circuited in the presence of paCA, potentially enhancing Hb-O_2 unloading and therefore β_b at the cardiac tissue (Rummer and Brauner, 2011). Therefore,

the physiological implications associated with the enhanced myocardial oxygenation offered by paCA in the heart are considerable. Future challenges will include quantifying the contribution of CA in enhancing β_b at the myocardium under stressful conditions, and to build this contribution into existing models of cardiac function. Of further interest, individual variation in cardiac paCA activity could offer a powerful scaffold for selective pressures to act upon and may contribute to observed fitness differences within populations (Eliason et al., 2011; Eliason et al., 2013). In the present study, variation in reaction velocities of either C18-inhibited or PI-PLC-treated atria were considerably lower than those of the control reactions (s.e.m. 5-12% versus 20-24%, respectively), which is consistent with reduced variability in the treatment groups due to substantial inhibition/removal of CA. The higher variability in the control groups may reflect inter-individual differences in luminal paCA abundance. Given the potential role of paCA in sustaining myocardial O_2 supply by enhancing β_b, this could dictate which salmon arrive at the spawning grounds and which perish on the way, failing to reproduce.

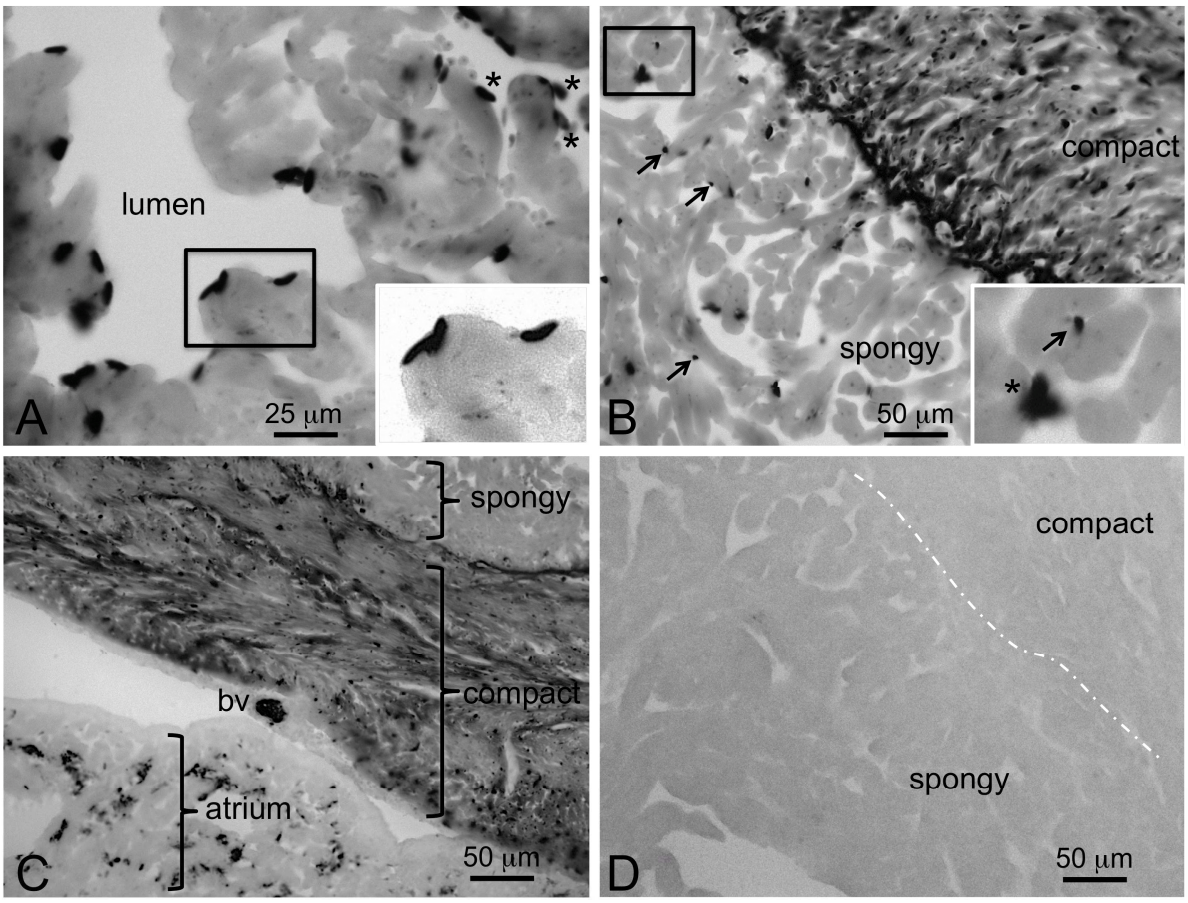


Figure 4-1 Distribution of carbonic anhydrase (CA) in the coho heart. CA activity in 16 μm transverse cryosections of the coho heart was visualised by enzyme histochemistry using cobalt sulphide precipitation (Hansson, 1967). (A) Staining in the atrium predominated in squamous endocardial-type cells (inset) lining the lumen, and in red blood cells (*) scattered throughout the lumen. (B) The compact layer of the ventricle was more heavily stained compared to the spongy layer. The inset in (B) contrasts a small round ventricular cell (arrow) with a cluster of 2-3 red blood cells (*) in the spongy ventricle. (C) Comparison of overall staining patterns in the atrium and ventricle, including a small blood vessel (bv) containing red blood cells in the epicardium. (D) Specificity of staining was confirmed by the addition of 10^{-5} M acetazolamide to the reaction solution, which completely abolished staining. The white dashed line indicates the boundary between the ventricular compact and spongy layers, which is deeply stained in (B).

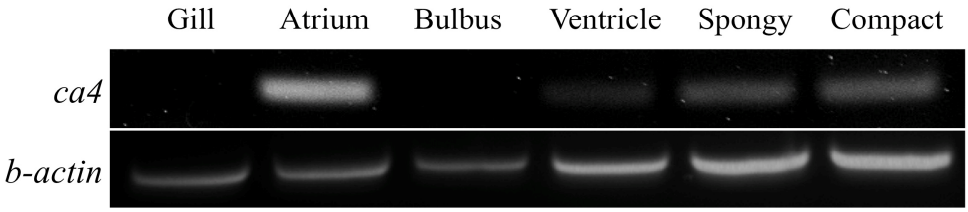
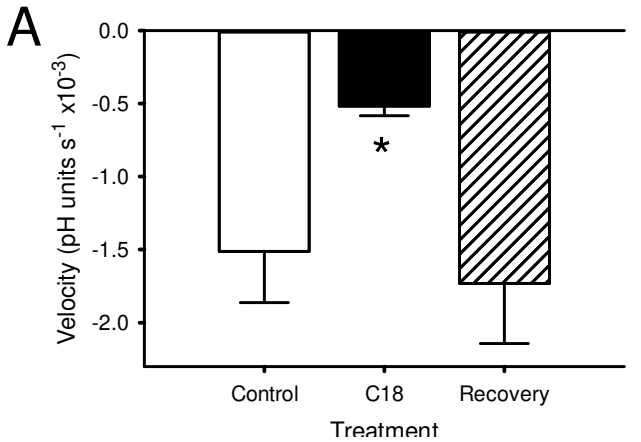


Figure 4-2 Expression of carbonic anhydrase 4 (*ca4*) in coho salmon tissues. The regional expression of c*a4* in the heart was compared by RT-PCR, with gill tissue included as a negative control (Georgalis et al., 2006) and *β-actin* as a positive control. The cardiac regions isolated were: atrium, bulbus arteriosus, whole ventricle, isolated spongy layer of the ventricle and isolated compact layer of the ventricle. All non-reverse transcribed templates failed to amplify products of either gene (not shown).


A
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-1.0
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Velocity (pH units s-1 x10-3)
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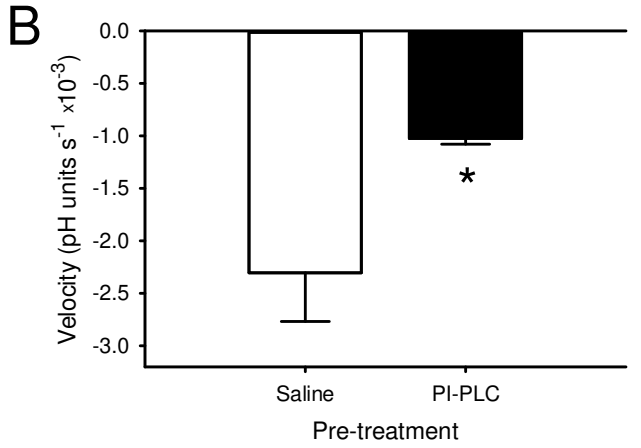
C18
Recovery
Treatment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Pre-treatment

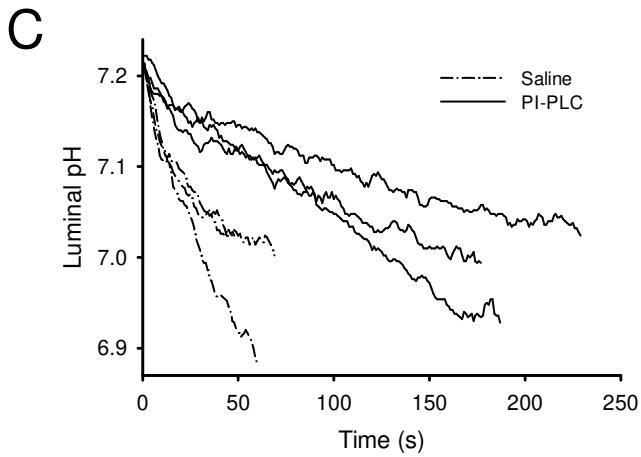
C
7.2
7.1
7.0
6.9
Luminal pH
Saline
PI-PLC
0
50
100
150
200
250
Time (s)

Figure 4-3 CO_2 hydration rates within atrial chambers of coho salmon. Carbonic anhydrase (CA) activity was measured within atrial chambers by quantifying the velocity of CO_2 hydration (pH units s^{-1}). (A) CO_2 hydration rates in atria perfused sequentially with assay buffer alone (control; white bar), buffer containing a membrane-impermeable CA inhibitor (C18; black bar), and again with assay buffer alone (recovery; hatched bar). The graph depicts the average reaction velocities for six atria. (B) In a separate experiment, atrial chambers were pre-treated with saline (white bar) or phosphatidylinositol-specific phospholipase C (PI-PLC; black bar) to cleave the membrane anchor of CA, then CO_2 hydration rate was measured in triplicate for each atrium (mean ± s.e.m..; $N = 6$). (C) Representative replicate traces from two atrial chambers, one pre-treated with saline (dashed lines) and the other with PI-PLC (solid lines). Asterisks indicate significant differences ($N = 6$; $P < 0.05$).

Chapter 5: A solution to Nature's haemoglobin knockout: a plasma-accessible carbonic anhydrase catalyses CO_2 excretion in Antarctic icefish gills[3]

5.1 Synopsis

In all vertebrates studied to date, CO_2 excretion depends largely on the enzyme carbonic anhydrase (CA) that catalyses the rapid conversion of HCO_3^- to CO_2 at the gas-exchange organs. The largest pool of CA is present within red blood cells (RBC) and, in some vertebrates, plasma-accessible CA (paCA) isoforms participate in CO_2 excretion. However, teleost fishes typically do not have paCA at the gills and CO_2 excretion is reliant entirely on RBC CA; a strategy that is not possible in icefishes. As the result of a natural knockout, Antarctic icefishes (channichthyids) are the only known adult vertebrates that do not express haemoglobin (Hb) as adults, and largely lack RBC in the circulation (haematocrit < 1%). Previous work has indicated the presence of high levels of membrane-bound CA activity in the gills of icefishes, but without determining its cellular orientation. Thus, we hypothesised that icefishes express a membrane-bound CA isoform at the gill that is accessible to the blood plasma. The CA distribution was compared in the gills of two closely-related notothenioid species, one with Hb and RBCs (*Notothenia rossii*) and one without (*Champsocephalus gunnari*). Molecular, biochemical and immunohistochemical markers indicate high levels of a Ca4 isoform in the gills of the icefish (but not the red-blooded *N. rossii*), in a plasma-accessible location that is consistent with a role in CO_2 excretion. Thus, in the absence of RBC CA, the icefish gill could exclusively provide the catalytic activity necessary for CO_2 excretion; a pathway that is unlike that of any other vertebrate.

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5.2 Introduction

The first scientific investigation of an Antarctic icefish, less than a century ago (Ruud, 1954), overthrew the common perception that haemoglobin (Hb) was a necessity to sustain vertebrate life. In fact, an entire family of teleosts, channichthyids (suborder notothenioids; perciforms) that comprise 16 species, do not express Hb as adults (Ruud, 1954; Eastman, 1993) and largely lack red blood cells (RBC) in their circulation; residual haematocrit (Hct) is typically <1% (Egginton, 1994). The implications for cardiovascular gas transport are tremendous. In the absence of Hb, icefish blood has a 10-fold lower O_2-carrying capacity compared to red-blooded notothenioids (Holeton, 1970), and without RBCs icefish lack the important pool of carbonic anhydrase (CA) that facilitates CO_2 transport and excretion in all vertebrates (Tufts and Perry, 1998). Those adaptations that address the dramatic impairment of O_2 transport in icefishes are largely known (Hemmingsen and Douglas, 1970; Holeton, 1970; Hemmingsen and Douglas, 1972); however, those needed to resolve the associated problem of CO_2 excretion are not.

Most vertebrates transport the majority of CO_2 that is produced in tissues as dissolved HCO_3^- in the blood plasma. In this regard icefishes are no exception, as indicated by venous blood pH and PCO_2 values (7.84 and 2.1 mmHg in *Chaenocephalus aceratus*; Hemmingsen and Douglas, 1972) that are in line with those found in other fishes. Under these conditions, and due to the low apparent pK of the CO_2-HCO_3^- reaction of ~6.2 (Boutilier et al., 1984), blood plasma is an effective sink for CO_2. While this greatly increases the capacitance for CO_2 transport in blood (Tufts and Perry, 1998; Henry and Swenson, 2000), it also requires a rapid conversion of CO_2 to HCO_3^- at the tissues and the reverse reaction at the gills for CO_2 excretion. However, the spontaneous rates of these reactions are slow relative to the residence time of blood at the respiratory surfaces and tissue capillaries and these rates further slow with decreasing temperature. At physiological temperatures in icefish, around -1.9°C (Littlepage, 1965), the $t_{1/2}$ of spontaneous HCO_3^- dehydration to CO_2 is ~300 s (Kern, 1960; Heming, 1984) and thus exceeds the residence time of blood at the gills (~1-3 s) by two orders of magnitude (Cameron and Polhemus, 1974; Hughes et al., 1981). Based on the arterial-venous differences in PCO_2 and pH in *C. aceratus* (Hemmingsen and Douglas, 1972), it can be estimated that in resting, normoxic icefish, about 68% of CO_2 excretion must depend on HCO_3^- dehydration at the gills, while the remainder is from physically dissolved CO_2 in the plasma. During aerobic exercise, where blood pH is largely maintained, HCO_3^- concentration may increase by 50% (Brauner et

al., 2000b) and the residence time at the gills will be reduced further, due to a higher $\dot{Q}$ (Randall, 1982a); an increase in cardiac output following exercise has recently been shown for *C. aceratus* (Joyce et al., 2018). Clearly, the uncatalysed rate of HCO_3^- dehydration is simply not rapid enough to support CO_2 excretion in any adult vertebrate, but in particular icefishes at these low temperatures.

The rate limitation of CO_2-HCO_3^- reactions in the blood of vertebrates is largely alleviated by the catalytic activity of CA. The major CA pools are: i) RBC intracellular CA (Maren, 1967), and plasma HCO_3^- has functional access to this CA pool *via* rapid Cl^-/HCO_3^- exchange across the RBC membrane (Romano and Passow, 1984); ii) soluble CA isoforms in the plasma (Henry et al., 1997b); and iii) plasma-accessible CA (paCA) isoforms that are anchored to the apical membranes of the endothelium (Henry and Swenson, 2000). At the tissue capillaries paCA is typically present and ensures a rapid conversion of CO_2 to HCO_3^- (Henry et al., 1997a). However, at the gas exchange surface, the contribution of different CA pools to CO_2 excretion varies largely among the major vertebrate groups. On one end of the spectrum are the basal hagfishes (Esbaugh et al., 2009) and chondrichthyans (Gilmour et al., 2002; Gilmour et al., 2007) that rely on RBC CA, soluble CA in the plasma and paCA at the gills for CO_2 excretion. All euteleostomes lack soluble CA activity in the plasma, and thus most tetrapods rely on RBC CA, and to a lesser degree (<10% of total CO_2 excretion) on paCA at the gas exchange surface (Bidani et al., 1983; Zhu and Sly, 1990; Stabenau and Heming, 2003). And finally, teleost fishes have also lost paCA activity at the gills (for review see Harter and Brauner, 2017), and thus HCO_3^- dehydration is shifted entirely into the RBC (Perry et al., 1982; Wood et al., 1982; Desforges et al., 2001; Desforges et al., 2002; for review see Perry and Gilmour, 2002), creating a strong coupling between O_2 and CO_2 transport; a hallmark of teleost gas exchange (Brauner and Randall, 1996). This strategy is clearly not available to icefishes, which are teleosts, but lack RBCs. Thus, with a clear need to catalyse HCO_3^- dehydration, some other CA pool must be present in icefishes to compensate for the loss of RBC CA.

