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GAS TRANSFER IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) : AN INVESTIGATION OF FACTORS CONTROLLING O_2 UPTAKE AND CO_2 EXCRETION

By

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GAS TRANSFER IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*): AN INVERSIGATION OF FACTORS CONTROLLING O_2 UPTAKE AND CO_2 EXCRETION

Abstract

The entry of bicarbonate ions (HCO_3^-) into the red blood cell (RBC) via the Cl^-/HCO_3^- exchanger for dehydration to CO_2 has long been regarded as the limiting step of CO_2 excretion in fish. The first component of this thesis was to test the hypothesis that proton availability may limit the catalysed dehydration of HCO_3^- within the extracellular compartment. The results presented in this thesis suggest that H^+ availability does not appear to limit plasma HCO_3^- dehydration *in vivo*. Furthermore, the enhancement of CO_2 excretion by means of the acceleration of HCO_3^- dehydration within the plasma following the administration of carbonic anhydrase (CA) demonstrated in this thesis was the first *in vivo* evidence ratifying the hypothesis of the chloride shift as the limiting factor on CO_2 excretion in teleost fish.

Using the knowledge acquired from the previous chapter, I tested the hypothesis that the slow entry rate of HCO_3^- into the RBC may be responsible for the apparent diffusion limitations on CO_2 excretion. The experiments examined the effect of blood transit time through the gills on gas transfer to test the hypothesis that the efficiency of CO_2 excretion is sensitive to changes in blood flow owing to chemical equilibrium limitations, whereas the efficiency of O_2 uptake is insensitive to changes in blood flow. The insensitivity of PaO_2 to changes in cardiac output *in vivo* reaffirms the theory that O_2 uptake behaves as a perfusion limited system. However, the sensitivity of CO_2 transfer efficiency to changes in cardiac output, coupled with the impact of (CA) availability on this sensitivity, argue strongly that the apparent diffusion limitations on CO_2 excretion are, in fact, chemical equilibrium limitations.

Résumé

L'entrée des ions de bicarbonate (HCO_3^-) dans les cellules rouges (RBC) via l'échangeur Cl^-/HCO_3^- pour sa déshydratation en CO_2 a longtemps été perçu comme étant le facteur limitant l'excrétion du CO_2 chez les poissons. La première partie de cette thèse était de vérifier l'hypothèse que la disponibilité des protons peut limiter la déshydratation du HCO_3^- catalysée dans le compartiment extracellulaire. Les résultats présentés dans cette thèse suggèrent que la disponibilité de H^+ ne limite pas la déshydratation de HCO_3^- dans le plasma *in vivo*. L'augmentation de l'excrétion du CO_2 par l'accelération de la déshydratation du HCO_3^- à l'intérieur du plasma à la suite d'une injection d'anhydrase carbonique (CA) confirme l'hypothèse selon laquelle l'échangeur Cl^-/HCO_3^- est le facteur limitant l'excrétion du CO_2 *in vivo* chez les poissons téléostéens.

En utilisant les connaissances acquises de ces expériences , on a mis a l'épreuve l'hypothèse supposant que le taux d'entrée lent du HCO_3^- dans les cellules rouges peut être responsable pour les limites de diffusions apparentes lors de l'excrétion du CO_2. La série d'expériences a porté sur l'effet qu'a eu le temps de passage du sang dans les branchies sur le transfert des gaz pour tester l'hypothèse que l'efficacité de l'excrétion du CO_2 est sensible aux changements du débit sanguin compte tenu des limites de l'équilibre chimique alors que l'efficacité du transfert d'O_2 est insensible aux changements du débit sanguin. L'insensibilité de PaO_2 aux changements de $\dot{V}b$ *in vivo* a réitéré la théorie que le transfert d'O_2 se comporte comme un système limité par la perfusion. Par contre, la sensibilité de l'efficacité du transfert du CO_2 aux changements de $\dot{V}b$ accompagnée de l'impact qu'a la disponibilité de CA suggèrent fortement que les limites de diffusions apparentes lors de l'excrétion du CO_2 sont en fait des limites d'équilibre chimique.

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Abbreviations

Δ, Greek letter delta

αCO_2, carbon dioxide solubility

β, non-bicarbonate buffering capacity

βNHE, β activated sodium-proton exchanger

A, surface area

AE, anion exchange

BSA, bovine serum albumin

CA, carbonic anhydrase

CCO_2, total carbon dioxide concentration

D, diffusion distance

Hb, haemoglobin

HPLC, High pressure liquid chromatography

K, Krogh's coefficient

$\dot{M}_{gas}$, gas exchange

N, number of samples or individuals

PCO_2, partial pressure of carbon dioxide

$PaCO_2$, arterial partial pressure of carbon dioxide

$PvCO_2$, venous partial pressure of carbon dioxide

PO_2, partial pressure of oxygen

PaO_2, arterial partial pressure of oxygen

PvO_2, venous partial pressure of oxygen

pHa, arterial pH

pHv, venous pH

RBC, red blood cell

SEM, standard error of the mean

V_{amp}, ventilation amplitude

$\dot{V}b$, cardiac output

V_f, ventilation frequency

$\dot{V}w$, ventilatory water flow

CHAPTER 1

GENERAL INTRODUCTION

Introduction

The fish gill is a complex organ involved in many functions including osmoregulation, ionoregulation, acid-base balance and its primary function, gas exchange. Gas transfer, especially carbon dioxide excretion, and acid-base regulation are closely linked (see review by Perry 1986; Gilmour 1997; Tufts and Perry 1998) and these areas will be the main focus of this thesis. The lamellae are the sites of gas transfer in the gills. Inspired water flows parallel to the lamellae, which are perfused with blood flowing in the opposite direction of the water flow; this results in a highly efficient countercurrent system of gas transfer. Gas transfer occurs by diffusion which is driven by the partial pressure gradient of the gases between the blood and the environment. Therefore, by maintaining partial pressure gradients, a countercurrent system (unlike a co-current system) allows gas exchange along a greater surface of the respiratory organ. Other than the partial pressure gradient between the blood and water (ΔP_{gas}), the exchange of gas also depends on the surface area (A) and the thickness of the diffusion barrier (D). The key principles for gas exchange can be summarised by Fick's equation: $\dot{M}_{gas} = K_{gas} * A * \Delta P_{gas}/D$. The two major gases transferred during respiration, oxygen and carbon dioxide, have different properties. The diffusion of a gas across a biological barrier can be determined by the Krogh's coefficient (K). The Krogh's coefficient takes into account the diffusion coefficient and the capacitance. The diffusion coefficient is inversely proportional to the square root of the molecular weight of the gas and the capacitance is defined as the change in the concentration of a gas for a given change in partial pressure. For instance, in the plasma, CO_2 has a high capacitance because it can be present as dissolved gaseous CO_2, HCO_3^- and CO_3^{2-} compared to oxygen, which is present only as

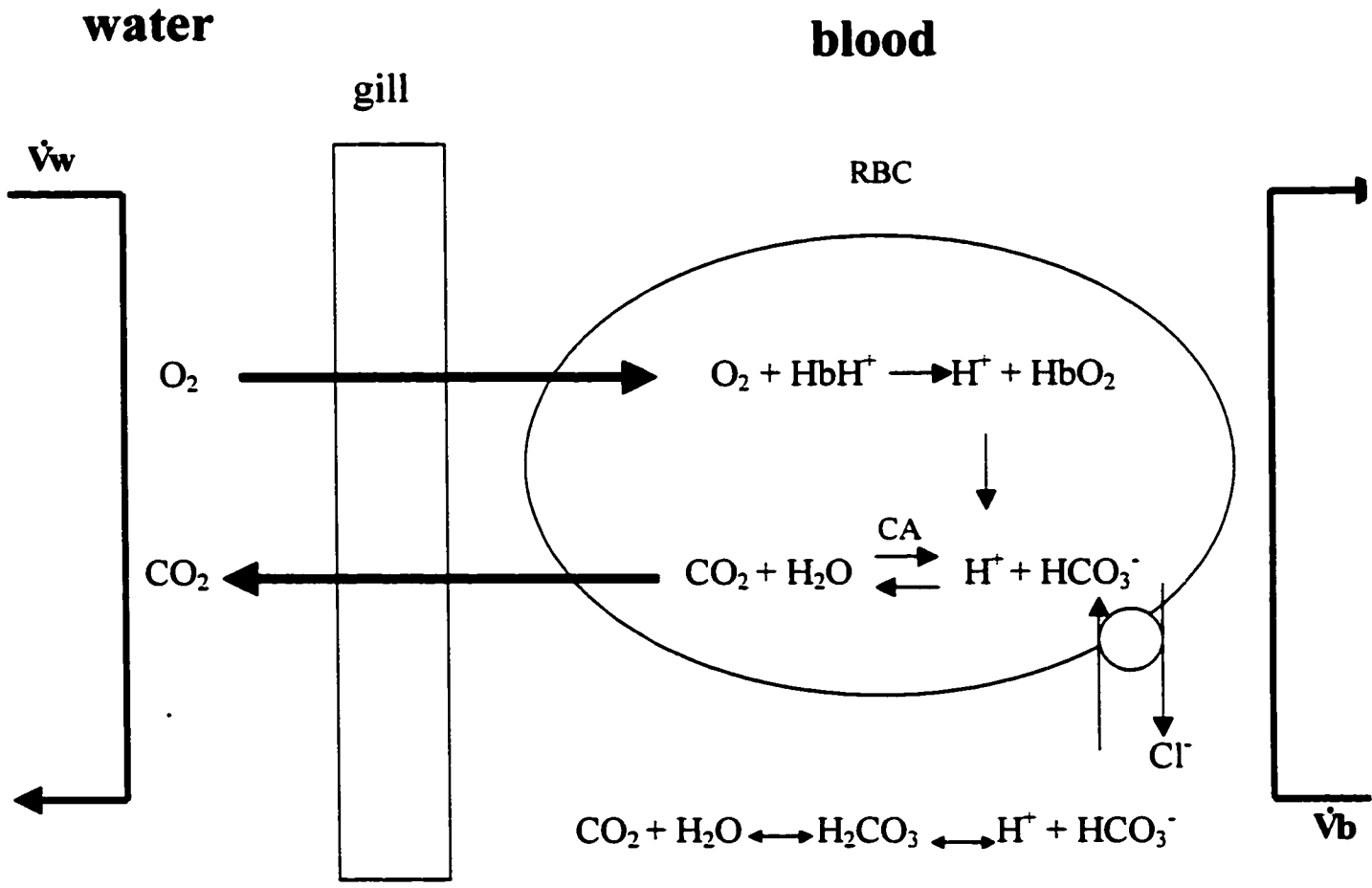
dissolved gaseous O_2. Because of its high capacitance, CO_2 should diffuse across the respiratory barrier more rapidly than O_2 even though CO_2 is a slightly larger molecule. However, this is not always the case during gas exchange *in vivo* (Piiper 1989; Swenson 1990).

Factors influencing gas exchange and transport in the blood

Figure 1-1 presents a general overview of gas transport in the blood along with gas exchange at the level of the gills. Oxygen diffuses across the lamellae and into the blood where it binds to haemoglobin in the erythrocytes. Haemoglobin plays a crucial role by transporting oxygen to the tissues and by allowing an increase in oxygen concentration without augmenting the partial pressure, thereby optimising the gradient for O_2 uptake (reviewed by Nikinmaa and Salama 1998). Carbon dioxide transport and excretion are considerably more complex than oxygen uptake. Metabolism produces molecular CO_2 at rates exceeding the carrying capacity of the blood for physically dissolved CO_2 (Boutilier et al. 1984). The ability of the blood to carry sufficient amounts of carbon dioxide relies on the conversion of CO_2 to HCO_3^-. In fact, over 90% of the carbon dioxide in the blood compartment is carried as HCO_3^-; the physiological pH of rainbow trout blood (<8.0) does not allow for significant formation of carbonate (CO_3^{2-}; reviewed by Tufts and Perry 1998). CO_2 produced by aerobic metabolism diffuses into the erythrocyte and is converted to HCO_3^- by carbonic anhydrase (CA). HCO_3^- exits the red blood cell (RBC) via an electroneutral Cl^-/HCO_3^- exchanger to be transported in the plasma. At the level of the gills, the same chain of reactions, however reversed, occurs

Figure 1-1. General diagram of gas exchange and transport at the level of the gill.

Arrows coupled with cardiac output ($\dot{V}b$) and ventilatory volume ($\dot{V}w$) represent the countercurrent system of water and blood. The uptake of oxygen by haemoglobin (Hb) releases protons (H^+) that are utilised in the dehydration of HCO_3^- catalysed by carbonic anhydrase (CA) in the red blood cell (RBC). Note that the same reaction in the plasma is slow owing to the absence of CA in the plasma of teleost fish.


water
blood
gill
$\dot{V}w$
RBC
O_2
$O_2 + HbH^+ \rightarrow H^+ + HbO_2$
CA
CO_2
$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$
Cl^-
$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$
$\dot{V}b$

for the excretion of CO_2 as shown in Figure 1-1. HCO_3^- enters the red blood cell through the same Cl^-/HCO_3^- exchanger to be dehydrated by CA and CO_2 is excreted by diffusion across the gills. The conversion of HCO_3^- to CO_2 is essential for the excretion of carbon dioxide across the gill epithelium because it is impermeable to HCO_3^- (Perry et al. 1982).

