The effects of water ionic composition on acid-base regulation in rainbow trout, *Oncorhynchus mykiss*, during hypercarbia at rest and during sustained exercise

by

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Abstract

Rising atmospheric carbon dioxide (CO_2) and by association pCO_2 in aquatic habitats (hypercarbia) has put increased focus on understanding the underlying mechanisms of acid-base regulation in fish. Aquatic hypercarbia results in a blood acidosis in fish, which is compensated for by the exchange of Na^+ and Cl^- for its acid/base counterpart (H^+, HCO_3^-) across the gill epithelium. Surprisingly, there are no studies on how a single species, capable of inhabiting both fresh and saltwater, responds to hypercarbia, and no existing studies examining how sustained exercise may affect hypercarbia recovery. The goal of this thesis was to examine how changes in ambient water ionic composition (soft-, hard-, and saltwater) affect the rate and degree of acid-base compensation in a rainbow trout, *Oncorhynchus mykiss*, during hypercarbia, at rest and during sustained exercise. Additionally, I sought to determine the effect of sustained exercise on the rate and degree of acid-base compensation during hypercarbia. Trout were acclimated to soft-, hard-, or saltwater and acid-base relevant blood parameters were measured during a 1% CO_2 hypercarbia exposure, both at rest and during sustained exercise (~60% U_{crit}). After 48h of hypercarbia, resting hard-, and saltwater acclimated trout had fully restored blood pH, however soft water acclimated trout were only 60.6±10.5% recovered, and in all fish recovery was associated with an increase in plasma [HCO_3^-] and a decrease in plasma [Cl^-] of similar magnitude. Trout exposed to hypercarbia during sustained exercise had a similar response, and following 8h the saltwater acclimated fish had fully restored blood pH, while soft-, and hard water fish were 42±18.1 and 64±6.8% recovered, respectively. Furthermore, following 8h of hypercarbia there was a significant effect of exercise on the degree of recovery compared

with resting fish, suggesting that sustained exercise results in a more rapid recovery from hypercarbia in trout, relative to rest. These results provide intra-specific support to previous studies that demonstrate marine fish compensate for hypercarbia faster than freshwater fish. This thesis not only demonstrates an important link between ambient water ion levels and the ability to recover from acid-base disturbances, and presents novel data suggesting sustained exercise enhances acid-base regulation.

Preface

Chapter 2 of this thesis is co-authored by Katelyn J. Tovey and Colin J. Brauner. I conducted all of the research under the supervision of Dr. Colin J. Brauner. I wrote all 3 chapters of the thesis and received editorial feedback from Drs. Colin J. Brauner, Jeffrey G. Richards, and Robert E. Shadwick. Treatment and experimental protocols involving animals were followed according to the University of British Columbia's Animal Care Committee, certificate A11-0235.

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List of abbreviations

αCO_2	carbon dioxide solubility coefficient
^oC	degrees Celsius
ANOVA	analysis of variance
ATPase	adenosinetriphosphatase
Ca^{2+}	calcium
$CaCO_3$	calcium carbonate
$CaCl_2$	calcium chloride
CFTR	cystic fibrosis transmembrane regulator
Cl^-	chloride
$[Cl^-]$	chloride concentration
CO_2	carbon dioxide
h	hour(s)
Hb	hemoglobin
H^+	proton
H^+ ATPase	proton ATPase
HCO_3^-	bicarbonate
$[HCO_3^-]$	bicarbonate concentration
K^+	potassium
Mg^{2+}	magnesium
MS-222	tricaine methane sulphonate (anaesthetic)
Na^+	sodium
$[Na^+]$	sodium concentration

NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
Na^+/K^+ ATPase	sodium-potassium ATPase
NKCC	sodium-potassium-2 chloride cotransporter
O_2	oxygen
pCO_2	partial pressure of carbon dioxide
p_wCO_2	partial pressure of carbon dioxide in water
pH_e	extracellular pH (blood pH)
pK’	apparent pK (dissociation constant) of carbonic acid in plasma
ppt	parts per thousand
TCO_2	total carbon dioxide
U_{crit}	critical swimming velocity

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For My Family

Chapter 1: General introduction

1.1 Overview

The purpose of this thesis was to demonstrate how the ionic composition of water and sustained exercise alters acid-base compensation during hypercarbia, within a single fish species. Saltwater is thought to promote recovery during hypercarbia compared with freshwater, however this relationship has never been characterized in a single fish species. Furthermore, previous studies on acid-base compensation were conducted at rest or following exhaustive exercise, whereas, the response during sustained aerobic swimming has yet to be characterized. Therefore, I performed several experiments in an effort to determine the combined responses of both water ion content and sustained exercise on the rate and degree of acid-base compensation during hypercarbia and to additionally distinguish the distinct role of each factor. The remainder of the general introduction will discuss what is known about: 1) the mechanisms used to maintain acid-base status; 2) how hypercarbia can be used to characterize acid-base regulatory responses; 3) what is known about the effects of ambient water ion composition on acid-base regulation; 4) the indirect evidence which suggests exercise may play a role in acid-base regulation; 5) the potential impact of the osmo-respiratory compromise on acid-base regulation during exercise. The introduction will conclude with my thesis objectives.

1.2 General acid-base regulation in fishes

Blood pH (pH_e) of animals is typically maintained within a narrow range to preserve function of essential physiological processes (Heisler, 1984). In particular,

enzymes operate optimally at a certain pH, where minute deviations can alter their charge and thus reduce functionality, in turn effecting whole animal performance (Putnam and Roos, 1997). Acid-base homeostasis is conserved when production and elimination of acids and/or bases (H^+ and HCO_3^-, respectively) are equal (Heisler, 1986). However, fishes are prone to acidotic disturbances of either metabolic or respiratory origin. The metabolism of numerous substances including fatty acids and carbohydrates results in the production of organic acids, and by extension release of H^+ ions. A metabolic acidosis occurs as a result of inefficient removal, and therefore accumulation of H^+ ions, often during hypoxia or strenuous exercise. Hypercarbia (elevated external CO_2) results in a respiratory acidosis, as aquatic CO_2 readily diffuses through the gill epithelium where it is hydrated, resulting in H^+ release and a blood acidosis. Regardless of the origin, the acidosis must be counteracted in order to maintain pH homeostasis.

In order to conserve extracellular acid-base status fish employ a number of strategies in response to an acidosis, involving both passive (i.e. buffering) and active (i.e. ion transport) mechanisms. Blood buffering *via* plasma HCO_3^- provides an initial passive compensatory response to an acid-base disturbance by consuming H^+ resulting in the formation of CO_2 and H_2O, thus limiting free H^+, and reducing the magnitude of the acidosis (Heisler, 1986). However, this mechanism is quickly overwhelmed due to the low extracellular [HCO_3^-] (~4mM) of fish, and, in order to combat detrimental pH fluctuations, fish must transfer acid-base relevant ions to/from the environment shortly thereafter (Heisler, 1999; Evans et al., 2005).

An increase in ventilation during an respiratory acidosis has been observed in several studies, and is typically associated with greater increases in ventilation volume

rather than ventilation frequency (Janssen and Randall, 1975; Smith and Jones, 1982; Thomas et al., 1983; Perry et al., 1999). However, fish maintain arterial pCO_2 similar to that of ambient water, often only ~1-2mmHg above p_wCO_2 (Cameron and Randall, 1972). Therefore use of gill ventilation as an effective strategy for CO_2 disposal during acidotic disturbances is unlikely, since the ability to create a large favourable CO_2 gradient is low (Janssen and Randall, 1975).

The specific mechanisms of acid-base relevant ion transfer are not completely described, however, the gill epithelium is widely accepted as the major site of ion transfer, with only minor contributions from the kidney and intestine. The current working model of the teleost gill suggests the exchange of environmental Na^+ and Cl^- with their acidic and basic counterparts (H^+ and HCO_3^-, respectively) to regulate acid-base balance (see review by Evans et al., 2005). Although the underlying transport mechanisms are still being investigated, the presence of apical HCO_3^-/ Cl^- anion exchangers on chloride cells has been noted in several studies (see reviews by Perry et al., 2003 and Evans et al., 2005). Apical transfer mechanisms of H^+ have not been fully elucidated, and appear to differ between freshwater and saltwater species. In general, acid extrusion in freshwater fish, such as salmonids, is thought to be *via* V-type H^+ ATPases that are associated with apical Na^+ channels (Lin et al., 1994; Sullivan et al., 1995). This mechanism appears to function cooperatively with osmoregulation, specifically, fish in this environment are prone to passive ion loss, and active H^+ extrusion can be used to drive Na^+ uptake (Randall and Lin, 1993). However, this pattern is not universal among freshwater fish, some of which rely on an electroneutral Na^+/H^+ ion exchanger

(NHE)(Hyde and Perry, 1989; Hirata et al., 2003), a strategy that is also thought to be the primary mechanism employed by marine fish species (Claiborne et al., 1999).

1.3 Why study hypercarbia?

Elevated environmental CO_2 (hypercarbia) naturally occurs in many aquatic environments, especially in areas rich in aquatic vegetation (Ultsch, 1996). Hypercarbic environments often occur in conjunction with O_2 depletion, making hypoxia a common occurrence in these environments as well (Ultsch, 1996). Recent elevations in atmospheric CO_2 associated with climate change have sparked renewed interest on hypercarbia tolerance. Aquatic environments have a large capacity for CO_2 removal from the atmosphere. With atmospheric CO_2 rising, aquatic environments will also experience elevated p_wCO_2 levels (ocean acidification), which have been shown to have adverse effects on aquatic animals (Wittmann and Pörtner, 2013).

The physiologic mechanisms responsible for acid-base regulation are often studied using injections of acid-base relevant chemicals, such as $NaHCO_3$ and NaCl. This technique is beneficial when studying acute effects, however, chemical injections dissipate rather quickly, and are less desirable when conducting studies over a longer term. In fish, hypercarbia can also be used as a tool to investigate the patterns of acid-base regulation. Hypercarbia is relatively easy to establish and maintain in laboratory settings, and it results in a sustained respiratory acidosis, as fish accumulate CO_2, thus reducing blood pH. Studies on acid-base regulation typically use CO_2 tensions much greater than projected ocean acidification CO_2 levels, however, it results in a large-scale acid-base disturbance and quantifiable physiological responses. Presumably, the

physiological mechanisms used to recover from high CO_2 tensions are similar to those employed during exposure to lower CO_2 tensions (i.e. ocean acidification levels) and thus studies on the former provide useful insight into the latter.

1.4 General ionoregulation in freshwater and saltwater

Water ionic composition is highly variable among aquatic environments, and the dispersion of fish species is limited to the ionic compositions they can tolerate. Life in freshwater or saltwater environments presents osmotic/ionic challenges that fish must cope with in order to maintain homeostasis. Conserving plasma osmolality in particular is a common problem for fish inhabiting either environment, since plasma osmolality is typically ~300-350 mOsmol/L, an intermediate between the two extremes. Below I will briefly discuss the main challenges of fish in each of these environments as well as the physiological mechanisms employed to combat them.

a) Challenges of inhabiting freshwater

Freshwater fish live in a hypotonic environment, with an osmolality less than ~10 mOsmo/L, and consequently are faced with passive inward water movement and outward ion movement (Scott et al., 2004). Maintaining plasma osmolality is due in large part to active ion uptake across the gill epithelium and excretion of dilute urine. Na^+ uptake at the gills is achieved either through a pathway involving the use of apical H^+ ATPase to maintain a favourable gradient for Na^+ uptake from the environment through Na^+ channels (Lin and Randall, 1991), or *via* apical Na^+/H^+ exchangers (Wilson et al., 2000). Na^+ is then pumped into extracellular fluid through basolateral Na^+/K^+ ATPase (Lin and

Randall, 1991, Wilson et al., 2000). Although the aforementioned Na^+ uptake strategy is generally accepted, the mechanisms behind Cl^- uptake in freshwater remain poorly understood. The current model suggests active H^+ excretion *via* apical H^+ ATPase maintains an electrochemical gradient promoting HCO_3^- excretion, which in turn drives Cl^- uptake *via* Cl^-/HCO_3^- exchangers (Wilson et al., 2000). Transportation of Cl^- into the extracellular fluid then occurs through basolateral Cl^- channels (Marshall et al., 2002).

Although the gills are the major site of osmo- and ionoregulation, the kidney also plays a role, albeit a minor, in ion regulation. Rather than drinking freshwater, fish in these environments obtain much of their water from either passive water uptake or from their foodstuffs. The kidneys high filtration rates and level of ion reabsorption result in relatively low levels of salt excretion (and thus ion loss) in the urine and high rates of water removal (Hickman and Trump, 1969).

b) Challenges of inhabiting saltwater

Saltwater osmolality is over 3 times that of fish plasma, therefore fish inhabiting these environments must combat passive water loss and ion gain (Evans, 2008). In these environments plasma osmolality is generally maintained by active ion excretion at the gills and high water absorption across the intestine. Chloride cells present in the gill epithelium of saltwater fish actively excrete salts through a complex pathway involving Na^+/K^+ ATPase; Na^+: K^+: $2Cl^-$ cotransporter (NKCC) and cystic fibrosis transmembrane conductance regulator (CFTR). Basolateral NKCC transports Na^+, K^+ and Cl^- into the cell (Marshall et al., 2002), secondarily relying on basolateral Na^+/K^+ ATPase to maintain a favourable inward Na^+ gradient by recycling Na^+ back to the extracellular fluid (Silva et

al., 1977). This process results in an accumulation of intracellular K^+ and Cl^-, the former is returned to the plasma through a K^+ channel and the latter is excreted along its electrochemical gradient through an apical CFTR (Marshall et al., 1995). Accumulation of Cl^- on the outer gill surface allows for paracellular Na^+ excretion through leaky tight junctions in the gill epithelium (Degnan and Zadunaisky, 1980).

In contrast to those in freshwater, marine species actively drink saltwater as a means to reduce osmotic water loss. In these species, intestinal uptake of ingested salts acts as a driving force for increased water reabsorption to combat passive water loss at the gills (Grosell, 2011). The added salt load resulting from this process is then eliminated by active ion excretion across the gill epithelium. (Grosell, 2011). Furthermore, the kidneys provide additional relief *via* efficient water reabsorption and increased ion excretion, thus producing small amounts of heavily concentrated urine (Hickman and Trump, 1969).

1.5 Hypercarbia and water ion composition

As previously discussed, acid-base compensation occurs predominantly at the gills, and is associated with acid-base relevant ion exchange. Therefore, altering ambient water ion composition may influence acid-base compensation.

Fish undergo additional morphological and physiological changes when living in ion-poor water (i.e. soft water). Ion-poor waters have been linked to increased cortisol levels early during acclimation (Perry and Laurent, 1989), a response commonly associated with an increase in chloride cell proliferation (Laurent and Perry, 1990). In fact, when transferred to ion-poor waters rainbow trout have exhibited extensive chloride

cell proliferation at the gills (Laurent et al., 1985; Greco et al., 1996), an increase in Na^+ and Cl^- transporter activity (McDonald and Rogano, 1986) and capacity (McDonald and Rogano, 1986; Perry and Wood, 1985), a response thought to enhance ion uptake in these environments. In addition to strategies designed to increase NaCl uptake, acclimation to ion-poor waters also results in reduced NaCl efflux, to levels approximately 25% of those in hard water (McDonald and Rogano, 1986). Presumably, such physiologic changes associated with ambient water ionic composition would alter both the time-course and degree of recovery from an acid-base disturbance in the blood. Larsen and Jensen (1997) demonstrated that availability of acid-base relevant counter-ions is likely a contributing factor in hypercarbia recovery. Specifically, this study examined the response to hypercarbia of *O. mykiss* acclimated to several water compositions, including soft water, soft water with additional $CaCl_2$, $NaHCO_3$ or NaCl, and hard water. Trout acclimated to hard water and soft water + $NaHCO_3$ had a similar recovery, characterized by a reduced blood acidosis and more rapid restoration of blood pH compared with those in soft water alone (Larsen and Jensen, 1997), implying that the level of acidosis and subsequent recovery is associated with acid-base relevant counter-ion availability, specifically Na^+ to drive H^+ excretion and HCO_3^- for Cl^- exchange.