Previous studies on gill homogenates from icefishes have provided biochemical evidence for a higher activity of membrane-associated CA when compared to red-blooded notothenioids (Feller et al., 1981; Maffia et al., 2001). Tufts et al. (2002) further characterised the branchial CA isoform distribution of notothenioids and found biochemical markers for the presence of a membrane-bound Ca4 isoform in the gills of an icefish species, but surprisingly, also in the gills

of a red-blooded notothenioid. A critical detail, the cellular orientation of putatively paCA isoforms remains unexplored and therefore the potential involvement of a Ca4 isoform in CO_2 excretion remains unresolved for icefishes. Building on these previous findings, we **hypothesised that icefishes express a membrane-bound CA isoform at the gill that is accessible to the blood plasma where it would catalyse CO_2 excretion in the absence of RBC CA.** To this end, biochemical, molecular and immunohistochemical techniques were used to compare the CA isoform distribution in the gills of the icefish *Champsocephalus gunnari* and the red-blooded *Notothenia rossii*. The obtained results shed new light on a divergent strategy of CO_2 excretion in icefishes, unlike that found in any other adult vertebrate.

5.3 Materials and Methods

5.3.1 Sample collection

Specimens of *Notothenia rossii* and *Champsocephalus gunnari* (average mass 343.4 ± 17.2 and 644.3 ± 70.1 g, and length 38.8 ± 0.7 and 37.7 ± 1.1 cm) were captured using otter trawls or baited pot traps deployed from the *U.S. ARSV Laurence M. Gould* at Low Island (63° 30' S, 62° 37' W) and North Dallmann Bay (63° 55' S, 62° 43' W), Antarctica. Animals were stunned by a blow to the head and a blood sampled was collected from the caudal vein. After sampling, animals were rapidly euthanised by severing the spinal cord and brain pithing. The blood was mixed with 3.2% sodium citrate (9:1 for *N. rossii* and 4:1 *C. gunnari*) to prevent clotting. Thereafter, samples were centrifuged at 5,300 x g for 10 min and plasma was decanted. Blood cells and plasma were frozen in liquid nitrogen and stored at -70°C. Gills and hearts were perfused with notothenioid Ringer (in mM: 260 NaCl, 2.5 $MgCl_2$, 5 KCl, 2.5 $NaHCO_3$, 5 NaH_2PO_4, at pH 8.0) and tissues were frozen at -70°C or fixed in 10% buffered formalin for 24 h and then transferred to 70% EtOH. Fixed tissues were shipped on ice and frozen tissues were shipped on dry ice, to The University of British Columbia (UBC), in Vancouver. All samples were collected opportunistically and in strict compliance with the guidelines of the Institutional Animal Care and Use Committee (IACUC Protocol no. 14-L-004, Ohio University).

5.3.2 Biochemical analysis of CA activity

Approximately 2 g of gill lamellae were homogenised (Polytron PT1200, Luzern, Switzerland) in 8 mL of assay buffer on ice (in mM: 225 mannitol, 75 sucrose, 10 TRIS base, and adjusted to pH 7.4 with 10% phosphoric acid). Differential centrifugation was at 4°C according to (Henry, 1988b; Henry et al., 1993): i) 800 x g for 20 min; ii) 8500 x g for 20 min

(Allegra 64R, Beckman Coulter, Brea, CA); iii) 100 000 x g for 90 min (Beckman L8-70M) to produce a microsomal pellet containing plasma membranes and a supernatant containing the cytosolic fraction. Pellets were re-suspended in 3 mL of assay buffer, by vortexing and mild sonication (5 W for 3 s). Protein concentration was measured spectrophotometrically at 595 nm using the Bradford assay (Sigma B6916) and bovine serum albumin standards (BioRad Quickstart 5000206; Hercules, CA).

The activity of CA in cellular fractions was measured using the electrometric ΔpH assay (Henry, 1991). Reactions were in 6 mL of assay buffer in a thermostatted vessel at 4°C using 100 μL CO_2 saturated water as a substrate. The reaction kinetics were assessed as the time for a 0.15 unit pH change, with a GK2401C electrode and PHM84 meter (Radiometer, Copenhagen, Denmark). Uncatalysed reaction rates (without sample addition) were subtracted from the enzymatic rates and absolute enzyme catalytic rates were calculated from the buffer curve of the assay buffer over the tested pH range (determined in separate titrations).

Membrane pellets were washed by an additional step of ultracentrifugation (100 000 x g for 90 min) and re-suspended in 3 mL of fresh buffer. Washed pellets were incubated with 1 I. U. phosphatidylinositol-specific phospholipase C (PI-PLC; Invitrogen P6466, Carlsbad, CA), an enzyme that cleaves the common glycosylphosphatidylinositol (GPI) membrane anchor, or with assay buffer as a control, for 90 min at 21°C. CA inhibition kinetics were assessed by: i) adding 0.005% sodium dodecyl sulfate (SDS) to the assay buffer; ii) titrations with 0.6-6 nM acetazolamide (Az) according to (Easson and Stedman, 1936; Dixon, 1953); and iii) adding 100 μL of plasma from either *C. gunnari* or *N. rossii* to the assay buffer. RBC lysates were produced from 50 μL packed RBCs from *N. rossii*, diluted 50-fold in distilled water and frozen in liquid nitrogen twice; CA activity was measured on 5 μL of lysate.

5.3.3 Plasma characteristics

Plasma protein concentration was measured in both species as described above. In addition, Hb concentration in plasma samples from *N. rossii* was measured spectrophotometrically at 540 nm using the cyanomethaemoglobin method with human Hb (Sigma H7379) dilutions as standards. The concentration of protein from Hb was then subtracted from total protein concentration measured in the plasma. The plasma non-bicarbonate buffer capacity (β_{plasma}) was measured with an automated titrator (TIM865, Radiometer, Copenhagen, Denmark). Plasma aliquots of 200 μL were added to 4.5 mL of deionised water in a magnetically

stirred glass titration vessel (4°C) that was continuously sparged with N_2. All results represent upward titrations from pH 4 to 9 with 0.01 M NaOH. β_{plasma} was calculated from the change in pH that corresponded to individual steps of base addition (10 µL) over the physiologically relevant pH range in notothenioids of pH 7.4 to 8.2 (Acierno et al., 1997). β_{plasma} was then calculated as the mean value over the tested pH range.

5.3.4 Immunohistochemistry

Localisation of Ca4 in the gills of *C. gunnari* and *N. rossii* was with a custom rabbit polyclonal antibody raised against rainbow trout (*Oncorhynchus mykiss*) Ca4, which has been described in detail (Gilmour et al., 2007) and has been successfully used in rainbow trout and spiny dogfish (*Squalus acanthias*). The antigenic sequence (TRRTLPDERLTPFTFTGY) corresponds to amino acids 57–74 of the rainbow trout Ca4 (GenBank AAR99330), which is 73% conserved in *N. corriceps*. The immunohistochemical results were later replicated using a custom chicken polyclonal antibody raised against the Ca4 of three elasmobranch species (*Squalus acanthias*, DQ092628.1; *Rhincodon typus*, XM_020514262.1; *Callorhinchus milii*, XP_007894777.1). The antigenic peptide sequence for *S. acanthias* Ca4 was GSEHTIDGEQYPMELHIVH (aa125-144), and the sequence in the notothenioid Ca4 is 100% conserved. Other sections were immunolabeled with a rabbit anti-bovine Ca2 antibody (bCa2, Abcam, Cambridge, UK); the cytosolic Ca2-like isoform in fishes was recently re-named Ca17 (Ferreira-Martins et al., 2016).

Ca4 and Ca2 antibodies were tested by western blot analysis using cytosolic and microsomal fractions of gill homogenates from both species. Subsamples containing 20 µg of protein were separated by SDS-page using 10% polyacrylamide gels (with 4% stacking gel). Proteins were then wet-transferred onto 0.2 µm PVDF membranes (Immun Blot, BioRad), rinsed and air dried. Transfer was assessed using total protein staining with 0.5% Ponceau S in 1% acetic acid and then imaged. Blots were rinsed with TTBS (Tris Buffered Saline with 0.05% tween 20, pH 7.4) and blocked with 5% blotto in TTBS overnight at 4°C. Thereafter, one membrane was probed with a 1:1,000 dilution of the rtCa4 and the other with a 1:2,500 dilution of the Ca2 antibody, overnight at room temperature on a rotisserie (Lab QuakeII, Thermo). Protein size was determined using a Precision Plus Protein Dual Color ladder (BioRad 1610374). All membranes were rinsed three times with TTBS and incubated with a 1:25,000 dilution of a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP; Genscript

Piscataway, NJ), for 1 h at room temperature. Finally membranes were rinsed with TTBS and proteins were visualised using a chemiluminescent HRP substrate (Clarity, BioRad). Images were acquired using the Azure C300 imaging system and provided software (Azure Biosystems, Dublin, CA).

To localise Ca4 and Ca17 in the gills, fixed tissues were stepwise dehydrated in EtOH, cleared in xylene and embedded in paraffin. Thin sections (5 µm) were cut on a microtome (Leica RM2500, Wetzlar, Germany) and mounted on aminopropylsilane (APS) coated microscope slides. A hydrophobic barrier (Sigma SuperPAP, St. Louis, MO) was created around the sections that were incubated in a blocking buffer (BLØK, Millipore, Burlington, MA) for 15 min. Incubation with the primary antibody (rtCa4 1:200 or bovCa2) in blocking buffer was overnight at 4°C in a humidified chamber. Negative controls were incubated with blocking buffer alone, or with normal rabbit serum. Detection of the primary antibody was done with a goat anti-rabbit IgG conjugated to Alexa 488 (Jackson Immunoresearch, West Grove, PA). Sections were then rinsed three times with 0.1 M phosphate buffered saline (PBS) for 5, 10 and 15 min and incubated with secondary antibody in a humidified chamber for 1 h, at 37°C. DAPI was added to the second wash step to visualise cell nuclei. Coverslips were mounted with 1:1 PBS glycerol containing 0.1% NaN_3 and imaging was done with a fluorescence photomicroscope (Leica DM5500; Orca Flash 4, Hamamatsu, Japan).

5.3.5 Sequencing and expression of ca4

Total RNA was extracted from approximately 100 mg of gill and ventricle tissue in 1 mL of Trizol, following the manufacturer's protocol (Invitrogen 15596018, Carlsbad, CA). Ventricles were used as a control tissue, in which the presence of Ca4 has been confirmed in several teleost species (Georgalis et al., 2006; Alderman et al., 2016). Tissues were homogenised with a Bullet Blender 24 with ~10 zirconium oxide beads (Next Advance, Averill Park, NY). The resulting RNA samples were treated with DNAse I (Thermo Scientific EN0521, Waltham, MA). RNA concentrations were measured using a nanodrop ND-2000 spectrophotometer (Thermo Scientific). First strand cDNA was synthesised from 2 µg of RNA using a high capacity reverse transcription kit (Applied Biosystems 4368814, Foster City, CA) and the cDNA product was diluted three-fold with molecular grade DEPC treated deionised water (Invitrogen 46-2224).

Degenerate PCR primers were designed by aligning available fish *ca4* sequences using the Clustal Omega web service (http://www.clustal.org), and identifying conserved sections

among the sequences (primer sequences were F 5'-GGA GAG CAG TAY CCC ATG G-3' and R 5'-TGG GCT TCT CAA ACA MRG TCC-3'). PCR products (40 cycles; 94°C for 2 min, 94°C for 30 s, 72°C for 1 min) were purified on a 1% agarose gel with a 1 kb ladder. Sections of the gel containing the 323 bp PCR product were cut out of the gel and purified using a GeneJet gel extraction kit (Thermo Scientific K0691). Purified PCR products were ligated into Topo2.1 plasmids and transformed in One-Shot Topo10 competent cells (Invitrogen C404010) following the manufacturer's protocol. Plasmids were extracted from ten different bacterial colonies with a GeneJet MiniPrep plasmid kit (Thermo Scientific K0503). Purified plasmids were sequenced at the UBC Nucleic Acid and Protein Service core facility (NAPS, Vancouver, Canada).

Primers for real-time quantitative PCR (RT-qPCR) analysis were designed by aligning the obtained partial coding sequences (CDS) for *C. gunnari ca4a* with the *N. corriceps ca4a*-like mRNA sequence (XM_010775657.1). The generated primers were used on gill and ventricle tissues of both species (primer sequences were F 5'-GGG AAG CAG AGA AGT GTT GC -3' and R 5'-TTT CAG ACG CAG AGG GAG TT-3'). Primers for the *ef1α* control gene were those reported by Urschel and O'Brien (2008), designed for three notothenioid species, and all results are reported relative to the expression of *ef1α*. RT-qPCR amplifications were with the SybrGreen kit (Applied Biosystems 4309155) on the Biorad CFX96 RT-PCR Detection System (Hercules, CA) with the following cycling conditions: 40 cycles, 95°C for 10 min, 95°C for 15 s, 55°C for 1 min; melt curve over 65-95°C at 0.5°C s^{-1}. No-amplification controls (no reverse transcriptase in the cDNA synthesis reaction) were run for each sample and showed no detectable amplification. Standard curves were run on each plate by serially diluting (1:5) pooled sample cDNA with molecular grade water in five steps. Primer pair efficiencies were within 100-120% and $R^2 > 0.99$ for all samples. To confirm the identity of the amplified products, RT-qPCR products were processed with a GeneJet PCR purification kit (Thermo Scientific K0701). The purified RT-qPCR products were cloned and plasmids were extracted as described above. Purified plasmids from ten colonies were sequenced using M13 forward and reverse primers (UBC NAPS).

5.3.6 Data analysis and statistics

All data were analysed in RStudio v1.1.383 (RStudioTeam, 2016) with R v3.4.1 (RCoreTeam, 2017) and figures were generated with the ggplot2 v.2.2.1 package (Wickham, 2009). Normality of distribution was tested with the Shapiro-Wilk test ($P < 0.05$) and by visually confirming the distribution of the residuals in quantile-quantile (q-q) plots (for dependent

samples t-tests, normality was tested on the differences between dependent scores). Homogeneity of variances was tested with the Levene's test ($P < 0.05$). To assess the CA inhibition kinetics of Az, titrations were carried out according to Dixon (1953) and the inhibition constant k_i was calculated as the slope of:

$$\frac{I_o}{i} = \frac{k_i}{1 - i} + E_0$$

where I_0 is the concentration of inhibitor, E_0 is the concentration of free enzyme and i is the fractional inhibition of enzyme activity at a given inhibitor concentration (Easson and Stedman, 1936). The effects of SDS and plasma on CA activity were tested with a dependent samples t-test against control measurements without these inhibitors, and the results are expressed as % inhibition. The effects of washing and Az on CA activity were tested using dependent samples t-tests, and an independent samples t-test for the effect of PI-PLC (due to an imbalance in replicates). Differences in relative gene expression between tissues, and plasma protein concentration between species, were tested using an independent samples t-test ($P < 0.05$). Differences in β_{plasma} between species and across the tested pH range were depicted by linear regression analysis. A linear mixed model (lme function in R) was run on the on the raw data to test for the effects of species (independent of individual), pH (nested within individual) and their interaction (species×pH). Average values of β_{plasma} were calculated for each individual over the entire pH range and significant differences in the average β_{plasma} between species were tested with a t-test ($P < 0.05$). All data are presented as means ± s.e.m., with $N = 6$ unless indicated otherwise.

5.4 Results

5.4.1 Immunohistochemistry

The results for the immunohistochemical localisation of Ca4 and Ca17 protein in the gills of *C. gunnari* and *N. rossii* are shown in Figure 5-1. In *C. gunnari*, reactivity for Ca4 protein was observed as a clear ring, lining the entire blood space of the secondary lamellae (marked with *). This staining pattern was associated with the apical membrane of pillar cells and the basolateral membrane of lamellar epithelial cells (panel a). In contrast, in *N. rossii*, reactivity for the Ca4 antibody was a diffuse staining pattern associated with the intracellular space of lamellar pillar

cells and epithelial cells and absent from the lamellar blood space (panel b). Nonetheless, probing for the cytosolic Ca17 protein showed a similar pattern for both species, with staining confined to the cytosol of all lamellar cell types and RBCs that remained in un-perfused areas within the lamellae (marked with * in panels c and d). The specificity of the antibodies was confirmed by the western blotting results. Probing of immunoblots from *C. gunnari* microsomal pellets with the Ca4 antibody revealed one band at ~37.5 kDa that was not observed in the pellets of *N. rossii* or the supernatants of either species. Probing for Ca17 protein showed immunoreactivity against a band at ~25 kDa in the cytosolic fractions from both species, but not in the pellets.