There are many crucial steps in the transport and exchange of the respiratory gases. Although this present thesis will consider both oxygen and carbon dioxide, emphasis will be placed on carbon dioxide. First, carbonic anhydrase (CA) is an important factor in CO_2 transport and excretion. It is an enzyme that catalyses the reversible hydration/dehydration reaction of carbon dioxide ($CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$). The time of the uncatalysed dehydration reaction of carbon dioxide is approximately 20 sec^{-1} (Edsall 1969) and exceeds the estimated 1 to 3 seconds residence time of blood in the gills (Cameron and Polhemus 1974). The high turnover rate type II isozyme of CA is present in high concentration in the erythrocytes of all vertebrates except agnathans and elasmobranches which possess the low turnover rate type I isozyme (Henry and Heming 1998). In contrast to air-breathing vertebrates, the membrane bound CA (type IV isozyme) does not provide CA activity to the plasma of fish at the level of the gills to facilitate the dehydration of HCO_3^- for CO_2 excretion, except in elasmobranchs (reviewed by Henry and Swenson 2000). Therefore, in the gills of teleost fish, the entry of HCO_3^- in the erythrocyte via the Cl^-/HCO_3^- exchanger is essential to access the CA activity for the excretion of molecular CO_2.

The Cl^-/HCO_3^- exchanger is also known as band 3 protein and is part of an anion exchanger (AE) gene family. This protein is the most abundant integral membrane protein in erythrocytes (Romano and Passow 1984). Lepke et al. (1976) estimated 10^6

molecules per cell, which is 25% of the total membrane protein. The band 3 protein permits the electroneutral exchange of HCO_3^- and Cl^- between the plasma and the red blood cell. This process is also called the chloride shift or the Hamburger shift. As mentioned previously, the absence of CA activity in the plasma of teleost fish requires the entry of HCO_3^- into the erythrocyte. Consequently, the Cl^-/HCO_3^- exchanger is also an important factor in the excretion of CO_2 by admitting HCO_3^- into the RBC. Perry et al. (1982) suggested that the slow entry rate of HCO_3^- into the RBC via the band 3 protein is the limiting factor in CO_2 excretion. The abundance of CA activity in the erythrocyte ensures a fast conversion of HCO_3^- to CO_2. Furthermore, the diffusion of CO_2 across the gill should not be a limiting factor since its diffusibility is 30 fold greater than the diffusibility of oxygen (Swenson 1990). This leaves the chloride shift as the slowest step in the cascade of events. This will be investigated and discussed further in chapters 2 and 3.

Another significant factor in CO_2 transport and excretion as well as acid-base balance is the buffering capacity of the blood and plasma. In fish blood, both bicarbonate and non-bicarbonate buffers contribute to overall buffering capacity. However, because the pK of the bicarbonate buffer system (~6.1) is far from blood pH (7.8-8.0), it is not an effective buffer. The most significant non-bicarbonate buffers are negatively charged proteins and because of the large concentration of haemoglobin in the blood, this makes it the principal buffer (see review by Tufts and Perry 1998). This causes the whole blood to have a higher buffering capacity (-9.7 mmol L^{-1} pH $units^{-1}$) compared to separated plasma (-2.6 mmol L^{-1} pH $units^{-1}$; Wood et al. 1982). In addition to trapping protons, haemoglobin also provides H^+ for the dehydration of HCO_3^- either through buffering or

the Haldane effect. As reviewed by Brauner and Randall (1998), the Haldane effect is the change in affinity between haemoglobin and protons resulting from a change in the partial pressure of oxygen. Thus, at constant PCO_2, deoxygenated blood contains a greater amount of CO_2 than oxygenated blood. As shown in Figure 1-1, oxygen uptake and carbon dioxide excretion are closely linked. The increase in PO_2 in the gills causes O_2 to bind to haemoglobin and this coincides with the release of protons. These protons are then used in the dehydration of HCO_3^- for the excretion of CO_2. Chapter 2 will concentrate on the role of plasma buffering capacity in the excretion of CO_2.

As mentioned previously, the exchange of gas is driven by the partial pressure difference between the blood and the environment. This is maintained by the convective processes which assures the movement of the media transporting the respiratory gases (reviewed by Perry 1986; Piiper, 1989; Gilmour 1997). The ventilation volume ($\dot{V}w$) dictated by ventilation amplitude (V_{AMP}) and frequency (V_f) assures the constant replenishment of water at the surface of the lamellae, delivering O_2 and removing the excreted CO_2. Meanwhile, cardiac output ($\dot{V}b$) mediates the perfusion of the gills with blood. This thesis will focus on the internal convective process (i.e.$\dot{V}b$). $\dot{V}b$ also determines the residence time of the blood in the gill and thus can impact on gas transfer efficiency depending on prevailing diffusion and perfusion limitations.

Diffusion *versus* perfusion limitations

Throughout chapter 3, the concept of diffusion and perfusion limitations will be discussed (see review by Perry 1986; Gilmour 1997). Diffusion limitations occur when

the time required for diffusion of the gas across the respiratory organ exceeds the blood residence time at the gas exchange surface, in this case, the lamellae. In a diffusion limited system, equilibrium between the blood and the respiratory medium cannot be attained within the blood transit time. Therefore, the efficiency of the gas exchange is inversely proportional to $\dot{V}b$ because a lower $\dot{V}b$ allows more time for gas exchange and a greater $\dot{V}b$ offers less time for the diffusion of the same gas across the respiratory organ. In opposition, perfusion limitations implies that the diffusion of the respiratory gas across the gas exchange area reaches equilibrium prior to the blood exiting the respiratory organ. The efficiency of gas transfer in a perfusion limited system will not be affected by changes in $\dot{V}b$ in the physiological range. However, a perfusion limited system may become diffusion limited when blood transit time in the respiratory organ approaches the equilibration time of the respiratory gas. The reverse is also true, a diffusion limited system may become perfusion limited if the blood residence time at the gas exchange surface is slower than the equilibration time of the gas. It is generally accepted that oxygen uptake acts as a perfusion limited system and carbon dioxide excretion behaves as a diffusion limited system even though CO_2 diffusibility is 30 fold greater than O_2 (Daxboeck et al. 1982; Pärt et al. 1984; Malte and Weber 1985; Piiper 1989; Swenson 1990; Brauner et al. 2000). The causes of the diffusion limitations for CO_2 excretion will be investigated and discussed in chapter 3.

Goals and hypothesis

In chapter 2, the hypothesis that proton availability limits the dehydration of HCO_3^- within the plasma, will be tested. The basic experimental protocol will be to increase the buffering capacity of rainbow trout plasma *in vivo* and *in vitro* in the presence or absence of exogenous CA within the extracellular compartment. An extracorporeal blood shunt will be used to measure blood gases *in vivo* and a radioisotopic assay will be employed to assess the dehydration rate of HCO_3^- on separated plasma *in vitro*. In addition, chapter 2 will examine the role of the Cl^-/HCO_3^- exchanger in the excretion of CO_2. The hypothesis is that the slow rate of the RBC Cl^-/HCO_3^- exchanger limits CO_2 excretion. If so, the administration of exogenous CA to plasma should enhance CO_2 excretion and if extracellular buffers are also limiting, the effect will be greatest in fish with increased plasma buffering capacity

In chapter 3, the sensitivity of CO_2 excretion and O_2 uptake to changes in blood flow will be evaluated using an extracorporeal blood shunt. $\dot{V}b$ will be manipulated via volume loading or plasma removal to modify the blood transit time through the gill. Moreover, utilising the findings of chapter 2, I will investigate the role of chemical equilibrium constraints in the apparent diffusion limitation for CO_2 excretion. I predict that CO_2 excretion, by acting as a diffusion-limited system, will be sensitive to changes in $\dot{V}b$ whereas O_2 uptake, as a perfusion-limited system, will be insensitive to changes in $\dot{V}b$. Finally, the addition of exogenous CA should eliminate the effects of altered $\dot{V}b$ on CO_2 excretion if the apparent diffusion limitation is caused by chemical equilibrium constraints.

CHAPTER 2

THE EFFECTS OF EXOGENOUS EXTRACELLULAR CARBONIC ANHYDRASE ON CO_2 EXCRETION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*): ROLE OF PLASMA BUFFERING CAPACITY

Chapter 2 is based on manuscripts published in Proceedings of an International Fish Physiology Symposium 2000: 61-76, and in the Journal of Comparative Physiology B 171: 465-473 (2001).

Abstract

The buffering capacity (ß) of rainbow trout (*Oncorhynchus mykiss*) plasma was manipulated prior to intravascular injection of bovine carbonic anhydrase (CA) to test the hypothesis that proton (H^+) availability limits the catalysed dehydration of HCO_3^- within the extracellular compartment. An extracorporeal blood shunt was employed to continuously monitor blood gases *in vivo* in fish exhibiting normal plasma β (-3.9 ± 0.3 mmol L^{-1} pH $unit^{-1}$), and in fish with experimentally (using HEPES) elevated plasma β (-12.1 ± 1.1 mmol L^{-1} pH $unit^{-1}$). An injection of 5 mg kg^{-1} CA equally reduced (after 90 min) the arterial partial pressure of CO_2 ($PaCO_2$) in trout with regular (-0.23 ± 0.05 Torr) or high (-0.20 ± 0.05 Torr) plasma β; saline injection was without effect. Because ventilation and venous blood gases were unaffected by CA, the effect of extracellular CA in lowering $PaCO_2$ was likely caused solely by a specific enhancement of CO_2 excretion owing to acceleration of HCO_3^- dehydration within the plasma.

The lowering of $PaCO_2$ in trout after injection of exogenous CA provides the first *in vivo* evidence that the accessibility of plasma HCO_3^- to red blood cell (RBC) CA constrains CO_2 excretion under resting conditions. Because the velocity of RBC Cl^- /HCO_3^- exchange governs HCO_3^- accessibility to RBC CA, the present study also provides evidence that CO_2 excretion at rest is limited by the relatively slow rate of Cl^- /HCO_3^- exchange. The effect of CA in lowering $PaCO_2$ was unrelated to plasma buffering capacity. However, using a radioisotopic assay, an increase in plasma β (from –4.9 to –12 mmol L^{-1} pH $unit^{-1}$) increased the *in vitro* HCO_3^- dehydration rate of separated plasma significantly in both the absence and presence of bovine CA. While these data could suggest that H^+ availability does not limit extracellular HCO_3^-

dehydration *in vivo* at resting rates of CO_2 excretion, it is more likely that the degree to which plasma β was elevated in the present study was insufficient to drive a substantially increased component of HCO_3^- dehydration through the plasma and the high availability of protons for HCO_3^- dehydration in the red cell *in vivo* compensates for the relatively slow step of HCO_3^- entry into the erythrocyte.

Introduction

Carbon dioxide excretion in teleost fish, as in mammals, relies extensively on the catalysed dehydration of HCO_3^- (Perry 1986; Gilmour 1997; Tufts and Perry 1998). According to current models (e.g. Tufts and Perry 1998), the vast majority of the CO_2 that is excreted across the teleost gill is derived from plasma HCO_3^-. The rate of the uncatalysed HCO_3^- dehydration reaction is slow (rate constant > 20 sec^{-1}; Edsall 1969) relative to gill transit time (1 – 3 sec; Cameron and Polhemus 1974). Thus, given the inaccessibility of carbonic anhydrase (CA) to catalyse plasma reactions (Gilmour 1998a), the principal site of CO_2 formation is generally believed to be the CA-rich red blood cell (RBC) rather than the plasma. The accessibility of plasma HCO_3^- to the RBC CA is governed by an electroneutral Cl^-/HCO_3^- exchanger (AE1 or Band-3 protein) on the RBC membrane (Cameron 1978; Romano and Passow 1984; Hubner et al. 1992). Because Cl^-/HCO_3^- exchange is the slowest in the series of reactions encompassing CO_2 excretion, it has been identified as the rate-limiting step in CO_2 excretion (Perry 1986). However, while there is *in vitro* evidence to support the theory that Cl^-/HCO_3^- exchange (and hence accessibility of HCO_3^- to RBC CA) constrains overall CO_2 excretion at rest (Perry and Gilmour 1993), the available *in vivo* evidence does not unequivocally support this view.

If the accessibility of plasma HCO_3^- to RBC CA in resting fish were indeed limited by Cl^-/HCO_3^- exchange, one would predict that injection of CA into the plasma would cause a transient increase in CO_2 excretion and be manifested by a reduction in arterial PCO_2 ($PaCO_2$). However, Wood and Munger (1994) reported that the effects of CA injection (10 mg kg^{-1}) into resting rainbow trout were similar to those of saline injection - very minor decreases in $PaCO_2$ were observed, changes that were close to the limit of reliable detection. Similar results (small increases in arterial pH (pHa), little or no change in $PaCO_2$ or blood total CO_2 content) were obtained in subsequent studies on resting trout (Gilmour et al. 1994; Lessard et al. 1995).

Under normal conditions, the protons required for HCO_3^- dehydration are provided primarily by haemoglobin, either through its buffering capacity or the Haldane effect (see Gilmour 1997; Henry and Heming 1998; Tufts and Perry 1998). The non-bicarbonate buffer capacity (β; $\Delta HCO_3^-/\Delta pH$) of rainbow trout plasma is low (-2.6 mmol L^{-1} pH $unit^{-1}$) compared to whole blood β (-9.7 mmol L^{-1} pH $unit^{-1}$; Wood et al. 1982). Thus, the results, and hence the conclusions of previous studies utilising injections of CA (see above) may have been confounded by an inadequate availability of protons (H^+) for HCO_3^- dehydration within the plasma. Indeed, Heming and Bidani (1992) showed that CO_2 excretion in a perfused rat lung (where CA activity is available to plasma HCO_3^-) was reliant on the buffer capacity of the perfusate. In lungs where the buffering capacity was increased with HEPES, CO_2 excretion was higher than in lungs with lower buffering capacity. If a similar situation (inadequate extracellular buffering) exists in fish, previous studies designed to assess RBC Cl^-/HCO_3^- limitations by injecting CA into resting fish may have been designed inadequately.