Furthermore, when faced with sustained hypercarbia, plasma [HCO_3^-] increased more rapidly and blood pH returned to pre-exposure levels much faster in marine salmon (*Onchorynchus kisutch*) relative to freshwater trout (*Salmo gairdneri*)(Perry, 1982). Studies by Iwama and Heisler (1991) yielded similar results in *O. mykiss* acclimated to varying [NaCl], however this study used concentrations up to 300mM, which is approximately 60% of full strength seawater. Clearly water ionic composition has a role

in acid-base compensation during sustained hypercarbia. These studies remain the few to date that examine the relationship between acclimation to a range of water ion compositions, and the rate and degree of compensation during exposure to hypercarbia.

Several fish species (e.g. salmon, trout, eels and killifish) possess the ability to move between freshwater and saltwater (termed euryhaline), although, this creates challenges for many physiological processes including osmo- and iono-regulation, and acid-base balance. In particular, the transition from freshwater to saltwater requires a complete reversal in the direction which ions must be transferred for ionoregulation (ie. ion uptake in freshwater and excretion in saltwater). However, the direction in which acid-base relevant counter-ions must be transported to compensate for an acidosis remains unchanged (ie. H^+ extrusion coupled to Na^+ uptake, or HCO_3^- uptake coupled with Cl^- extrusion). To date, no studies have examined the ability of a single fish species capable of inhabiting a range of water ion compositions, from freshwater through to seawater, to compensate for hypercarbia. Thus, the dogma that marine fish compensate for hypercarbia more effectively than freshwater fish is based upon the comparison of different species, such as trout relative to eel, which have different physiological mechanisms associated with acid-base regulation, as described below (Heisler, 1984; Toews et al., 1983).

Trout respond to a hypercarbia disturbance in a manner similar to many other fish species (Fig 1.1). Increased ambient CO_2 diffuses through the gill epithelium and dissociates into H^+ and HCO_3^-, resulting in a respiratory acidosis the magnitude of which is determined by the buffering capacity of the blood (A-B, Fig 1.1). As previously mentioned, compensation to hypercarbia is characterized by a net increase in plasma

[HCO_3^-] (in exchange for Cl^-), increasing blood pH back towards pre-exposure levels (B-C, Fig 1.1). However, plasma [HCO_3^-] plateaus at 23-33mM in most fish species therefore limiting hypercarbia recovery (Heisler, 1988). Although the nature of this HCO_3^- limitation remains elusive, the basis is more likely a physiological restriction rather than environmental, since elevating environmental HCO_3^- during hypercarbia exposure does not alter the plasma [HCO_3^-] threshold (Heisler, 1999).

Studies on acid-base compensation in waters of differing ion content are limited, and therefore have forced comparisons between trout in freshwater and eels in seawater. However, eels have a somewhat distinctive mechanism of acid-base regulation. The contribution of Cl^-/HCO_3^- exchange is suggested to be almost nonexistent, and these species may rely more heavily on Na^+/H^+ exchange mechanisms (Hyde and Perry, 1987). In fact, eels acclimated to low and high [NaCl] did not have significantly different recovery during hypercarbia (Hyde and Perry, 1987). However, when environmental [Na^+] was limited recovery during hypercarbia was severely reduced (Hyde and Perry, 1987). The inherent differences in acid-base regulatory mechanisms between these species illustrate the need for an intra-specific study to directly determine how water ionic composition, from freshwater through to saltwater, influences acid-base regulation during exposure to hypercarbia. Therefore the first objective of my thesis was to understand the role water ion composition plays on acid-base regulation during hypercarbia, using rainbow trout as a model species capable of inhabiting several water ion compositions.

1.6 Fish physiology during sustained exercise

Studies of swimming ability are an important method in determining the effects of external stressors on a whole animal performance. These studies typically focus on measurements such as critical swimming speed (U_{crit}), which is considered a representative measure of sustained/ prolonged cruising behaviour. First noted in Brett (1964), U_{crit} is determined using a series of step-wise water velocity increases until the fish fatigues, and is calculated as:

$$U_{crit} = u_i + [(t_i/t_{ii})(u_{ii})]$$

where, u_i is the highest velocity maintained for the whole time period increment, u_{ii} is the step-wise increase in water velocity, t_i is the amount of time spent at the fatigue velocity, and t_{ii} is the time period. U_{crit} studies have become an important measurement as they are easily conducted in a laboratory setting and results have implications both ecologically and physiologically, often representing overall fitness level.

Exercise, is a common stressor experienced by fish, and, is maintained by a coordinated series of physiological adjustments. Greater O_2 demand of working muscles during bouts of exercise requires enhanced respiratory gas exchange and delivery. During bouts of exercise, O_2 demand is met in part by an increase in gill ventilation volume which allows for greater O_2 extraction by increasing water flow over the gills (Kiceniuk and Jones, 1977).

The response of the circulatory system to exercise is remarkably complex. In fact, changes in heart rate alone are quite fluid, for example, low speeds do not appear to result in a measurable change in heart rate, suggesting there may be a threshold, above which heart rate increases (Kiceniuk and Jones, 1977). Additionally, following the onset

of exercise, rainbow trout initially respond by decreasing heart rate, however this response is short-lived and quickly reversed (Kiceniuk and Jones, 1977). Overall, it appears that heart rate has a relatively minor role in increasing cardiac output during exercise. Stroke volume, however, can increase up to 5-fold during moderate levels of exercise alone (Stevens and Randall, 1967a), coupled with increased blood pressure (Stevens and Randall, 1967b), this allows for perfusion of distal gill lamellae by overcoming the greater frictional resistance associated with their narrower structure. Seemingly, lamellar recruitment during exercise enhances O_2 uptake by increasing the functional surface area of the gills (Booth, 1978).

An increase in O_2 delivery to the tissues during exercise occurs in part through an increase in cardiac output, but also through increased venous O_2 extraction, as demonstrated by a decrease in venous Hb-O_2 saturation during exercise despite similar arterial Hb-O_2 saturation levels (Kiceniuk and Jones, 1977). Decreased venous Hb-O_2 saturation secondarily helps drive O_2 uptake at the gills by creating an increased gradient for O_2 diffusion from the water into the blood (Jones and Randall, 1979). The physiological changes that occur during sustained exercise likely alter acid-base compensation during hypercarbia, a process that also relies heavily on ventilation and perfusion. However, to date the mechanisms of acid-base regulation during hypercarbia and periods of sustained aerobic exercise have not been examined. Thus, the second objective of my thesis was to determine the rate and degree of acid-base compensation during hypercarbia in fish acclimated to different water ionic composition during sustained exercise.

1.7 The osmo-respiratory compromise

Fish gills play a major role in many physiological processes including respiration, acid-base regulation, osmo- and iono- regulation and nitrogenous waste disposal. This multifunctional nature creates several trade-offs among those physiological processes. The osmo-respiratory compromise suggests the characteristics of the gill that maximize respiration counteract those required for efficient osmoregulation (Nilsson, 1986). Optimizing respiratory exchange requires minimizing diffusion distance and maximizing functional surface area of the gill (as per the Fick equation). However these characteristics increase the surface area over which ions can be passively lost in freshwater or gained in seawater and thus may compromise ion regulation. Environmental or physiological conditions impact this system by adding additional stress to either osmoregulation (i.e. changes in ambient water ion content) and/or respiration (i.e. exercise).

During bouts of exercise the influence of the osmo-respiratory compromise would be expected to increase, as there is greater demand for O_2 at working muscles and a need to minimize CO_2 buildup. Increased gill perfusion and ventilation volume due to exercise enhance gas exchange by increasing the functional surface area available for gas exchange and water flow over the gills. However, although these characteristics aid respiration they may impair ion and acid-base regulation. Therefore the third objective of my thesis was to determine the effects of sustained exercise on the rate and degree of acid-base compensation during hypercarbia in comparison with resting conditions.

1.8 Thesis objectives and hypotheses

Objective 1: To perform an intra-specific comparison to quantify the rate and degree of acid-base compensation during hypercarbia in fish acclimated to differing water ionic compositions at rest. Acid-base regulation has been well studied in many fish species living in a variety of water ion compositions. However, few studies have been performed within a single fish species, which may experience fluctuating water ionic compositions throughout its lifecycle. I acclimated rainbow trout to water of varying ionic compositions (soft-, hard- and saltwater) and used a 1% CO_2 (7.5 mmHg) hypercarbia exposure to shed light on the underlying pattern of acid-base regulation in these environments. It was hypothesized that as concentration of ambient water ions increased the rate and degree of acid-base compensation from hypercarbia would also increase due to greater counter-ion availability.

Objective 2: To perform an intra-specific comparison to quantify the rate and degree of acid-base compensation during hypercarbia in fish acclimated to differing water ionic compositions during sustained exercise. Few studies regarding acid-base regulation have been conducted on fish during sustained exercise, and given their active lifestyle studies conducted at rest may not be an accurate representation of their ability to compensate for acid-base disturbances. Trout were acclimated to the previously mentioned water ion compositions and exposed to 1% CO_2 during sustained aerobic exercise (~60% U_{crit}). Fish acclimated to water of high ion content were hypothesized to have a greater rate and degree of hypercarbia recovery compared with those acclimated to water of lower ion content due to greater counter-ion availability.

Objective 3: To determine the effect of sustained exercise on the rate and degree of acid-base compensation during hypercarbia in the rainbow trout relative to resting fish. To our knowledge this is the first experiment attempting to shed light on how fish respond to hypercarbia during sustained exercise. Exercise puts pressure on the respiratory system to increase O_2 delivery to working muscles, suggesting that as O_2 demand increases the ability to recover from acid-base disturbances will be hindered due to the osmo-respiratory compromise. Therefore, exercising fish were hypothesized to have a lower rate and degree of hypercarbia recovery compared with their resting counterparts.

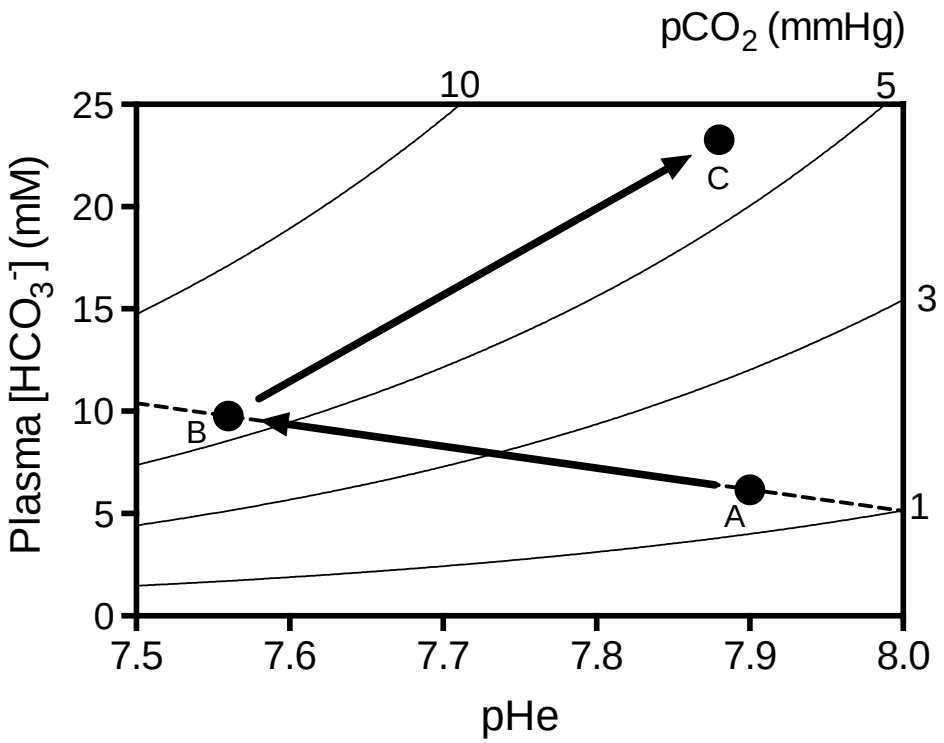

Figure 1.1: A representation of a pH/HCO_3^- plot illustrating the typical response of a teleost during exposure to sustained hypercarbia. The dashed line represents the buffering capacity of typical rainbow trout blood from Wood and LeMoigne (1991), and A, B and C represent hypothetical times of hypercarbia exposure.

Chapter 2: The effects of water ionic composition on acid-base regulation in rainbow trout, Oncorhynchus mykiss, during hypercarbia at rest and during sustained exercise[1]

2.1 Introduction

Acid-base status is tightly regulated, as small deviations in pH can alter protein charge resulting in profound impacts on physiological processes including metabolism and cell-signaling (Putnam and Roos, 1997). Typical disturbances to acid-base status may be internal (i.e. exhaustive exercise) or external (i.e. hypercarbia, hypoxia etc.) in origin, regardless, they must be swiftly corrected in order to avoid negative physiological effects. Hypercarbia is a useful tool to study acid-base regulation in fishes because it results in a sustained respiratory acidosis to which the fish must compensate in order to maintain acid-base status.

Fish employ a number of mechanisms to limit internal pH changes. Small-scale pH deviations are counteracted primarily by bicarbonate and non-bicarbonate blood buffers (Heisler, 1986). However, fish have a relatively low blood buffering capacity (Heisler, 1999) and rely more heavily on acid-base relevant ion exchange during pH disturbances of greater magnitude. The specific ion transfer mechanisms are still being described, and vary both environmentally (i.e. between freshwater and marine species) and among species (see review by Evans et al., 2005). Many studies cite the primary method of H^+ extrusion to be *via* apical Na^+ channels linked to V-type H^+ ATPases in

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freshwater fish (Lin et al., 1994), and electroneutral Na^+/H^+ ion exchangers in marine fish (Claiborne et al., 1999), however some freshwater species may employ NHEs as well (Hyde and Perry, 1989; Hirata et al., 2003). Mechanisms of HCO_3^- uptake are generally thought to be associated with Cl^-/HCO_3^- anion exchangers (see reviews by Perry et al., 2003 and Evans et al., 2005). However, this route varies among species, eels, for example, are thought to lack Cl^-/HCO_3^- anion exchangers and thus rely more heavily on H^+ extrusion mechanisms during acidotic disturbances (Hyde and Perry, 1987). During periods of hypercarbia, aquatic CO_2 is elevated and diffuses into the blood resulting in a respiratory acidosis that must be combatted in order to maintain homeostasis. Typical acid-base compensation during hypercarbia is thought to be achieved, in large part, due to either H^+ extrusion and/or HCO_3^- uptake across the gill epithelium, in exchange for Na^+ or Cl^-, respectively (see review by Evans et al., 2005), and thus is dependent on available counter-ions.

Fish inhabiting environments of different water ion content demonstrate varying levels and rates of recovery during hypercarbia, which may not be surprising, as recovery relies heavily on ion exchange and thus counter-ion availability. A previous study demonstrated the importance of counter-ion availability by acclimating rainbow trout to water of various ion compositions that ranged from soft- to hard water, and included soft water with additional acid-base relevant ions (Larsen and Jensen, 1997). During hypercarbia, trout acclimated to soft water with additional $NaHCO_3$ and those acclimated to hard water responded with a reduced acidosis and faster compensation compared with those in soft water (Larsen and Jensen, 1997). Additionally, it has been shown that saltwater fish species are able to compensate for acid-base disturbances more quickly

than freshwater species (Perry, 1982). However, this study focused on the response of a marine salmon (*Onchorynchus kisutch*) compared with freshwater trout (*Salmo gairdneri*), rather than an intra-specific comparison. These studies shed light on how ambient water ion composition may impact acid-base regulation during hypercarbia. However, to date no study documents acid-base compensation during hypercarbia in a single fish species capable of inhabiting environments ranging from the challenging soft water environment to the similarly challenging saltwater.

Salmonids maintain a very active lifestyle, whether it is aerobic exercise, typically associated with routine swimming, or anaerobic, which is characterized by periods of burst swimming associated with prey capture/predator escape. Exercise presents fish with several physiological demands that must be met in order to sustain activity. At the gills, demands for increased gas exchange are met by an increase in ventilation volume, allowing for greater O_2 extraction from ambient water (Kiceniuk and Jones, 1977). Elevated stroke volume during exercise results in increased cardiac output (Stevens and Randall, 1967a), and blood pressure which increases the functional surface area of the gills by overcoming the frictional resistance of narrow distal gill lamellae, and increasing perfusion (Jones and Randall, 1979). Additionally, the efficiency of O_2 extraction at the level of the muscles increases with exercise (Kiceniuk and Jones, 1977). Furthermore, increased cardiac output, coupled with elevated blood pressure during exercise also drives greater O_2 delivery and CO_2 removal to/from working muscles.