5.4.2 Gene expression

Homology cloning yielded a CDS for *C. gunnari ca4a* of 323 bp (uploaded to Genbank: MG561387) that was blasted against the stickleback (*Gasterosteus aculeatus*) and cod (*Gadus morhua*) genomes from the Ensembl genome browser (http://www.ensembl.org). BLAST results returned a ~90% sequence homology with the *ca4a* gene in both stickleback and cod (E-values were $1e^{-29}$ and $4e^{-9}$, respectively). The CDS of *C. gunnari* codes for a deduced protein of 103 amino acids, most closely resembling Ca4 and sharing 95% identity with *N. corriceps* Ca4 (XM_010775657.1) and 67% with *O. mykiss* Ca4 (XP_021479942.1).

Control gene expression for *ef1α* did not differ between ventricles and gills of *C. gunnari* ($P = 0.338$) or *N. rossii* ($P = 0.203$), and the expression of *ca4a* is reported relative to that of the control gene in Figure 5-2. The relative expression of *ca4a* mRNA in the gills of *C. gunnari* was not different from that of the ventricle ($P = 0.610$), a tissue in which *ca4* expression has been reported in other teleosts (Georgalis et al., 2006; Alderman et al., 2016), and expression values were comparable to the expression of the control gene. Likewise, in the ventricle of *N. rossii*, *ca4a* was expressed at levels comparable to the control gene; however, expression in the gills was significantly lower compared to the ventricle ($P = 0.044$).

5.4.3 Biochemical analysis of CA activity

All cellular fractions obtained by differential centrifugation of gill homogenates showed significant CA activity. In both species, CA activity was highest in the supernatant containing the cytosolic fraction, and in microsomal pellets that contain plasma membranes, and averaged over species values were 529 ± 50 and 99 ± 28 μmol H^+ mg^{-1} min^{-1}, respectively. The effects of washing on microsomal CA activity for both notothenioids are shown in Figure 5-3. Washing

significantly increased CA activity in pellets of *C. gunnari* (when expressed per unit protein), from 17 ± 1 to 109 ± 9 µmol H^+ mg^{-1} min^{-1} ($P < 0.001$). Whereas washing significantly decreased CA activity in pellets of *N. rossi*, from 181 ± 27 to 31 ± 2 µmol H^+ mg^{-1} min^{-1} ($P = 0.002$). However, washing significantly reduced total CA activity in both species (when expressed per volume of fraction) from 244 ± 17 to 149 ± 11 µmol H^+ mL^{-1} min^{-1} in *C. gunnari* ($P < 0.001$), and from 453 ± 69 to 94 ± 9 µmol H^+ mL^{-1} min^{-1} in *N rossii* ($P = 0.003$).

To assess whether microsomal CA isoforms were membrane-bound by a GPI anchor, membrane pellets were incubated at 21°C for 90 min, in the absence (Ctrl) or presence or of PI-PLC. In the Ctrl incubations of both species, CA activities per unit of protein were reduced by about half compared to initial values (67 ± 9 and 15 ± 1 2 µmol H^+ mg^{-1} min^{-1} for *C. gunnari* and *N. rossii*, respectively). The effects of PI-PLC on microsomal CA activity of both notothenioids are shown in Figure 5-4. Treatment of *C. gunnari* pellets with PI-PLC significantly decreased CA activity compared to Ctrl values, to 20 ± 1.5 µmol H^+ mg^{-1} min^{-1} ($P = 0.031$), and a corresponding increase was observed in the CA activity of the supernatant ($P = 0.002$). Likewise, a significant effect of PI-PLC was detected on CA activity in pellets of *N. rossii* that decreased to 10 ± 1 µmol H^+ mg^{-1} min^{-1} ($P < 0.001$); however, no significant change in CA activity was observed in the supernatant ($P = 0.450$).

Figure 5-5 shows the inhibitory effect of SDS, a surfactant, on the CA activity in cellular fractions of both notothenioids. In the pellets of *C. gunnari*, CA activity was unaffected by SDS (0.7 ± 2.5% inhibition; $P = 0.813$), whereas CA activity in the supernatant was significantly inhibited by 55.1 ± 6.6% ($P = 0.003$). In contrast, in *N. rossii*, SDS significantly inhibited CA activity in the pellets by 38.5 ± 4.4% ($P = 0.003$), and in the supernatant by 44.8 ± 3.9% ($P = 0.001$). In addition, titrations with increasing concentrations of Az resulted in inhibition constants (k_i) that were significantly different ($P = 0.032$) between CA isoforms derived from microsomal pellets or supernatants of *C. gunnari* gills; and the average k_i were 0.74 ± 0.11 and 1.18 ± 0.13 nM, respectively.

As expected, RBC lysates from *N. rossii* had a high CA activity of, on average, 39 ± 1 µmol H^+ mg^{-1} min^{-1} (despite the high protein content of this fraction) and the inhibitory effects of 100 µL of plasma from either species are shown in Figure 5-6. The addition of plasma from *N. rossii* significantly inhibited CA activity in the RBC lysate, by 92.3 ± 2.8% ($P < 0.001$). Likewise, the addition of plasma from *C. gunnari* significantly inhibited CA activity in the RBC

lysates of *N. rossii*, by 81.7 ± 3.7% ($P < 0.001$). However, the CA activity in microsomal pellets of *C. gunnari* gills was unaffected in the presence of endogenous plasma (0.9 ± 1.1% inhibition; $P = 0.809$); whereas, CA activity in pellets of *N. rossii* was significantly inhibited in the presence of endogenous plasma, by 77.2 ± 4.3% ($P < 0.001$). In both species, CA activity in the supernatant was significantly inhibited by the addition of endogenous plasma; by 73.1 ± 6.3% in *C. gunnari* ($P < 0.001$) and by 90.2 ± 3.5% in *N. rossii* ($P < 0.001$).

5.4.4 Plasma characteristics

Plasma protein concentration was significantly higher ($P = 0.001$) in *C. gunnari* compared to *N. rossii* (18.3 ± 1.4 and 10.8 ± 0.3 mg mL^{-1}, respectively). Plasma protein concentrations were corrected for Hb protein from RBC lysis, and Hb concentration in the plasma was typically low and close to the detection limit of the assay (25 µg mL^{-1}). However, a single sample had an elevated Hb concentration of 5.49 mg mL^{-1}, and this sample was excluded from the analysis of β_{plasma} (see symbol "×" in Fig. 5-7). Figure 5-7A shows β_{plasma} for both notothenioids over the physiological pH range and a significant interaction effect in the linear mixed model (species×pH; $P < 0.001$) indicated that *C. gunnari* had a higher β_{plasma} at the low end of the physiological pH spectrum, compared to *N. rossii*. However, the average β_{plasma} over the entire pH range were not significantly different between the two species and average values for *C. gunnari* and *N. rossii* were 5.01 ± 0.68 and 4.26 ± 0.42 mmol L pH^{-1}, respectively.

5.5 Discussion

Taking advantage of the natural Hb-knockout model provided by Antarctic icefishes, we tested the hypothesis that, in the absence of RBC CA, icefish gills express a paCA isoform that can provide the catalytic activity necessary for CO_2 excretion. To determine the cellular orientation of the putatively plasma-accessible Ca4 isoform in the gills of notothenioids (Tufts et al., 2002), gill sections of the icefish *C. gunnari* and the red-blooded *N. rossii* were immuno-labelled with an antibody raised against rainbow trout Ca4 (Gilmour et al., 2007). In gills of *C. gunnari*, a clear immunohistochemical signal (Fig. 5-1a) placed Ca4 protein in association with the apical plasma membranes of pillar cells and the basolateral membrane of some lamellar epithelial cells. Thus, Ca4 appears to line the entire lamellar blood space (marked with *), a pattern that is consistent with a plasma-accessible orientation of the enzyme, which has not been observed previously in a teleost. A similar pattern has been described in the gills of dogfish, an elasmobranch (Gilmour et al., 2007), where the presence of Ca4 has been linked to functional

measurements that infer a role of the enzyme in CO_2 excretion (Gilmour et al., 2001). Western analysis revealed a Ca4 protein of ~37.5 kDa in *C. gunnari* that matches closely the size of dogfish Ca4, of ~40 kDa (Gilmour et al., 2007). Our immunohistochemical finding was corroborated by the pattern of gene expression in the gills of *C. gunnari*, where expression of *ca4a* was detected at high levels, comparable to those in the ventricle (Fig. 5-2). This is unlike the situation in other teleosts, such as rainbow trout, where *ca4* is expressed in the ventricle but not in the gills (Georgalis et al., 2006). Surprisingly, the gills of *N. rossii* also showed detectable expression of *ca4a*, albeit at a significantly lower level compared to those in the ventricle, and without a corresponding immunohistochemical signal. Reactivity for the Ca4 antibody was clearly absent in the lamellar blood space of *N. rossii* (marked with *; Fig. 5-1b), but some intracellular reactivity was detected. These immunohistochemical results were later confirmed using a second antibody, raised against elasmobranch Ca4, for which the antigenic peptide sequence of the notothenioid Ca4 was 100% conserved (data not shown). It is possible that the low expression of *ca4a* mRNA in gills of *N. rossii* is not translated into a protein, or perhaps that it is translated into a small pool of protein that is anchored to intracellular membranes and does not undergo the post-translational modifications required for export to a plasma-accessible location (Waheed et al., 1996). This issue was clarified by the biochemical characterisation of this CA pool as follows.

To characterise the CA-isoform distribution in gills of *C. gunnari* and *N. rossii*, gill homogenates were fractionated by differential centrifugation and CA activity was measured in the supernatant, comprising the cytosolic fraction, and in microsomal pellets that contain plasma membranes. In both species CA activity was highest in the supernatant, compared to microsomal pellets (averaged over species 529 ± 50 and 99 ± 28 $\mu mol\ H^+\ mg^{-1}\ min^{-1}$, respectively). This result is in line with immunohistochemical data showing reactivity for a soluble Ca17 protein in the gills of both species that is clearly confined to the cytosol of both pillar- and lamellar epithelial cells, although more abundant in the latter (Fig. 5-1c and d). This prevalence of cytosolic over membrane-associated CA activity is consistent with previous findings on the CA isoform distribution in the gills of notothenioids (Maffia et al., 2001) and other fish species (Harter and Brauner, 2017) and highlights the importance of this CA pool for iono- and acid-base regulation and the sensing of CO_2 and pH in neuro-epithelial cells (for review see Gilmour,

2012). However, this soluble cytoplasmic CA is not plasma-accessible and thus cannot participate in plasma HCO_3^- dehydration and CO_2 excretion.

Membrane-bound Ca4 isoforms were identified by using four common biochemical markers: i) resistance to washing of microsomal pellets, ii) liberation of CA by PI-PLC, iii) resistance to SDS and iv) resistance to plasma CA inhibitors. Washing significantly reduced the CA activity in the pellets of *N. rossii*, but, when expressed per unit of protein, washing increased CA activity in the pellets of *C. gunnari* (Fig. 5-3). This was likely due to the washout of non-CA proteins from the microsomal fraction, and washing significantly reduced total CA activity in the pellets of both species. Importantly, after washing, the pellets of *C. gunnari* retained a three-fold higher CA activity, compared to *N. rossii* (109 ± 9 and 31 ± 2 μmol H^+ mg^{-1} min^{-1}, respectively). PI-PLC treatment significantly reduced CA activity in the microsomal pellet of *C. gunnari*, releasing CA activity into the supernatant (Fig. 5-4); this is a clear indication for the presence of a GPI membrane-bound Ca4 and/or Ca15 isoform in the icefish. A statistically significant, but numerically small effect of PI-PLC was also detected on CA activity in the pellets of *N. rossii*, however, without a corresponding increase in supernatant CA activity. This is in line with the data of Tufts et al. (2002), who found a significant effect of PI-PLC on CA activity in microsomal pellets of *C. aceratus* and *N. coriiceps*, while other studies have found no effect of PI-PLC in non-notothenioid teleosts (Gilmour et al., 2001; Gilmour et al., 2002). In combination, these results corroborate the finding of a CA isoform that is linked to membranes by a GPI anchor in the gills of the icefish *C. gunnari* and *C. aceratus*, and provide equivocal indications for the presence of a similar, but perhaps less abundant, isoform in red-blooded notothenioids, that may be restricted to intracellular membranes or may be associated with epithelial cells; and thus, is membrane-associated, but not plasma-accessible.

To further determine whether the observed CA activity in the gills of *C. gunnari* was derived from Ca4 protein, microsomal pellets were treated with SDS. Mammalian studies show that CA4 isoforms have two additional disulfide bonds that stabilise the enzyme against denaturation by SDS (Waheed et al., 1996) and thus, SDS-resistant CA activity is often described as Ca4-like in fishes and other non-mammalian vertebrates (Gervais and Tufts, 1998; Gilmour et al., 2002; Stabenau and Heming, 2003; Gilmour et al., 2007; Esbaugh et al., 2009). CA activity in the pellet of *C. gunnari* was unaffected by SDS (Fig. 5-5), while CA activity was significantly reduced in *N. rossii* (by 38.5 ± 4.4%). As expected, cytosolic CA activity in the

supernatant of *C. gunnari* and *N. rossii*, which are typically SDS-sensitive, soluble CA isoforms, was significantly reduced in the presence of SDS (by 55.1 ± 6.6 and 44.8 ± 3.9%, respectively). These findings corroborate previous data that indicate Ca4-like enzyme activity in gill membranes of the icefish *C. aceratus*, but not in those of *N. coriiceps* or in the supernatants of either species (Tufts et al., 2002).

The inhibition characteristics for Az, a common sulfonamide CA inhibitor are well studied in mammals and allow further differentiation among CA isoforms (Baird et al., 1997). Tufts et al. (2002) found no difference between the inhibition constant (k_i) for Az in pellets and supernatants of *C. aceratus*, indicating similar CA isoforms in both fractions. However, here, in *C. gunnari*, the k_i for Az was 0.74 ± 0.11 nM in gill microsomal pellets, compared to 1.18 ± 0.13 nM in the supernatant; a significant difference, indicating that different CA isoforms are present in the two fractions. The discrepancy with previous data may indicate the presence of two isoforms with similar k_i in *C. aceratus*, or perhaps that a low number of replicates in the earlier study ($N = 4$; Tufts et al., 2002), was insufficient to resolve the small numerical difference observed here.

An intriguing finding was the discovery of a CA inhibitor in the plasma of the icefish, *C. gunnari*. In fact, the CA activity in RBC lysates from *N. rossii* was significantly reduced in the presence of 100 µl of plasma from either *N. rossii* (by 92.3 ± 2.8%; Fig. 5-6) or *C. gunnari* (by 81.7 ± 3.7%), providing strong evidence that both species possess a CA inhibitor in their plasma. The putative role of plasma CA inhibitors is to either inactivate or recycle CA from RBC lysis (Henry and Heming, 1998), but neither role would be relevant for icefishes that largely lack RBCs. A plasma CA inhibitor has also been described in the icefish *C. aceratus* (Tufts et al., 2002) and because the phylogenetic distance between *C. aceratus* and *C. gunnari* spans nearly the entire clade of channichthyids (Near et al., 2003) it is plausible that plasma inhibitors of CA are present in all icefishes. Whether the plasma CA inhibitor in icefishes is an evolutionary relic from a red-blooded ancestry, or whether its role should include the scavenging of cytoplasmic CA shed by the lysis of other cell types, remains unclear. Regardless, the presence of an endogenous plasma CA inhibitor can be used as a powerful diagnostic for Ca4 that, in mammals, is largely unaffected by the inhibitor, and this safeguards its function in plasma-accessible locations (Hill, 1986; Heming et al., 1993). A critical finding, thus, was that CA activity in pellets of *C. gunnari* was unaffected by the presence of endogenous plasma (Fig. 5-6), whereas

CA activity in pellets of *N. rossii* was significantly inhibited by 77.2 ± 4.3%. Further, the supernatant of both species was significantly inhibited by plasma addition (in *C. gunnari* by 73.1 ± 6.3% and in *N. rossii* by 90.2 ± 3.5%). Thus, CA activity in membranes of *C. gunnari* displays Ca4-like characteristics that are not seen in membranes of *N. rossii* or in those fractions containing soluble CA isoforms.

Four biochemical criteria are commonly used to characterise membrane-bound Ca4: i) resistance to washing of pellets, ii) liberation by PI-PLC, iii) resistance to SDS and iv) resistance to plasma CA inhibitors. CA activity in the pellets of *C. gunnari* conformed to all four criteria and this was supported by the expression of *ca4a* mRNA at the gills and the immunohistochemical detection of Ca4 protein, in a subcellular location that indicates a plasma-accessible orientation. CA activity in the pellets of *N. rossii* was largely removed by washing and inhibited by SDS. A significant effect of PI-PLC and low levels of *ca4a* expression may indicate the presence of some Ca4 protein that appears to be localised to intracellular membranes. Regardless of the isoform identity, the fact that microsomal CA activity in *N. rossii* was susceptible to the plasma CA inhibitor prohibits this CA pool from participating in HCO_3^- dehydration in the plasma. In combination, these data support the hypothesis that *C. gunnari* possess plasma-accessible Ca4 at the gills that should facilitate CO_2 excretion, while gills of *N. rossii* appear to lack a CA pool that could participate in this role. Thus, in the absence of RBC CA, icefish may be the only adult vertebrate in which CO_2 excretion is driven exclusively by the paCA activity provided by the gill.