Thus, the objective of the present study was to test the hypothesis that an increase in plasma non-bicarbonate buffering capacity would increase the extent to which exogenous CA activity enhances plasma HCO_3^- dehydration in rainbow trout both *in vivo* and also *in vitro*. Furthermore, the experiments were designed to test, under more appropriate conditions, the hypothesis that RBC Cl^-/HCO_3^- exchange constrains CO_2 excretion at rest.

Materials and methods

Experimental animals

Rainbow trout (*Oncorhynchus mykiss*; 447 ± 20 g; experimental N = 91) of either sex were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario). Fish were maintained on a 12L:12D photoperiod in large fibreglass aquaria supplied with flowing, aerated and dechlorinated City of Ottawa tap water at 13°C. Trout were fed to satiation on alternate days on a diet of commercial trout pellets; food was withheld for 24 h prior to experimentation. Fish were allowed at least two weeks to acclimate to the holding conditions before any experiments were performed. Two groups of fish were used; one group (N = 37) acted as blood donors for *in vitro* measurements of plasma buffering capacity, CA activity and for the radioisotopic assay, while blood respiratory variables were measured *in vivo* using an extracorporeal blood circulation on the second group of trout (N = 54).

Fish were anaesthetised by immersion in an oxygenated solution of benzocaine (ethyl-*p*-aminobenzoate; 0.1 g L^{-1}), then placed on a surgical table that allowed continuous irrigation of the gills with the same anaesthetic solution. For the fish used as

blood donors for *in vitro* measurements, a single indwelling cannula (Clay-Adams PE50 polyethylene tubing) was implanted into the dorsal aorta according to the basic method of Soivio et al. (1975). For continuous measurements of blood respiratory variables *in vivo* using the extracorporeal blood shunt, the caudal vein and caudal artery were cannulated. Briefly, a lateral incision was made at the level of the caudal peduncle to allow the epaxial and hypaxial musculature to be separated and the haemal arch to be exposed. Catheters (PE50) were then inserted into the caudal vein and caudal artery in the anterior direction, the incision was closed with silk sutures and both cannulae were secured with ligatures to the skin. Small brass plates (1 cm^2) were sutured to the external surface of each operculum to allow the measurement of ventilation parameters using an impedance converter. After surgery, fish were placed in individual opaque acrylic boxes supplied with flowing, aerated water (flow rate > 2.5 L min^{-1}) for a 24 h recovery period. Cannulae were flushed with heparinised (100 i.u. mL^{-1} sodium heparin) Cortland saline (Wolf 1963).

Extracorporeal circulation and experimental protocol *in vivo*

To assess the effects of exogenous CA injection on CO_2 excretion *in vivo*, an extracorporeal blood circulation was used to monitor arterial or venous blood CO_2 tension ($PaCO_2$ and $PvCO_2$, respectively) and pH (pHa and pHv, respectively) for 90 min following the injection of either 5 mg kg^{-1} bovine CA (in saline) or the saline vehicle alone. The effects of these treatments in fish at their naturally-occurring plasma non-bicarbonate buffering capacity (control β) were compared with those in fish that had

received an injection of HEPES approximately 2 h prior to CA or saline injection to elevate plasma β (high β).

Blood respiratory variables were continuously monitored using an extracorporeal blood shunt in which blood was withdrawn from the caudal artery or caudal vein using a peristaltic pump and passed through an external circuit containing PO_2, PCO_2 and pH electrodes (Thomas 1994). The flow rate through the external loop, which contained approximately 1 mL of blood, was 0.6 mL min^{-1}. To prevent clotting, the circuit was rinsed for 10-15 min with heparinised (540 i.u. mL^{-1}) saline before initiating blood flow. Arterial or venous blood pH, PCO_2 and PO_2 were measured using Metrohm (pH) and Radiometer or Cameron Instruments (CO_2, O_2) electrodes housed in thermostatted cuvettes and connected to a blood gas analyser (Cameron Instruments). Prior to each experiment, the pH electrode was calibrated by pumping precision buffer solutions through the circuit; water equilibrated with appropriate gas mixtures (supplied by a GF-3/MP gas mixing flowmeter; Cameron Instruments) was used to calibrate the blood gas electrodes. The frequency and amplitude of opercular displacements were assessed as indices of ventilation using a custom-built impedance converter that detected and quantified the changes in impedance between the brass plates attached to the opercula. All analog signals (blood gases, pH and impedance) were converted to digital data and stored by interfacing with a data acquisition system (Biopac Systems Inc.; sampling frequency 10 – 40 samples sec^{-1}) using Acknowledge™ data acquisition software (v 3.03) and a Pentium™ PC.

Three series of experiments were carried out. In the first series (high β), blood withdrawal was via the caudal artery cannula and thus arterial blood gases and pH were

recorded. Following initialisation of the extracorporeal circulation and stabilisation of the measured ventilatory and blood gas variables (usually within 20 – 30 min of starting the blood shunt), fish were given an intra-arterial injection of HEPES solution (2 mL kg^{-1} of 1.5 mol L^{-1} stock solution over 4-5 min) to achieve a nominal final circulating concentration of 10 mmol L^{-1} HEPES, and were then monitored for 80 – 160 min to allow the blood gas and ventilatory readings to re-stabilise. At the end of this period, the measured variables were recorded for 10 min, following which either bovine CA (5 mg kg^{-1} in saline) or the saline vehicle alone (1 mg kg^{-1}) was administered via the caudal vein. Blood gas and ventilatory variables were recorded for 90 min post CA or saline injection, and plasma β was measured at the end of the experiment (see below). The experimental protocol followed in the second series of experiments (control β) was identical to that used in the first series, with the exception that the fish were not treated with HEPES. The third series of experiments (venous loops) repeated the second series, but with blood withdrawal via the caudal vein cannula such that venous blood gases and pH were recorded. In this experimental series, the CA or saline was injected into the caudal artery, and blood gas and ventilatory variables were recorded for 60 min post-injection.

Validation experiments

Two additional series of experiments were carried out, one to validate the procedure used to elevate the plasma non-bicarbonate buffering capacity, and one to assess the longevity of elevated plasma CA activity following bovine CA injection. In

both cases, fish fitted only with a dorsal aortic cannula were used and measurements were made *in vitro* on blood samples withdrawn from the fish.

The efficacy of HEPES injection in elevating the plasma non-bicarbonate buffering capacity was assessed by constructing buffer curves for separated plasma samples obtained from rainbow trout 1, 2 or 6 h after the administration of HEPES solution (2 mL kg^{-1} of 1.5 mol L^{-1} stock solution over 4-5 min via the dorsal aorta); plasma samples were also obtained from untreated fish as a control. In all cases, 5 mL of blood was withdrawn from the dorsal aorta, heparinised (250 i.u. mL^{-1}), and immediately centrifuged to yield separated plasma. The plasma samples were gassed first with 1% CO_2 for approximately 5 min, and then five times briefly with N_2. Between bouts of gassing, the plasma samples were kept on ice under airtight conditions in glass scintillation vials and swirled intermittently. Following each bout of gassing, approximately 0.7 mL of plasma were withdrawn and analysed for total CO_2 concentration (CCO_2; 20 μL in triplicate; Capni-Con 5 total CO_2 analyser; Cameron Instruments) and pH (E301 glass pH and reference electrodes in a thermostatted blood gas cell connected to a blood gas analyser; Cameron Instruments). Total CO_2 concentration was plotted against pH and the slope of the resultant linear regression line was taken to be the separated plasma non-bicarbonate buffering capacity (β).

To quantify the extent to which bovine CA injection into rainbow trout elevated plasma CA activity and to monitor the longevity of elevated plasma CA activity post-injection, the electrometric ΔpH method described by Henry (1991) and modified by Gervais and Tufts (1998) was used to measure the CA activity in plasma samples. Blood samples (0.2 mL) were withdrawn from the dorsal aorta of rainbow trout prior to, and 5,

15, 30, 60 and 90 min following a bolus injection of bovine CA (5 mg kg^{-1} in 1 mL saline). Blood samples were immediately centrifuged to yield plasma, which was diluted 100-fold with reaction medium (in mmol L^{-1}, 10 Tris-PO_4, 225 mannitol, 75 sucrose, pH 7.40) and held on ice until all samples from a single fish could be assayed at the same time. Samples (25 μL) of the diluted plasma were added to a reaction vessel (4°C) containing 10 mL of the reaction medium, and the reaction was initiated by the addition of CO_2-saturated water (400 μL, 4°C). The initial velocity of the reaction was measured over a change of approximately 0.15 pH units. To obtain the true catalysed rate, the uncatalysed rate (addition of CO_2-saturated water in the absence of any CA source) was subtracted from the observed rate. A Metrohm combined pH electrode connected to a Radiometer PHM73 blood gas analyser was used to measure pH.

Radioisotopic assay *in vitro*

The objective of the final series of experiments was to assess HCO_3^- dehydration rates *in vitro* in separated plasma under various conditions of β, CA activity and plasma HCO_3^- concentrations ($[HCO_3^-]$). HCO_3^- dehydration rates were measured using the radioisotopic assay of Wood and Perry (1991). Approximately 20 mL of separated plasma was required for a typical single experimental run (*i.e.* N = 1). Thus, it was necessary to use pooled blood obtained by slow withdrawal from the dorsal aortic cannulae of 2-3 fish. Following blood withdrawal/pooling and separation by brief centrifugation to obtain plasma, 0.6 mL of 180 mmol L^{-1} HEPES was added to one half of the plasma pool to achieve a nominal final concentration of 10 mmol L^{-1} HEPES; β was measured for both the control (*i.e.* naturally-occurring β) and HEPES-treated plasma

(see above). For each β, three levels of CA activity (0, 0.001 and 1 mg mL^{-1}) and two levels of HCO_3^- (nominally 10 and 15 mmol L^{-1}) were tested. Bovine CA was added (50 µl injection volume, bovine CA dissolved in saline) immediately prior to assaying the sample. Plasma HCO_3^- levels were elevated abruptly at the start of the assay by 'spiking' the plasma with 10 or 20 µL of isotope prepared in 500 mmol L^{-1} $NaHCO_3$. The nominal HCO_3^- levels were verified by analysing the plasma total CO_2 concentration (CCO_2); actual HCO_3^- concentrations were 11.0 ± 0.3 (48) and 15.5 ± 0.3 (48) mmol L^{-1} [mean ± SEM (N)]. Plasma pH was measured prior to and following the assay (pH glass and reference electrodes in a thermostatted blood gas cell connected to a blood gas analyser; Cameron Instruments).

The radioisotopic HCO_3^- dehydration assay was carried out as described by Wood and Perry (1991). In brief, plasma samples (0.8 mL) were equilibrated with a humidified gas mixture of 0.5% CO_2 in air (GF-3/MP gas mixing flowmeter; Cameron Instruments) for 60 min in a shaking water bath held at 10°C. To start the assay, 74 kBq of sodium [^{14}C] bicarbonate was added to each sample and the vial containing the sample was then immediately sealed with a cap containing a CO_2 trap (a filter paper impregnated with 150 µL hyamine hydroxide). At the end of the 3 min assay period, filter paper and plasma ^{14}C activities were determined by liquid scintillation counting (Packard TR 2500) with automatic quench correction. Filter papers were counted in 10 mL of Bio-Safe NA (Research Products Int.) while 50 µL of plasma was counted in 10 mL of ACS (Amersham) scintillation cocktail. Plasma CCO_2 was measured on 20 µL duplicate samples (Capni-Con 5 total CO_2 analyser; Cameron Instruments). The HCO_3^-

dehydration rate for each vial was then calculated by dividing filter paper ^{14}C activity by plasma specific activity and time.

Statistical analyses

Data are presented as mean values ± 1 standard error of the mean (SEM) (N). For *in vivo* experiments using the extracorporeal blood shunt, mean blood gas, pH and ventilatory data were compiled for 2 min periods for 10 min prior to, and 60 or 90 min post CA or saline injection. Owing to inter-individual variation and the small magnitude of the observed effects, blood gas and pH data for individual fish were normalised by subtracting from each data point the value at time = 0, the point of CA or saline injection. The effects of time and CA *versus* saline injection on blood gases, pH and ventilatory parameters within series 1 (high β) and 2 (control β) were analysed statistically using a two-way repeated measures analysis of variance (ANOVA) followed by the Bonferroni post-hoc multiple comparisons test, as appropriate. Pre-injection values for blood gases, pH and ventilatory variables were compared for the fish used in series 1 and 2 by means of a one-way ANOVA followed by the Bonferroni post-hoc multiple comparisons test, as appropriate; a similar procedure was used to analyse statistically the effect of HEPES treatment on separated plasma non-bicarbonate buffering capacity. A one-way repeated measures ANOVA followed by the Bonferroni post-hoc multiple comparisons test was used to analyse the effect of time on venous blood gases and pH (series 3 – venous loops), as well as on plasma CA activity, following administration of bovine CA. A two-way repeated measures ANOVA followed by Tukey's post-hoc multiple comparisons test (as appropriate) was used to analyse the *in vitro* HCO_3^- dehydration data for statistically-

significant differences. The fiducial limit of significance in all statistical analyses was 5%, and a commercial software package (Sigmastat v2.03) was used to perform all statistical analyses.