Presumably, optimizing the gills for gas exchange during exercise may create additional stress for osmo/iono regulation and acid-base balance since morphological alterations that benefit respiration may impair the latter, a paradigm known as the osmo-

respiratory compromise (Nilsson, 1986). For example, an increase in gas exchange is accomplished by an increase in the functional surface area of the gills, and a reduction in blood-water diffusion distance, both of which would be expected to result in increased passive ion loss in freshwater and gain in saltwater. Additionally, during exercise, gill perfusion increases providing a greater surface area available for gas exchange, thereby aiding in respiration, however this may impair ion and acid-base regulation. Therefore, periods of hypercarbia (recovery from which relies on ion exchange) and exercise (which increases O_2 demand) present the fish with opposing stressors. To date, the response to hypercarbia during sustained exercise has not been investigated in fish.

The purpose of this thesis was to provide insight into the pattern of acid-base regulation employed by a single fish species capable of inhabiting waters of different ionic compositions that span the range from freshwater to saltwater. The primary objectives were two-fold, to determine the effects of water ionic composition on the rate and degree of acid-base compensation during hypercarbia within a single species: a) at rest and b) during sustained exercise. A third objective was to determine the effect of activity level on the rate and degree of acid-base regulation. I hypothesized that water with higher ion content would allow for a faster and more complete recovery during hypercarbia, and that exercise relative to rest would impede the rate and degree of hypercarbia recovery due to the nature of the osmo-respiratory compromise

2.2 Methodology

2.2.1 Experimental animals

Domesticated adult rainbow trout *Oncorhynchus mykiss* (approx. 350-450g) were obtained from Miracle Springs Trout Farm (Mission, British Columbia, Canada). Following arrival at the University of British Columbia (Vancouver, British Columbia, Canada) fish were held in an outdoor holding tank (3000 L) supplied with flow-through dechlorinated Vancouver tap water (in mM: Na^+, 0.08; Cl^-, 0.05; HCO_3^-, unknown; Ca^{2+}, 0.03; Mg^{2+}, 0.006; K^+ 0.004; alkalinity as $CaCO_3$ 3.0mg/L; hardness as $CaCO_3$ 3.43mg/L; pH 6.5; Metro Vancouver, 2012). Fish were then divided into three experimental groups (*n*=5-10, per group), soft water, artificial hard water and saltwater. The soft water group was maintained in flow-through dechlorinated Vancouver tapwater (as described above), while fish in the other two groups were transferred to separate recirculating systems (550 L, 9-11ºC, 12:12 photoperiod) and given a two-day acclimation period in dechlorinated Vancouver tapwater before water ion composition was manipulated. A standardized artificial hard water treatment (EPA, 2007) was created by adding salts to dechlorinated Vancouver tapwater (in mM: Na^+, 2.4; Cl^-, 0.2; HCO_3^-, 2.3; Ca^{2+}, 0.9; Mg^{2+}, 1.0; K^+, 0.12; alkalinity as $CaCO_3$ 110-120 mg/L; hardness as $CaCO_3$ 160-180 mg/L; pH 7.6-8.0) and fish were given a minimum of 2 weeks to acclimate prior to experimentation. The saltwater trial was created by increasing the salinity 3-4 parts per thousand (ppt) every two days using Instant Ocean® until a final salinity of 28 ppt was reached. At this point fish were given a minimum of 2 weeks to acclimate prior to experimentation. Half water changes were done at a minimum of once daily during the acclimation period. Fish were fed a typical diet of commercial fish

pellets (Taplow, 3.5mm) thrice weekly to satiation (approximately 1% body mass/feeding). To eliminate metabolic effects of feeding, fish were starved 24 hours prior to experimentation.

2.2.2 Surgical procedure

Fish were anaesthetized in the water to which they were acclimated using MS-222 (0.2 g/L, buffered with $NaHCO_3$) and weighed prior to being transferred to an operating =table. During surgery, gills were irrigated with well-oxygenated acclimation water with a lower dose of MS-222 (0.05 g/L MS-222, buffered with $NaHCO_3$). The dorsal aorta was cannulated with PE 50 tubing (Clay Adams PE 50, i.d. = 0.58mm, o.d. = 0.965mm) as outlined by Soivio et al (1975). Following surgical procedures fish were revived by irrigating gills with aerated acclimation water and fish were then left to recover for approximately 24 hours in aerated acclimation water prior to experimentation as outlined below.

2.2.3 Experimental procedure

2.2.3.1 Series I (resting fish)

Following surgery fish were transferred to a temperature controlled environmental chamber (10^oC, 12:12 photoperiod) and held in a black Perspex box supplied with their respective acclimation water (1.5 L/min) within a 170 L recirculating system. Fish were left to recover for approximately 24 hours. An initial blood sample (500µL) was taken following recovery. Fish were then exposed to 1% CO_2 (7.5mm Hg), which was achieved in under 15 minutes and then maintained for the duration of the experiment. The target

CO_2 tension was achieved using predetermined flow rates (generated with Sierra Instruments mass flow controllers) of air and 100% CO_2 bubbled into the recirculating system water. Water pH was used as an indirect measure of p_wCO_2 based upon preliminary studies relating p_wCO_2 with the pH of the corresponding water type (soft-, hard-, or saltwater). In all water ion compositions all 1% CO_2 reduced water pH by approximately 1 unit (Table 2.1). Subsequent blood samples (500μL) were taken at 1, 2, 4, 8, 24 and 48 hours following initiation of hypercarbia. Following removal of the final blood sample, fish were removed from the Perspex box and killed by a severe blow to the head. A sample ($1cm^3$) of dorsal epaxial muscle was extracted, weighed, and then dried (at 65ºC) to constant weight for determination of muscle water content.

2.2.3.2 Series II (during sustained exercise)

After surgery, fish were transferred to a black Perspex box as described above and left to recover for 36 hours (preliminary studies determined that additional recovery time was required for fish to sustain exercise). Following recovery, fish were transferred to a swim tunnel (90 L, 70x20x20 cm dimensions), which contained their respective acclimation water, and fish were left to acclimate overnight at a low flow rate of 2cm/s. Preliminary studies were conducted to determine maximal critical swimming velocity (U_{crit}) using a step-wise procedure as demonstrated in Beamish (1978) and U_{crit} was calculated as per Brett (1964). Following the overnight acclimation, water velocity was gradually increased using a ramp-U_{crit} protocol as outlined by Farrell (2008), using a $0.029cm/s^2$ acceleration to reach 60% of U_{crit} (52cm/s) where water velocity was maintained for the following 8h. 60% of U_{crit} was selected because it is a velocity that the

fish could likely sustain indefinitely and thought to be wholly aerobic (Kiceniuk and Jones, 1977; Burgetz et al., 1998). Initial blood samples (500μL) were taken in normocarbia following overnight acclimation and then p_wCO_2 was increased to 1% CO_2 as described above. Subsequent blood samples were taken at 0.5, 1, 2, 4, and 8 hours during sustained swimming in hypercarbia. Following the final blood sample fish were netted and sacrificed by a severe blow to the head. A sample of dorsal epaxial muscle was taken for the determination of muscle water content as described above.

2.2.4 Analysis

Haematocrit was measured by centrifuging 60μL samples at 15,000 rev/min for 5 minutes. Blood pH was measured using a thermostatted glass pH electrode (Radiometer G299A) housed within a Radiometer BMS Mk 2 blood microsystem and displayed on a Radiometer PHM84 research pH meter. Remaining blood was centrifuged at 10, 000G for 5 minutes. Total plasma CO_2 (TCO_2) was measured using a TCO_2 Analyzer, Corning, Model 965 and plasma bicarbonate was calculated using the Hendersen-Hasselbalch equation and previously reported CO_2 solubility coefficient (α CO_2) and pK' of rainbow trout (Boutilier et al., 1984). Remaining plasma was used to determine plasma osmolality (Westcor Vapor Pressure Osmometer, Model 5520), [Na^+] (flame spectrophotometer, Varian AA 240 FS) and [Cl^-] (HBI digital chloridometer, Model 4425000).

2.2.5 Relevant calculations

For the purpose of this thesis the percent (%) of hypercarbia recovery was calculated using two different methods. The first calculated percent recovery per individual as follows:

$$\% \text{ Recovery} = (pH_x - pH_{min})/(pH_0 - pH_{min}) \times 100$$

where pH_x is the pH at time X, pH_{min} is the minimal pH reached and pH_0 is the initial pH (at time 0). The second method consisted of the same basic formula, however, pH_0 was calculated as the mean initial blood pH of fish per water ionic composition at rest. This value was used as pH_0 for both resting and exercising fish (per water ionic composition), as it was deemed a more accurate representation of resting blood pH, and compensated for the effect of the blood acidosis that occurred prior to the onset of hypercarbia, which was likely due to the initiation of swimming (see Results for further elaboration).

2.2.6 Statistical analysis

All data is reported as means ± standard error of mean. Data was tested for normality and homogeneity of variances. In each Series blood parameters of trout in different water ionic compositions were analyzed using repeated measures analysis of variance (ANOVA). Differences among water compositions were determined using two-way ANOVAs and Tukey post hoc tests. Within a given water ionic composition, significance between Series I and II was determined by two-way ANOVAs on time-points consistent between the Series and Tukey post hoc tests. One-way ANOVAs were used to determine the significance of time and/or water ionic composition when there was

a significant interaction effect. A 5% probability level was used to determine significance. All statistics were performed using R.

2.3 Results

2.3.1 Water analysis

Water ion content of soft-, hard- and saltwater treatments are shown in Table 2.1. In each water composition there were no significant differences in water pH between Series I and Series II experiments ($p>0.05$, Table 2.1). Initial (normocarbic) water pH was significantly lower in soft water compared with hard- and saltwater ($p<0.05$, Table 2.1). Water pH during hypercarbia was significantly reduced by 0.8–1 pH unit relative to normocarbia in all water compositions ($p<0.05$, Table 2.1), and once established remained stable for the duration of exposure. However, water pH during hypercarbia was significantly different among all water compositions ($p<0.05$, Table 2.1).

2.3.2 Series I

In soft water acclimated trout, exposure to 1% CO_2 resulted in a significant reduction in pH_e at 1, 2, 4, and 8h ($p<0.05$, Fig 2.1 A) and a significant increase in plasma [HCO_3^-] with time during hypercarbia exposure, having higher plasma [HCO_3^-] at 24 and 48h of hypercarbia ($p<0.05$, Fig 2.1 A). Plasma [Cl^-] was significantly decreased at 48h following exposure to hypercarbia ($p<0.05$, Fig 2.2 A). Soft water acclimated trout exhibited no significant change in plasma [Na^+] or plasma osmolality during exposure to hypercarbia (Fig 2.2 B, C).

Hard water acclimated trout exhibited a significant reduction in blood pH after 1, 2 and 4h ($p<0.05$, Fig 2.1 B) and a significant increase in plasma [HCO_3^-] at 8, 24 and 48h of hypercarbia ($p<0.05$, Fig 2.1 B). Plasma [Cl^-] was significantly reduced at 48h of hypercarbia exposure ($p<0.05$, Fig 2.2 A), however, there were no statistically significant changes in either plasma [Na^+] or plasma osmolality (Fig 2.2 B, C). Hard water acclimated trout hematocrit at rest was significantly elevated following 2h of hypercarbia ($p<0.05$, Table 2.2), but otherwise was not significantly different from initial values ($p>0>05$, Table 2.2).

Saltwater acclimated trout exhibited a statistically significant reduction in blood pH at 1 and 2h of hypercarbia exposure ($p<0.05$, Fig 2.1 C). Plasma [HCO_3^-] increased significantly at 2, 4, 8, 24, and 48h of hypercarbia exposure ($p<0.05$, Fig 2.1 C). Saltwater acclimated trout exhibited a statistically significant reduction in plasma [Cl^-] at 24 and 48h of hypercarbia ($p<0.05$, Fig 2.2 A), but there were no significant changes in plasma [Na^+], osmolality or hematocrit (Fig 2.2 B, C, Table 2.2).

Blood pH among the different water compositions was not significantly different 0, 1, 2 and 4h after the onset of hypercarbia (Fig 2.1). However, pH_e was significantly greater in hard-, and saltwater acclimated trout relative to soft water at 8, and 48h of hypercarbia ($p<0.05$, Fig 2.1). Plasma [HCO_3^-] was not significantly different among trout acclimated to soft-, hard-, and saltwater at 0, 1 and 2h of hypercarbia ($p<0.05$, Fig 2.1). Plasma [HCO_3^-] was significantly greater in saltwater acclimated trout at 4h of hypercarbia compared with hard water acclimated trout ($p<0.05$, Fig 2.1), however soft water did not significantly differ from hard- or saltwater acclimated trout ($p>0.05$, Fig 2.1). Plasma [HCO_3^-] was significantly greater in saltwater acclimated trout after 8 and

24h of hypercarbia compared with both soft water and hard water acclimated trout ($p<0.05$, Fig 2.1); however, after 48h of hypercarbia hard water trout were no longer significantly different from saltwater acclimated trout ($p>0.05$, Fig 2.1). During hypercarbia, plasma [Cl^-] was greater in saltwater acclimated trout relative to soft water acclimated trout ($p<0.05$, Fig 2.2 A). Plasma [Cl^-] of saltwater acclimated trout was also greater at 0, 1 and 2h relative to hard water acclimated trout ($p<0.05$, Fig 2.2 A), however at 4, 8, 24 and 48h they were not significantly different (Fig 2.2 A). Plasma [Na^+] was also significantly greater in saltwater acclimated trout for the duration of hypercarbia relative to both soft-, and hard water acclimated trout ($p<0.05$, Fig 2.2 B). Plasma osmolality among all water compositions was significantly different during hypercarbic exposure ($p<0.05$, Fig 2.2 C), however there was no significant interaction with time. During hypercarbia, hematocrit was not significantly different between soft-, hard-, and saltwater acclimated trout at rest ($p>0.05$, Table 2.2). The mass of soft water acclimated trout at rest was significantly greater than hard water trout ($p>0.05$, Table 2.2), but neither differed significantly from saltwater acclimated trout ($p>0.05$, Table 2.2). Muscle water content was not significantly different among trout acclimated to soft-, hard-, or saltwater at rest ($p<0.05$, Table 2.2). At 48h of hypercarbia all water compositions had a significantly greater degree of recovery relative to 8h, however saltwater acclimated trout at 48h were significantly more recovered than soft water acclimated trout ($p<0.05$, Table 2.3). In fact, with a standard initial blood pH all water compositions have a significantly greater degree of recovery after just 24h of hypercarbia exposure and saltwater acclimated trout exhibited greater recovery than soft water acclimated trout at 24h ($p<0.05$, Table 2.4).

2.3.3 Series II

Soft water acclimated trout showed no significant changes in pH_e during the time course of hypercarbic exposure during sustained exercise (Fig 2.3 A). However, at 8h of hypercarbia exposure there was a significant elevation of plasma [HCO_3^-] ($p<0.05$, Fig 2.3 A). There was also no significant change in plasma [Cl^-], [Na^+], osmolality or hematocrit during hypercarbic exposure (Fig 2.4, Table 2.2).

Hard water acclimated fish exhibited a no significant differences in pH_e or plasma [HCO_3^-] during hypercarbia ($p>0.05$, Fig 2.3 B). Plasma [Cl^-], [Na^+], osmolality and hematocrit during hypercarbia were not significantly different from normocarbia (Fig 2.4, Table 2.2).

Saltwater acclimated fish exhibited no significant changes in blood pH during hypercarbia and sustained exercise ($p>0.05$, Fig 2.3 C). Plasma [HCO_3^-] was significantly elevated following 4 and 8h of hypercarbia ($p<0.05$, Fig 2.3 C), however there were no significant changes in plasma [Cl^-], [Na^+], osmolality or hematocrit over the duration of hypercarbic exposure (Fig 2.4, Table 2.2).