Why most other teleosts lack paCA activity at the gills, despite its potential benefit for CO_2 excretion is still debated. One powerful argument relates to the evolution of highly pH-sensitive Hbs that required the active regulation of RBC intracellular pH to safeguard branchial O_2 uptake during a blood acidosis (Nikinmaa et al., 1984; Berenbrink et al., 2005); this protective mechanism requires an absence of CA activity in the plasma (Jacobs and Stewart, 1942; Motais et al., 1989; Rummer et al., 2013). If the presence of paCA at the teleost gill was functionally constrained by the characteristics of teleost Hb and RBC function, perhaps these constraints were released in icefishes, which lack both. Assessing the presence of paCA in the gills of the closest red-blooded relatives of the channichthyids (the bathydraconids; Near et al., 2004) and confirming the absence of paCA in other notothenioid families, would strengthen the functional link between the loss of Hb and the expression of paCA. In addition, teleost plasma is

an unfavourable medium to support high CA activities, mainly due to its low buffer capacity (β_{plasma}), as HCO_3^- dehydration requires equimolar amounts of H^+s (Bidani and Heming, 1991; Gilmour et al., 2002; Szebedinszky and Gilmour, 2002). In the presence of RBCs with fast Cl^-/HCO_3^- exchange, an abundant pool of CA and buffers on Hb, paCA activity may be largely inconsequential for CO_2 excretion in teleosts (Desforges et al., 2001). Notably, it is those fishes with the highest β_{plasma} that also have paCA activity; conditions that, to varying degrees, contribute to CO_2 excretion in *Squalus acanthias* (Lenfant and Johansen, 1966; Graham et al., 1990; Gilmour et al., 2001) and *Eptatretus stoutii* (Esbaugh et al., 2009).

The β_{plasma} in *C. gunnari* was 5.01 ± 0.68 mmol L pH^{-1} and, at the lower end of the physiological pH range, significantly higher compared to that in *N. rossii*, of 4.26 ± 0.42 mmol L pH^{-1} (Fig. 5-7). Previous studies that measured β_{plasma} in other icefish species over the same pH range reported average values of 3.4 ± 0.2 mmol L pH^{-1} in *Pagetopsis macropterus* (Wells et al., 1988) and 9.7 ± 0.9 mM L pH^{-1} in *Chionodraco hamatus* (Acierno et al., 1997). While these values vary largely between studies and species, the β_{plasma} reported here exceed, by about two-fold, typical teleost values (2-3 mmol L pH^{-1}; Tufts and Perry II, 1998), perhaps with the exception of some catfishes (Cameron and Kormanik, 1982; Szebedinszky and Gilmour, 2002). Plasma proteins in *Channichthys rhinoceratus*, another icefish, are rich in imidazole-based histidines, a residue capable of reversibly binding H^+s, which likely contribute to the high β_{plasma} in this species (Feller et al., 1994). Similarly, histidine-rich proteins (in this case albumins) appear to underlie the unusually high β_{plasma} in *Ameiurus nebulosus*, a catfish (Szebedinszky and Gilmour, 2002). And in fact, plasma protein concentration in *C. gunnari* was 18.3 ± 1.4 mg mL^{-1} and significantly higher compared to that in *N. rossii*, of 10.8 ± 0.3 mg mL^{-1}; a finding that may correlate with the higher β_{plasma} in the icefish at low pH. The protein concentration in *N. rossii* plasma conforms with the range typically reported in teleosts (Acierno et al., 1997), however values in *C. gunnari* are lower compared to other icefishes studied (Egginton, 1994; Acierno et al., 1997; Feller and Gerday, 1997); the reason for this discrepancy is unknown.

Due to the large blood volume of icefishes (about 7.6% of body weight; Hemmingsen and Douglas, 1970) compared to 2-3% in other teleosts (Thorson, 1961; Houston and DeWilde, 1969), and their low Hct (<1% compared to >25% in other teleosts; Holeton, 1970), the total volume of plasma in icefishes is at least three-times higher than in most teleosts (Feller et al., 1994). The plasma of red-blooded teleosts contributes 20-40% to whole blood buffer capacity,

typically <10 mmol L pH^{-1}, which is largely determined by the buffer capacity of Hb (Wood et al., 1982; Tufts and Perry, 1998; Gilmour et al., 2002; Szebedinszky and Gilmour, 2002). Although the measured β_{plasma} in *C. gunnari* is only half that of typical teleost whole blood, this is clearly overcompensated by the three-fold higher plasma volume of icefishes. Thus, per unit of animal mass, icefishes have a greater capacity to buffer metabolically produced H^+s in their blood compared to most teleosts, despite lacking Hb. In combination with a low metabolic rate (Hemmingsen et al., 1969), hence a lower release of CO_2 and H^+ into the plasma, β_{plasma} in icefishes would seem adequate to sustain arterial-venous pH homeostasis and HCO_3^- dehydration (and this is supported by experimental data; Hemmingsen and Douglas, 1972), which is catalysed by the paCa4 isoform at the gill.

The evolutionary time-course over which RBCs were lost from the circulation in the common ancestor of channichthyids, and whether this coincided with the loss of transcriptionally active Hb genes, is presently unknown. However, Hb is the largest H^+ buffer within the RBC cytosol (in teleosts largely through the Bohr-Haldane effect) and the absence of Hb will have severely restricted the functional significance of RBC CA. Thus, the time-course over which icefishes had to acquire paCA at the gill, to compensate for the reduction of RBC CA function, may have corresponded closely to the loss of Hb. The molecular mechanism by which icefishes catalyse HCO_3^- dehydration in the plasma is analogous to that in all other non-teleost vertebrates, where CA4 is GPI-anchored to the apical membrane at the gas exchange organs. Therefore, it seems likely that paCA was never "lost" at the teleost gill, but functional constraints related to the pH-sensitivity of teleost Hb prevented a significant expression of the trait, until the loss of Hb in icefishes simultaneously released functional constrains and created a need to catalyse HCO_3^- dehydration in the plasma. Possible scenarios may include: i) natural selection favoured phenotypes with higher paCA activity at the gill, which requires that there was standing variation in this trait in the common ancestor of channichthyids; ii) phenotypic plasticity induced an up-regulation of *ca4a* gene expression at the icefish gill, which may be supported by the presence of the transcript in *N. rossii*; or iii) neoteny allowed for branchial paCA to be retained throughout icefish ontogeny, a mechanism that underlies other adult characters in notothenioids (Montgomery and Clements, 2000), and which would place branchial paCA as an embryonic trait in teleosts; a scenario that could be tested experimentally.

In conclusion, the natural knockout of Hb in Antarctic icefishes had profound consequences for cardiovascular O_2 transport and resulted in fascinating adaptations that compensate for the reduction in O_2 carrying capacity of the blood. In addition, results from the present study show that the reduction of RBCs and the associated loss of CA catalytic activity in the blood of icefishes led to a divergent strategy of CO_2 excretion. While paCA is functionally absent at the gills of teleosts, icefishes may have re-acquired this trait, and unlike the situation in any other vertebrate studied to date, in icefishes, the CA catalytic activity required for CO_2 excretion may be provided exclusively by the gills. Therefore, the study of Antarctic icefishes may reveal a previously unidentified evolutionary plasticity in the vertebrate CO_2 excretion pathway and perhaps provide a framework to address more general questions on the evolutionary dynamics of vertebrate gas exchange.

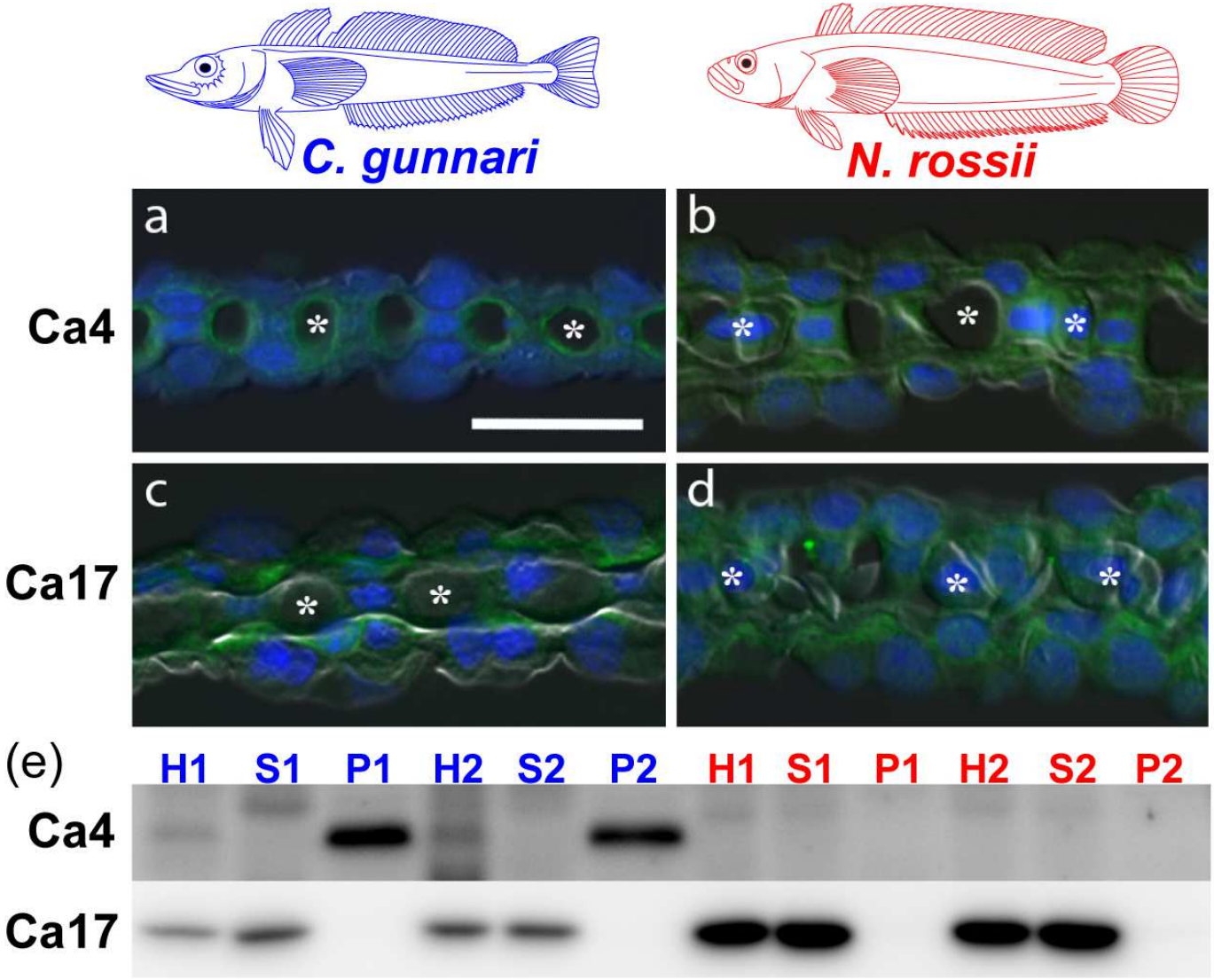
C. gunnari
N. rossii
a
b
c
d
Ca4
Ca17
(e)
H1 S1 P1 H2 S2 P2 H1 S1 P1 H2 S2 P2
Ca4
Ca17

Figure 5-1 Immunohistochemical localisation of Ca4 and Ca17 protein in the gills of the icefish *C. gunnari* and the red-blooded *N. rossii*. All CA antibodies were labelled with a green secondary antibody and nuclei were stained blue with DAPI (4',6'-diamidino-2-phenylindole). In *C. gunnari*, immunoreactivity for Ca4 resulted in a circular staining pattern associated with the apical membrane of pillar cells, lining the entire lamellar blood space (marked with * in panel a), consistent with a plasma-accessible orientation of the enzyme (white scale bar = 25 µm). No immunoreactivity for Ca4 was detected in the lamellar blood space of *N. rossii* gills (marked with * in panel b), but some intracellular reactivity was detected that does not appear to be plasma-accessible. Ca17 protein was detected in the cytosol of pillar and epithelial cells of both notothenioid species (panels c and d). The lower panel (e) shows representative western blots for crude gill homogenates (H), supernatants (S, cytosolic fraction) and pellets (P, membranes fraction), obtained by differential centrifugation from two individuals of *C. gunnari* and *N. rossii*, respectively. Probing with the Ca4 antibody produced a strong band at ~37.5 kDa in the pellets of *C. gunnari* that was not observed in the supernatants or in any fraction of *N. rossii*. Probing for Ca17 protein produced bands at ~25 kDa in the supernatants but not the pellets, of both species.

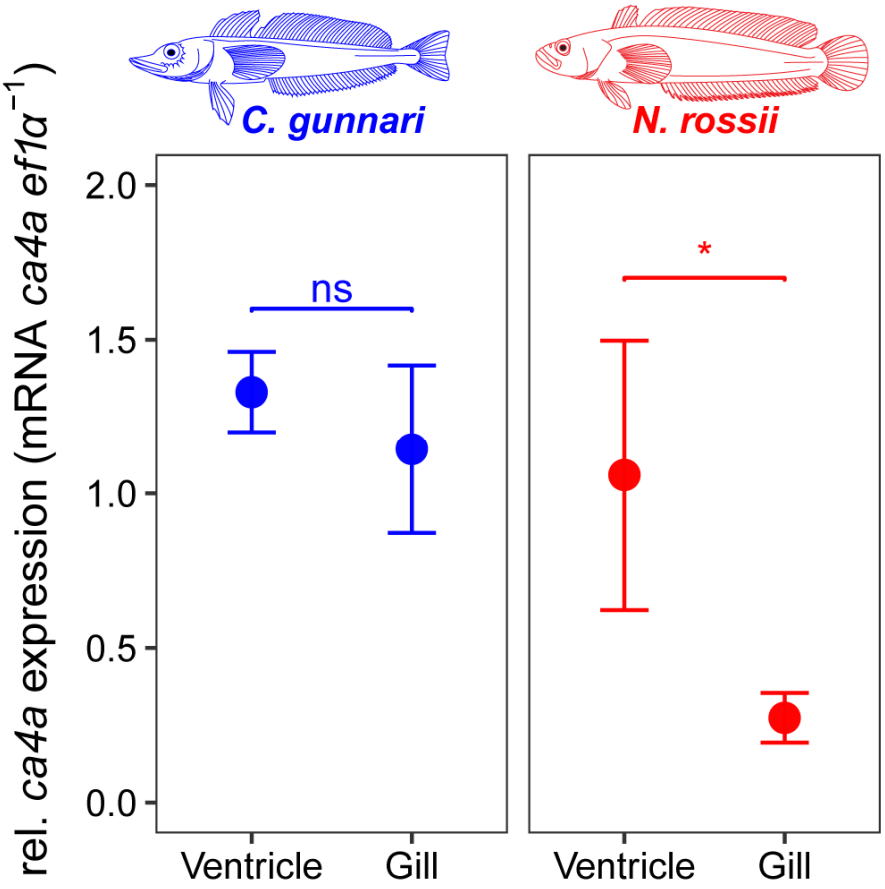

Figure 5-2 Relative expression of *ca4a* mRNA in ventricle and gill homogenates of *N. rossii* and *C. gunnari*. Measurements were by real-time quantitative PCR (RT-qPCR) and all expression levels are standardised to that of *ef1α*. Differences in relative gene expression between tissues, were assessed with independent t-tests within species ($N = 5$, except for *C. gunnari* ventricle where $N = 2$; $P < 0.05$) and are indicated as: $P < 0.05$*; 0.01**; 0.001***; or "ns" for non-significant. All data are mean ± s.e.m..