Results

Validation experiments

Measurements of the non-bicarbonate buffering capacity of separated plasma from untreated trout and from trout injected with HEPES to achieve a final (nominal) circulating concentration of 10 mmol L^{-1} revealed that HEPES treatment was successful in raising plasma β approximately three-fold *in vivo* (Fig. 2-1). Plasma β remained significantly elevated for at least 6 h after the administration of HEPES, providing ample time for the *in vivo* experiment to be carried out (~ 4 h was required to complete the CA-injection protocol in HEPES-treated fish).

The injection of bovine CA was similarly successful in elevating the CA activity associated with rainbow trout plasma (Fig. 2-2). As expected (Henry et al. 1993, 1997), no CA activity was detected in trout plasma under control conditions (Fig. 2-2). Addition of 5 mg kg^{-1} bovine CA to the circulation raised the plasma CA activity 5 min after injection to levels that were comparable to those associated with the RBC (500 000 μmol CO_2 min^{-1} mL^{-1}; Henry et al. 1993, 1997). While this very high level of plasma CA activity was achieved only transiently, plasma CA activities remained significant for at least 90 min following CA injection (Fig. 2-2), again providing sufficient time for the experimental protocol (fish were monitored for 90 min post-CA injection).

Effects of CA injection on blood gases, acid-base status and ventilation *in vivo*

Injection of bovine CA into trout that had previously (2 h earlier) been treated with HEPES to elevate plasma β (Fig. 2-1) resulted in a small, but significant lowering of arterial PCO_2 (Fig. 2-3). The decrease in $PaCO_2$ developed over 90 min to a maximum value of 0.20 ± 0.05 Torr, was not accompanied by a corresponding change in pHa, and was not observed in fish injected with the saline vehicle alone; the latter group exhibited no significant changes in $PaCO_2$ over the 90 min experimental period (Fig. 2-3). A slight, but significant, fall in PaO_2 was observed in HEPES-treated fish following either CA or saline injection (Fig. 2-3). The decrease was similar in both groups, amounting to 10 ± 3.0 Torr at 90 min.

Very similar results were obtained following CA or saline injection into fish at their naturally-occurring plasma β (Fig. 2-4). Again, $PaCO_2$ decreased significantly following CA injection but was unaffected by saline injection, and the magnitude of the $PaCO_2$ fall (0.23 ± 0.05 Torr at 90 min) was similar to that measured for HEPES-treated fish. In these fish, the lowering of $PaCO_2$ with CA injection was accompanied by a significant increase in pHa that occurred rapidly in comparison with the $PaCO_2$ response (Fig. 2-4). PaO_2 was essentially unaffected by either CA or saline injection in fish of normal β (Fig. 2-4).

Absolute values for blood gases, acid-base status and ventilation parameters (frequency and amplitude) for fish of normal and elevated plasma β prior to CA or saline injection are presented in Table 2-1. In general, there were no significant differences among the four treatment groups. The one exception was pHa, which was significantly higher (by about 0.1 pH units) in fish of normal plasma β than in HEPES-treated fish that

were subsequently injected with saline (Table 2-1). Ventilation frequency and amplitude were unaffected by saline or CA injection, remaining at the pre-injection values throughout the experimental period (Table 2-1). Venous blood gases were similarly unaffected by CA injection in fish of normal plasma buffering capacity (Table 2-2), although a small increase in venous pH, which was significant only 60 min after CA injection, was observed.

Effects of plasma β on HCO_3^- dehydration rates *in vitro*

HCO_3^- dehydration rates of separated plasma at the natural buffering capacity of the fish or at elevated buffering capacity (HEPES-treated plasma) were measured at three levels of CA activity and two HCO_3^- concentrations using the radioisotopic assay of Wood and Perry (1991). As expected on the basis of previous studies (e.g. Wood and Perry, 1991; Perry and Gilmour, 1993), the HCO_3^- dehydration rate for separated plasma was low in the absence of added CA activity and increased in a dose-dependent fashion with added bovine CA (Fig 2-5). Manipulation of the plasma HCO_3^- concentration had little effect on plasma HCO_3^- dehydration rates at any CA level (Fig 2-5). Addition of 10 mmol L^{-1} HEPES to separated plasma to elevate the non-bicarbonate buffer capacity significantly from -4.86 ± 0.73 to -12.1 ± 1.37 mmol L^{-1} pH $unit^{-1}$, approximately a 2.5 fold rise, increased the HCO_3^- dehydration rate significantly at all CA levels (Fig 2-5). The higher HCO_3^- dehydration rates in HEPES-treated plasma samples were achieved with plasma pH increases that were generally smaller, although not significantly so, than those in plasma samples at the naturally-occurring buffer capacity (data not shown).

Figure 2-1. The effects of an intravascular injection of HEPES (2 mL kg^{-1} of 1.5 mol L^{-1} stock solution to achieve a final nominal circulating concentration of 10 mmol L^{-1}) on the non-bicarbonate buffering capacity (β) of separated plasma from rainbow trout. Plasma samples were obtained 1, 2 or 6 h following HEPES injection (N = 6 for each group). Values for HEPES-treated trout were compared with those for untreated fish ("control"; N = 17). The plasma buffering capacity of the HEPES-treated fish used in extracorporeal experiments is also presented ("post-experiment"; N = 21). Data are presented as means ± 1 SEM. * indicates a significant difference from the control value ($P < 0.05$).

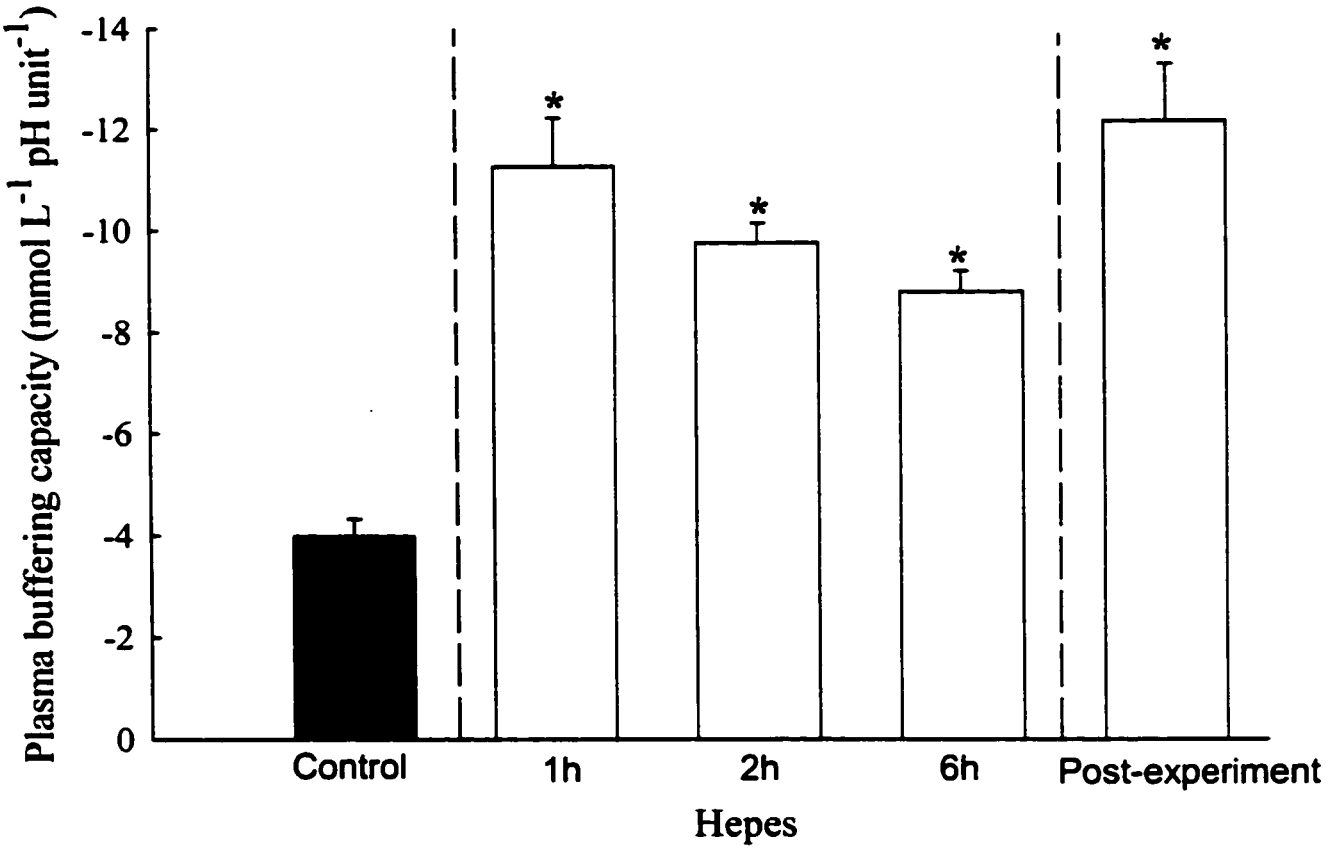

Plasma buffering capacity (mmol L-1 pH unit-1)
-14
-12
-10
-8
-6
-4
-2
0
*
*
*
*
Control
1h
2h
6h
Post-experiment
Hepes

Figure 2-2. Carbonic anhydrase (CA) activity associated with rainbow trout plasma before ("pre") and 5, 15, 30, 60 and 90 min after the intravascular injection of 5 mg kg^{-1} bovine CA (N = 6). Data are presented as mean values ± 1 SEM. ND signifies an undetectable level of CA activity. * indicates a significant difference from the "pre" value ($P < 0.05$).

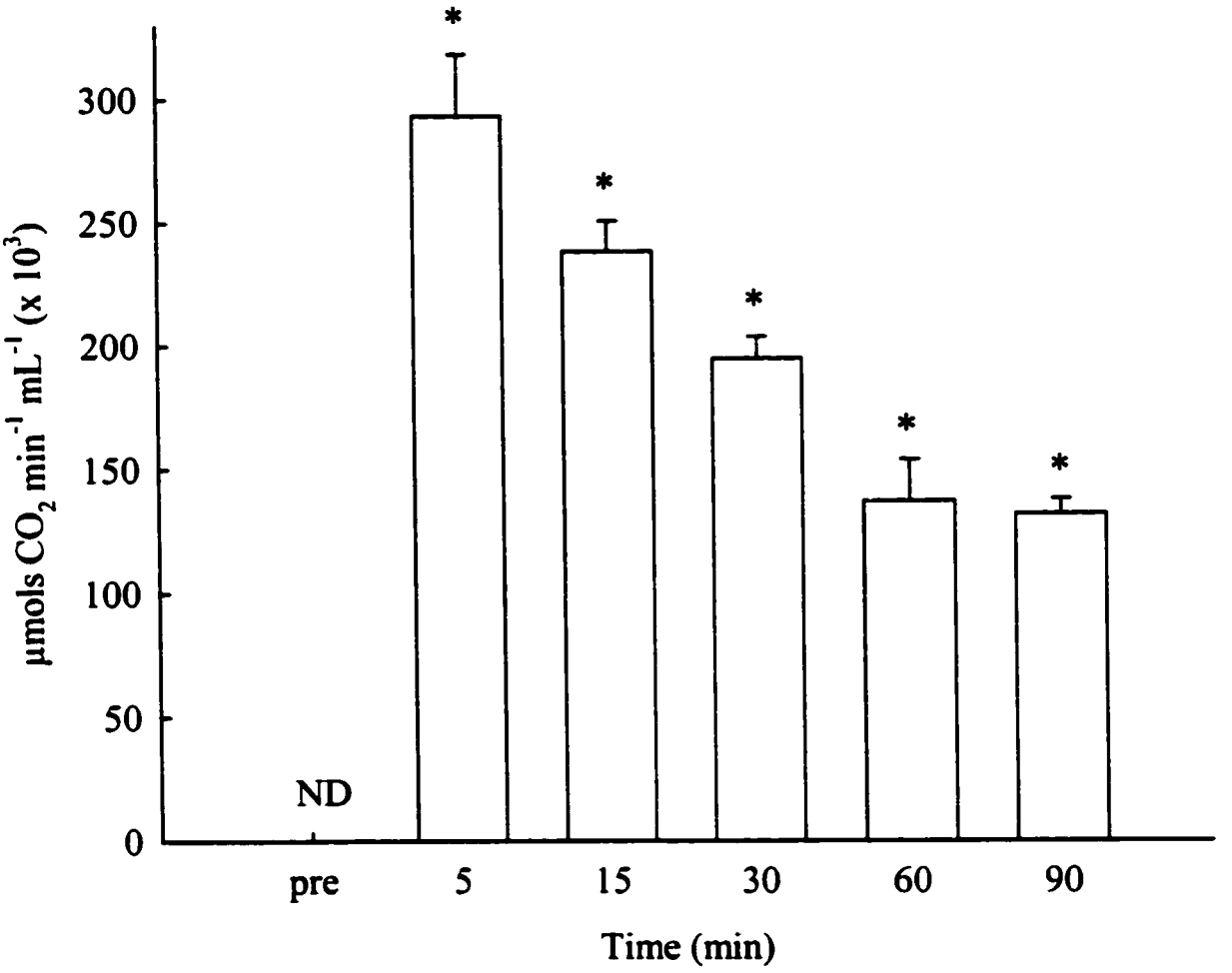

*
*
*
*
*
300
250
200
150
100
50
0
ND
pre
5
15
30
60
90
µmols CO2 min-1 mL-1 (x 10-3)
Time (min)

Figure 2-3. The effects of an intravascular injection of 5 mg kg^{-1} bovine carbonic anhydrase (CA) (filled circles; N = 8) or the saline vehicle alone (unfilled circles; N = 6) on changes in (A) arterial PCO_2 ($PaCO_2$), (B) arterial pH (pHa), and (C) arterial PO_2 (PaO_2) in rainbow trout treated with HEPES to elevate the plasma non-bicarbonate buffer capacity. The dashed vertical line at time = 0 min indicates the point of CA or saline injection. Data are presented as delta values which were obtained by subtracting from each point in the response the value at time = 0 min. * denotes a significant difference within a treatment group from the value prior to CA or saline injection ($P < 0.05$), while the double headed arrows indicate a significant difference between the CA- and saline-injected groups. Data are presented as mean values ± 1 SEM.