Blood pH of saltwater acclimated trout was significantly greater than soft water and hard water acclimated trout following 8h of sustained exercise in hypercarbia ($p<0.05$, Fig 2.3). Plasma [HCO_3^-] was significantly higher in soft water and saltwater acclimated trout relative to hard water acclimated trout at 4 and 8h of hypercarbia ($p<0.05$, Fig 2.3). Plasma [Cl^-] and [Na^+] was significantly greater in saltwater acclimated trout relative to both soft-, and hard water acclimated trout ($p<0.05$, Fig 2.4 A). Plasma osmolality was not significantly different between water compositions during hypercarbia

and sustained exercise ($p>0.05$, Fig 2.4 C). During sustained exercise there was no significant difference in hematocrit between soft-, hard-, and saltwater acclimated rainbow trout ($p>0.05$, Table 2.2). Soft water acclimated trout mass was significantly greater than hard-, and saltwater acclimated trout ($p<0.05$, Table 2.2). During sustained exercise, saltwater acclimated trout had significantly greater muscle water content compared with soft water acclimated trout ($p<0.05$, Table 2.2), however neither was significantly different from hard water acclimated trout ($p>0.05$, Table 2.2). The degree of recovery was significantly greater at 4 and 8h of hypercarbia exposure when initial blood pH was standardized, and at 8h, saltwater acclimated trout had a significantly greater degree of recovery relative to soft- and hard water acclimated trout ($p<0.05$, Table 2.4).

2.3.4 Series I versus series II

Initial blood pH of exercising trout was lower than resting fish in all water ionic compositions (Fig 2.5). This is likely due to a metabolic acidosis associated with the onset of swimming which was initiated 30 minutes prior to the first blood sample. Therefore to make more accurate comparisons between resting and exercising fish the degree of hypercarbic recovery in Table 2.4 was calculated using the average initial blood pH (calculated per water ion composition), as it is likely a more accurate representation of blood pH during normocarbia.

Soft water acclimated trout exhibited no significant differences in pH_e between resting and exercising fish, however there was a significant interaction between the two variables ($p<0.05$, Fig 2.5 A). There were also no significant differences in plasma

[HCO_3^-], hematocrit or mass between resting and exercising soft water acclimated trout ($p>0.05$, Fig. 2.5 A, Table 2.2). Following 8h of hypercarbia exercising soft water acclimated fish had a greater degree of recovery from hypercarbia compared with resting (82.3± 32.91 and 12.18± 9.0, respectively)(Table 2.3).

Blood pH of exercising hard water acclimated trout was not significantly different relative to resting fish (Fig 2.5 B). Plasma [HCO_3^-] of exercising hard water fish was significantly lower than that of resting fish throughout hypercarbia ($p<0.05$, Fig 2.5 B). Hematocrit and mass did not differ significantly between resting and exercising hard water acclimated trout ($p>0.05$, Table 2.2). The degree of recovery of hard water acclimated trout was greater in exercising fish relative to resting fish (Table 2.3), however this trend was not observed when initial blood pH is standard (Table 2.4).

Blood pH of exercising saltwater acclimated trout during hypercarbia was not significantly different relative to resting trout, however there was a significant interaction ($p<0.05$, Fig 2.5 C). Interestingly, resting saltwater acclimated trout had significantly elevated plasma [HCO_3^-] at 4 and 8h of hypercarbia exposure compared with exercising trout ($p<0.05$, Fig 2.5 C). Saltwater acclimated trout showed no significant differences in hematocrit or mass between rest and exercise ($p>0.05$, Table 2.2). Exercising saltwater acclimated trout had a greater degree of recovery relative to those at rest (Table 2.3), although the degree of recovery is reduced when initial blood pH is standardized it remains greater in exercising trout (Table 2.4).

2.4 Discussion

The results of this study demonstrate that rainbow trout acclimated to water of high ion content (i.e. saltwater) have a greater rate and degree of recovery during hypercarbia, compared with those in soft water, both at rest and during sustained exercise. At rest, trout acclimated to soft-, hard-, and saltwater experienced a similar blood acidosis in response to hypercarbia (1% CO_2), however at 48h, hard-, and saltwater acclimated trout fully recovered blood pH, even demonstrating some overcompensation, whereas soft water acclimated trout only achieved ~60% pH recovery. During sustained exercise, the effect of water ion composition during hypercarbia was similar to that observed at rest. Following 8h of hypercarbia and sustained exercise, saltwater acclimated rainbow trout had fully restored blood pH, once again with some overcompensation, while soft-, and hard water acclimated trout had only recovered by ~40 and 60%, respectively. These data indicate that sustained exercise may enhance hypercarbia recovery over resting conditions, however, there were some confounding conditions in this study, which are discussed below.

2.4.1 Effect of water ionic composition on hypercarbia recovery

By acclimating a single fish species to a range of ecologically relevant water ion compositions, ranging from soft- to saltwater, I was able to demonstrate the importance of ambient water ion composition on acid-base compensation in resting rainbow trout exposed to hypercarbia. During hypercarbia, blood pH showed similar significant reductions in trout acclimated to soft-, hard-, and saltwater and, following 48h of exposure, blood pH of trout in all water ion compositions showed some measure of

recovery. Recovery in all treatments was associated with an increase in plasma [HCO_3^-], in exchange for Cl^-. However, at 48h of CO_2 exposure, saltwater acclimated trout had fully recovered blood pH, whereas those acclimated to soft water were only ~60% recovered, suggesting that reduced availability of acid-base relevant counter-ions in soft water may pose limitations on hypercarbia recovery.

Distinguishing between HCO_3^- uptake and H^+ extrusion at the gill as the route for acid-base compensation is not possible from the data collected here. However, the results of this study show an almost equimolar plasma [Cl^-] decrease, that is coupled with the significant plasma [HCO_3^-] increase, a result which has been shown in many other studies (Iwama and Heisler, 1991; Larsen and Jensen, 1997). Larsen and Jensen (1997) found similar results in their experiment examining the importance of acid-base relevant ions (HCO_3^-, Na^+, Cl^- etc.) in ambient water. However, Larsen and Jensen (1997) noted that during hypercarbia fish acclimated to soft water with additional NaCl did not demonstrate a response similar to those acclimated to hard water, suggesting that sufficient HCO_3^- in ambient water is required for compensation from an acid-base disturbance. Additionally, a similar response to, and, recovery during hypercarbia was noted between fish in the hard water treatment and the soft water treatment with added $NaHCO_3$, thus providing further evidence for HCO_3^- uptake as a route for acid-base compensation (Larsen and Jensen, 1997).

Interestingly, the pattern of hypercarbia recovery in hard- and saltwater acclimated trout was remarkably similar, despite the hard water treatment having much lower [Na^+] and [Cl^-] concentrations, but similar [HCO_3^-]. At first glance, this may imply that acid-base regulation is associated with HCO_3^- uptake from the water, however, this is

not as clear when one considers the gradient for Cl^- extrusion (which must occur in exchange for HCO_3^- uptake) is dramatically greater in saltwater trout. Moreover, the gradient for Na^+ influx, presumably coupled with H^+ efflux, is also more favourable in saltwater, but does not appear to speed up recovery. This would suggest that the associated gradients for Na^+ and Cl^- may not have a large influence over the magnitude of HCO_3^- uptake, however, the mechanism behind this is unclear. Ion transporter saturation may account for the similarity between hard-, and saltwater trout hypercarbia recovery, since transporters are operating close to their maximum affinity during normocarbia (Goss and Wood, 1990). Furthermore, the water ion composition used by Goss and Wood (1990) was similar to the hard water treatment performed in this study, therefore if ion transporters are fully saturated in hard water, increased [NaCl] would have minimal impact on further enhancing recovery in saltwater. However, water [HCO_3^-] was similar between trout in hard- and saltwater treatments and, given the similarity in their response to hypercarbia, the importance of available water HCO_3^- may exceed that of NaCl.

Morphological features of the gill epithelium may also play a role in regulating the transfer of acid-base relevant ions, a feature that is independent of counter-ion availability. In fact, after just 6h of hypercarbia exposure, the surface area of chloride cells in the brown bullhead is reduced to less than half that of normocarbia (Goss et al., 1992). Furthermore, following 48h of hypercarbia, there was also a noticeable reduction in exposed chloride cells on the gill epithelium, which resulted in chloride cell fractional area being reduced to just 5% of pre-exposure values (Goss et al., 1992). However, these morphological changes were only correlated with a decrease in the rate of Cl^- influx and not Na^+ influx (Goss et al., 1992). In terms of acid-base regulation, this would infer a

reduced rate of Cl^- influx, and thus HCO_3^- efflux, while Na^+ influx remained unchanged and thus H^+ efflux also unchanged, with the net result being H^+ efflux. This would account for the increase in plasma [HCO_3^-] and restoration of blood pH observed during my study. Furthermore, in ion-poor water, there is a proliferation in chloride cells (Laurent et al., 1985; Greco et al., 1996), therefore the aforementioned reduction in chloride cell fractional area may not be as pronounced in these fish or require more time to fully achieve. This may account for the greater rate and degree of hypercarbia recovery, associated with a greater plasma [HCO_3^-] increase in hard and saltwater acclimated trout relative to soft water acclimated trout.

Additional studies on barramundi noted that at high salinities (22.5 and 32 ppt) peak H^+ excretion rates were observed at 4h of hypercarbia exposure, compared with 8-12h in those acclimated to lower salinities (Weakley et al., 2012). The authors suggest that greater environmental Na^+ may lead to preferential H^+ excretion *via* NHEs (Weakley et al., 2012) driven by the high Na^+ gradients saltwater. However, H^+ ATPase expression was also greater during hypercarbia compared with normocarbia, an effect only observed transiently at 15 and 32 ppt in this study (Weakley et al., 2012). A similar effect was also demonstrated in freshwater trout by Perry et al (2000), which would suggest that H^+ ATPase also plays a role in hypercarbia recovery in these environments. Therefore, regardless of the exact mechanism responsible for ion transfer during hypercarbia, the results of this experiment demonstrate the importance of counter-ion availability as a regulating factor in hypercarbia recovery.

Exercising rainbow trout acclimated to different water ion compositions had a similar response to those at rest during hypercarbia exposure. However, the initial blood

pH of all exercising trout was noticeably less than those at rest, which likely contributed to the lack of significant differences in pH_e over the time course of hypercarbia exposure. This response was likely the result of the experimental procedure, in which exercise was initiated prior to the initial blood pH sampling. Therefore, it is possible that a metabolic acidosis occurred due to exercise, masking the fall in blood pH due to hypercarbia. Despite this, at 8h of hypercarbia and sustained exercise blood pH of saltwater trout was fully restored, again with some overcompensation, whereas soft-, and hard water acclimated trout only achieved ~40 and ~65% pH recovery respectively. This result was similar to that of the resting trials, therefore providing additional support for the relationship between increased counter-ion availability and greater hypercarbia recovery.

In general, these results are consistent with other studies on acid-base disturbances in freshwater and marine fish species. Ambient [NaCl] is thought to be a regulating factor to maintain acid-base status as sufficient Na^+ and Cl^- are essential to maintain electroneutral exchange of their acidic or basic counterparts (H^+ and HCO_3^-, respectively)(Goss and Wood, 1990). The results of this study are generally in agreement, as saltwater acclimated trout recovered from hypercarbia earlier than those in soft water, both at rest and during sustained exercise. Further research should focus on understanding the mechanisms behind acid-base relevant ion transporters in each environment. Specifically, studying mRNA expression of NHEs in saltwater during hypercarbia would help determine whether ion transport mechanisms or morphological changes are driving recovery. Additionally, manipulating water [Na^+] and [Cl^-] independently may shed light on the importance of each acid-base relevant ionic gradient, and ion availability during hypercarbia.

2.4.2 The effect of exercise on hypercarbia recovery

The osmo-respiratory compromise suggests that gill morphology can be optimized for either respiration or iono/osmoregulation. In short, at the gill, the diffusion distance over which respiratory gases are exchanged must be minimized, and the functional surface area maximized to optimize respiration during periods of aerobic exercise in order to meet the elevated metabolic requirements of contracting muscles. These morphological changes, although beneficial for respiration, increase the surface area over which ions are lost in freshwater, or gained in saltwater. Therefore, the addition of a sustained exercise component during hypercarbia may impair recovery from the acid-base disturbance, which relies on ion exchange mechanisms. However, data from this study, specifically the saltwater acclimated trout, suggests the opposite; that exercise may enhance a fishes ability to compensate for an acid-base disturbance.

This is the first study to investigate hypercarbia recovery during sustained exercise, however, during successive half hour increases in swimming speed of seawater acclimated rainbow trout, Brauner et al (2000) noted a significant increase in plasma PCO_2 and $[HCO_3^-]$, the latter of which appeared to prevent a reduction in blood pH. Thus, during sustained exercise at relatively high swimming speeds (i.e. >90% U_{crit}), acid-base relevant ion transfer processes may be activated very quickly and effectively, at least in seawater, which is consistent with the data presented here.

Postlethwaite and McDonald (1995) demonstrated changes in ionoregulation during sustained exercise, indirectly supporting the trend observed here. Following 3h of sustained exercise, at speeds similar to those in this study, rainbow trout exhibited a

significant decrease in plasma [ion] (Postlethwaite and McDonald, 1995), likely associated with the osmo-respiratory compromise and impaired ability to regulate plasma ion levels during high levels of activity. Furthermore, the rate of Na^+ efflux had decreased to less than half of control values by 6h of sustained exercise, and by two-thirds by 12h, with an associated tripling of ion influx (Postlethwaite and McDonald, 1995). The aforementioned results follow a comparable time course to my results, suggesting that during hypercarbia and sustained exercise there is likely a similar change in acid-base relevant ion transfer rates. While this was not measured in my study, clearly this is an area worthy of further exploration.

What might be some of the changes associated with exercise that increase the capacity for acid-base recovery during exercise? During exercise there is an increase in ventilation volume and an increase in blood flow to the distal portions of the gills (Cameron et al., 1971; Booth, 1978). This may increase the functional surface area available for acid-base relevant ion exchange. Therefore, it is conceivable that increased gill perfusion coupled with an increase in ventilation amplitude would allow for greater acid-base relevant ion transfer between the blood and interlamellar water.

Interestingly, in my study, blood pCO_2 values of exercising fish were below that of ambient water and it is not clear how that could be since teleosts typically maintain internal pCO_2 between 1-2 mmHg above ambient (Cameron and Randall, 1972). If fish experienced lower p_wCO_2 levels during exercise it would account for the magnitude of the plasma [HCO_3^-] increase being greater in fish at rest, since they experienced an acidosis of greater magnitude compared with those during sustained exercise. Although highly speculative, this novel finding may be associated with changes in the gill

microenvironment that occur during exercise. Specifically, previous studies suggest the gill mucus layer is thinner during exercise, which may increase the efficiency of respiratory gas exchange by reducing diffusion distance (Randall and Wright, 1989). Therefore, at rest fish may be less efficient at CO_2 clearance due to a thicker gill mucus layer, moreover, this would narrow the interlamellar water channel, resulting in less water volume passing over the lamellae for gas and ion exchange.

It is possible that sustained exercise increased the level of circulating hormones, although the function of many hormones are still being elucidated, few have been proposed to have a role in acid-base regulation, including somatolactin and cortisol. Somatolactin, for example, has been implicated in regulating plasma [HCO_3^-] (Kakizawa et al., 1997) and [Ca^{2+}] levels (Kakizawa et al., 1996), and is released rapidly during periods of stress (Rand-Weaver et al., 1993). Somatolactin release appears to be driven predominantly by metabolic acidosis (i.e. exhaustive exercise), rather than a respiratory acidosis (Kakizawa et al., 1997). During sustained exercise initial blood pH values (i.e. prior to the onset of hypercarbia) were noticeably lower than initial blood pH values at rest. Therefore, due to the methodology of this study, exercising fish likely experienced a metabolic acidosis due to the initiation of swimming, thus, it is possible that somatolactin release played a role in recovery. Additionally, increased cortisol has been shown to increase H^+ ATPase activity (Lin and Randall, 1993) and chloride cell proliferation (Laurent and Perry, 1990), thus increasing the functionality and abundance of acid-base regulatory cells.

At this point, the mechanisms behind the trend of enhanced hypercarbia recovery during sustained exercise are highly speculative. However, this presents several routes for

future research in this area. Firstly, relatively little is known about the composition and regulation of the gill mucus layer, its role in respiration is of critical importance since it presents an additional obstacle for diffusion of respiratory gases. Additional research on the affinity and rate of acid-base relevant ion transport during sustained exercise would be of utmost importance. To date there is very little information regarding ion transport during sustained exercise, since fish maintain an active lifestyle, determining the typical energetic cost of acid-base balance in behaviourally relevant conditions is important.