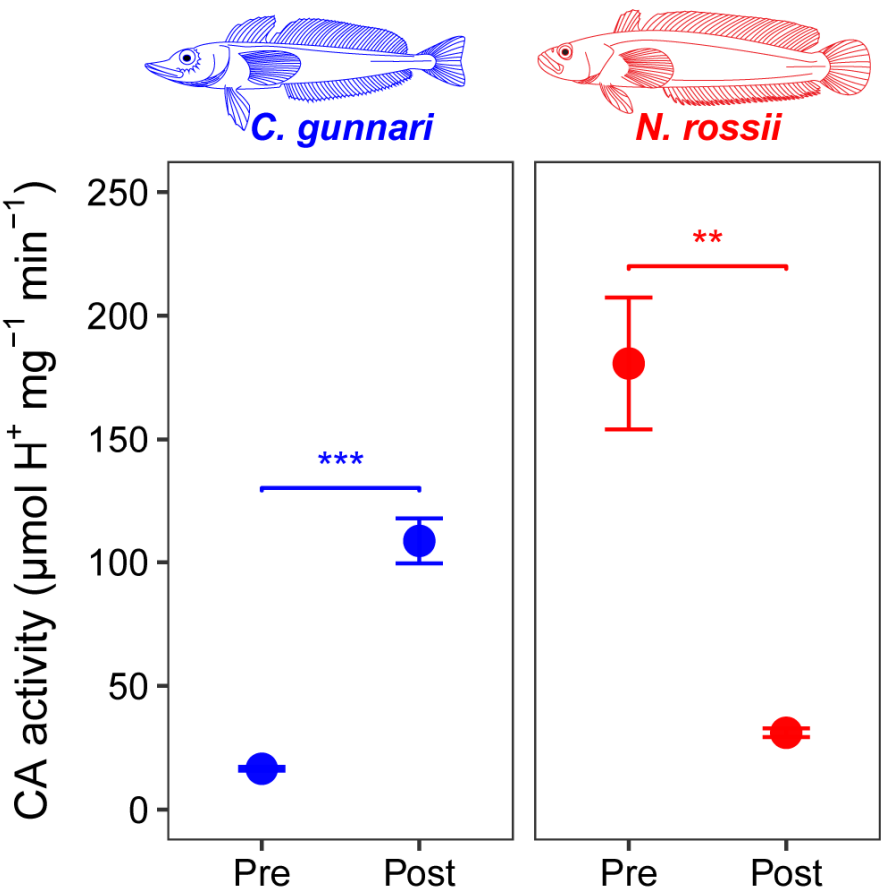

Figure 5-3 Carbonic anhydrase (CA) activity (µmol H^+ mg $protein^{-1}$ min^{-1}) in the microsomal fraction of gill homogenates from the icefish *C. gunnari* and the red-blooded *N. rossii*. Membrane pellets were obtained by differential centrifugation and a final step of ultracentrifugation. Samples were measured before (Pre) and after (Post) a washing step with fresh assay buffer and measurements were with the electrometric ΔpH assay (Henry, 1991). The effects of washing on CA activity were assessed with a depend t-test within species ($P < 0.05$, $N = 6$) and are indicated as: $P < 0.05$*; 0.01**; 0.001***; or "ns" for non-significant. All data are mean ± s.e.m..

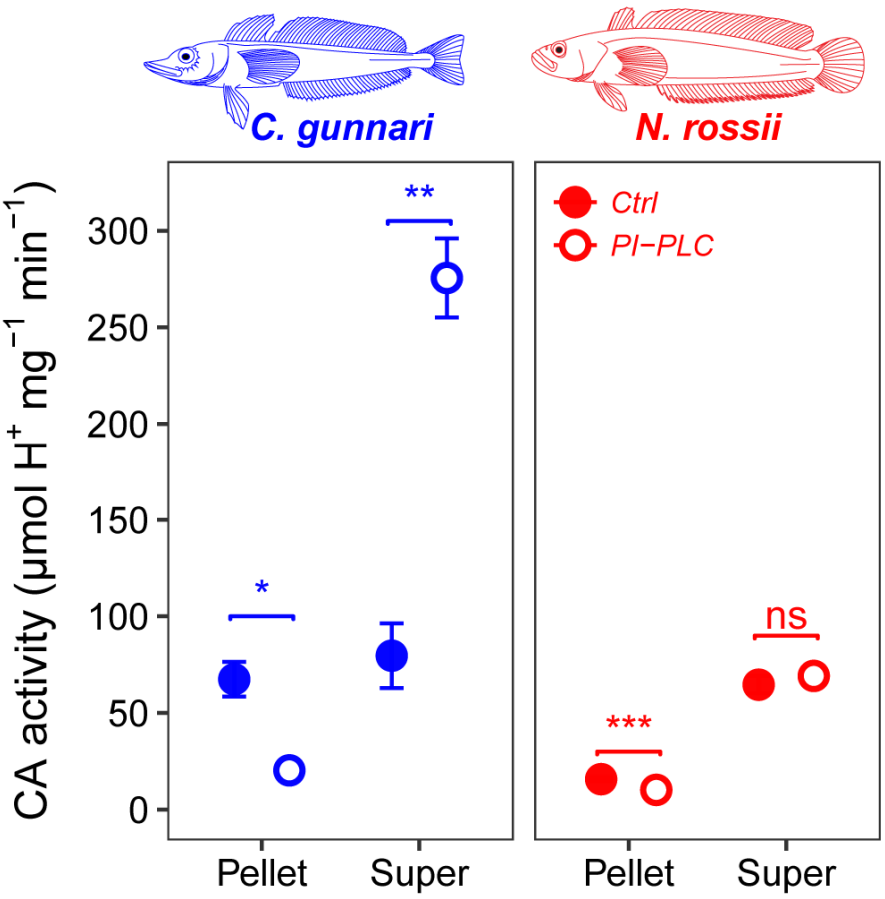

Figure 5-4 Carbonic anhydrase (CA) activity (µmol H^+ mg $protein^{-1}$ min^{-1}) in cellular fractions of gill homogenates from the icefish *C. gunnari* and the red-blooded *N. rossii*. Membrane pellets and supernatants (Super) were obtained by differential centrifugation and a final step of ultracentrifugation. Measurements were with the electrometric ΔpH assay (Henry, 1991) after incubation of samples with saline (Ctrl) or phosphatidylinositol-specific phospholipase C (PI-PLC), an enzyme that cleaves the membrane anchors of Ca4 and Ca15. The effect of PI-PLC on CA activity was assessed with an independent t-tests for each cellular fraction within species ($P < 0.05$, $N = 6$, except for *C. gunnari* Ctrl where $N = 3$). Significant differences are indicated as: $P < 0.05$*; 0.01**; 0.001***; or "ns" for non-significant. All data are mean ± s.e.m..

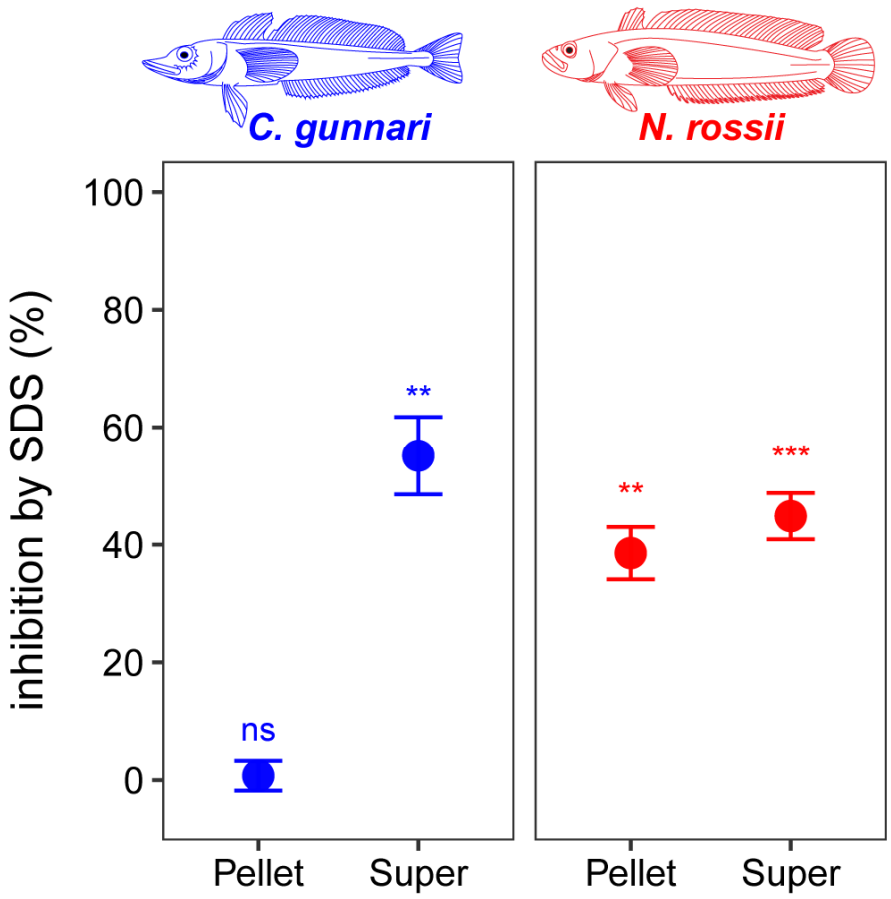

Figure 5-5 Inhibition of carbonic anhydrase (CA) activity (in %), by sodium dodecyl sulfate (SDS) in cellular fractions of gill homogenates from two notothenioid species, the icefish *C. gunnari* and the red-blooded *N. rossii*. Membrane pellets and supernatants (Super) were obtained by differential centrifugation and a final step of ultracentrifugation, and CA activity was measured with the electrometric ΔpH assay (Henry, 1991), in the absence (Ctrl) or presence of 0.005% SDS, a surfactant that inhibits CA activity, but is less potent for Ca4 isoforms. The effect of SDS on CA activity was assessed with a dependent t-tests comparing Ctrl and SDS treated samples, for each cellular fraction within species ($P < 0.05$, $N = 6$). Inhibition of CA activity that is significantly different from zero is indicated as: $P < 0.05$*; 0.01**; 0.001***; or "ns" for non-significant. All data are mean ± s.e.m..

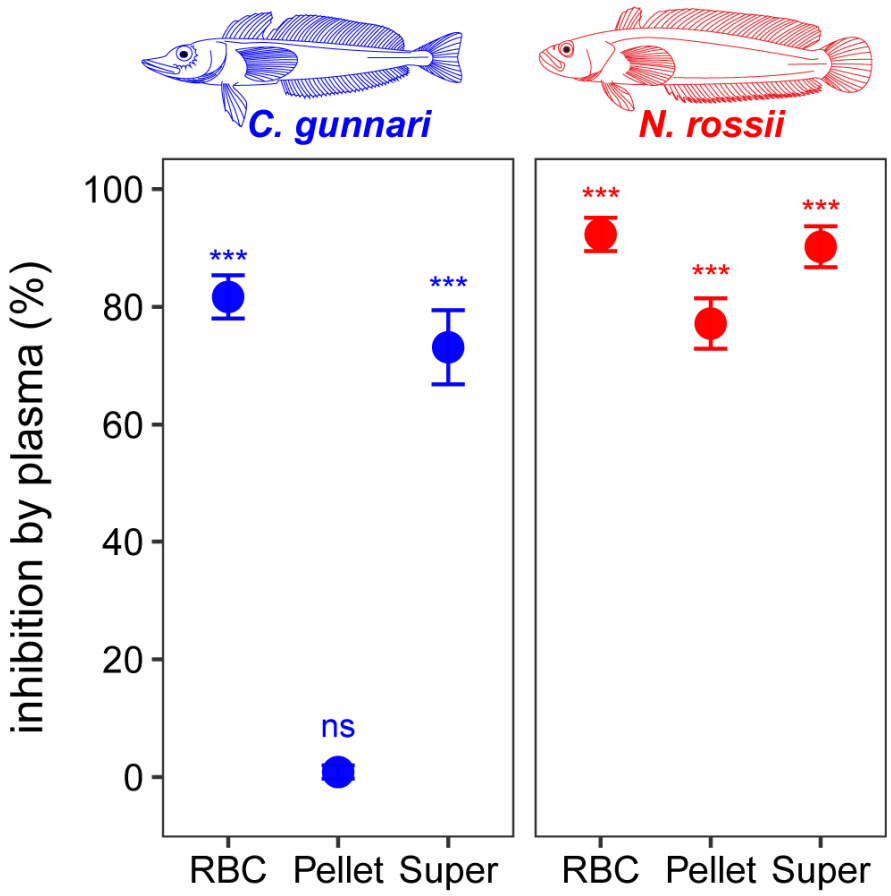

Figure 5-6 Inhibition of carbonic anhydrase (CA) activity (in %), by blood plasma, in cellular fractions of gill homogenates from two notothenioid species, the icefish *C. gunnari* and the red-blooded *N. rossii*. Membrane pellets and supernatants (Super) were obtained by differential centrifugation and a final step of ultracentrifugation. Red blood cell (RBC) lysates were obtained from *N. rossii* and CA activity was measured with the electrometric ΔpH assay (Henry, 1991), in the absence (Ctrl) or presence of 100 µL plasma from either *N. rossii* or *C. gunnari*. The effect of plasma on CA activity was assessed with a dependent t-tests comparing Ctrl and plasma treated samples, for each cellular fraction within species ($P < 0.05$, $N = 6$). Inhibition of CA activity that is significantly different from zero is indicated as: $P < 0.05$*; 0.01**; 0.001***; or "ns" for non-significant. All data are mean ± s.e.m..

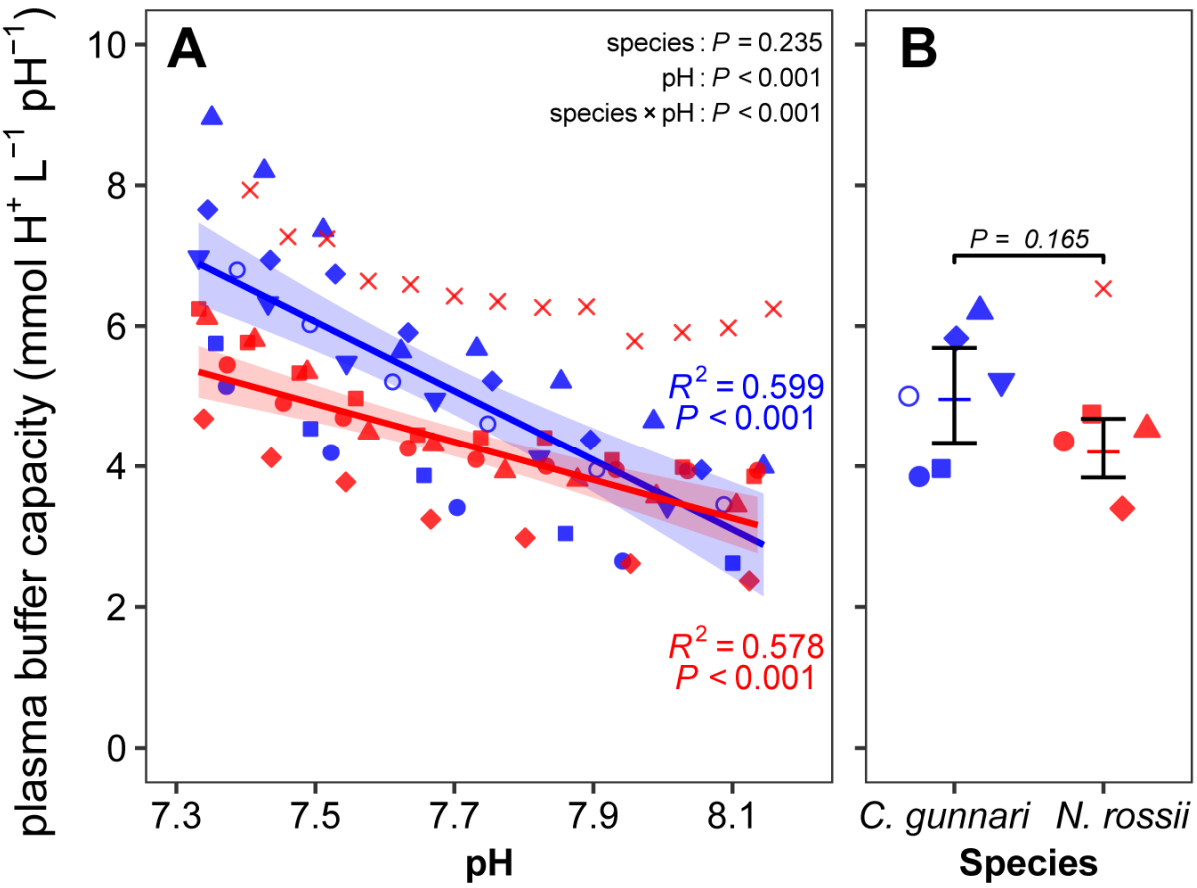

Figure 5-7 (A) Plasma buffer capacity (β_{plasma}; mmol H^+ L sample^{-1} pH^{-1}) of two notothenioid species. The icefish *C. gunnari* is indicated in blue and the red-blooded *N. rossii* in red. Measurements were performed with an automated titrator over a pH range of 7.4-8.2. Linear regression models were fitted to the data, where individual animals are represented by different symbols and 95% confidence intervals are indicated by shaded areas. Haemoglobin (Hb) concentration was measured in all samples from *N. rossii* and was low (around the detection limit of the assay; 25 µg mL^{-1}) except in one individual where 5.49 mg mL^{-1} Hb were detected; this individual was excluded from the analysis (see symbol "×"). A linear mixed model was used to test for significant effects of species, pH and their interaction (species×pH) on β_{plasma}. (B) Average values of β_{plasma} were calculated for each individual over the entire pH range and are plotted for both species as mean ± s.e.m.. A t-test did not reveal a significant difference in the average β_{plasma} between species (*N. rossii*, $N = 4$; *C. gunnari*, $N = 6$, $P > 0.05$).

Chapter 6: General Discussion and Conclusion

My dissertation provides strong evidence that β-NHE short-circuiting, an active cellular mechanism that is under adrenergic control, increases β_b and is critical for cardiovascular O_2 transport in salmonids, and potentially, in most teleost species. If this proves to be the case, our understanding of O_2 transport in nearly half of all vertebrates would change profoundly.