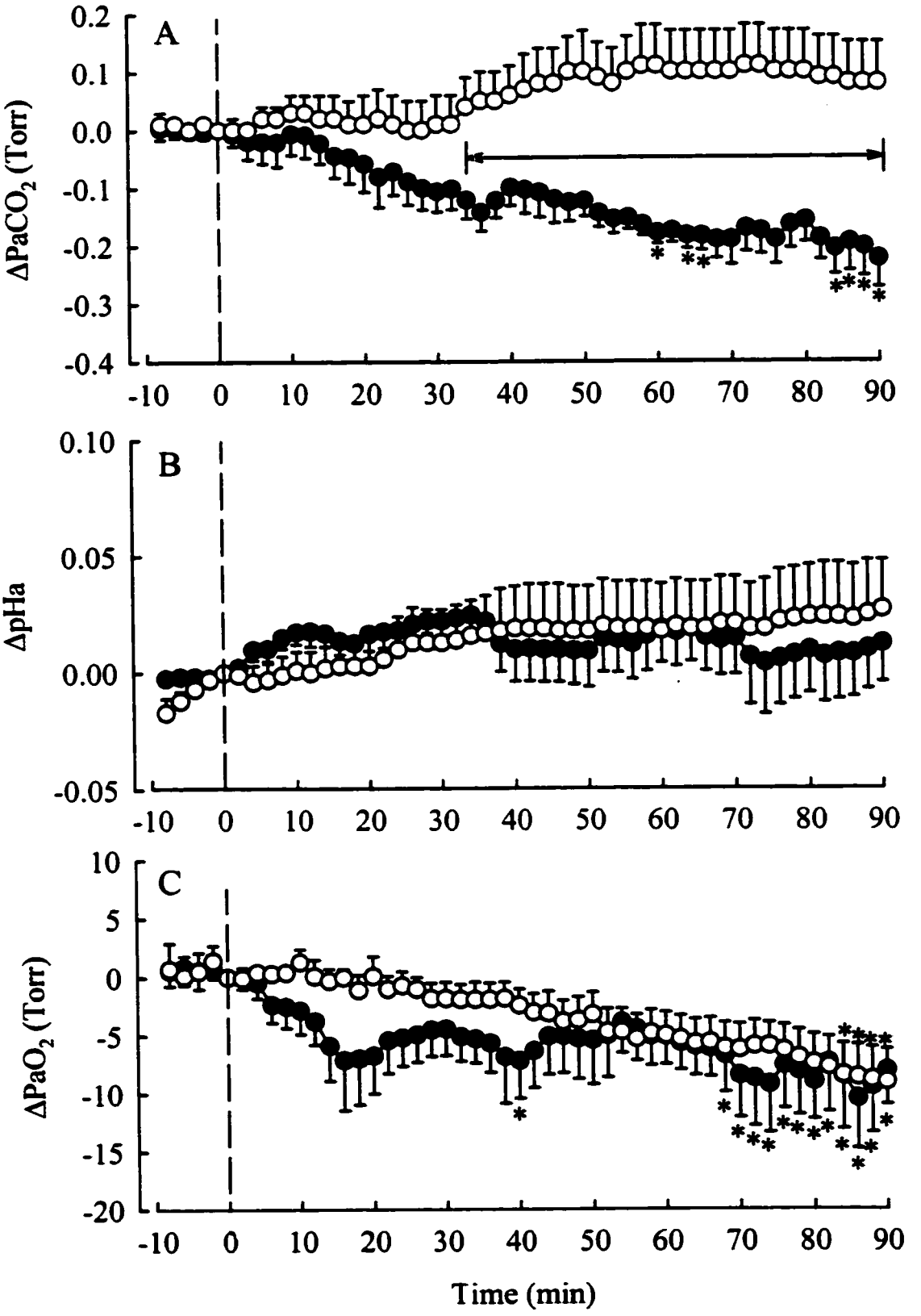

A
B
C
ΔPaCO2 (Torr)
ΔpHa
ΔPaO2 (Torr)
Time (min)
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
0.10
0.05
0.00
-0.05
10
5
0
-5
-10
-15
-20
-10
0
10
20
30
40
50
60
70
80
90

Figure 2-4. The effects of an intravascular injection of 5 mg kg^{-1} bovine carbonic anhydrase (CA) (filled circles; N = 22) or the saline vehicle alone (unfilled circles; N = 12) on changes in (A) arterial PCO_2 ($PaCO_2$), (B) arterial pH (pHa), and (C) arterial PO_2 (PaO_2) in rainbow trout at their naturally-occurring plasma non-bicarbonate buffer capacity. The dashed vertical line at time = 0 min indicates the point of CA or saline injection. Data are presented as delta values which were obtained by subtracting from each point in the response the value at time = 0 min. * denotes a significant difference within a treatment group from the value prior to CA or saline injection ($P < 0.05$), while † or double headed arrows indicate a significant difference between the CA- and saline-injected groups. Data are presented as mean values ± 1 SEM.

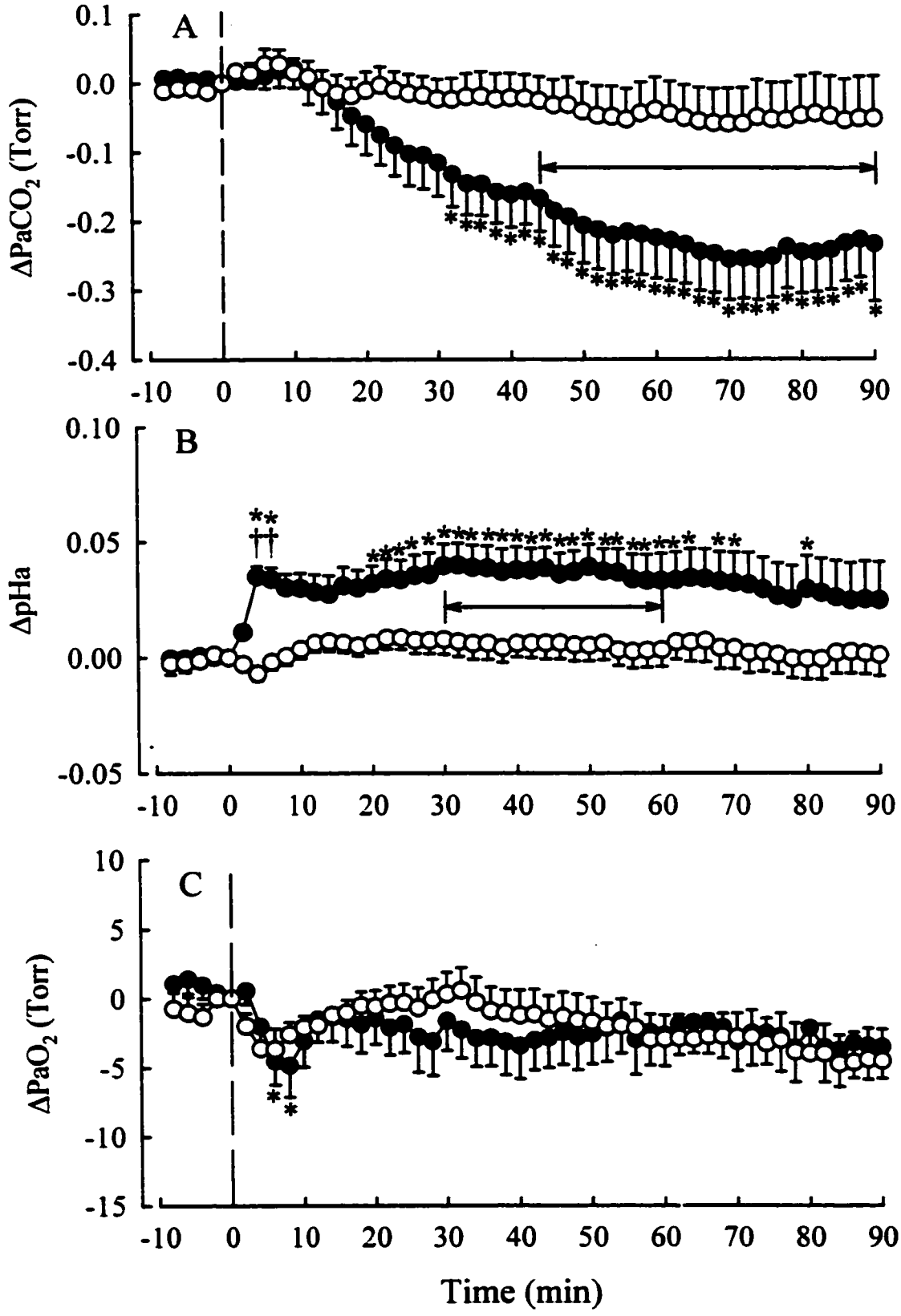

A
B
C
ΔPaCO2 (Torr)
ΔpHa
ΔPaO2 (Torr)
Time (min)
0.1
0.0
-0.1
-0.2
-0.3
-0.4
0.10
0.05
0.00
-0.05
10
5
0
-5
-10
-15
-10
0
10
20
30
40
50
60
70
80
90

Figure 2-5. Mean *in vitro* HCO_3^- dehydration rates (N = 6 for all groups) for separated plasma samples from rainbow trout under various conditions of non-bicarbonate buffer capacity (β), CA activity and plasma HCO_3^- concentration ($[HCO_3^-]$). * indicates a significant effect of $[HCO_3^-]$ while † indicates a significant effect of β on the HCO_3^- dehydration rate (two-way repeated measures ANOVA followed by Tukey's all-pairwise post-hoc multiple comparisons test at each CA level, $P < 0.05$). Use of the bracket denotes that no significant interaction occurred between the two factors, $[HCO_3^-]$ and β, such that multiple comparisons were carried out only within a factor and not among all four groups in a treatment.

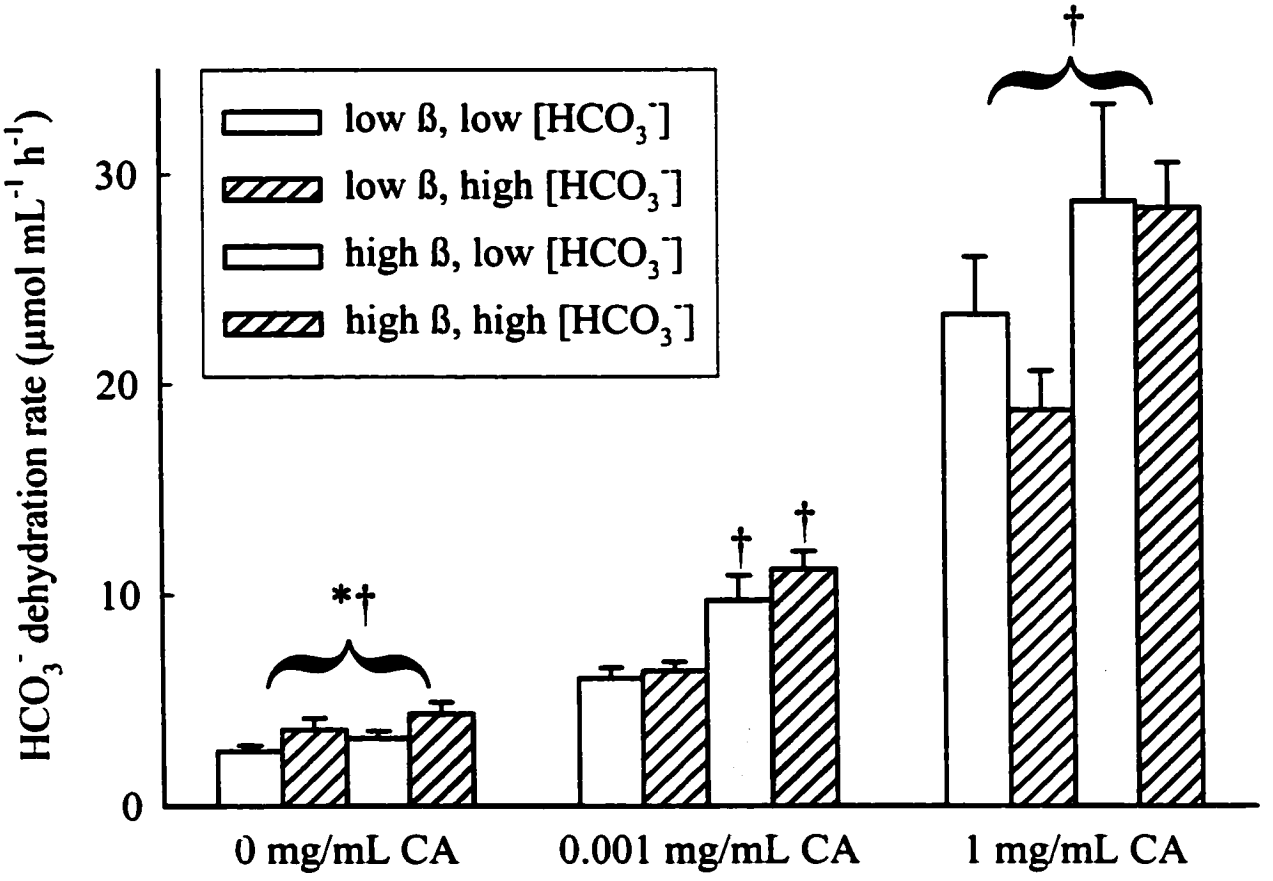

low ß, low [HCO3-]
low ß, high [HCO3-]
high ß, low [HCO3-]
high ß, high [HCO3-]
HCO3- dehydration rate (µmol mL-1 h-1)
0
10
20
30
*†
†
†
†
0 mg/mL CA
0.001 mg/mL CA
1 mg/mL CA

Table 2-1. Absolute values for arterial blood gases ($PaCO_2$ and PaO_2) and pHa, and ventilation parameters (frequency and amplitude), for rainbow trout of normal and elevated plasma non-bicarbonate buffering capacity prior to saline or bovine carbonic anhydrase (5 mg kg^{-1}) injection and 90 min post-injection (ventilation only).