2.4.3 Summary

To date there is surprisingly little known about how euryhaline species respond to acid-base disturbances, particularly during sustained exercise. This study demonstrates the capacity of a single fish species to respond to an acid-base disturbance when acclimated to water of varying ion composition. Additionally, we provide intra-specific evidence that supports the hypothesis of marine fish having an enhanced rate and degree of hypercarbia recovery compared with those in freshwater. Furthermore, this is the first study to examine the effects of sustained exercise on recovery during hypercarbia. Contrary to my hypothesis, sustained exercise appears to enhance hypercarbia recovery, however the specific mechanisms involved remain are unknown and require future investigation.

Table 2.1: Water ionic composition of soft-, hard-, and saltwater treatments. Soft water ion content is data from Metro Vancouver (2012), hard water ion content from EPA (2007) and saltwater values were calculated from typical Instant Ocean® concentrations at 32 ppt. pH values are presented as the pooled mean of Series I and II experiments ± S.E.M, letters indicate significant differences between treatments.

	Soft water	Artificial Hard Water	Saltwater (28 ppt)
$[Na^+]$ (mM)	0.08	2.4	410
$[Cl^-]$ (mM)	0.05	0.2	476
$[Ca^{2+}]$ (mM)	0.03	0.9	8.8
$[Mg^{2+}]$ (mM)	0.006	1.0	54.3
$[K^+]$ (mM)	0.004	0.12	10.7
$[HCO_3^-]$ (mM)	unknown	2.3	3.0
hardness as $CaCO_3$	3.43 mg/L	160-180 mg/L	-
pH (normocarbia)	6.7 ± 0.13^a	8.0 ± 0.05^b	7.86 ± 0.05^b
pH (hypercarbia (1% CO_2))	5.92 ± 0.02^a	6.97 ± 0.01^b	6.8 ± 0.01^c

Table 2.2: Mass, muscle water content and pooled hematocrit of rainbow trout exposed to acute hypercarbia at rest (48h) and during sustained exercise (8h). Values are presented as means ± S.E.M. Letters indicate significant differences between groups.

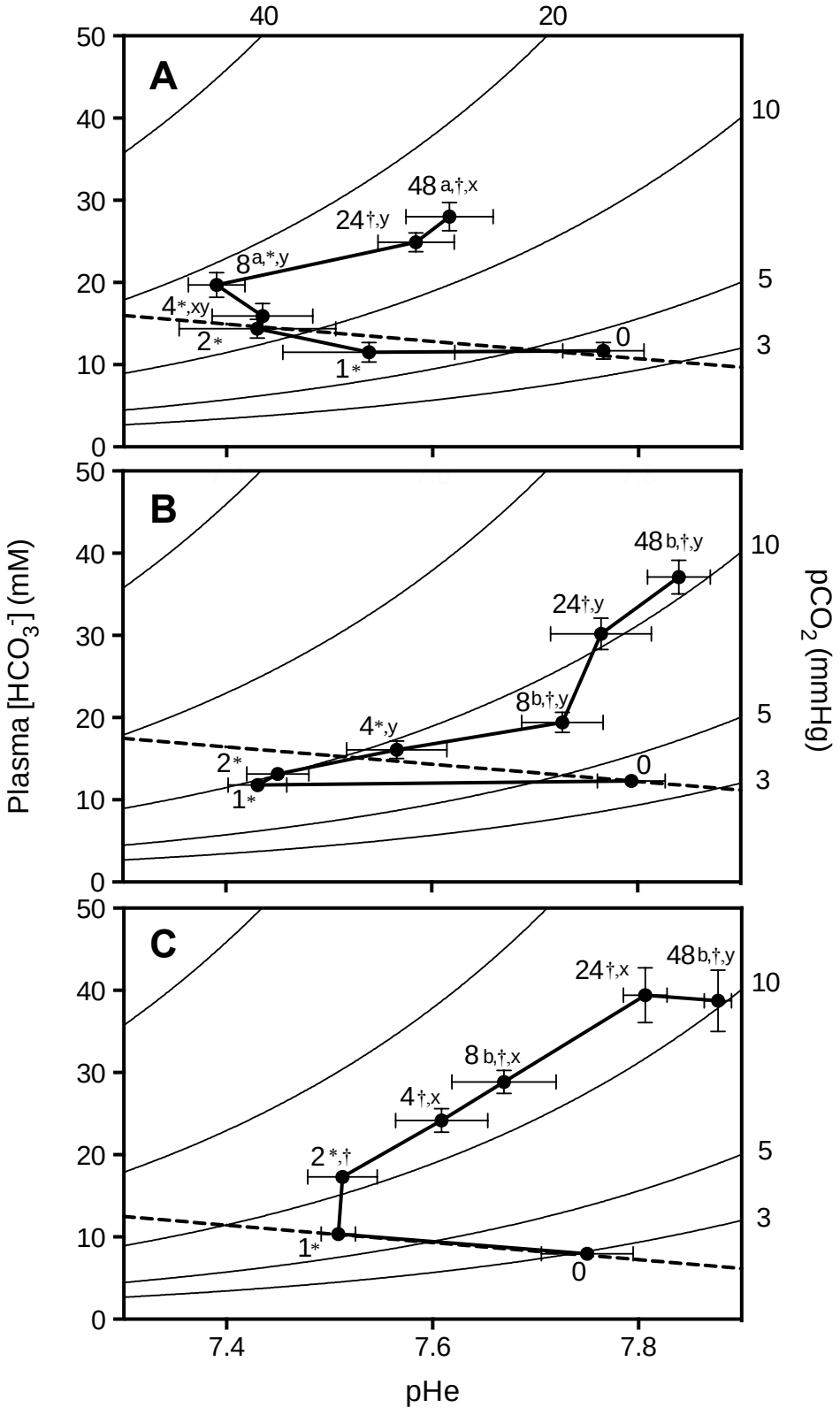
	Soft water	Hard water	Saltwater
Rest			
Mass (g)	379.0± 29.5[a]	268.0± 16.8[b]	341.3± 26.8[ab]
Muscle Water Content	79.2± 4.3	80.7± 0.7	80.3± 0.9
Hematocrit	28.4± 0.8	26.1± 0.8	22.4± 0.7
Sustained Exercise			
Mass (g)	403.8± 26.8[a]	291.7± 11.7[b]	308.8± 29.6[b]
Muscle Water Content	76.7± 1.4[a]	79.0± 0.3[ab]	80.2± 0.7[b]
Hematocrit	24.8± 0.6	27.4± 0.8	25.0± 0.7

Table 2.3: Degree of recovery (%) from hypercarbia of rainbow trout at rest and during sustained exercise. Values are calculated on an individual basis using the minimum blood pH and initial blood pH of each fish. Values are presented as means± S.E.M; letters denote significant differences between treatments; an asterisk represents a significant difference from 8h. There was a significant difference at 8h between fish at rest and fish during sustained exercise ($p<0.05$).

At Rest	2h	4h	8h	24h	48h*
Soft water			12.18± 9.0	49.24± 10.06	60.64± 10.47[a]
Hard water			84.1± 9.76	95.26± 13.3	115.3± 11.46[ab]
Saltwater			83.42± 20.8	150.17± 36.65	191.17± 54.64[b]
Sustained Exercise					
Soft water	132.29± 51.41	95.8± 38.39	82.3± 32.91		
Hard water	111.64± 18.32	149.9± 33.06	171.36± 47.84		
Saltwater	68± 12.26	127.83± 23.61	303.2± 107.63		

Table 2.4: Degree of recovery (%) from hypercarbia of resting and exercising rainbow trout. The average initial blood pH in each water treatment was calculated and kept consistent between resting and exercising fish for comparative purposes. Values are presented as means± S.E.M; letters denote significant differences between treatments; a dagger denotes a significant difference from 2h; an asterisk denotes a significant difference from 8h.

At Rest	2h	4h†	8h†	24h*	48h*
Soft water			13.88± 10.42[a]	50.36± 13.18[a]	58.53± 12.53[a]
Hard water			83.46± 9.54[b]	93.18± 12.06[ab]	111.63± 7.62[b]
Saltwater			72.85± 16.91[ab]	122.14± 8.59[b]	148.82± 5.86[b]
Sustained Exercise					
Soft water	53.0± 12.44	36.74± 7.73	42.01± 18.13[b]		
Hard water	45.59± 9.18	59.02± 3.5	64.30± 6.76[b]		
Saltwater	44.41± 7.09	61.53± 11.38	118.53± 6.72[a]		


40
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A
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C
48 a,†,x
24†,y
8a,*,y
4*,xy
2*
1*
0
48 b,†,y
24†,y
8b,†,y
4*,y
2*
1*
0
24†,x
48b,†,y
8 b,†,x
4†,x
2*,†
1*
0
Plasma [HCO3-] (mM)
pCO2 (mmHg)
pHe
7.4
7.6
7.8

Figure 2.1: A pH/HCO_3^- plot of the effect of hypercarbia (7.5mmHg, 1% CO_2) on blood acid-base status in resting A) soft water B) hard water and C) saltwater acclimated rainbow trout. Values indicate means ± S.E.M (*n*= 5-9). Numbers on plot indicate time (in hours) of hypercarbia exposure; the dashed line represents the buffer value of rainbow trout blood (Wood and LeMoigne, 1991) plotted through initial blood pH. An asterisk denotes a significant difference in pH_e from normocarbic (0h) values, a dagger denotes a significant difference in plasma [HCO_3^-] from normocarbia values, letters denote significant differences in pH_e (a,b) and plasma [HCO_3^-] (x,y) between water ionic compositions.

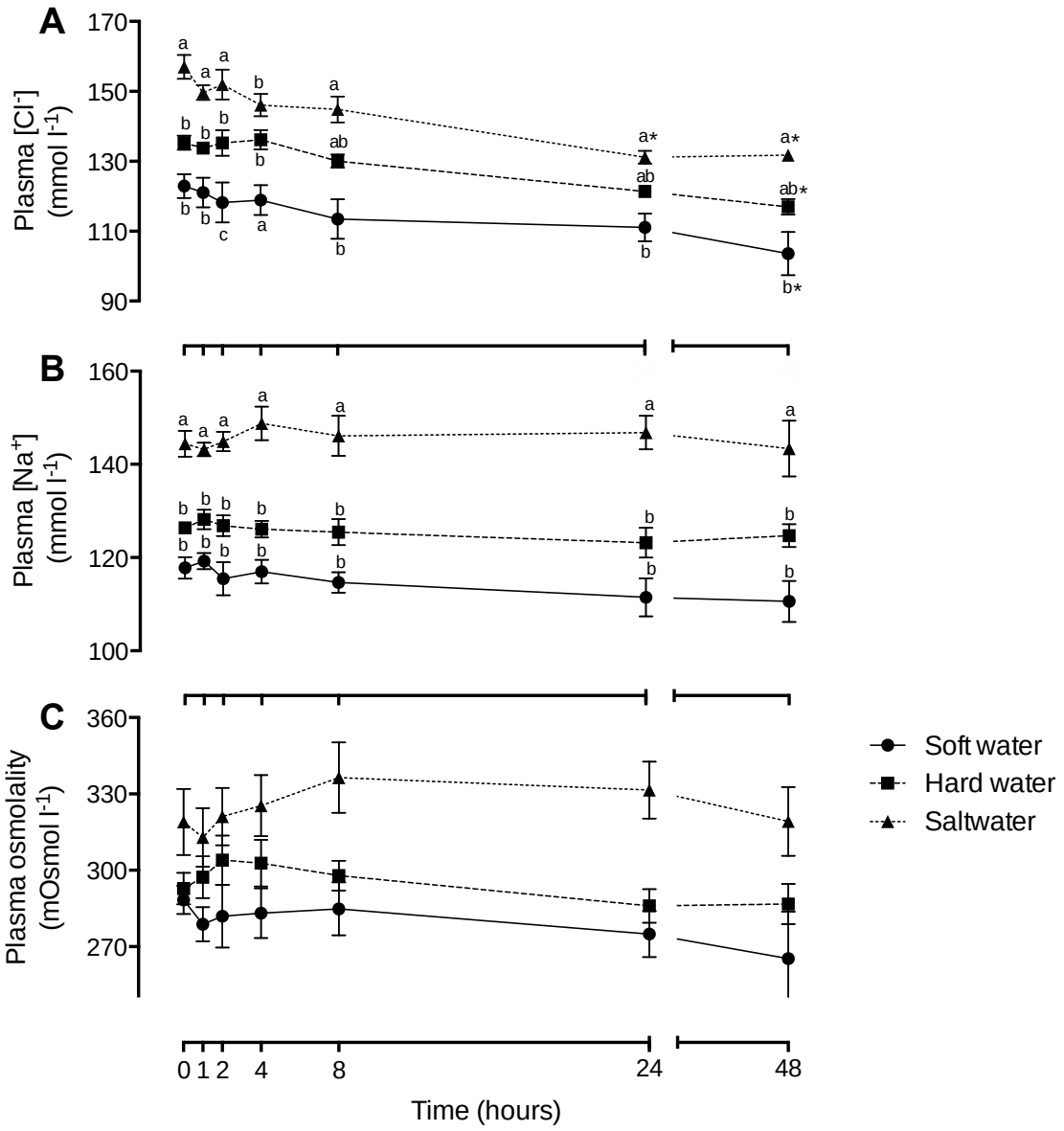
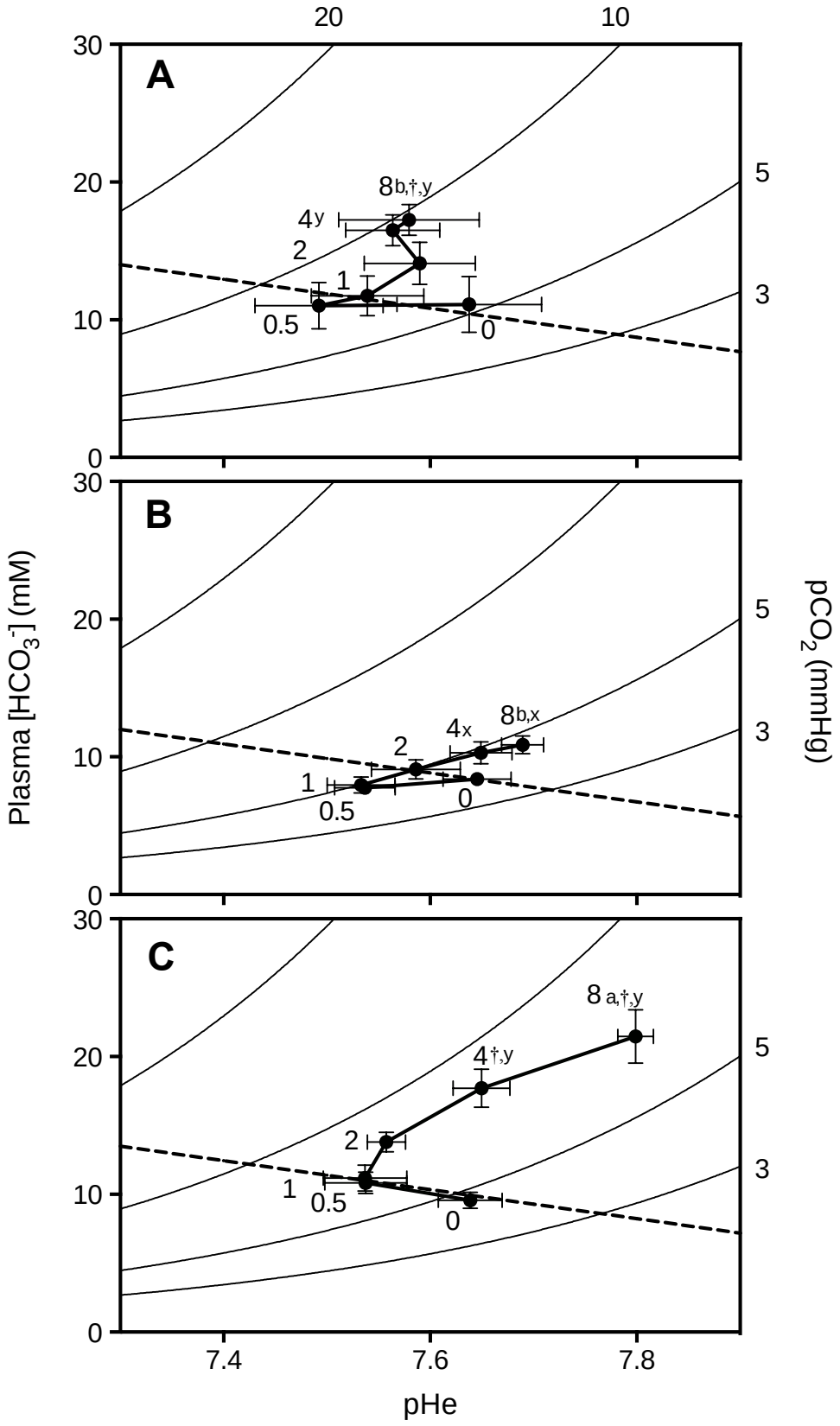


Figure 2.2: The effect of acute (48h) exposure to hypercarbia (7.5 mmHg, 1% CO_2) in resting trout acclimated to different water ionic compositions on A) plasma [Cl^-], B) plasma [Na^+] and C) plasma osmolality. There was a significant effect of water composition on plasma [Cl^-], [Na^+] and osmolality ($p<0.05$). See Fig 2.1 legend for further information.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8b,†,y
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8b,x
4x
8a,†,y
4†,y
Plasma [HCO3-] (mM)
pCO2 (mmHg)
7.4
7.6
7.8
pHe

Figure 2.3: A pH/HCO_3^- plot of the effect of hypercarbia (7.5mmHg, 1% CO_2) on blood acid-base status during sustained exercise in A) soft water B) hard water and C) saltwater acclimated rainbow trout. Values indicate means ± S.E.M (*n*=7-8). See Fig 2.1 legend for further information.