The overarching objective of the present dissertation was to increase our mechanistic understanding of a novel mechanism of enhanced Hb-O_2 unloading in salmonids, and to generate evidence for its *in vivo* importance for cardiovascular O_2 transport and to sustain exercise performance in teleosts. Thus, I tested the hypothesis that **teleosts increase the O_2 capacitance of their blood (β_b) by a mechanism of active RBC pH_i regulation that is modulated through a heterogeneous distribution of paCA, which has functional significance for O_2 transport *in vivo*.** The major findings of my work support this hypothesis. Data collected in Chapter 2 indicate that the time-course of RBC short-circuiting at the capillaries and the recovery of RBC pH_i during venous transit fit the requirements of a system that can sustainably enhance β_b with every pass through the circulation. Results in Chapter 3 show, for the first time, a role of paCA in cardiovascular O_2 transport in a teleost *in vivo*, at rest, during exercise and during recovery from exercise. Chapter 4 describes the finding of paCA in the heart lumen of a teleost that, in light of previous results for skeletal muscle in teleosts, may play a role in supplying O_2 to the myocardium. And finally, Chapter 5 determined that icefishes have paCA at the gills, a trait that in all other teleosts is likely prohibited by the characteristics of Hb and RBC function.

Before this dissertation, *in vitro* work (Rummer and Brauner, 2011) and a single *in vivo* study on hypercapnic, paralysed rainbow trout (Rummer et al., 2013), indicated that paCA plays a role in sustaining elevated red muscle PO_2. This important discovery pointed towards a novel mechanism that could potentially change our understanding of O_2 transport in all teleosts; however, the use of a single species and very specific study conditions precluded from a broader interpretation of the finding. Until now. The data presented in this dissertation, collectively, indicate that salmonids can enhance tissue O_2 extraction by selectively creating and eliminating H^+ gradients across the RBC membrane, through a mechanism of active RBC pH_i regulation that is modulated by a heterogeneous distribution of paCA in the circulation and that increases β_b. Importantly, the data highlight the importance of this system for sustaining O_2 transport *in vivo* and for, in part, enabling the renowned exercise performance that is typical of salmonids.

Further, the reliance on paCA and an elevated β_b was altered by hypoxia acclimation, pointing towards some plasticity in the mechanism of β-NHE short-circuiting that is otherwise entirely unexplored. The requirements for this mechanism are met by all salmonids and probably most teleost species, further increasing the relevance of these findings. Finally, evidence for paCA in the hearts of coho salmon, the red muscle of Atlantic salmon and rainbow trout, and the gills of icefishes but not those of other teleosts, may indicate that the heterogeneous distribution of paCA is a dynamic adaptation that may have played an important role in the evolutionary success of the teleost clade. Thus, the present dissertation extends the earlier discovery by showing that the "novel system of enhanced Hb-O_2 unloading" is not simply a specialised mechanism that is recruited by some species under certain conditions. It is likely an integral part of the mode of cardiovascular O_2 transport in teleosts that may apply to most species in the clade, is recruited routinely over a wide range of conditions, and may respond plastically to changes in environmental or metabolic conditions.

The following sections of the discussion will: i) highlight the major findings of this dissertation (Chapter 6.1); ii) explore the implications of these findings for teleost and non-teleost species (Chapter 6.2); iii) identify areas that are worthwhile investigating in future studies to advance our understanding of the unique mode of O_2 transport in teleosts (Chapter 6.3); and finally, iv) provide a general conclusion of the collective work presented here (Chapter 6.4).

6.1 Thesis highlights and main contributions

6.1.1 paCA plays a role in O_2 transport in vivo

Results from the present dissertation show, for the first time, a role of paCA in cardiovascular O_2 transport in a teleost *in vivo*, under conditions that are ecologically relevant. In Atlantic salmon swimming at a moderate speed (50% U_{crit}), the specific inhibition of paCA required a compensatory increase in $\dot{Q}$, of ~30%, that allowed the animals to maintain $\dot{M}O_2$ (Chapter 3). Plasma adrenaline and noradrenaline concentrations were elevated during exercise, supporting an involvement of β-NHE activity. Therefore, the present work is the first to show a functional link between β-NHE and paCA activity and the enhancement in β_b that supports cardiovascular O_2 transport in Atlantic salmon; results that are consistent with β-NHE short-circuiting. A large scope to further increase plasma catecholamines may indicate that the system can be upregulated to meet an increasing $\dot{M}O_2$. Strikingly, the inhibition of paCA in resting fish also increased $\dot{Q}$ by ~30%, thus indicating that over a broad range of conditions, Atlantic salmon

maintain an elevated β_b that allows cardiac function to be reduced by about a third. Thus, β-NHE short-circuiting in teleosts may facilitate a more efficient transport of O_2 in the blood relative to $\dot{Q}$, compared to other vertebrates. Foraging in salmonids is associated with continuous swimming at moderate speed, such as in lakes, streams or in the oceanic stages of migratory salmon (Hartt et al., 1966; Royce et al., 1968). A system that reduces the requirements on $\dot{Q}$ may allow salmonids to allocate blood flow to other critical physiological functions, such as digestion during exercise (Thorarensen et al., 1993). β-NHE short-circuiting may also represent a significant energetic advantage in terms of cardiac power output that in fish has been estimated at 1-4% of $\dot{M}O_2$ (Farrell and Steffensen, 1987b). However, the cost of maintaining paCA and actively generating H^+ gradients across the RBC membrane are presently unknown and must be considered when estimating the energetic trade-offs that govern β-NHE short-circuiting.

If salmonids routinely maintain an elevated β_b that allows for a reduction in $\dot{Q}$ by 30%, then inhibiting paCA must set limits to maximal aerobic performance. When paCA was inhibited in Atlantic salmon swimming at 50% U_{crit}, $\dot{M}O_2$ was sustained by a $\dot{Q}$ that was 2.25-fold higher than resting. Therefore, even at this moderate swimming speed the animals may have largely utilised their scope to increase $\dot{Q}$. In fact, Atlantic salmon swimming at 75% U_{crit} collapsed after injection of C18 and were unable to sustain the higher level of exercise in the absence of paCA. It appears that animals swimming at the higher speed did not have the scope to increase $\dot{Q}$ by the additional ~30% that is required to offset the reduction in β_b. Therefore, the exercise performances that are typically attributed to Atlantic salmon are not, even remotely, possible without the enhancement of β_b that is facilitated by paCA. As a word of caution, Atlantic salmon used here were farmed fish and thus corroborating these findings in wild populations will prove indispensable to assess their ecological implications. If substantiated, these finding may be relevant for all salmonids, having critical ecological implications especially for those species where reproductive success depends on the ability to maximally increase $\dot{Q}$, such as shown for sockeye salmon (Eliason et al., 2013). Thus, in the face of increasing river temperatures, future conservation efforts may benefit greatly from a better understanding of the physiology underlying β-NHE short-circuiting that may enable a successful spawning migration in these iconic animals (Eliason et al., 2011).

During recovery from exercise, $\dot{Q}$ in Atlantic salmon decreased below resting levels, while $\dot{M}O_2$ remained elevated for some time. When paCA was inhibited, $\dot{Q}$ increased relative to

control fish, providing strong evidence for a role of paCA in supporting the higher $\dot{M}O_2$ that is required to reverse acid-base, osmotic and energetic imbalances that result from strenuous exercise. The release of catecholamines that was observed during exercise may up-regulate the system of β-NHE short-circuiting and the benefits of a higher β_b appear to be available even post-exercise. An elevated catecholamine concentration has been implied to support the recovery from exercise in teleosts (Wood and Perry, 1985; Wood, 1991) and β-NHE short-circuiting may provide a mechanistic explanation for this observation. The benefits of β-NHE short-circuiting during recovery from exercise may be critical for all salmonids, to endure repeated bouts of maximal exercise and recovery that are experienced during spawning migrations, or during prey capture and predator avoidance in non-migratory species.

Clearly, the ability to maintain swimming performance is an indispensable requirement during nearly all life-stages and in all species of salmonids. β-NHE short-circuiting and the related increase in β_b may enable a more efficient cardiovascular O_2 transport under routine conditions, increase the scope for maximal O_2 transport, and promote the recovery from exercise; thus, indicating a mechanism that is recruited over a broad range of conditions. The cardiovascular adjustments by which vertebrates offset an increase in $\dot{M}O_2$ during exercise include increases in $\dot{Q}$, O_2 carrying capacity and O_2 extraction at the tissues by recruiting additional capillaries (Stevens and Randall, 1967; Kiceniuk and Jones, 1977; Brauner et al., 2000a). However, through β-adrenergic stimulation teleosts may recruit an additional mechanism that increases β_b and that reduces the requirements on other cardiovascular adjustments proportionally, *via* active RBC pH_i regulation and a heterogeneous distribution of paCA. It appears that maintaining an elevated β_b is a fundamental aspect of the salmonid mode of O_2 transport that enables the astounding exercise performances that are typical of this group. The validation of this mechanism in other teleosts may profoundly change our understanding of the cardio-respiratory physiology of nearly half of all vertebrates.

6.1.2 β-NHE short-circuiting increases β_b

Previous data on the role of paCA in teleost internal O_2 transport was generated by short-circuiting β-NHE *in vitro* (Rummer and Brauner, 2011) and by inhibiting paCA in a hypercapnic rainbow trout preparation *in vivo* (Rummer et al., 2013), with both studies measuring changes in PO_2. Not surprisingly, the previous model of β-NHE short-circuiting in teleosts revolved around the increase in tissue PO_2 that is facilitated by paCA. This is only part of the story and the

findings of the present dissertation allow us to extend this putative model to *in vivo* relevant conditions of O_2 transport. In the tissue capillaries, β-NHE short-circuiting causes a right-shift of the OEC that enhances Hb-O_2 unloading. In previous studies, PO_2 may have increased because β-NHE short-circuiting was in a closed vial (Rummer and Brauner, 2011), or in a paralysed trout preparation where red muscle $\dot{M}O_2$ was likely low (Rummer et al., 2013) and where the capillaries behave more like a closed system (Brill and Bushnell, 2006). However, under *in vivo* conditions where $\dot{M}O_2$ is elevated, capillaries behave (largely) like an open system where the O_2 content of the blood decreases and PO_2 decreases due to the reduction in Hb-O_2 saturation. Thus, during sub-maximal exercise, a large right-shift in the OEC allows for a higher tissue O_2 extraction at a given PO_2 and consequently $\dot{Q}$ may be reduced proportionally. Indeed, results in Chapter 3 indicate that paCA in Atlantic salmon facilitated an elevated β_b through a higher O_2 extraction at the tissues and when paCA was inhibited, $\dot{Q}$ increased to compensate. Whereas at maximal $\dot{Q}$, the greater tissue O_2 extraction that is facilitated by β-NHE short-circuiting can extend maximal exercise performance in teleosts, before a decreasing P_vO_2 sets a limit to O_2 diffusion to the mitochondria. This is in line with previous studies that have proposed an O_2 diffusion limitation at the fish muscle during exercise (Farrell, 2002) that is supported by indirect experimental evidence (Davie et al., 1986; Sänger, 1992; Gallaugher et al., 1995; Taylor et al., 1997). If so, exercise performance in teleosts is tightly linked to their ability to maintain tissue PO_2 within narrow limits, and *in vivo* measurements of red muscle PO_2 and P_vO_2 in rainbow trout support that this is the case over a wide range of *in vivo* conditions (Farrell and Clutterham, 2003; McKenzie et al., 2004).

Due to the focus on tissue PO_2, the previous model of β-NHE short-circuiting largely overlooked the need to recover RBC pH_i in the venous system. However, only if Hb-O_2 loading at the gills is protected, can β-NHE short-circuiting enhance β_b on a systemic level. Thus, the present findings resolve one of the major mechanistic uncertainties in the teleost system of β-NHE short-circuiting. Data collected in Chapter 2 show that the residence time of blood at the capillaries is sufficient to short-circuit β-NHE activity and importantly, that the $t_{1/2}$ of RBC pH_i recovery (~17 s) falls well within the time available during venous transit in rainbow trout (30-90 s). In addition, after short-circuiting RBCs re-establish the initial H^+ gradient across the membrane and thus, can potentially undergo repeated cycles of short-circuiting and recovery. These temporal kinetics are in line with a system of β-NHE short-circuiting that can sustainably

enhance O_2 extraction at the tissues with every pass through the circulation. In combination, these findings corroborate the mechanistic underpinnings of a system that enhances β_b in salmonids and decreases the requirements on cardiac function by about a third over a wide range of metabolic conditions.

6.1.3 paCA in the heart

The finding of paCA in the heart lumen of coho salmon opens the door to an entirely new avenue in the research of cardiac function in teleosts (Chapter 4). The capacity of the teleost heart to increase $\dot{Q}$ during aerobic challenges depends on an adequate supply of O_2 to the myocardium. In salmonids a coronary supplies the compact myocardium with arterial blood, however, the spongy myocardium that represents ~70% of the heart, relies exclusively on the O_2 supplied by the venous return (Davie and Farrell, 1991a). Cardiac $\dot{M}O_2$ is less than 5% of whole animal $\dot{M}O_2$ in fish, and thus C_vO_2 is not expected to limit cardiac function, as long as P_vO_2 can maintain adequate diffusion gradients (Farrell and Steffensen, 1987b). Therefore, the O_2 supply to the spongy myocardium may set the limits on O_2 extraction at the tissues upstream and this has been identified as a potential bottleneck that may predict exercise performance and thermal tolerance in teleosts (Davie and Farrell, 1991a; Farrell, 1997; Gallaugher et al., 2001). However, the teleost mechanism of β-NHE short-circuiting may alleviate this limitation, by: i) an increase in β_b that reduces the requirements on $\dot{Q}$ proportionally; ii) enabling a higher O_2 extraction at the tissues while maintaining P_vO_2; and iii) increasing O_2 extraction in the heart itself. Experimental evidence provided throughout this dissertation and in previous work supports these conclusions as follows.

Data presented in Chapter 3 indicate that paCA allows Atlantic salmon to maintain an elevated $\Delta\dot{M}O_2/\dot{Q}$, over a broad range of conditions, including rest and recovery from exercise; and when paCA was inhibited, $\Delta\dot{M}O_2/\dot{Q}$ decreased. These findings are in line with previous studies that found an elevated $\dot{M}O_2/\dot{Q}$ in hypoxia-acclimated steelhead trout and Atlantic salmon during exercise and a thermal challenge (Petersen and Gamperl, 2010a; Motyka et al., 2017). Therefore, β-NHE short-circuiting and the related increase in β_b can reduce cardiac work and alleviate the requirements on the O_2 supply to the spongy myocardium, and does so broadly across teleost species and conditions. Further, results from previous studies support that teleosts can increase tissue O_2 extraction substantially, while maintaining P_vO_2 and thus protect the O_2 supply to the spongy myocardium until near-maximal levels of exercise (Farrell and Clutterham,

2003; McKenzie et al., 2004). Finally, however, a role paCA in enhancing O_2 extraction in the heart itself remains to be substantiated. Important data may be generated in isolated heart preparations, where cardiac power output and $\dot{M}O_2$ are measured in the presence and absence of C18.

6.1.4 Plasticity of β-NHE short-circuiting

The present findings indicate some plasticity in the mechanism that elevates β_b in Atlantic salmon. The effects of hypoxia acclimation in Atlantic salmon were less prominent than those previously observed in steelhead trout or Atlantic cod (Petersen and Gamperl, 2010a; Motyka et al., 2017). However, data in Chapter 3 indicate that hypoxia-acclimated Atlantic salmon relied on paCA and the related increase in β_b to a larger extent, compared to normoxia-acclimated fish. This was most evident during recovery from exercise, where hypoxia-acclimated fish maintained significantly higher $\dot{Q}$ in the absence of paCA. Also during exercise, the injection of C18 caused a numerically larger increase in $\dot{Q}$ in hypoxia-acclimated fish (34.4%) compared to normoxia-acclimated fish (20.2%) and this was reflected in a lower $\dot{M}O_2/\dot{Q}$. The pathways by which hypoxia acclimation may up-regulate the mechanism of β-NHE short-circuiting in teleosts is yet to be determined; however, plasticity in the three principal components of the system may allow a modulation as follows.

The activity of RBC β-NHE determines the H^+ gradient across the membrane and thus the ΔpH_{a-v} that can be achieved at the tissues. The RBC β-adrenergic response in teleosts is highly variable (Perry et al., 1991; Reid et al., 1998) and may be modulated through changes in circulating catecholamine levels (Petersen and Gamperl, 2011), the density and binding affinity of the RBC β-adrenergic receptors (Perry and Reid, 1992; Motyka et al., 2017), by internalising receptors (Reid and Perry, 1991) or by modulating the signalling pathway down-stream of the receptor (Perry et al., 1996). In addition, the Bohr shift that can be accomplished for a given ΔpH_{a-v} is largely determined by the Hb-O_2 binding characteristics. Hb P_{50}, n_H or the Bohr coefficient may change through allosteric modulation or the expression of different Hb isoforms (Jensen et al., 1998) in response to hypoxia acclimation, temperatures changes and exercise training (Harter and Brauner, 2017). Finally, the mechanism of β-NHE short-circuiting may be up-regulated by increasing the expression and activity of paCA at the tissue capillaries or in the heart. The distribution of paCA is a vastly under-studied area, not only in teleosts, and whether, enzyme activities are modulated *in vivo* remains to be assessed.