	Normal β		High β	
	Saline	**CA**	**Saline**	**CA**
$PaCO_2$ (Torr)	2.01 ± 0.15 (12)	2.54 ± 0.17 (22)	1.73 ± 0.16 (6)	2.22 ± 0.20 (8)
pHa	7.92 ± 0.02 (12)*	7.90 ± 0.02 (22)*	7.80 ± 0.05 (6)	7.94 ± 0.03 (8)
PaO_2 (Torr)	98.3 ± 7.9 (12)	109.4 ± 3.8 (22)	93.2 ± 14.0 (6)	112.1 ± 9.4 (8)
V_f (min^{-1})	77.2 ± 1.1 (6)	80.5 ± 5.7 (7)	69.5 ± 1.9 (6)	65.5 ± 2.7 (6)
V_f at 90 min (min^{-1})	68.4 ± 2.4 (6)	81.2 ± 7.3 (7)	67.0 ± 3.2 (6)	66.5 ± 2.5 (6)
V_{amp} (cm)	0.38 ± 0.03 (6)	0.38 ± 0.04 (7)	0.39 ± 0.03 (6)	0.41 ± 0.04 (6)
V_{amp} at 90 min (cm)	0.35 ± 0.03 (6)	0.36 ± 0.05 (7)	0.39 ± 0.03 (6)	0.41 ± 0.05 (6)

Data are presented as means ± 1 SEM (N).

* denotes a statistically significant difference ($P < 0.05$) from the value for fish of high plasma β injected with saline.

Ventilation data were analysed by repeated measures ANOVA; no significant effect of time or treatment (CA *versus* saline injection) on ventilation frequency or amplitude was detected. CA, carbonic anhydrase; β, non-bicarbonate buffering capacity; V_f, ventilation frequency; V_{amp}, ventilation amplitude.

Table 2-2. Effects of bovine carbonic anhydrase injection (5 mg kg^{-1}) on venous blood gases ($PvCO_2$ and PvO_2) and pHv in rainbow trout of normal plasma non-bicarbonate buffering capacity.

	Pre	5 min	10 min	20 min	40 min	60 min
$PvCO_2$ (Torr)	4.05 ± 0.47	4.06 ± 0.49	4.05 ± 0.48	4.02 ± 0.52	3.92 ± 0.55	3.75 ± 0.57
pHv	7.85 ± 0.03	7.88 ± 0.03	7.88 ± 0.04	7.88 ± 0.04	7.89 ± 0.03	7.91 ± 0.03 *
PvO_2 (Torr)	29.0 ± 3.0	27.6 ± 3.9	23.2 ± 3.1	24.8 ± 3.7	23.1 ± 3.1	24.5 ± 2.6

Data are presented as means ± 1 SEM, N = 6.

* denotes a statistically significant difference from the corresponding "pre" value ($P < 0.05$).

The "pre" value was taken as the average value 10 min prior to carbonic anhydrase injection.

Discussion

The key finding of the present study, namely that a significant lowering of $PaCO_2$ occurs in trout after injection of exogenous bovine CA, constitutes the first *in vivo* evidence that CO_2 excretion in teleost fish under resting (steady-state) conditions is constrained by the accessibility of RBC CA to plasma HCO_3^-. The premise upon which the experiment was based was that exogenous CA would provide an extracellular site for CO_2 excretion, allowing the requirement for HCO_3^- entry into the red cell to be bypassed and hence enhancing CO_2 excretion which, in turn, would be detectable as a lowering of $PaCO_2$. Arterial PCO_2 is dependent upon a host of factors, including the rate of CO_2 production (metabolic rate), ventilation, gill diffusing capacity, and post-branchial CO_2-H^+-HCO_3^- equilibration reactions. Ideally, it would be desirable to measure the PCO_2 of blood leaving the gills as this value most accurately reflects CO_2 excretion. Owing to the lack of plasma-accessible CA activity in rainbow trout gills (see reviews by Gilmour 1998a,b), CO_2-H^+-HCO_3^- reactions come into equilibrium as blood flows away from the gills, resulting in small changes in PCO_2 (approximately 0.03 Torr) and pH (approximately 0.04 units), changes that are eliminated by the addition of CA to the circulatory system (Gilmour et al. 1994). Measurement of $PaCO_2$ rather than the PCO_2 of blood leaving the gills therefore, while necessary from a practical standpoint, introduces measurement error into the comparison of treatment effects on CO_2 excretion. However, the magnitude of this error (~ 0.03 Torr) is negligible with respect to the treatment effect of CA on $PaCO_2$ (~0.2 Torr). Moreover, because PaO_2 (Figs. 2-3 and 2-4), ventilation (frequency and amplitude) and venous blood gases (Table 2-2) were unaffected by CA treatment, changes in gill diffusing capacity or rate of CO_2 production also likely did not

occur and therefore the significant decrease in $PaCO_2$ with CA injection probably reflected a specific enhancement of CO_2 excretion owing to acceleration of HCO_3^- dehydration within the plasma.

Plasma HCO_3^- ions gain access to erythrocyte CA by means of the RBC membrane band 3 anion exchanger, and hence the present study also affords the first *in vivo* support for the widely-held belief that CO_2 excretion in teleost fish is limited by the relatively slow velocity of Cl^-/HCO_3^- exchange. Numerous previous studies have provided indirect (Wood et al. 1982; Heming and Randall 1982) or direct (Cameron 1978; Obaid et al. 1979; Perry et al. 1982, 1991, 1996; Tufts et al. 1988; Perry and Gilmour 1993; Jensen and Brahm 1995; Gervais and Tufts 1999) evidence for the involvement of RBC Cl^-/HCO_3^- exchange in CO_2 excretion in fish. Moreover, on the basis of the slow velocity of the band 3 exchanger in relation to the other steps involved in excreting CO_2, Cl^-/HCO_3^- exchange was identified as the rate-limiting factor for CO_2 excretion (Perry 1986). The significant decrease in $PaCO_2$ following bovine CA injection into resting rainbow trout in the present study validates experimentally this model of CO_2 excretion for teleost fish. Thus, these *in vivo* data confirm and extend the findings of Perry and Gilmour (1993), who utilised an *in vitro* approach to demonstrate that the rate of RBC HCO_3^- dehydration was increased significantly by lysis of the red cells, and that except at very low haematocrits (< 5%) no further increase was achieved by adding a saturating level of bovine CA to the (lysed) blood.

The slow rate at which plasma HCO_3^- enters the red cell to be converted to CO_2 is thought to be the basis of the apparent diffusion limitations on CO_2 transfer across the fish gill. Although CO_2 diffusion in biological media is rapid and should never be rate-

limiting (Swenson 1990), it has been argued on the basis of theory and mathematical modelling that CO_2 excretion will behave as a diffusion-limited system owing primarily to the requirement for Cl^-/HCO_3^- exchange (Cameron and Polhemus 1974; Malte and Weber 1985; Swenson 1990). Recently, this hypothesis was confirmed experimentally by Julio et al. (2000), who found that ligation of two gill arches to reduce the functional surface area of the gills by 30% caused a significant increase in $PaCO_2$ in rainbow trout. $PaCO_2$ not only rose in response to the lowering of diffusion conductance (the hallmark of a diffusion-limited system), but the increase in $PaCO_2$ was relieved by addition of bovine CA to the plasma of ligated fish (Julio et al. 2000). Furthermore, this hypothesis was also confirmed Desforges et al. (2001) and will be discussed further in chapter 3 of this thesis. Thus, the chemical reaction kinetics of Cl^-/HCO_3^- exchange were revealed to be the source of the apparent diffusion limitations on CO_2 excretion. The results of the present study confirm those of Julio et al. (2000) and demonstrate that, even in rainbow trout of normal branchial diffusion conductance, the apparent diffusion limitations on CO_2 excretion reflect chemical equilibrium limitations.

In vivo support for Cl^-/HCO_3^- exchange as the rate-limiting factor for CO_2 excretion in teleost fish has, until now, been elusive. Previous studies in which rainbow trout under steady-state conditions were injected with bovine CA failed to find any consistent effect of exogenous CA in lowering $PaCO_2$ or blood total CO_2 content (Gilmour et al. 1994; Wood and Munger 1994; Lessard et al. 1995). Given the small magnitude (~0.2 Torr) and slow time course (full development at ~70 – 90 min post-injection) of the $PaCO_2$ response to CA injection under resting conditions (Fig. 2-4), it seems likely that the use of the extracorporeal preparation contributed to the detection of

the significant decrease in $PaCO_2$ with CA injection in the present study. Unlike standard blood sampling techniques (such as those used by Wood and Munger 1994), the extracorporeal preparation enables continuous monitoring of blood gases and therefore even subtle changes in $PaCO_2$ can be measured.

It is also unlikely that the lack of effect of exogenous CA injection on CO_2 excretion in previous studies resulted from insufficient proton (H^+) availability for HCO_3^- dehydration within the plasma. In the present study, the extent of the $PaCO_2$ decline following CA injection was essentially identical in rainbow trout at the naturally-occurring plasma non-bicarbonate buffering capacity (Fig. 2-4), and in fish treated with HEPES so as to elevate plasma β approximately three fold (Fig. 2-3). This lack of correlation between plasma buffering capacity and the CA-induced $PaCO_2$ decrease would suggest that, at least at resting rates of CO_2 excretion, extracellular HCO_3^- dehydration is not constrained by proton availability. Nevertheless, there are strong grounds, both theoretical and empirical, for proposing proton availability as a limiting factor on HCO_3^- dehydration in the plasma of rainbow trout.

For each mole of HCO_3^- ions dehydrated, one mole of H^+ ions is consumed. While the majority of CO_2 is carried as HCO_3^- ions in the plasma (Perry 1986; Tufts and Perry 1998), plasma non-bicarbonate buffer capacities are low, reflecting the low (~3%) plasma protein levels (Tufts and Perry 1998). Typically, the arterial-venous difference in blood total CO_2 concentration in rainbow trout is about 1.5 mmol L^{-1} (Brauner, 1995). This clearance of CO_2 as the blood passes through the gills is accompanied by a decrease in PCO_2 from about 3.7 Torr in the pre-branchial blood to about 2.3 Torr in the post-branchial blood (Tufts and Perry, 1998). The arterial-venous pH difference is dependent

upon a variety of environmental factors (Brauner and Randall, 1998), but tends to be small under resting, normoxic conditions (arterial pH > venous pH) (Brauner, 1995). Thus, under normal conditions, ~1.5 mmol L^{-1} HCO_3^- is lost from the plasma with little change in pH (Fig. 2-6); the 1.5 mmol L^{-1} H^+ required for HCO_3^- dehydration is provided by the buffering capacity of haemoglobin and by Bohr protons released from haemoglobin during oxygenation (Tufts and Perry, 1998; Brauner and Randall, 1998). However, were HCO_3^- dehydration to take place only in the plasma, where β is -2.6 mmol L^{-1} pH $unit^{-1}$ (rather than the whole blood value of -9.7 mmol L^{-1} pH $unit^{-1}$; Wood et al. 1982), a decrease in PCO_2 from 3.7 to 2.3 Torr would elicit only an approximately 0.5 mmol L^{-1} decrease in plasma HCO_3^-; a 1.5 mmol L^{-1} change in the plasma HCO_3^- concentration would require an approximately 6 Torr decrease in PCO_2 together with a 0.5 unit pH change (Fig. 2-6). Clearly, by theory the low buffering capacity of the plasma will limit proton availability and hence constrain the extent of HCO_3^- dehydration that can take place in this compartment. The data from Wood et al. (1982) was used since a lot of assumptions are made constructing these pH-HCO_3^- diagrams.