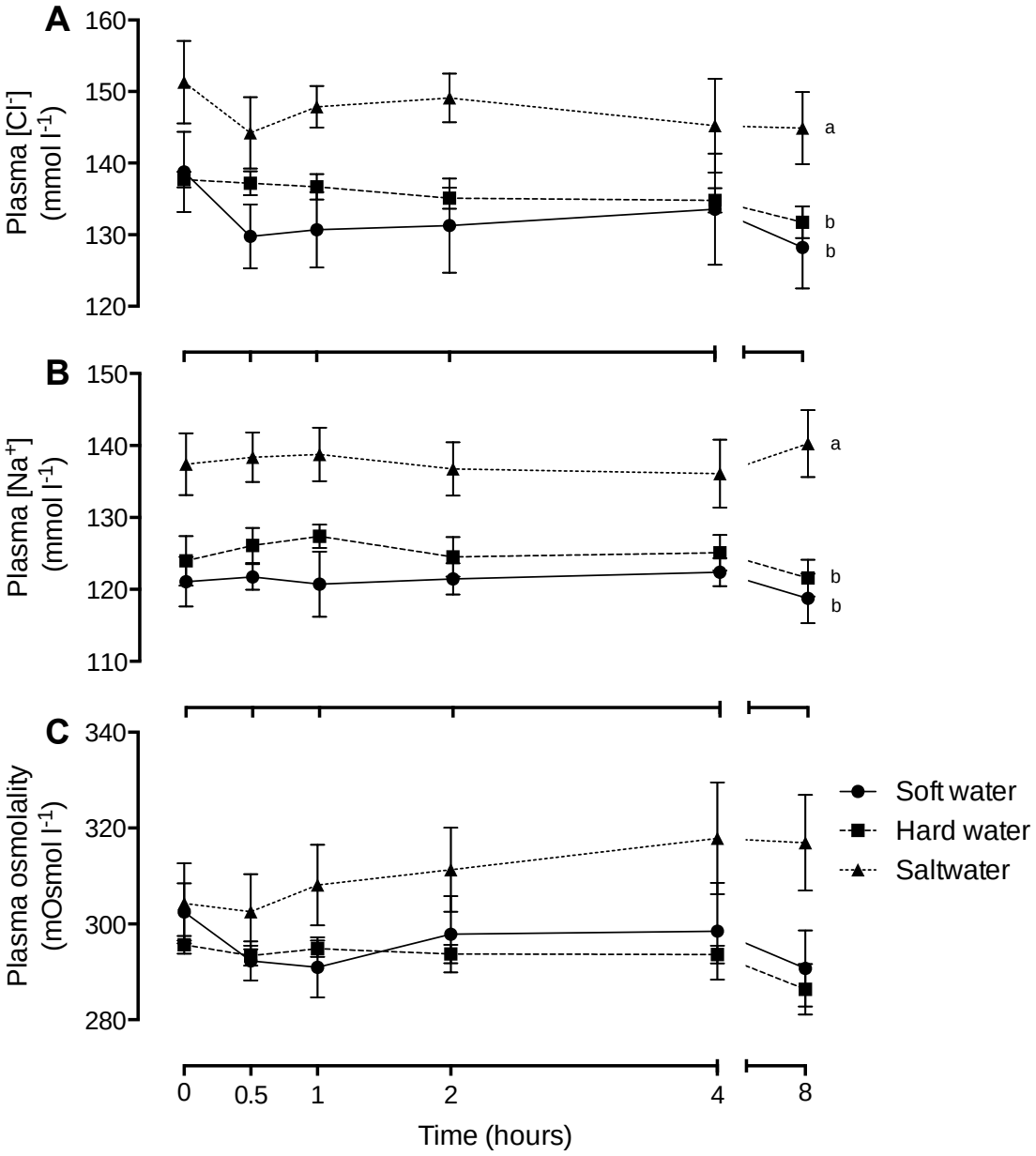
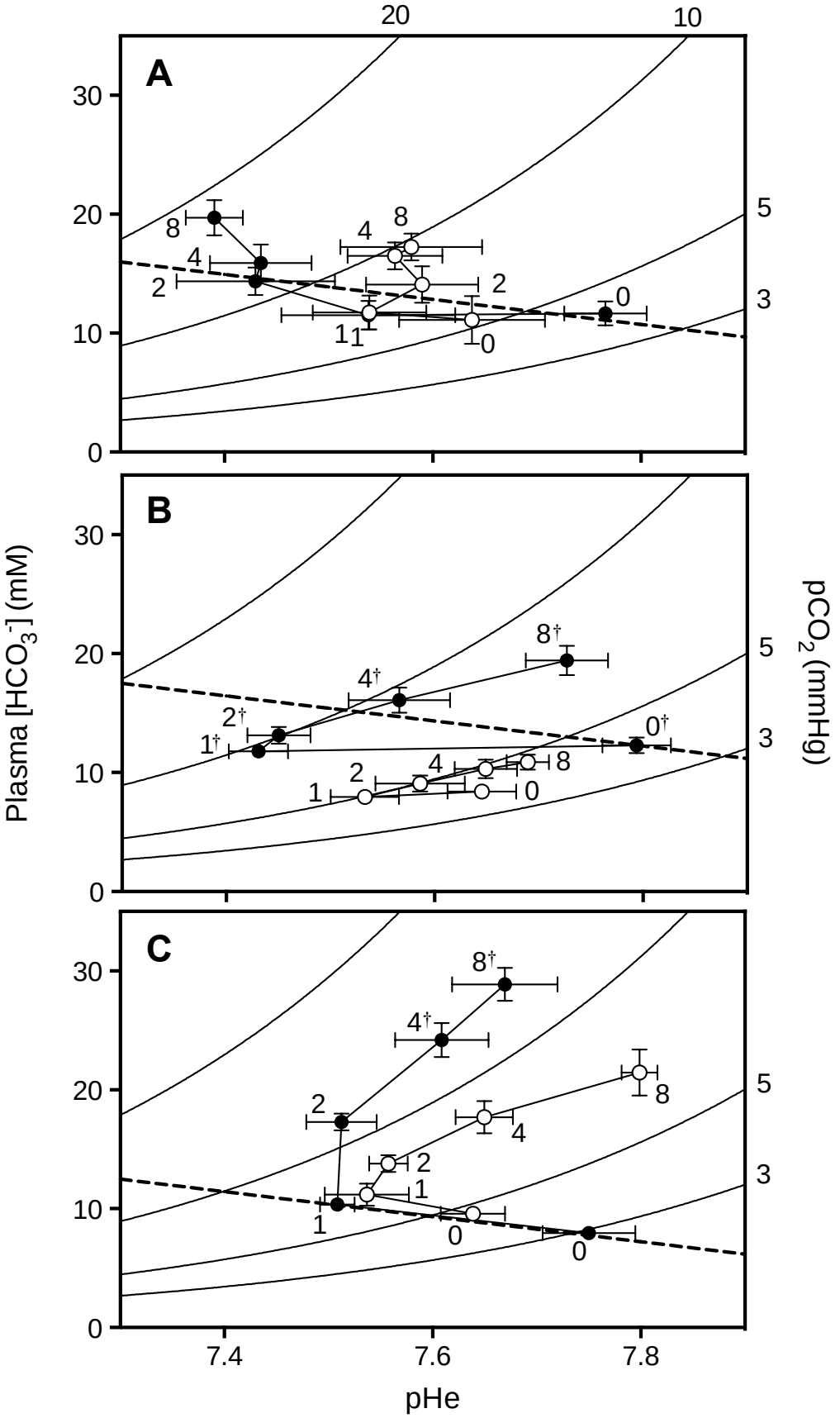


Figure 2.4: The effect of acute (8h) exposure to hypercarbia (7.5 mmHg, 1% CO_2) during sustained swimming in trout acclimated to different water ionic compositions on A) plasma [Cl^-], B) plasma [Na^+] and C) plasma osmolality. Values represent mean ±S.E.M. (*n*=7-8). There was a significant effect of water ionic composition plasma [Cl^-] and [Na^+], but no significant interaction. See Fig 2.2 legend for further information.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0†
Plasma [HCO3-] (mM)
pCO2 (mmHg)
7.4
7.6
7.8
pHe

Figure 2.5: A pH/HCO_3^- plot of the effect of hypercarbia (7.5mmHg, 1% CO_2) on blood acid-base status at rest (black circles) and during sustained exercise (open circles) in A) soft water B) hard water and C) saltwater acclimated rainbow trout. Values indicate means ± S.E.M (*n*=5-9). Only time points common to resting and exercising trout were plotted. Daggers denote a significant difference in plasma [HCO_3^-] in resting fish, compared with exercising fish. See Fig 2.1 legend for further information.

Chapter 3: General discussion

3.1 Thesis summary

This study sheds light on the important role of water ion composition on recovery from acid-base disturbances, which appears to either enhance or limit acid-base regulation through counter-ion availability. Many fish species are capable of inhabiting waters of varying ion content, a characteristic that is often coupled with numerous physiological changes, many of which occur at the gill, and counteract iono/osmo-regulatory pressures. However, to date, no studies have focused on the mechanisms of acid-base regulation during hypercarbia in a single species that is capable of inhabiting waters ranging from soft water to saltwater. Specifically, I examined how adult rainbow trout respond to hypercarbia when acclimated to water compositions ranging from soft- to saltwater. Both at rest and during sustained exercise, fish acclimated to hard- and saltwater restored blood pH earlier than those in soft water. Furthermore, fish seldom exercise maximally or are truly at rest, instead they are often swimming aerobically, however, there are no studies focused on acid-base regulation during periods of sustained aerobic exercise. Given that the gill is the main site of respiration and iono/osmoregulation, recovery during hypercarbia was hypothesized to be impaired during sustained exercise due to the morphological changes required for respiration. However, in all water ion compositions, there was a trend suggesting that exercise may enhance hypercarbia recovery. This is the first study to demonstrate that sustained exercise may enhance the ability of fish to respond and recover from an acid-base disturbance. Furthermore, this finding would suggest that the capacity for acid-base

compensation in fish may continually be underestimated since studies thus far have been conducted at rest.

3.2 Life in freshwater versus life in saltwater

As demonstrated by this study, hypercarbia may be more stressful for fish inhabiting water with low ionic content. Additionally, periods of hypercarbia are naturally occurring, particularly in environments rich in aquatic vegetation, whose respiration depletes O_2 stores and increases aquatic CO_2. Data from this study suggests that fish inhabiting waters of low ion content are less able to recover from hypercarbia, compared with those experiencing similar conditions in waters of greater ion content. Given these results, euryhaline fish, if able, may avoid these adverse conditions by moving to saltwater, the greater counter-ion content of which may allow for improved hypercarbia recovery.

Migrating to marine environments is not without its drawbacks, as it presents numerous stressors of its own, particularly osmo/ionoregulation, that require many energetically costly physiological changes (particularly at the gills). However, acid-base regulatory processes during hypercarbia can also incur large metabolic costs, and divert energy away from other physiologic processes. Studies during developmental life stages have demonstrated adverse effects due to hypercarbia, including a reduction in the size of perch eggs (Vinogradov and Komov, 1985) and reduced growth of juvenile sturgeon (Crocker and Cech, 1996). Presumably, escape from such conditions would be a favourable alternative for euryhaline species, however, this raises the question, given the

costs associated with migration: at what point does moving to saltwater become a more beneficial alternative?

3.3 Ocean acidification and CO_2 disposal

The CO_2 tension used in this study was well above projected aquatic CO_2 increases due to climate-change related atmospheric CO_2 concentrations. However, these large-scale hypercarbia tensions are useful in determining the underlying physiological strategies (Hayashi et al., 2004), presumably, also employed to combat small-scale (i.e. ocean acidification level) increases in ambient CO_2 (Esbaugh et al., 2012). Determining the energetic costs associated with elevated water CO_2 is becoming increasingly more important, and large-scale CO_2 tensions elicit a rapid blood acidosis in fishes which can be easily studied (Hayashi et al., 2004). Furthermore, many proposed disposal methods for atmospheric CO_2 center around using the ocean as a disposal site, and one particular strategy includes injecting its liquid form directly into the deep sea resulting in what are essentially underwater CO_2 lakes (Seibel and Walsh, 2003).

The deep-sea environment is unique due to its stability relative to many other environments, in particular, fluctuations in temperature, salinity, and dissolved gases are limited (Kennett and Ingram, 1995), and fishes inhabiting the deep-sea are thought to be relatively intolerant to such abiotic variation (Seibel and Walsh, 2003). Furthermore, the scale of CO_2 change resulting from this oceanic disposal strategy may result in pH reductions reported to range anywhere from 0.01 (Drange et al., 2001) to 4.0 (Adams et al., 1997) pH units, and dispersal distance may extend hundreds of kilometres from the injection site (Caulfield et al., 1997). The physiology of deep-sea fishes is inherently

different from rainbow trout, however, rainbow trout are used as a model species for many aquatic studies, and the results presented here provide useful background information regarding the effects of ambient water ion composition on acid-base balance. Furthermore, given the active lifestyle of fishes, the results from the sustained exercise trials demonstrate that fish may not be as vulnerable to acid-base disturbances as previously thought.

3.4 Limitations of research

Due to the nature of the setup and equipment, CO_2 levels during hypercarbia were not directly measured during the experiment, rather water pH was used during experimentation as a proxy for CO_2. Reasoning for this method was two-fold:

1) In order to directly measure CO_2 in the water using a pCO_2 meter the tension must be greater than 1.5% CO_2. Increasing the CO_2 tension was not a viable option because early observations noted reduced sustained swimming ability at 60% U_{crit} and this CO_2 tension.
2) It takes ~20 minutes for the pCO_2 meter to equilibrate with the water, therefore using a pH meter is a faster measurement which becomes increasingly important during the short term swimming exposure.

In all water compositions the change from normocarbia to hypercarbia in the water was very similar (~1 pH unit), and following the onset of hypercarbia the pH did not differ significantly through the remainder of the experiment. To mitigate these errors

water pH at 1% CO_2 was determined in each water ion composition prior to experimentation using a variety of techniques including premixed gases, and a Wosthöff gas mixer. However there does exist the possibility that a pCO_2 tension of 1% was not consistent among trials. This would explain the responses observed during the exercise trials as the fish may have experienced a reduced pCO_2 tension, thus making a comparison between resting and exercising trials difficult.

It would be an oversight not to mention the potential role water pH may have played in the results of this experiment. Low pH is associated with hypercarbia as H^+ release, and thus acidic conditions are coupled with elevated CO_2, making it difficult to distinguish between responses solely related to high CO_2 and those associated with low pH. Although the relative pH changes during hypercarbia in this study were consistent among water compositions (~ 1 pH unit) the initial pH of soft water was much lower than both hard- and saltwater. Furthermore, Lin and Randall (1991) noted that when water pH was 2.5 units lower than blood pH, H^+ excretion was completely eliminated. The possibility of this effect would have been the most pronounced on soft water acclimated fish as blood-water pH differed by ~2 units in this water ionic composition.