Likewise, different teleost species may have adapted to exploit a higher β_b to different degrees. Clearly, the β-NHE short-circuiting mechanism of teleosts needs to be explored on a larger number of species, and the tremendous ecological and physiological diversity of teleosts, may be viewed as both an exciting opportunity and a daunting challenge. However, a roadmap to identify promising species that are likely to differ in their reliance on β-NHE short-circuiting may already exist (Berenbrink et al., 2005). Large differences in β-NHE activity and Hb pH sensitivity are found across teleost species and these are primary loci that may determine a species' capacity to enhance tissue O_2 extraction *via* β-NHE short-circuiting. For example, β-NHE activity was reduced independently in four teleost groups that have also lost a Root effect and a *choroid rete*. Many of these clades are notoriously sluggish and in these animals the benefit of maintaining an elevated β_b may be largely inconsequential (siluriforms, *Monopterus*, *Botia*, *Misgurnus* and *Apteronotus*).

Species in the genus *Anguilla* also appear to have a reduced sensitivity of RBCs to β-adrenergic stimulation. However, RBC NHE in American and European eel respond strongly to osmotic disturbances, while some adrenergic activation does occur in hypoxia (Hyde and Perry, 1990; Perry and Reid, 1992; Reid and Perry, 1994; Romero et al., 1996). Many species of eel are active swimmers with catadromous life-cycles, where animals migrate up to 5500 km to their spawning grounds; whether these animals also enhance β_b by NHE short-circuiting remains to be investigated. However, the anguilliform mode of swimming appears to be more efficient compared to that of many other teleosts and even during sustained exercise over 6 months, $\dot{M}O_2$ is well-below that of migrating salmonids (van Ginneken et al., 2005). Unlike salmonids that continue to feed during their oceanic migrations, eel fast during their entire spawning migration. Thus, perhaps eel have been selected more strongly for efficiency of locomotion, to mete out their limited energy reserves, compared to maximal $\dot{M}O_2$ and $\dot{Q}$ that are important for the up-river migrations of salmon; if so, a higher β_b may be less important for eel.

In contrast, other species may greatly benefit from an elevated β_b, such as carp that frequently experience hypoxia in their habitat, or tunas that seem to exploit all physiological avenues that enable a higher exercise performance; and in fact many of these species have highly pH sensitive Hb and a well-developed β-NHE response (Jensen and Weber, 1982; Lowe et al., 1998; Berenbrink et al., 2005). Clearly, a broader survey of β-NHE short-circuiting across teleosts may shed some light on the adaptations that enable an enhanced β_b, and the study of

single species across different conditions may reveal plasticity in a mechanism that may be critical to support cardiovascular O_2 transport in half of all vertebrates.

6.1.5 The distribution of paCA

The elegant work of Berenbrink et al. (2005) unravelled the sequence of events that led to the evolution of the Bohr- and Root effects, O_2 secreting *retes* at the eyes and the swimbladder, and RBC pH_i regulation by β-NHE. However, in light of the present findings, the evolution of a heterogeneous distribution of paCA is a missing element in this story. If one accepts that a homogeneous distribution of paCA in the circulatory system represents the ancestral state, the question arises as to why some fish lineages have lost paCA in parts of their circulation (possibly independently). The selective pressures that may have led to a loss of paCA in these groups are largely unresolved and Table 6-1 represents a conceptual summary of the findings on the distribution of paCA in fishes in relation to other known blood characteristics (for a detailed discussion see Harter and Brauner, 2017).

Antarctic icefishes have paCA at the gills, a trait that in all other teleosts is likely prohibited by the characteristics of their Hb and RBC function. These findings shed light on the mechanistic trade-offs that underlie the expression of paCA at the gills, and that may have shaped the evolution of the teleost mode of O_2 transport. Notothenioids derive from a perciform ancestor (Near et al., 2003, 2004; Eastman, 2005) that likely had the full extent of teleost blood characteristics and these are retained in the red-blooded representatives of the clade. However icefishes, that have lost Hb and largely reduced RBCs in the circulation (Hct < 1%; Egginton, 1994), obviously lack a Root effect, Bohr effect, β-NHE activity or a *choroid rete* (Eastman and Lannoo, 2003, 2004; Wells, 2005; Sidell and O'Brien, 2006; Wujcik et al., 2007). Like all other vertebrates, icefish primarily transport CO_2 as HCO_3^- in the plasma (Hemmingsen and Douglas, 1972) and CA activity is required to catalyse the dehydration reaction at the gill, especially at low temperatures. In the absence of RBC CA, that drives CO_2 excretion in all other teleost, the gills of icefishes take on this catalytic role by expressing a paCa4 isoform (Chapter 5; Maffia et al., 2001; Tufts et al., 2002). Interestingly, other teleosts that have secondarily reduced their Root effect and β-NHE activity and increased their β_{plasma}, such as siluriforms (Cameron and Kormanik, 1982; Gilmour et al., 2002; Szebedinszky and Gilmour, 2002; Berenbrink et al., 2005), do not express paCA at the gills (Henry et al., 1988; Gilmour et al., 2002). And based on the work of Desforges et al. (2001) it appears that “re-gaining” branchial paCA may be of little

benefit to CO_2 excretion in the presence of a rapid AE and sufficient H^+ and CA availability within the RBC. While the reduction in RBC CA in icefishes and the need to catalyse CO_2 excretion may have driven the expression of branchial paCA, the absence of pH-sensitive Hb or β-NHE activity was likely a permissive requirement. Further insight may be gained by studying the relationship between Hb concentration and branchial paCA in basal notothenioids, as these traits may correlate and change gradually towards the icefish lineage; work that is currently underway.

Lamprey have independently lost paCA at the gills (Henry et al., 1993) and have otherwise converged on many of the typical teleost blood characteristics by distinctly different mechanisms, such as a marked Bohr-Haldane effect and RBC pH_i regulation (monomeric vs. tetrameric Hb and non-adrenergic vs. β-NHE; Nikinmaa et al., 1995; Tufts and Perry, 1998). The early divergence of the teleost and lamprey lineages (Arnason et al., 2004; Janvier, 2007) may represent a unique opportunity to study the functional relationships between these traits and selective pressures that may have led to a loss of paCA at the gill. Data on *Amia* indicate that the loss of paCA (Gervais and Tufts, 1998) pre-dated the evolution of a β-NHE (Berenbrink et al., 2005) or a Root effect that may have been expressed in the general circulation (Regan and Brauner, 2010b); however, it may have released the functional constraint on a later evolution of β-NHE activity. Alternative selective pressures for the loss of branchial paCA may have included: i) uncoupling of pH_e and pH_i to protect Hb-O_2 affinity during an acidosis (Randall et al., 2014); ii) strengthening the coupling between O_2 and CO_2 transport in the presence of a Bohr effect (Brauner, 1995); iii) or it may have been a by-product of the reduction in β_{plasma} that rendered paCA activity inconsequential for CO_2 excretion (Desforges et al., 2001; Gilmour et al., 2004).

A heterogeneous distribution of paCA enabled RBC pH_i regulation and a high pH sensitivity of Hb, and was certainly a key innovation in the evolutionary trajectory that resulted in a system of β-NHE short-circuiting that supports cardiovascular O_2 transport in modern teleosts. Hb- and plasma buffer capacities, the Bohr effect and the onset pH of the Root effect changed substantially in the basal actinopterygians and in particular the common ancestor of teleosts and *Amia* (Berenbrink et al., 2005; Regan and Brauner, 2010a; Regan and Brauner, 2010b). Thus, resolving the evolutionary dynamics that led to a loss of paCA at the gills of some fishes will require a broader study of other basal actinopterygians and teleosts, and that should

include non-airbreathing representatives of these groups; a circumstance that may well influence the observed relationships (Graham, 1997; Brauner and Berenbrink, 2007).

6.1.6 Active enhancement of O_2 diffusion in teleosts

Perhaps one of the most exciting findings of the present dissertation is that teleosts may possess an active, adrenergically regulated mechanism that enhances O_2 diffusion at the tissues. Christian Bohr was familiar with the high PO_2 in the swimbladder of teleosts that exceeds arterial PO_2 and thus, contradicted a mechanism that is driven solely by diffusion. He imagined, incorrectly, that an active cellular mechanism must drive O_2 into the swimbladder and that O_2 uptake at the mammalian lung would follow a similar principle that he described as "O_2 secretion" (Bohr, 1909). Eventually, Bohr's idea was disproven by his own student, August Krogh, who showed that O_2 uptake at the mammalian lungs is a passive process that is driven by diffusion alone (Krogh, 1910). Today we know that also the teleost swimbladder is filled by diffusion of O_2, however, the diffusion gradients are enhanced by active acidification of the blood in the *retes*. A few years later, Krogh went on to show that an increase $\dot{M}O_2$ at the tissues is met by an increase in blood flow through the recruitment of additional capillaries and thus too can be explained by diffusion alone (e.g., Krogh, 1919a, b); findings that ultimately won him the Nobel Prize in 1920. Still today, Krogh's seminal work dictates our understanding of O_2 transport in all vertebrates, and teleosts are no exception. However, my dissertation provides evidence for an active mechanism in teleosts that enhances the O_2 diffusion gradients outside of the *retes*, at all tissues, and that allows for a flux of O_2 that exceeds what can be achieved by the passive process alone.

There are clear similarities between enhanced O_2 unloading in the teleost *retes*, and β-NHE short-circuiting, as both are active mechanisms that create large, localised $\Delta pH_{a\text{-}v}$ that increase PO_2 by a Bohr shift. In the *retes*, the high PO_2 are necessary to overcome large diffusion distances in the avascular retina of the eye, and high hydrostatic pressure in the swimbladder at depth. Whereas in other tissues, such as the exercising muscle, a right-shift of the OEC will enable a higher O_2 extraction, before a minimum critical P_vO_2 sets limits to diffusion. As β_b increases, fewer capillaries need to be recruited to maintain tissue $\dot{M}O_2$ and this reduces the requirements on local blood flow and eventually $\dot{Q}$. Within a species' blood characteristics, the magnitude of the Bohr shift that can be accomplished by β-NHE short-circuiting, will largely be a function of the H^+ gradient across the RBC membrane. This H^+ gradient is a store of potential

energy that is actively generated by β-NHE activity and that in turn, is driven by the ATP consuming NKA. In the presence of paCA and a pH sensitive Hb, this potential energy is then translated into a higher O_2 extraction and β_b. Of course, the transfer of O_2 from Hb to the mitochondria in the tissues of teleosts is still entirely driven by diffusion, August Krogh remains correct (Krogh, 1919a, b). However, teleosts invest ATP into changing these diffusion gradients to their advantage. Outside of the *retes*, there is no other account of a similar mechanism in vertebrates that uses energy to enhance O_2 diffusion, and that matches so closely the predictions of an active cellular mechanism of "O_2 secretion" that Christian Bohr had envision in the mammalian lungs (Bohr, 1891), perhaps he would have enjoyed learning about these findings today.

6.2 Implications of the present findings

6.2.1 Implications for teleosts in general

The present dissertation assessed different aspects of the teleost O_2 transport system in three salmonid species (and two notothenioids). It should be emphasised that even among salmonids, critical differences in the ecology and physiology of study species may confound experimental results. Perhaps most importantly, rainbow trout and coho salmon (Chapters 1 and 4) were acclimated to freshwater, while Atlantic salmon (Chapter 3) were acclimated to seawater; differences in $\dot{M}O_2$ and swimming performance have been reported in other salmonids that were acclimated to different salinities (Rao, 1968; Morgan and Iwama, 1991). Further variability may be introduced by studying hatchery-raised fish that are typically selected for higher stress tolerance and often show reduced exercise performance compared to wild conspecifics (Brauner et al., 1994; Eliason et al., 2013). In addition, while Chapter 4 investigated only female coho salmon, all other chapters investigated mixed-sex populations of white sturgeon, rainbow trout, Atlantic salmon and Antarctic notothenioids; sex steroids have been shown to alter cardiovascular physiology in salmonids (Thorarensen et al., 1996a; Davie and Thorarensen, 1997; Farrar and Rodnick, 2004). And finally, experiments were carried out on animals of different stages of maturity; white sturgeon were juveniles, whereas all salmonids were studied as non-spawning adults and no detailed information is available for the specimens of Antarctic notothenioids used in Chapter 5. It is important to consider these potentially confounding factors when comparing cardiovascular O_2 transport across salmonid and other teleost species, and critically, also when comparing results across studies. However, at present,

no study has investigated how factors such as sex, life-stage or salinity acclimation may affect the system of β-NHE short-circuiting in teleosts, and this is an area ripe for exploration.

Importantly however, and despite the investigation of several salmonid and teleost species, across sexes and life-stages, all results in the present dissertation are in remarkable agreement with the initial hypotheses and generally in line with our current understanding of β-NHE short-circuiting. One may argue that these consistent results on different species strengthen the idea that β-NHE short-circuiting is a common trait, at the very least in salmonids. The extension of these results to teleosts in general is rooted in the mechanistic basis for β-NHE short-circuiting, and indirect evidence from one other non-salmonid, Atlantic cod (Butler et al., 1989; Petersen and Gamperl, 2010a). An overwhelming number of studies have indicated that all salmonids and most teleosts appear to meet the necessary requirements to enhance tissue O_2 extraction by β-NHE short-circuiting, namely: i) pH sensitive Hbs, ii) β-NHE activity, and iii) a heterogeneous distribution of paCA (Berenbrink et al., 2005; Harter and Brauner, 2017; Shu et al., 2017). The evolutionary history of these traits lends further support for a ubiquitous presence across teleosts, except for the few clades that have reversed these trends. But clearly, additional work is required to substantiate that these three requirements for β-NHE short-circuiting are met by most teleosts. In particular, the distribution of paCA in the vasculature is yet to be resolved in, essentially, all vertebrates.

6.2.2 Implications for non-teleosts

Whether other, non-teleost species, may enhance tissue O_2 extraction by β-NHE short-circuiting or related mechanisms, remains to be thoroughly assessed. However, few vertebrate groups seem to meet the necessary requirements. A moderate Bohr effect and some ΔpH_{a-v} at the tissues are commonly found across all vertebrates (Berenbrink et al., 2005). However, outside of teleosts the potential to increase β_b by short-circuiting H^+ gradients is severely limited by the lower pH sensitivity of Hb (Rummer and Brauner, 2015). In addition, ΔpH_{a-v} that match those generated by teleost *retes* or RBC β-NHE have not been described in other vertebrates. The presence of other transporters that can generate H^+ gradients across the RBC membrane, is worthwhile investigating (Rummer et al., 2010; Rummer and Brauner, 2011). However, results in Chapter 2 indicate that no such H^+ gradients exist across the RBC membrane of white sturgeon; and thus, there is currently no evidence for a comparable transporter in non-teleosts. The only exception are lamprey; they can generate H^+ gradients across the RBC membrane by

non-adrenergic NHEs, have a large Bohr effect and appear to have a heterogeneous distribution of paCA in the circulation. Lamprey, like teleosts, may enhance β_b by short-circuiting H^+ gradients across the RBC membrane and if so, may share yet another convergently evolved trait of their respiratory physiology with teleosts.

In the same way as eliminating H^+ disequilibria across the RBC membrane may increase β_b, eliminating pH disequilibria in the plasma itself, may be of similar benefit (Randall et al., 2014). There is ample evidence for such pH disequilibrium states at the gill of teleosts (Henry et al., 1988; Gilmour and Perry, 1994; Gilmour et al., 1994; Gilmour and Perry, 1996; Gilmour et al., 1997), but also in the venous system (Perry et al., 1997). In the absence of paCA, CO_2 excretion at the gill will create an excess of HCO_3^- and H^+ in the post-branchial blood and a pH that is lower than at equilibrium. If paCA is present in post-branchial arteries the pH disequilibrium will be quickly eliminated, leading to a sudden increase in PCO_2 and an acidification of the RBC *via* the Jacobs-Stewart cycle that may increase P_aO_2 (Randall et al., 2014). In fact, Foster and Steen (1969) showed that the addition of CA to eel RBCs suspended in a solution that is in pH disequilibrium will release O_2 from Hb *via* the Bohr effect. Like β-NHE short-circuiting, this mechanism may increase $\Delta pH_{a\text{-}v}$ and would extend the benefits of an enhanced β_b to species and conditions without β-NHE activity; at present an untested idea that is worthwhile investigating.

The formation of pH disequilibrium states in the plasma still hinges on a heterogeneous distribution of paCA in the vasculature; a requirement that doesn't appear to be met outside of the teleost clade (Stabenau and Heming, 2003; Harter and Brauner, 2017). Perhaps this is the reason why β-NHE short-circuiting did not evolve in other clades, such as the elasmobranchs or tetrapods, where high metabolic rates may be supported by other physiological adaptations. However, many basal actinopterygians have a substantial Bohr effect and at least *Amia* appear to have a heterogeneous distribution of paCA. Therefore, *Amia* may enhance β_b by short-circuiting pH disequilibria in the plasma. If substantiated, this may change the current view on the evolution of β-NHE short-circuiting in teleosts that may simply be a variation of a system that already enhanced β_b in all tissues of basal actinopterygians and preceded the evolution of the *retes*. In many respects, a further investigation of cardiovascular O_2 transport in basal actinopterygians will increase our understanding of the mechanistic underpinnings and the evolution of the unique mode of O_2 transport of modern teleost.