The more sophisticated model of Bidani and Heming (1991) clearly delineated the importance of the non-bicarbonate buffer capacity in determining the magnitude of CA-catalysed HCO_3^- dehydration in mammalian pulmonary capillaries. Corresponding to their theoretical predictions, Heming and Bidani (1992) used a saline-perfused rat lung to demonstrate that CO_2 excretion was strongly dependent on perfusate non-bicarbonate buffering capacity. CA activity (isozyme IV) is associated with endothelial cell membranes in mammalian pulmonary capillaries and is available to catalyse plasma CO_2 reactions (e.g. see review by Henry and Swenson 2000). In the perfused rat lung

preparation of Heming and Bidani (1992), the ability of pulmonary capillary CA IV to enhance CO_2 excretion increased significantly with increasing perfusate β. Thus, proton availability becomes increasingly important as the rate of HCO_3^- dehydration increases. Similar results were obtained in our *in vitro* approach to assess the impact of non-bicarbonate buffering capacity on HCO_3^- dehydration in rainbow trout plasma. While addition of HEPES to separated plasma increased the HCO_3^- dehydration rate of all samples significantly, the effect tended to be more pronounced in samples to which bovine CA had been added (50-75% increase) than under uncatalysed conditions (24% increase; Fig. 2-5).

Yet, even though theoretical considerations would predict, and *in vitro* experiments have confirmed, that proton availability is a limiting factor on HCO_3^- dehydration in rainbow trout plasma, the effect of CA injection in lowering $PaCO_2$ *in vivo* in rainbow trout in the present study was unrelated to plasma β. This apparent discrepancy is probably a reflection of two factors; the high buffering capacity of haemoglobin, and the tight linkage that is thought to exist in teleost fish between O_2 uptake and CO_2 excretion owing to the Haldane effect (Brauner 1995; Brauner and Randall 1996, 1998; Brauner et al. 2000). The whole blood buffering capacity in fish is generally only 2-3 times that of the separated plasma (Tufts and Perry 1998). However, when haematocrit (~20 - 25%) and the low plasma β (-3 to -4 mmol L^{-1} pH $unit^{-1}$) are taken into account, RBC buffering capacity can probably be conservatively estimated to be -30 to -40 mmol L^{-1} pH $unit^{-1}$.

In addition to the buffering properties of RBC haemoglobin, protons for HCO_3^- dehydration in the red cell are provided by H^+ release from haemoglobin during

oxygenation through the Haldane effect (Perry and Gilmour 1993; Brauner et al. 1996; Perry et al. 1996). Indeed, the protons required for red cell HCO_3^- dehydration in teleost fish may be derived predominantly through the Haldane effect, owing to the low (relative to mammals) buffering capacity and large Haldane effect characteristically exhibited by teleost haemoglobins (Brauner and Randall 1998). This reliance on the Haldane effect for protons for HCO_3^- dehydration is thought to be the physiological basis of the tight coupling that appears to exist between O_2 uptake and CO_2 excretion in teleost fish (Brauner 1995; Brauner and Randall 1996, 1998; Brauner et al. 2000). In the context of the present study, these considerations suggest that the relatively small three-fold increase in plasma β achieved through HEPES treatment was probably insufficient to drive a substantial component of HCO_3^- dehydration through the plasma. That is, CO_2 excretion in HEPES-treated fish continued to occur primarily via the RBC following CA injection, despite the rate-limiting constraint of Cl^-/HCO_3^- exchange, because of the high RBC proton availability. Thus, while the results of the present study could be interpreted as evidence that HCO_3^- dehydration in rainbow trout plasma is *not* limited by proton availability *in vivo*, it seems more likely that plasma HCO_3^- dehydration *in vivo* is so limited by proton availability in comparison to that in the red cell that even increasing plasma β three-fold has little impact.

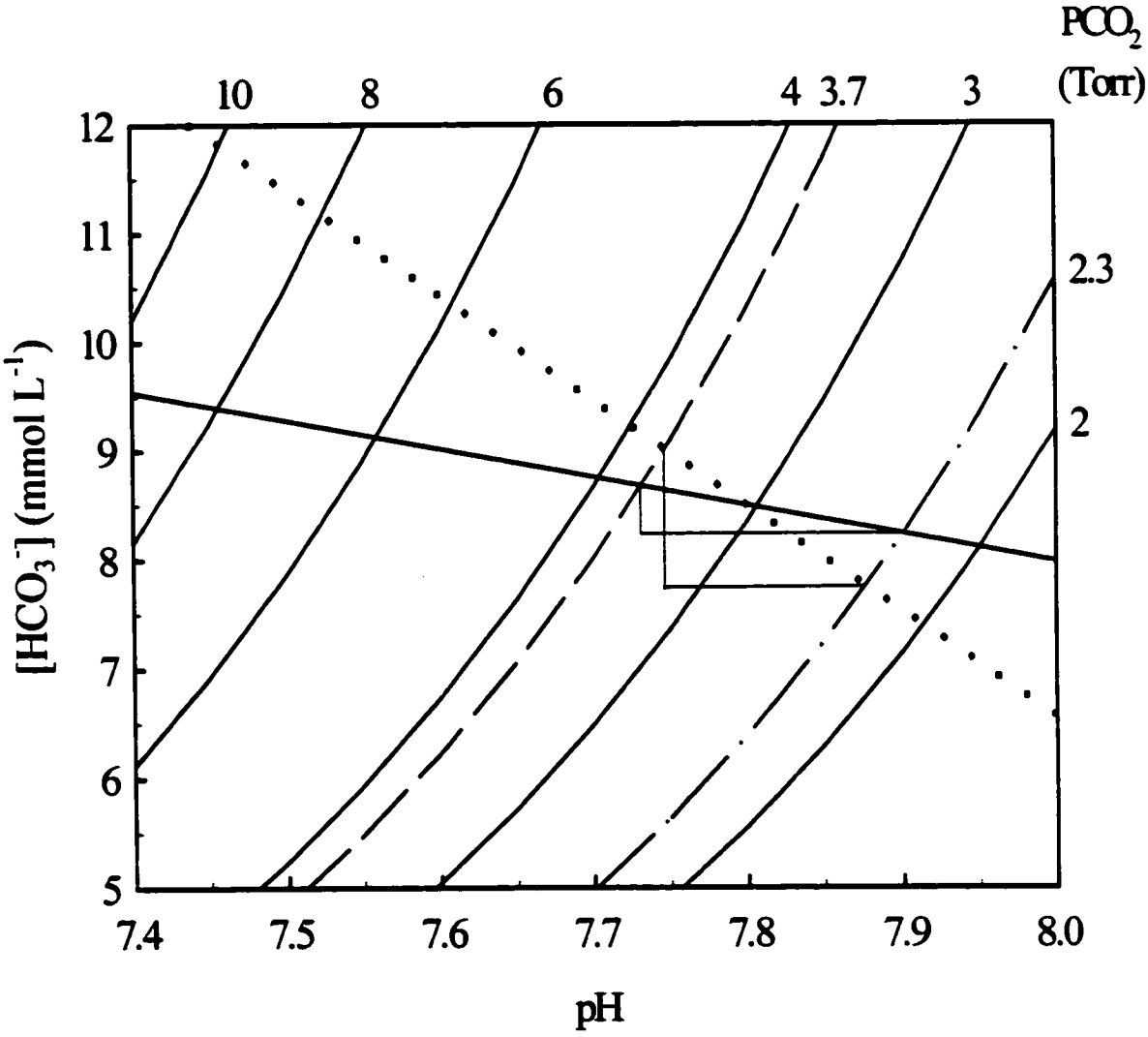
Increasing the plasma buffering capacity did, however, affect the pH response to bovine CA injection; whereas pHa increased significantly following CA injection into fish of normal plasma β (Fig. 2-4), no significant change in pHa occurred with CA treatment in HEPES-treated fish (Fig. 2-3). The loss of CO_2 from the blood to the ventilatory water during the passage of blood through the gills creates a disequilibrium in

plasma CO_2-HCO_3^--H^+ reactions that cannot be rapidly corrected in teleost fish because no CA activity is available to catalyse the plasma hydration/dehydration reactions (see reviews by Gilmour 1998a,b). Consequently, changes in pH lag behind the change in PCO_2, and pHa in teleost fish increases slowly in the postbranchial blood towards the new equilibrium value (Gilmour et al. 1994). The addition of exogenous CA to the circulation abolishes the postbranchial disequilibrium by catalysing plasma CO_2 reactions, such that the pHa of blood leaving the gills is already at the equilibrium value (Gilmour et al. 1994). This establishment of an equilibrium condition in the postbranchial blood accounts for the significant, rapid increase in pHa measured in fish of normal plasma β following CA injection (Fig. 2-4); similar effects have been reported previously (Gilmour 1998a). The significant increase in pHv at 60 min after CA injection (Table 2) probably also reflected the establishment of an equilibrium condition as an acid-base disequilibrium has also been documented in the venous blood of rainbow trout (Perry et al. 1997). In fish treated with HEPES to elevate plasma β, injection of bovine CA similarly would be expected to abolish the postbranchial acid-base disequilibrium. In this case, however, no increase in pHa was observed, probably because of the effect of buffering capacity on the magnitude and time-course of the disequilibrium. At high β, the half-time of pH equilibration is increased, but the magnitude of the pH change is reduced (Bidani and Heming 1991).

In conclusion, by demonstrating that $PaCO_2$ in rainbow trout was decreased significantly by bovine CA injection, the results of the present study have provided experimental confirmation of the widely-held view that CO_2 excretion under resting conditions is limited in teleost fish *in vivo* by the slow rate of Cl^-/HCO_3^- exchange. A

similar conclusion was reached for mammals by Swenson et al. (1993a) on the basis of experiments in which dogs treated with the anion exchange inhibitor 4,4' dinitrostilbene-2,2'-disulfonate exhibited a reduction in pulmonary CO_2 excretion. Furthermore, the data of the present study reaffirm the view that the apparent diffusion limitations on CO_2 transfer across the fish gill (Malte and Weber 1985) are in reality a reflection of chemical equilibrium limitations arising from the slow rate at which plasma HCO_3^- enters the red cell to be converted to CO_2 (Swenson 1990; Julio et al. 2000).

Figure 2-6. A pH-HCO_3^- diagram for rainbow trout at 13°C. The PCO_2 for a given combination of pH and [HCO_3^-] was calculated using the Henderson-Hasselbalch equation and the appropriate values for pK' and αCO_2 (Boutilier et al. 1984). Venous (dashed line) and arterial (dot-dash line) PCO_2 values for rainbow trout were drawn from Tufts and Perry (1998). Buffer lines for rainbow trout whole blood (dotted line) and separated plasma (solid line) were constructed using buffer values, β, derived by Wood et al. (1982). Δ[HCO_3^-] and ΔpH for the arterial-venous PCO_2 decrease at each β are drawn.


PCO2
(Torr)
10
8
6
4
3.7
3
2.3
2
12
11
10
9
8
7
6
5
[HCO3-] (mmol L-1)
7.4
7.5
7.6
7.7
7.8
7.9
8.0
pH

CHAPTER 3

THE SENSITIVITY OF CO_2 EXCRETION TO CHANGES IN BLOOD FLOW IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) IS DETERMINED BY CARBONIC ANHYDRASE AVAILABILITY

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Abstract

The blood transit time through the gills of rainbow trout (*Oncorhynchus mykiss*) was modified by manipulation of cardiac output ($\dot{V}b$). The experiments were designed to test the hypothesis that the efficiency of CO_2 excretion is sensitive to changes in blood flow owing to chemical equilibrium limitations, whereas the efficiency of O_2 uptake is insensitive to changes in blood flow. An extracorporeal blood shunt was used to continuously monitor blood gases *in vivo* in fish in which $\dot{V}b$ was elevated (by 13.3 ± 2.4 mL min^{-1} kg^{-1}) by intravascular saline injection or reduced (by 10.8 ± 1.8 mL min^{-1} kg^{-1}) by removal of plasma. The arterial partial pressure of CO_2 ($PaCO_2$; an index of CO_2 excretion efficiency) was increased with elevated $\dot{V}b$ and was decreased with reduced $\dot{V}b$ such that the changes in $PaCO_2$ exhibited a significant positive sigmoidal relationship with the changes in $\dot{V}b$ ($r^2 = 0.75$; $P < 0.05$). In contrast, there was no significant relationship between changes in the arterial partial pressure of O_2 (PaO_2; an index of O_2 uptake efficiency) and changes in $\dot{V}b$ ($r^2 = 0.07$; $P > 0.05$). The intravenous administration of carbonic anhydrase (CA; 10 mg kg^{-1}) prior to vascular volume loading eliminated the increase in $PaCO_2$ with increased $\dot{V}b$ that was observed in control fish. The insensitivity of PaO_2 to changes in $\dot{V}b$ reaffirms the theory that O_2 uptake through the respiratory barrier is perfusion limited. However, the sensitivity of CO_2 excretion efficiency to changes in blood flow, coupled with the impact of CA availability on this sensitivity, argue strongly that the apparent diffusion limitations on CO_2 excretion are, in fact, chemical equilibrium limitations.