3.5 Future directions

This thesis creates the foundation for future work on the relationship between hypercarbia and sustained exercise. Although the capacity for hypercarbia recovery may be elevated during sustained exercise in rainbow trout, the underlying mechanisms by which this may occur were not determined. In this thesis I have outlined the possibility of lamellar recruitment as a likely candidate, however, further research into this area would be beneficial. There is evidence that distal lamellae of American eel (*Anguilla*

rostrata) gills may be perfused at rest (Evans et al., 2005), therefore limiting the capacity for hypercarbia recovery during sustained exercise in this species. Studies on the relationship between hypercarbia and sustained exercise on this species may be beneficial in determining the mechanisms by which exercise may enhance hypercarbia recovery.

A novel and unexpected result arising from this study was the low blood PCO_2 value in exercising fish. The mechanism behind this observation remains to be seen, as fish typically maintain internal PCO_2 ~1-2 mmHg above ambient (Cameron and Randall, 1972). Therefore, in order to achieve the values observed here, fish must maintain internal alkaline conditions relative to the ambient water. Presumably, this could be the result of changes in the gill microenvironment, as the gill is the primary site of both iono/osmoregulation and respiration. Studies on the gill microenvironment are startlingly few, nevertheless, Shephard (1992) noted that when ambient water was acidified, mucus pH at the mucus/tissue interface was maintained at levels higher than surrounding water. However, this occurrence seems to be confined to severe acidification of ambient water, as many studies bring water pH quite low (i.e. below 5) (Lin and Randall, 1990; Shephard, 1992). Similar studies focusing on the gill microenvironment of fish in several water ionic compositions may resolve the elevated PCO_2 levels we observed, as water ion content has a role determining ion gradients between the mucus layer and water. Studies on the gill microenvironment would also be useful in determining the possibly enhanced ion exchange mechanisms that may aid in hypercarbic recovery during sustained exercise. Additionally, the possible contribution of the gill mucus layer may be of particular importance to determine the capacity of fish to recover from acid-base disturbances, particularly because it is thought to be rich in carbonic anhydrase, the

enzyme responsible for catalyzing the conversion of CO_2 to H^+ and HCO_3^- (Wright et al., 1986).

For this study, fish were swum at an absolute speed, which was determined during preliminary trials. However, these trials were conducted on un-cannulated fish, furthermore, it was determined that fish for this trial needed an extra 24 h in order to maintain exercise during hypercarbia. Therefore, stress of the surgical procedure may have reduced their swimming ability and the swimming speed may have been more stressful than intended. Additionally, during exercise, fish exhibited a lower blood pH at the initial (pre-hypercarbia) time point than resting fish, suggesting they may have experienced an acidosis due to the initiation of swimming. Therefore, although there may have been some unforeseen effects during the swimming trials, all fish experienced the same conditions. Further studies in this area should mitigate these effects by determining the individual U_{crit} of cannulated fish and give fish more time to acclimate to swimming conditions prior to the onset of hypercarbia and blood sampling. However, the trend of enhanced hypercarbia recovery observed during exercise present an interesting area for further research. These results suggest that studies at rest may underestimate the ability of fish to recover from acid-base disturbances, and thus, further studies on hypercarbia should include exercise in order to correctly determine typical acid-base regulatory mechanisms and responses.

References

Adams, E. E., Caulfield, J. A., Herzog, H. J., and Auerbach, D. I. (1997). Impacts of reduced pH from ocean CO_2 disposal: sensitivity of zooplankton mortality to model parameters. *Waste Man.* 17: 375-380.

Beamish, F. W. H. (1978). Swimming capacity. In *Fish Physiology Volume 7* (eds. W. S. Hoar and D. J. Randall), pp. 101-187, New York: Academic Press Inc.

Booth, J. H. (1978). The distribution of blood flow in the gills of fish: application of a new technique to rainbow trout (*Salmo gairdneri*). *J. Exp. Biol.* 73: 119-129.

Boutilier, R. G., Heming, T. A., and Iwama, G. K. (1984). Appendix: Physicochemical parameters for use in fish respiratory physiology. In *Fish Physiology Volume 10A* (eds. W. S. Hoar and D. J. Randall), pp 403- 430, New York: Academic Press Inc.

Brauner, C. J., Thorarensen, H., Gallaugher, P., Farrell, A. P. and Randall, D. J. (2000). CO_2 transport and excretion in rainbow trout (*Oncorhynchus mykiss*) during graded sustained exercise. *Resp. Physiol.* 119: 69-82.

Brett, J. R. (1964). The respiratory metabolism and swimming performance of young sockeye salmon. *J. Fish. Res. BD. Canada.* 21(5): 1183- 1226.

Burgetz, I. J., Rojas-Vargas, A., Hinch, S. G., and Randall, D. J. (1998). Initial recruitment of anaerobic metabolism during sub-maximal swimming in rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* 201: 2711-2721.

Cameron, J. N. and Randall, D. J. (1972). The effect of increased ambient CO_2 on arterial CO_2 tension, CO_2 content and pH in rainbow trout. *J. Exp. Biol.* 57: 673-680.

Cameron, J. N., Randall, D. J. and Davis, J. C. (1971). Regulation of the ventilation-perfusion ratio in the gills of *Dasyatis sabina* and *Squalus suckleyi*. *Comp. Biochem. Physiol.* 39A: 505-519.

Caulfield, J. A., Auerbach, D. I., Adams, E., and Herzog, H. J. (1997). Near field impacts of reduced pH from ocean CO_2 disposal. *Energy Convers. Man.* 38: 343-348.

Claiborne, J. B., Blackston, C. R., Choe, K. P., Dawson, D. C., Harris, S. P., Mackenzie, L. A., and Morrison-Shetlar, A. I. (1999). A mechanism for branchial acid excretion in marine fish: identification of multiple Na^+/H^+ antiporter (NHE) isoforms in gills of two seawater teleosts. *J. Exp. Biol.* 202: 315-324.

Crocker, C. E., and Cech, J. J. (1996). The effects of hypercapnia on the growth of juvenile white sturgeon, *Acipenser transmontanus*. *Aquaculture.* 147: 293-299.

Degnan, K. J., and Zadunaisky, J. A. (1980). Passive sodium movements across the opercular epithelium: the paracellular shunt pathway and ionic conductance. *J. Membr. Biol*. 55: 175-185.

Drange, H., Alendal, G., and Johannessen, O. M. (2001). Ocean release of fossil fuel CO_2: a case study. *Geophys. Res. Lett.* 29: 2637-2640.

EPA. (2007). Short term methods for estimating the chronic toxicity of effluents and receiving water to freshwater. http://water.epa.gov/scitech/methods/cwa/wet/upload/2007_07_10_methods_wet_disk2_atx7-10.pdf

Esbaugh, A. J., Heuer, R., and Grosell, M. (2012). Impacts of ocean acidification on respiratory gas exchange and acid-base balance in a marine teleost, *Opsanus beta*. *J. Comp. Physiol. B.* 182(7): 921-934.

Evans, D. H. (2008). Teleost fish osmoregulation: what we have learned since August Krogh, Homer Smith and Ancel Keys. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 295: R704- R713.

Evans, D. H., Piermarini, P. M. and Choe, K. P. (2005). The multifunctional gill: dominant site of gas exchange, osmoregulation, acid-base regulation and excretion of nitrogenous waste. *Physiol. Rev.* 85: 97-177.

Farrell, A. P. (2008). Comparisons of swimming performance in rainbow trout using constant acceleration and critical swimming speed tests. *J. Fish. Biol.* 72: 693-710.

Goss, G. G., and Wood, C. M. (1990). Na^+ and Cl^- uptake kinetics, diffusive effluxes and acidic equivalent fluxes across the gills of rainbow trout. II. Responses to bicarbonate infusion. *J. Exp. Biol.* 152: 549-571.

Goss, G. G., Laurent, P. and Perry, S. F. (1992). Evidence for a morphological component in the regulation of acid-base balance in hypercapnic catfish (*Ictalurus nebulosus*). *Cell Tiss. Res.* 268: 539-552.

Greco, A. M., Fenwick, J. C., and Perry, S. F. (1996). The effects of soft-water acclimation on gill structure in the rainbow trout *Oncorhynchus mykiss*. *Cell. Tissue. Res.* 285: 75-82.

Grosell, M. (2011). The role of the gastrointestinal tract in salt and water balance. In *Fish Physiology Volume 30* (eds. M. Grosell, A. P. Farrell and C. J. Brauner), pp 136- 164, New York: Academic Press Inc.

Hayashi, M., Kita, J., and Ishimatsu, A. (2004). Acid-base responses to lethal aquatic hypercapnia in three marine fishes. *Mar. Biol.* 144: 153-160.

Heisler, N. (1984). Acid-base regulation in fishes. In *Fish Physiology*, vol. X (ed. W. S. Hoar and D. J. Randall), pp. 315-401. San Diego: Academic Press.

Heisler, N. (1986). Buffering and transmembrane ion transfer processes. In *Acid-base regulation in animals* (ed. N. Heisler), pp 3-47. Amsterdam: Elsevier Science Publishers.

Heisler, N. (1988). Ion transfer processes as mechanisms for acid-base regulation. In *Lung biology in health and disease – comparative pulmonary physiology: current concepts* (ed. S.C. Wood), pp 539-583. New York: Marcel Dekker.

Heisler, N. (1999). Limiting factors for acid-base regulation in fish: branchial transfer capacity versus diffusive loss of acid-base relevant ions. In: *Regulation of acid-base status in animals and plants* (eds. S. Egginton, E.W. Taylor and J.A. Raven), pp 125-154. Cambridge: Cambridge University Press.

Hickman, C. P. and Trump, B. F. (1969). The kidney. In: *Fish physiology*, vol. I (ed. W. S. Hoar and D. J. Randall), pp. 91-239. San Diego: Academic Press.

Hirata, T. Kaneko, T., Ono, T., Nakazato, T., Furukawa, N., Hasegawa, S., Wakabayashi, S., Shigekawa, M., Chang, M. H., Romero, M. F., and Hirose, S. (2003). Mechanism of acid adaptation of a fish living in a pH 3.5 lake. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 284: R1199-R1212.

Hyde, D. A. and Perry, S. F. (1987). Acid-base and ionic regulation in the American eel, (*Anguilla rostrata*) during and after prolonged aerial exposure: branchial and renal adjustments. *J. Exp. Biol.* 133: 429-447.

Hyde, D. A. and Perry, S. F. (1989). Differential approaches to blood acid-base regulation during exposure to prolonged hypercapnia in two freshwater teleosts: the rainbow trout (*Salmo gairdneri*) and the American eel (*Anguilla rostrata*). *Physiol. Zool.* 62(6):1164-1186.

Iwama, G. K. and Heisler, N. (1991). Effect of environmental water salinity on acid-base regulation during environmental hypercapnia in the rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* 158: 1-18.

Janssen, R. G., and Randall, D. J. (1975). The effects of changes in pH and pCO_2 in blood and water on breathing in rainbow trout, *Salmo gairdneri*. *Resp. Physiol.* 25: 235-245.

Jones, D. R., and Randall, D. J. (1979). The respiratory and circulatory systems during exercise. In *Fish Physiology Volume 7* (eds. W. S. Hoar and D. J. Randall), pp 425- 501, New York: Academic Press Inc.

Kakizawa, S., Ishimatsu, A., Takeda, T., Kaneko, T., and Hirano, T. (1997). Possible involvement of somatolactin in the regulation of plasma bicarbonate for the compensation of acidosis in rainbow trout. *J. Exp. Biol.* 200: 2675-2683.

Kakizawa, S., Kaneko, T., and Hirano, T. (1996). Elevation of plasma somatolactin concentrations during acidosis in rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* 199: 1043-1051.

Kennett, J. P., and Ingram, B. L. (1995). A 20,000-year record of ocean circulation and climate change from the Santa Barbara basin. *Nature*. 377, 510-514.

Kiceniuk, J. W., and Jones, D. J. (1977). The oxygen transport system in trout (*Salmo gairdneri*) during sustained exercise. *J. Exp. Biol.* 69: 247-260.

Larsen, B. K., and Jensen, F. B. (1997). Influence of ionic composition on acid-base regulation in rainbow trout (*Oncorhynchus mykiss*) exposed to environmental hypercapnia. *Fish. Physiol. Biochem.* 16(2): 157-170.

Laurent, P., and Perry, S. F. (1990). Effects of cortisol on gill chloride cell morphology and ionic uptake in the freshwater trout, *Salmo gairdneri*. *Cell. Tissue. Res.* 259: 429-442.

Laurent, P., Hõbe, H., and Dunel-Erb, S. (1985). The role of environmental sodium chloride relative to calcium in gill morphology of freshwater salmonid fish. *Cell. Tissue. Res.* 240: 675-692.

Lin, H., and Randall, D. (1990). The effect of varying water pH on the acidification of expired water in rainbow trout. *J. Exp. Biol.* 149: 149-160.

Lin, H., and Randall, D. (1991). Evidence for the presence of an electrogenic proton pump on the trout gill epithelium. *J. Exp. Biol.* 161, 119-134.

Lin, H., and Randall, D. J. (1993). H^+-ATPase activity in crude homogenates of fish gill tissue: inhibitor sensitivity and environmental and hormonal regulation. *J. Exp. Biol.* 180: 163-174.

Lin, H., Pfeiffer, D. C., Vogl, A. W., Pan, J., and Randall, D. J. (1994). Immunolocalization of H^+-ATPase in the gill epithelia of rainbow trout. *J. Exp. Biol.* 195: 169-183.

Marshall, W. S., Bryson, S. E., Midelfart, A., and Hamilton, W. F. (1995). Low-conductance anion channel activated by cAMP in teleost Cl^-secreting cells. *Am. J. Physiol. Integr. Comp. Physiol.* 268: R963-R969.

Marshall, W. S., Lynch, E. M., and Cozzi, R. R. F. (2002). Redistribution of immunofluorescence of CFTR anion channel and NKCC cotransporter in chloride cells during adaptation of the killifish *Fundulus heteroclitus* to sea water. *J. Exp. Biol*. 205: 1265.

McDonald, D. G. and Rogano, M. S. (1986). Ion regulation by the rainbow trout, *Salmo gairdneri,* in ion-poor water. *Physiol. Zool.* 59(3): 318-331.

Metro Vancouver. (2012). Water: the Greater Vancouver water district quality control annual report 2012 Volume 1. http://www.metrovancouver.org/about/publications /Publications/2012WaterQualityControlAnnualReportVolume1.pdf

Nilsson, S. (1986). Control of gill blood flow. In: *Fish Physiology: Recent advances* (eds. S. Nilsson and S. Holmgren), pp. 87-101. London: Croom Helm.

Perry, S. F. (1982). The regulation of hypercapnic acidosis in two salmonids, the freshwater trout (*Salmo gairdneri*) and the seawater salmon (*Oncorhynchus kisutch*). *Mar. Behav. Physiol.* 9: 73-79.

Perry, S. F. and Laurent, P. L. (1989). Adaptational responses of rainbow trout to lowered external NaCl concentration: contribution of the branchial chloride cell. *J. Exp. Biol.* 147: 147-168.

Perry, S. F., and Reid, S. G. (2002). Cardiorespiratory adjustments during hypercarbia in rainbow trout *Oncorhynchus mykiss* are initiated by external CO_2 receptors on the first gill arch. *J. Exp. Biol.* 205: 3357-3365.

Perry, S. F. and Wood, C. M. (1985). Kinetics and branchial calcium uptake in the rainbow trout: effects of acclimation to various external calcium levels. *J. Exp. Biol.* 116: 411-433.

Perry, S. F., Fritschie, R., Hoagland, T. M., Duff, D. W., and Olson, K. R. (1999). The control of blood pressure during external hypercapnia in the rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol*. 202: 2177-2190.

Perry, S. F., Beyers, M. L., and Jonhson, D. A. (2000). Cloning and molecular characterisation of the trout (*Oncorhynchus mykiss*) vacuolar H^+-ATPase B subunit. *J. Exp. Biol.* 203: 459-470.

Perry, S. F., Shahsavarani, A., Georgalis, T., Bayaa, M., Furimsky, M., and Thomas, S. L. Y. (2003). Channels, pumps, and exchangers in the gill and kidney of freshwater fishes: their role in ionic and acid-base regulation. *J. Exp. Zool.* 300a: 53-62.