6.2.3 Implication for the teleost heart

In Atlantic salmon at rest, when f_h is ~60 bpm, the average residence time of blood within the heart is < 1 s, and this value may halve during maximal exercise. Since short-circuiting of β-NHE seems feasible at the tissue capillaries within a similar timeframe (Chapter 2), the same may be possible in the lumen of the heart. Unlike tissue capillaries, the lumen of the heart has a relatively small surface area to volume ratio and most RBCs may not encounter paCA on trabeculae or the walls of the chamber. However, conditions in the contracted ventricle may be beneficial to facilitate β-NHE short-circuiting, as the surface area to volume ratio will be largest, at a time when blood flow is low for a brief period. In a fish swimming at 1 bl s^{-1} the ventricular ejected fraction is ~90% (Franklin and Davie, 1992), thus the volume of the contracted ventricle will be decreased by an order of magnitude during systole. Conceptually, the presence of paCA in the heart (Chapter 4) may right-shift the OEC in the 10% of blood that remains in the ventricle (end systolic volume), promoting the diffusion of O_2 to mitochondria in the myocardium (Davie and Farrell, 1991a).

The idea that β-NHE short-circuiting in the teleost heart is limited by an unfavourable surface area to volume ratio, may be important in safeguarding Hb-O_2 loading at the gills. β-NHE activity in rainbow trout can recover RBC pH_i after short-circuiting at the tissues, within the time available during venous transit ($t_{1/2}$ ~ 17 s; Chapter 2). However, the short transit time of blood in the ventral aorta (~50-250 ms, assuming that a ventral aorta in a 1 kg fish is: L = 1 cm; D = 0.3 cm; and resting and maximal $\dot{Q}$ are 20 and 60 mL kg^{-1} h^{-1}) will prohibit complete RBC pH_i recovery. If only 10% of RBCs are effectively short-circuited at the heart, this may prevent a detrimental reduction in C_aO_2. In fact, a small reduction in C_aO_2 may be offset by the benefits of a higher O_2 extraction in the ventricle that may enable a higher $\dot{Q}$.

The available data on paCA distribution in the hearts of teleosts is limited and the complex haemodynamics within the heart prohibit from accurately predicting the fraction of RBCs that will interact with paCA in the lumen. Future studies may address this problem by modelling blood flow in the hearts of teleosts and by careful measurements of C_aO_2 in the ejected fraction of isolated hearts in the presence of absence of specific CA inhibitors. Results from these experiments may significantly advance our understanding of cardiac physiology in teleosts that is critical for making accurate predictions on the impacts of climate change on fishes.

6.2.4 Implications in a warming world

In an attempt to formulate a unifying hypothesis that explains upper thermal limits in aquatic ectotherms, and thus the boundaries of their latitudinal distribution, the oxygen and capacity limited thermal tolerance (OCLTT) theory identified limitations on cardiac performance and an inability to increase tissue O_2 delivery, as the main driver for a deterioration of whole animal performance at elevated temperatures (Pörtner, 2001; Clark et al., 2008; Pörtner and Farrell, 2008; Pörtner and Lannig, 2009). The framework of the OCLTT has been applied successfully to explain thermal tolerance in a number of fish species (Pörtner, 2001; Pörtner, 2010; Eliason et al., 2011), however, criticism arises due to a disconnect between aerobic scope and fitness in other studies (Healy and Schulte, 2012; Clark et al., 2013; Gräns et al., 2014; Norin et al., 2014). In fact, the idea that aerobic scope is a universal predictor of ecologically relevant limitations in animal performance may be the basis for some of the controversy surrounding the OCLTT (Clark et al., 2013; Schulte, 2015; Jutfelt et al., 2018), however its mechanistic underpinnings represent a useful framework to test hypotheses regarding the physiology of thermal tolerance in fish.

Most fish species that have been investigated to date, to validate or refute the OCLTT, were teleosts that may have some capacity to maintain an elevated β_b *via* β-NHE short-circuiting, which alleviates the requirements on the heart. Thus, models that are based on teleost physiology may grossly underestimate thermal tolerance in fish species that are incapable of β-NHE short-circuiting, such as those that lack RBC β-NHE or a significant Bohr effect; these may include all chondrichthyans, sarcopterygians and basal actinopterygians. Based on the findings of the present dissertation, mechanisms that increase β_b, such as β-NHE short-circuiting, need to be incorporated into working models of the physiological basis underlying thermal tolerance in fishes. Manipulative studies may address the issue by inhibiting β-NHE or paCA activity in teleosts and non-teleost species; the OCLTT would predict a decrease in thermal tolerance only in the former. Teleost representative from thermal extremes, such as the lake Magadi tilapia (*Alcolapia grahami*) and red-blooded notothenioids offer further opportunities to address these questions experimentally.

6.3 Future directions

Results from the present dissertation indicate that β-NHE short-circuiting may greatly enhance β_b in salmonids with important ecological implications. If the system were present in the

majority of teleosts this finding may change, fundamentally, our understanding of O_2 transport in nearly half of all vertebrates. However, clearly, the next step will be to address the importance of β-NHE short-circuiting for cardiovascular O_2 transport more broadly across teleost species, before sweeping conclusions can be drawn. Whether some variability in common aerobic performance metrics is explained by the capacity to enhance β_b in teleosts, is yet another exciting avenue for future work. Based on the present findings, one may predict that measurements of aerobic scope in the absence of paCA activity (C18 injection) and using thermal and exercise challenges, is likely to reveal the ecological importance β-NHE short-circuiting in teleosts.

6.3.1 Measurements of P_vO_2

Additional *in vivo* measurements may substantiate the mechanistic underpinnings of β-NHE short-circuiting, such as careful measurements of arterial-venous changes in PO_2 and C_vO_2. Data on Atlantic cod indicate that hypoxia acclimation caused a relative increase in tissue O_2 extraction of ~21% (from 50.9 to 61.5%) when measured in normoxia, and this was reflected in a higher $\dot{M}O_2/\dot{Q}$ (Petersen and Gamperl, 2011). In a preliminary trial in Chapter 3, the dorsal and ventral aortae were cannulated in Atlantic salmon ($N = 3$), fish were swum at 1 bl s^{-1} and injected with a sham and with C18, while C_aO_2 and C_vO_2 were measured. Unfortunately, the venous cannula clogged in one fish that was subsequently included in the main experiment. Of the other two individuals, one collapsed after the injection of C18, and was unable to continue swimming. Therefore, a complete data set was only obtained for one individual and this precluded a mention in the manuscript. O_2 extraction at rest was 32 ± 4% ($N = 2$) and increased to 58 ± 1% during exercise ($N = 2$); results that match previous measurements in salmonids (Kiceniuk and Jones, 1977; Brauner et al., 2000a; Eliason et al., 2013). Upon injection of C18, O_2 extraction decreased to 46% ($N = 1$), representing a relative reduction in O_2 extraction of 20% that was entirely due to an increase in C_vO_2. A reduction in O_2 extraction of this magnitude must be compensated by an increase in $\dot{Q}$ of ~25% (i.e., 125% × 46% = ~58%) that matches closely that observed *in vivo*, of 27% (Chapter 3). During recovery from exercise, O_2 extraction was 34% ($N = 1$). Clearly, due to the lack of replication, these results should not be over-interpreted and I will refrain from drawing any conclusions at this point. But, if nothing else, these preliminary findings point towards a fruitful avenue for future studies that may corroborate the role of paCA in maintaining a higher tissue O_2 extraction at constant P_vO_2, and that may shed some insight into the regulation of the system.

6.3.2 Does branchial paCA impair arterial O_2 transport?

Teleost rely on β-NHE activity to prevent a reduction in RBC pH_i during a blood acidosis and to protect C_aO_2 (Nikinmaa et al., 1984; Primmett et al., 1986; Tetens and Christensen, 1987; Vermette and Perry, 1988; Perry and Kinkead, 1989). One may predict that the injection of soluble CA in teleosts would short-circuit β-NHE activity on a systemic level and compromise C_aO_2 during an acidosis. However, two studies that injected bovine CA into rainbow trout, exposed to hypoxia (Lessard et al., 1995) or exercised to exhaustion (Wood and Munger, 1994), found no effect on C_aO_2 despite evidence for β-NHE activity. Perhaps the major confounding factor in the latter studies was a blunted blood acidosis in CA injected fish compared to saline injected controls, which may have masked a detrimental effect of β-NHE short-circuiting on C_aO_2. Injection of CA catalyses HCO_3^- dehydration in the plasma and CO_2 excretion becomes more efficient, consistent with the idea that access of HCO_3^- to RBC intracellular CA is the rate limiting step in CO_2 excretion (Perry and Gilmour, 1993). In addition, and perhaps as a consequence of a higher pH_e, the release of catecholamines was reduced in CA-injected fish (Wood and Munger, 1994). Clearly, whether β-NHE short-circuiting on a systemic level may impair C_aO_2 deserves a renewed investigation, specifically within this framework, and using an experimental design that overcomes the confounding effects of CA injection. A respiratory acidosis induced by hypercarbia may create conditions that reliably stimulate β-NHE and create a sufficiently severe acidosis to decrease RBC pH_i, and this approach should be robust to the catalysis of HCO_3^- dehydration in the plasma. The obtained results may answer conclusively whether the absence of paCA at the teleost gill is a necessary requirement to safeguard C_aO_2 *via* β-NHE activity during an acidosis.

6.3.3 The teleost intestine

The intestine of teleosts is yet another tissue that may greatly enhance β_b *via* pH-sensitive Hb-O_2 unloading. Marine teleosts must drink continuously to compensate for the passive loss of water and gain of ions from the hyperosmotic seawater. Water uptake in the intestine is largely driven by HCO_3^- secretion into the lumen (Wilson, 1999; Grosell and Genz, 2006; Grosell et al., 2009; Tresguerres et al., 2010) and maintaining enterocyte pH_i requires an equimolar secretion of H^+ into the blood (Grosell and Genz, 2006; Grosell and Taylor, 2007). In addition, PCO_2 in the intestinal lumen is high (Wood et al., 2010) and CO_2 may diffuse across the intestinal epithelium into the blood. The vasculature in the intestinal *villi* seems to function as a counter-current

exchanger (Hallbäck et al., 1978; Jodal et al., 1978) that, in mammals, is conductive to O_2 (Kampp et al., 1967). Furthermore, the high osmolyte concentrations in these capillaries, related to nutrient or ion absorption, may cause a "salting out" effect by decreasing O_2 solubility in the plasma (Pelster et al., 1990). Due to these obvious similarities to the situation in the *retes*, an expression of the Root effect may be possible at the intestine of marine teleosts. The implication may be critical for this tissue, which must greatly increase $\dot{M}O_2$ after feeding (Taylor and Grosell, 2009; Seth et al., 2011) and where both $\dot{M}O_2$ and splanchnic blood flow vary substantially and not always in concert with one another (Holmgren et al., 1992; Thorarensen et al., 1993; Axelsson et al., 2000; Crocker et al., 2000; Farrell et al., 2001). The H^+ load into intestinal capillaries is proportional to the osmoregulatory activity of the tissue. Therefore, pH sensitive Hb-O_2 unloading may not only meet the high $\dot{M}O_2$ of the intestine, but also provide some auto-regulation of β_b based on ΔpH_{a-v} and blood flow. In addition, β-NHE short-circuiting may provide a further increase in β_b that would by far exceed the values estimated by Cooper et al. (2014). In the marine stages of many salmonids that must simultaneously allocate blood flow to digestion and exercise, mechanisms that increase β_b may be of ecological importance and are clearly worthwhile studying.

6.3.4 The coronary

A coronary circulation first evolved in the early gnathostomes, and while agnathans, such as hagfish and lamprey, rely exclusively on the venous return to supply O_2 to their hearts, most elasmobranchs have a well-developed arterial O_2 supply to the compact and spongy layers of myocardium. However, the reliance on a coronary supply decreased in the transition from basal actinopterygians to early teleosts (Farrell et al., 2012). In fact, many teleosts do not have a coronary circulation and those that do, often lack a coronary supply to the spongy myocardium (Tota, 1983; Santer, 1985; Farrell, 1991). The evolutionary timeframe, over which teleosts have lost a coronary supply to the spongy myocardium, is in line with the innovation of β-NHE short-circuiting. By this mechanism, paCA in the heart lumen (Chapter 4) may maintain diffusion gradients even if larger diffusion distance need to be overcome in thicker trabeculae that are required for larger hearts. This may be especially relevant for species that have a high affinity Hb, a requirement in many aquatic habitats, and where P_vO_2 is necessarily low, such as carp (Brauner et al., 2001). Also athletic teleosts that have lost a coronary supply to the spongy myocardium may benefit greatly from a higher P_vO_2, such as salmonids. Finally, those teleost

species that have a reliable coronary supply throughout the heart may further enhance exercise performance by β-NHE short-circuiting in the heart lumen and the coronary itself; such as tuna. In aquatic vertebrates, where high arterial blood pressures are not necessary, reversal to a largely spongy myocardium may have energetic advantages and a lower reliance on the arterial O_2 supply may dissipate the risks involved with a failure of the coronary supply (Farrell and Steffensen, 1987a). A potential role of paCA in the cardiac O_2 supply of teleosts remains to be validated, and the current lack of experimental data may offer a rich avenue for future work. If substantiated, a heterogeneous distribution of paCA and RBC pH_i regulation in teleosts may represent key innovations that shaped the evolution of cardiac function in half of all vertebrates; and that may provide valuable insight into the evolutionary dynamics of cardiac function in vertebrates in general.

6.4 Conclusion

At the time I started working on this dissertation, a novel mechanism of enhanced Hb-O_2 unloading had been recently described, and i*n vitro* and *in vivo* studies had revealed the great potential of this system to increase PO_2 at the tissues of rainbow trout. However, the system was viewed by many (including myself) as an exotic mechanism that may be relevant for some teleosts under extreme conditions. Based on the data generated in this dissertation, my opinion has changed. Collectively, my findings indicate that salmonids, and potentially most teleosts, may enhance tissue O_2 extraction through a mechanism of active RBC pH_i regulation that is modulated by a heterogeneous distribution of paCA, which increase β_b and reduces the requirements on the heart. Analogous to the situation in the teleost *retes*, β-NHE short-circuiting is an active mechanism that creates large $\Delta pH_{a\text{-}v}$ locally, and promotes Hb-O_2 unloading *via* the Bohr effect. However, the resulting increase in β_b is available to all tissues and likely enables the astounding exercise performance, and perhaps thermal tolerance, of modern teleosts. Importantly, the present data highlight the *in vivo* relevance of the system, which is an integral part of the mode of cardiovascular O_2 transport in teleosts and is recruited routinely, over a broad range of conditions, including rest, exercise and recovery from exercise. The teleost system of β-NHE short-circuiting showed some plasticity in response to hypoxia acclimation; this, and the tremendous diversity in the ecology and physiology of teleosts, opens exciting avenues for future comparative studies. Further, the distribution of paCA within individuals, and across teleost and other vertebrate species, is largely unexplored. It appears that CA, which is usually implicated in

acid-base regulation and CO_2 excretion, may have shaped the evolution of the teleost O_2 transport system and played an important role in the extensive adaptive radiation of this clade. The present dissertation advances our understanding of a novel mechanism of cardiovascular O_2 transport that has important implications for future work on the physiology, the conservation, and the evolutionary history, of nearly half of all vertebrates.

Table 6-1 Conceptual summary of blood characteristics in select fish lineages, relative to the distribution of carbonic anhydrase (CA) activity at the gas exchange surface.

Species	Circul. CA[1]	pICA[2]	paCA[3]	β_{plasma}[4]	β Hb[5]	Bohr-Hb[6]	NHE[7]
Chondrichthyans	+	-	+	+	+	-	-
Hagfish	+	-	+	+	+	-	-
Lamprey	+	-	-	-	-	+	+
Amia	-	+	-	-	-	+	-
Salmonids	-	+	-	-	-	+	+
Flounder	-	+	-	-	-	+	+
Siluriforms	-	+	-	+	+	-	-
Red-blooded notothenioids	-	+	-	-	-	+	+
Icefish	-	+	+	+			

[1]Circulating CA activity in the plasma; soluble isoforms (+ indicates presence, - absence)

[2]Plasma inhibitor of CA (+ indicates presence, - absence)

[3]Plasma-accessible CA at the gill; membrane-bound isoforms (+ indicates presence, - absence)

[4]Non-bicarbonate buffer capacity of plasma (+ indicates relatively high, - relatively low)

[5]Haemoglobin buffer capacity (+ indicates relatively high, - relatively low)

[6]Bohr-Haldane effect of haemoglobin (+ indicates relatively high, - relatively low)

[7]Sodium-proton exchangers activity on the red blood cell membrane; adrenergic β-NHE in teleosts and non-adrenergic NHE in lamprey (+ indicates presence, - absence)

See Harter and Brauner (2017) for a discussion of the original data

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