Postlethwaite, E. K., and McDonald, D. G. (1995). Mechanisms of Na^+ and Cl^- regulation in freshwater-adapted rainbow trout (*Oncorhynchus mykiss*) during exercise and stress. *J. Exp. Biol.* 198: 295-304.

Putnam, R. W. and Roos, A. (1997). Intracellular pH. In *Handbook of Physiology. Cell Physiology* (eds. J. Hoffman and J. Jamieson), pp. 389–440. Oxford: Oxford University Press.

Rand-Weaver, M., Pottinger, T. G., and Sumpter, J. P. (1993). Plasma somatolactin concentrations in salmonid fish are elevated by stress. *J. Endocrinol.* 138(3): 509-515.

Randall, D. J. and Lin, H. (1993). Effects of water pH on gas and ion transfer across fish gills. In *Fish Ecophysiology* (eds. J. C. Rankin and F. B. Jensen), pp. 265-275. London: Chapman and Hall.

Randall, D. J., and Wright, P. A. (1989). The interaction between carbon dioxide and ammonia excretion and water pH in fish. *Can. J. Zool.* 67: 2936-2942.

Scott, G. R., Rogers, J. T., Richards, J. G., Wood, C. M. and Schulte, P. M. (2004). Intraspecific divergence of ionoregulatory physiology in the euryhaline teleost *Fundulus heteroclitus*: possible mechanisms of freshwater adaptation. *J. Exp. Biol.* 207: 3399-3410.

Seibel, B. A., and Walsh, P. J. (2003). Biological impacts of deep-sea carbon dioxide injection inferred from indices of physiological performance. *J. Exp. Biol.* 206: 641-650.

Shephard, K. L. (1992). Studies on the fish-gill microclimate: interactions between gill tissue, mucus and water-quality. *Environ. Biol. Fish.* 34: 409-420.

Silva, P., Solomon, R., Spokes, K., and Epstein, F. H. (1977). Ouabain inhibition of gill Na-K-ATPase: relationship to active chloride transport. *J. Exp. Zool.* 199: 419-425.

Smith, F. M., and Jones, D. R. (1982). The effect of changes in blood oxygen-carrying capacity on ventilation volume in the rainbow trout (*Salmo gairdneri*). *J. Exp. Biol.* 97: 325-334.

Soivio, A., Nynolm, K., and Westman, K. (1975). A technique for repeated sampling of the blood of individual resting fish. *J. Exp. Biol.* 62: 207-217.

Stevens, E. D., and Randall, D. J. (1967a). Changes of gas concentrations in blood and water during moderate swimming activity in rainbow trout. *J. Exp. Biol.* 46: 329-337.

Stevens, E. D., and Randall, D. J. (1967b). Changes in blood pressure, heart rate, and breathing rate during moderate swimming activity in rainbow trout. *J. Exp. Biol.* 46: 307-315.

Sullivan, G. V., Fryer, J. N., and Perry, S. F. (1995). Immunolocalization of proton pumps (H^+-ATPase) in pavement cells of rainbow trout gill. *J. Exp. Biol.* 198: 2619-2629.

Thomas, S., Fievet, B., Barthelemy, L., and Peyraud, C. (1983). Comparison of the effects of exogenous and endogenous hypercapnia on ventilation and oxygen uptake in the rainbow trout (*Salmo gairdneri* R.). *J. Comp. Physiol.* 151: 185-190.

Toews, D. P., Holeton, G. F., and Heisler, N. (1983). Regulation of the acid-base status during environmental hypercapnia in the marine teleost fish *Conger conger*. *J. Exp. Biol.* 107: 9-20.

Ultsch, G. R. (1996). Gas exchange, hypercarbia and acid-base balance, paleoecology, and the evolutionary transition from water-breathing to air-breathing among vertebrates. *Palaeogeog. Palaeoclim. Palaeoecol.* 123, 1-27.

Van Den Thillart, G., Randall, D. and Hoa-Ren, L. (1983). CO_2 and H^+ excretion by swimming coho salmon, *Onchorynchus kisutch*. *J. Exp. Biol.* 107: 189-180.

Vinogradov, G. A., and Komov, V. T. (1985). Ion regulation in the perch, *Perca fluvuatilis*, in connection with the problem of acidification of water bodies. *J. Ichthyol.* 25: 53-61.

Weakley, J. C., Claiborne, J. B., Hyndman, K. A., and Edwards, S. L. (2012). The effect of environmental salinity on H^+ efflux in the euryhaline barramundi (*Lates calcarifer*). *Aquaculture*. 338-341, 190- 196.

Wilson, J. M., Laurent, P., Tufts, B. L., Benos, D. J., Donowitz, M., Wayne Vogl, A., and Randall, D. J. (2000). NaCl uptake by the branchial epithelium in freshwater teleost fish: an immunological approach to ion-transport protein localization. *J. Exp. Biol.* 203: 2279-2296.

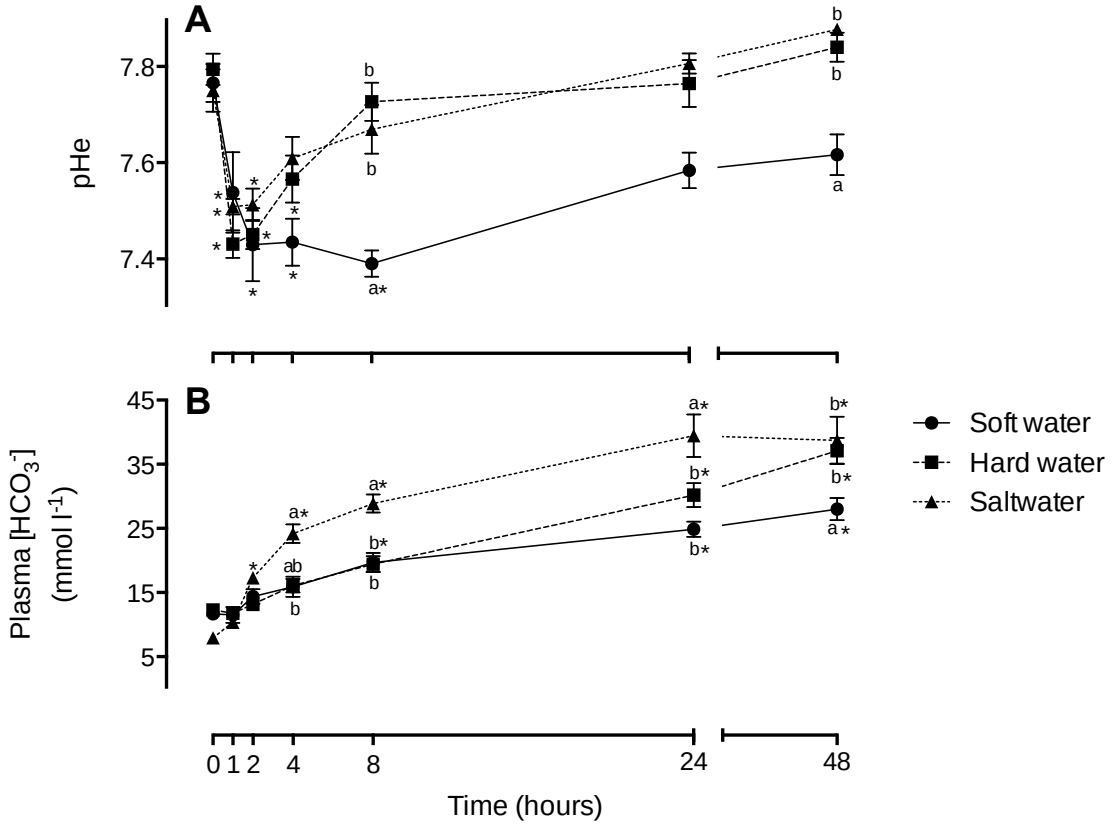
Wittmann, A. C. and Pörtner, H-O. (2013). Sensitivities of extant animal taxa to ocean acidification. *Nat. Clim. Change*. 3: 995-1001.

Wood, C. M. and LeMoigne, J. (1991). Intracellular acid-base responses to environmental hyperoxia and normoxic recovery in rainbow trout. *Respir. Physiol.* 55(2): 155-173.

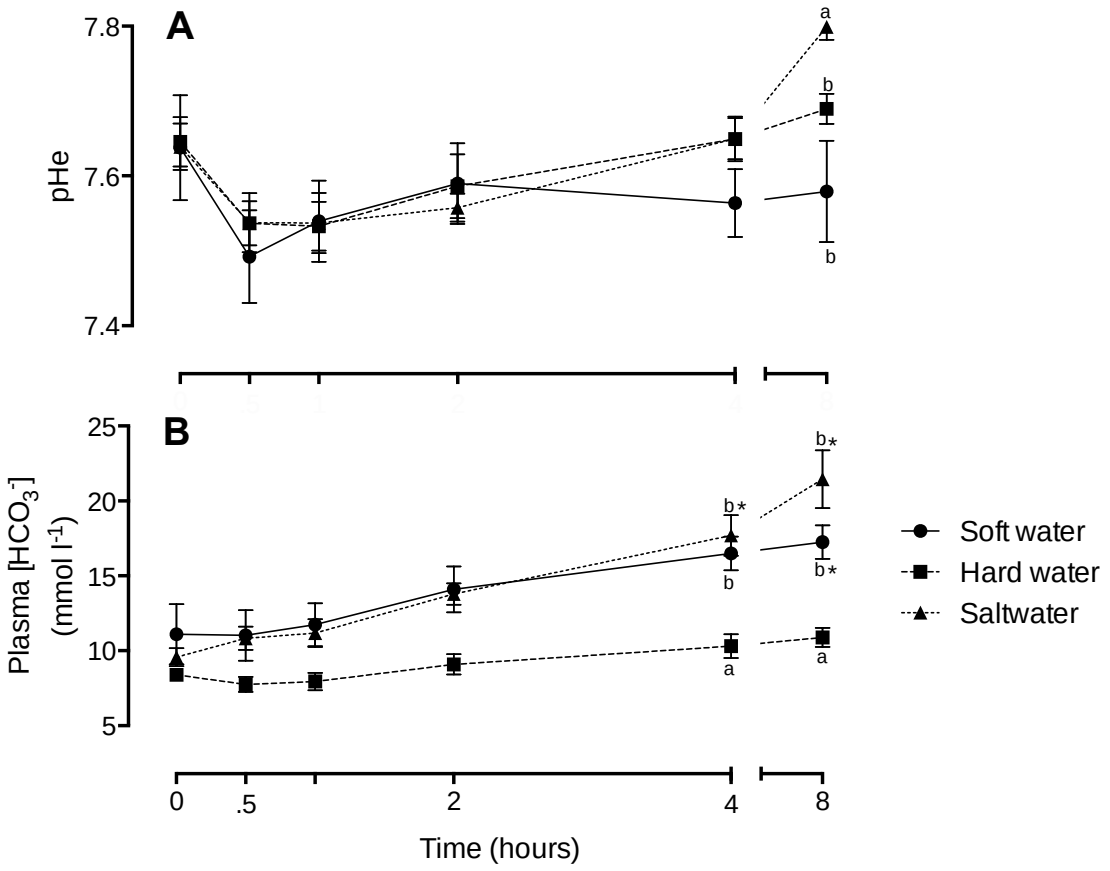
Wright, P., Heming, T., and Randall, D. (1986). Downstream pH changes in water flowing over the gills of rainbow trout. *J. Exp. Biol.* 126: 499-512.

Appendix

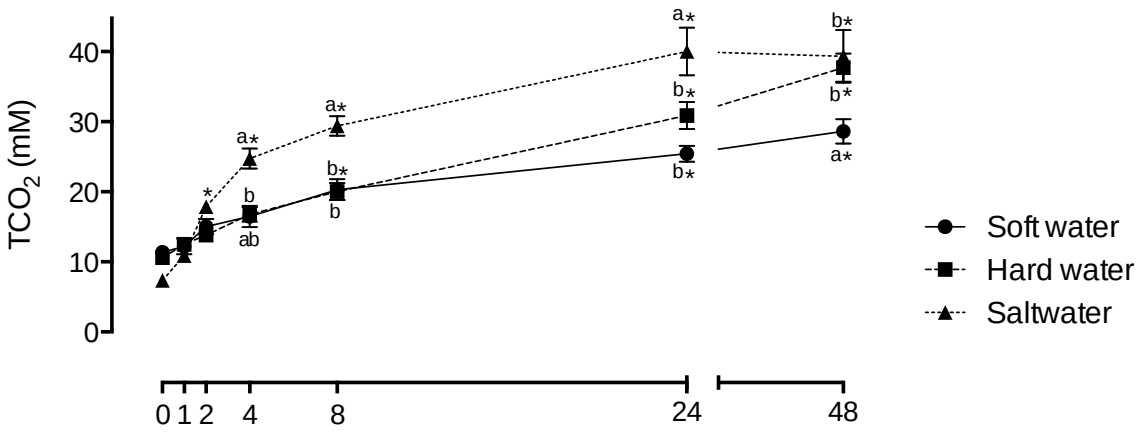
Appendix A: supplementary figures



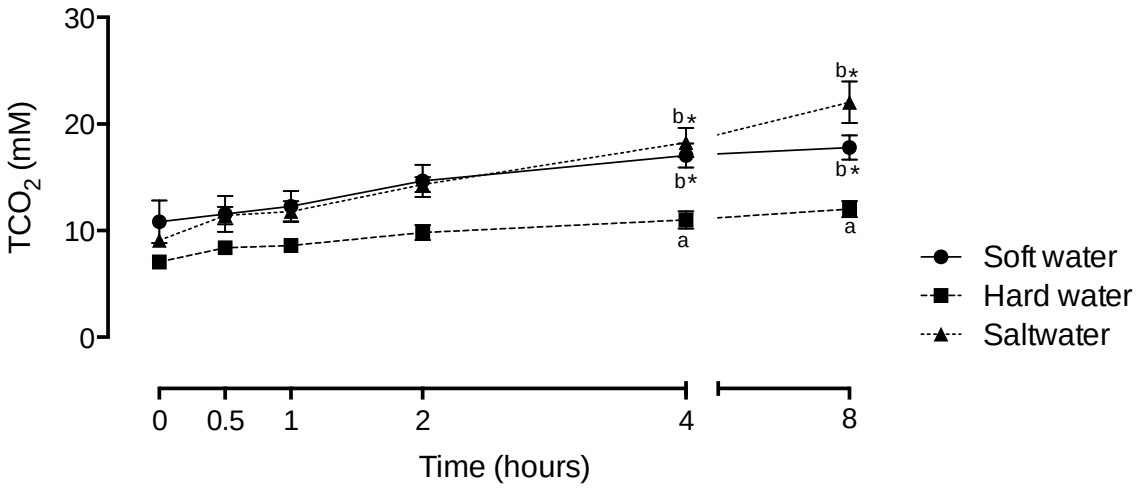
A. 1: The effect of acute (48h) exposure to hypercarbia (7.5 mmHg, 1% CO_2) in resting trout acclimated to different water ionic compositions on A) blood pH (pH_e) and B) plasma [HCO_3^-]. Circles represent soft water, squares represent hard water and triangles represent saltwater (28 ppt) acclimated trout. Values represent mean ±S.E.M. (*n*=5-9). Letters denote a significant difference among water compositions, an asterisk denotes a significant difference from normocarbic (0h) values within a given water composition.



A. 2: The effect of acute (8h) hypercarbia (7.5 mmHg, 1% CO_2) in trout acclimated to different water ionic compositions during sustained exercise on A) blood pH (pH_e) and B) plasma [HCO_3^-]. Values represent mean ±S.E.M. (*n*=7-8). Letters denote a significant difference among water compositions, an asterisk denotes a significant difference from normocarbic (0h) values within a given water composition.



A. 3: The effect of acute (48h) hypercarbia exposure on total carbon dioxide (TCO_2) concentration of fish acclimated to soft-, hard-, or saltwater at rest. Data is presented as means± S.E.M (*n*=5-9); letters denote significant differences between treatments, an asterisk denotes a significant change from initial (0h) levels.



A. 4: The effect of acute (8h) hypercarbia exposure on total carbon dioxide (TCO_2) concentration of fish acclimated to soft-, hard-, or saltwater during sustained exercise. Data is presented as means± S.E.M (*n*=7-8); letters denote significant differences between treatments, an asterisk denotes a significant change from initial (0h) levels.