Introduction

On the basis of permeation coefficients alone, CO_2 should be more diffusible in tissue than O_2 (Swenson 1990). Yet, in teleost fish, O_2 uptake appears to be perfusion limited whereas CO_2 excretion behaves as a diffusion-limited system (Cameron and Polhemus 1974; Daxboeck et al. 1982; Pärt et al. 1984; Malte and Weber 1985; Perry 1986; Piiper 1989). This apparent discrepancy likely reflects chemical equilibrium limitations on CO_2 excretion. The majority of CO_2 excreted is transported in the plasma as bicarbonate ions owing to the low solubility of plasma for CO_2 (Boutilier et al. 1984). Because the rate of the uncatalysed dehydration of HCO_3^- (rate constant > 20 sec^{-1}; Edsall 1969) is low relative to the gill transit time (1-3 sec; Cameron and Polhemus 1974), effective CO_2 excretion depends largely on the dehydration of HCO_3^- within the red blood cell (RBC), where it is catalysed by carbonic anhydrase (CA) (Perry 1986; Gilmour 1997; Henry and Heming 1998; Tufts and Perry 1998). HCO_3^- ions gain access to the RBC via the electroneutral Cl^-/HCO_3^- exchanger (AE1 isoform, band 3 protein) on the RBC membrane (Cameron 1978; Obaid et al. 1979; Romano and Passow 1984; Jensen and Brahm 1995). The entry of HCO_3^- into the RBC via this process is believed to be the rate-limiting step in CO_2 excretion (Perry 1986; Desforges et al. 2001). In addition to the CA present in vertebrate RBC's, mammals possess a pulmonary membrane-associated CA (CA IV) with an extracellular orientation which is absent from the gills of teleost fish (see review by Henry and Swenson 2000). Consequently, in teleosts, the slow rate of conversion of HCO_3^- to CO_2 (chemical equilibrium limitations) in plasma may result in apparent diffusion limitations.

By theory, in a diffusion-limited system, gas transfer efficiency will be affected by changes in blood transit (residence) time within the respiratory organ. Thus, for a gas where the time to diffusion equilibrium across the respiratory barrier exceeds the residence time, a decrease in transit time would be expected to lower the efficiency of transfer whereas an increase in transit time would be expected to increase gas transfer efficiency. In contrast, gas transfer efficiency in a perfusion-limited system will be maintained despite changes in blood flow until the residence time of blood at the gas exchange surface approaches the diffusion equilibrium time of the respiratory gas.

While several studies have investigated the sensitivity of O_2 uptake to manipulation of cardiac output ($\dot{V}b$) to assess perfusion/diffusion limitations on O_2 transfer in fish (Daxboeck et al. 1982; Pärt et al. 1984), few have measured the effects of changes in $\dot{V}b$ on CO_2 excretion. Utilising a spontaneously ventilating blood perfused trout preparation, Perry (1986) reported an increase in $PaCO_2$ with elevation of $\dot{V}b$. Recently, Brauner et al. (2000) observed that when $\dot{V}b$ was increased during sustained exercise in rainbow trout, there was an associated elevation of arterial blood PCO_2 ($PaCO_2$). These data suggest that transit time limitations exist for CO_2 excretion in rainbow trout.

With this background, the objective of the present study was to test the hypothesis that CO_2 transfer efficiency in rainbow trout would be sensitive to changes in blood transit time in the gill because CO_2 excretion behaves as a diffusion limited system, and that this sensitivity would reflect chemical equilibrium constraints. $\dot{V}b$ was manipulated

by vascular volume loading or by blood withdrawal; changes in $\dot{V}b$ were assumed to inversely modify the blood residence time in the gills. Gas transfer efficiency was estimated by continuous online monitoring of arterial blood gases (because efficiency is defined as the difference between arterial and venous gas tensions divided by the difference between venous and inspired gas tensions). Intravenous administration of bovine CA, to reduce the need for RBC HCO_3^- dehydration (chapter 2; Desforges et al. 2001), was used to assess the role of chemical equilibrium constraints in any apparent CO_2 transfer diffusion limitations. Rainbow trout was used as a model teleost species because teleost fish are thought to be unique in lacking plasma-accessible CA activity and therefore, unlike in mammals, there is no capacity for catalysed HCO_3^- dehydration in the plasma.

Materials and methods

Experimental Animals

Rainbow trout (*Oncorhynchus mykiss*) of either sex were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario). Fish were maintained on a 12L:12D photoperiod, in large circular fibreglass aquaria supplied with flowing, aerated and dechlorinated city of Ottawa tap water at 13°C. Fish were allowed at least two weeks to acclimate to the holding conditions before any experiments were performed and were fed to satiation on alternate days with a commercial trout pellet diet until 24 h prior to experimentation. Three groups of fish were used; in one group [N = 60; 555 ± 16 g (mean ± 1 SEM)], blood respiratory variables were measured *in vivo* by means of an extracorporeal blood shunt (see below). A second group of fish (N = 14; 611 ± 36 g)

acted as blood donors for blood removal experiments, and a third group of smaller fish (N = 22; 266 ± 11 g) were used for experiments designed to examine the effects of increasing blood volume on plasma catecholamine levels. All experimental protocols were previously approved by the University of Ottawa Animal Care Committee in accordance with guidelines provided by the Canadian Council on Animal Care.

Fish were anaesthetised by immersion in an oxygenated solution of benzocaine (ethyl-*p*-aminobenzoate; 0.1g L^{-1}) and placed on a surgical table that allowed irrigation of the gills with the same anaesthetic solution. For continuous measurements of blood respiratory variables *in vivo*, the caudal vein and caudal artery were cannulated. Briefly, a lateral incision (~ 2 cm in length) was made at the level of the caudal peduncle to allow the epaxial and hypaxial musculature to be separated and the haemal arch to be exposed. Catheters (Clay Adams PE 50 polyethylene tubing) were inserted into the caudal vein and caudal artery in the anterior direction. The incision was closed with silk sutures and both cannulae were secured to the skin with ligatures. To enable measurement of cardiac output, a small (1.5 cm) midline ventral incision was made to expose the pericardial cavity and the pericardium was dissected away to expose the bulbus arteriosus. A 3S or 4S ultrasonic flow probe (Transonic Systems, Ithaca, N.Y.) was placed non-occlusively around the bulbus. Lubricating jelly was used with the perivascular flow probe as an acoustic couplant. Silk sutures were used to close the ventral incision and to anchor the cardiac output probe lead to the skin. Small brass plates (1 cm^2) were sutured to the external surface of each operculum to allow the measurement of ventilation parameters by means of an impedance converter.

Fish used for measurements of catecholamines, as blood donors or in blood withdrawal experiments received an indwelling cannula (Clay Adams PE 50) in the dorsal aorta following the technique of Soivio et al. (1975). After surgery, fish were transferred to individual opaque acrylic boxes supplied with aerated flowing water (flow rate > 2.5 L min^{-1}) and were left to recover for approximately 24 h before experimentation. Cannulae were flushed with heparinised (100 i.u. mL^{-1} sodium heparin) Cortland saline (Wolf 1963).

Experimental protocol

An extracorporeal blood shunt (Thomas 1994) was used to continuously monitor arterial or venous blood O_2 tension (PaO_2 and PvO_2, respectively), CO_2 tension ($PaCO_2$ and $PvCO_2$ respectively), and pH (pHa and pHv, respectively). Blood withdrawn from the caudal artery or vein using a peristaltic pump was passed through an external circuit containing PO_2, PCO_2 and pH electrodes before being returned to the fish via the other cannula. The flow rate through the external loop, which contained approximately 1 mL of blood (< 4% of fish's blood volume), was 0.6 mL min^{-1}. Immediately prior to experimentation, the extracorporeal shunt was rinsed for 10-15 min with heparinised (540 i.u. mL^{-1}) saline to prevent blood from clotting in the tubing and electrode chambers. After initiating the extracorporeal blood shunt, ~ 20 min were required to obtain stable readings for blood gas variables, as well as blood flow and ventilation parameters. Upon stabilisation, experiments commenced with a 10 min period of baseline recording followed by one of four procedures.

In the first series of experiments, the effects of an increase in blood flow were investigated. Fish received an injection into the caudal vein of Cortland saline (10 mL kg^{-1}) containing 3% bovine albumin (BSA), when arterial blood was monitored (N = 11), or the caudal artery, when venous blood was monitored (N = 6). The PCO_2 of the saline solution was measured using the extracorporeal loop prior to injection and was always equal to or slightly lower than the resting blood PCO_2 of the fish. Cardiorespiratory variables were monitored for 60 min post-injection. The second series of experiments investigated the effects of decreasing blood flow. Blood (8-12 mL) was withdrawn from the dorsal aorta to obtain a decrease in cardiac output in the range of 5-10 mL kg^{-1} min^{-1} (N = 7). To avoid possible changes in blood gases associated with the loss of RBC's rather than volume, 25% of the volume of blood removed was immediately replaced with loosely packed red blood cells provided by a donor fish. Again, recording continued for 60 min after blood withdrawal; arterial blood only was monitored in this series. In the associated control experiments, fish were simply monitored for 70 min in total; no injection or blood removal was carried out (N = 6).

The impact of carbonic anhydrase (CA) availability on blood gas and pH changes associated with increases in blood volume was investigated in a third series of experiments. Following baseline recording, CA (10 mg kg^{-1} using a volume of 1 mL kg^{-1}) was administered and 30 min later, fish received an injection into the caudal vein of saline (10 ml kg^{-1}) containing 3% bovine albumin (N = 9). Data were recorded for 60 min after the saline injection; arterial blood only was monitored in this series. In the control experiments (N = 9), fish received a saline injection (1 mL kg^{-1}) rather than the CA but the experimental protocol was otherwise identical to that for the CA-treated fish.

Because of the possible role of elevated circulating catecholamine levels in eliciting the observed responses (see results), a fourth series of experiments was carried out using the non-specific β-adrenoreceptor antagonist sotalol to block possible ß-adrenergic effects induced by elevated catecholamine levels. Fish (N = 6) were injected with both CA (10 mg kg^{-1} using a volume of 1 mL kg^{-1}) and sotalol (9.9 μm kg^{-1}) into the caudal vein. After stabilisation of blood gas variables (40-110 min later), 10 mL kg^{-1} of Cortland saline containing 3% bovine albumin (BSA) was administered. Data were recorded for 60 min after the saline injection, and this was followed by the administration of isoproterenol (0.03 μmol kg^{-1}), a selective β-adrenoreceptor agonist, to confirm that β-receptor blockade had been achieved. Previous studies have established that a sudden and pronounced decrease of blood pH *in vivo* is an effective index of ß-adrenergic activation of red blood cell Na^+-H^+ exchange (Motais et al. 1989). Thus, in the present study, the absence of a pH drop following isoproterenol administration was deemed sufficient demonstration of β-blockade. In the control group for this series, isoproterenol was injected into fish that had not received the sotalol treatment.

A final series of experiments was performed on fish surgically fitted with only a dorsal aortic cannula to assess the impact of volume heading on circulating catecholamine levels. After withdrawing a pre-injection blood sample (0.4 mL), fish were injected with 10 mL kg^{-1} of Cortland saline containing 3% bovine albumin (BSA) via the dorsal aortic cannula (N = 12), whereas no injection was carried out on the control group (N = 10). Additional blood samples were withdrawn 5, 10, 15, 20, 30, and 60 min after the injection. All blood samples were immediately centrifuged (16,000 *g*). The

separated plasma was frozen in liquid N_2 and stored at -80°C until subsequent analysis of catecholamine levels.

Analytical procedures

In experiments utilising the extracorporeal blood shunt, arterial or venous blood pH, PCO_2 and PO_2 were monitored using Metrohm (pH) and Cameron Instruments (CO_2 and O_2) electrodes housed in thermostatted cuvettes and connected to a blood gas analyser (Radiometer PHM 73 or Cameron Instruments). The O_2 electrode was calibrated by pumping a zero solution (2 g L^{-1} sodium sulfite) or air-saturated water continuously through the circuit until stable readings were recorded. The CO_2 electrode was calibrated in a similar manner using mixtures of 0.5 and 1.0% CO_2 in air that were provided by a gas mixing flowmeter (GF-3/MP Cameron Instruments). The pH electrode was calibrated by pumping precision buffer solutions through the circuit. All electrodes were calibrated prior to each experiment. The frequency and amplitude of opercular displacements were assessed as indices of ventilation using a custom-built impedance converter that detected and quantified the changes in impedance between the brass plates attached to the opercula (Peyraud and Ferret-Bouin 1960). Cardiac output was determined by connecting the ultrasonic flow probe to a small animal blood flow meter (T106, Transonic Systems, Ithaca, NY). All analog signals (blood gases, pH, impedance values and $\dot{V}b$) were converted to digital data and stored by interfacing with a data acquisition system (Biopac Systems Inc.) using Acknowledge™ data acquisition software (sampling rate set at 30 Hz) and a Pentium™ PC. Haematocrit was determined in duplicate by centrifuging microcapillary tubes at 5000 *g* for 10 min.

Plasma catecholamines (adrenaline and noradrenaline) were determined on alumina extracted samples (200 μl) using high pressure liquid chromatography (HPLC) with electrochemical detection. The extracted samples were passed through an Ultratechsphere ODS-C18 5 μm column (HPLC Technology Ltd. Macclesfield, U.K.) and separated amines were integrated with the Star Chromatography software program (version 4.0, Varian, Walnut Creek, Calif.). Concentrations were calculated relative to appropriate standards, and with 3,4-dihydroxybenzylamine hydrobromide (DHBA) as an internal standard in all determinations. Detection limits for adrenaline and noradrenaline were 0.1 nmol L^{-1}.

Statistical analyses

All data are presented as means ± 1 standard error of the mean (SEM). For experiments using the extracorporeal blood shunt, means for blood gases, pH, ventilatory data and $\dot{V}b$ were compiled for 2 min periods from 10 min prior to, until 60 min post intravascular volume changes. Owing to the variation within the population and the small magnitude of the observed effects, data for individual fish for blood gases, pH, ventilatory variables and $\dot{V}b$ were normalised by subtracting from each data point the value at time = 0 (the point of blood volume modification). In the fifth series, the total catecholamine concentrations were obtained by adding noradrenaline and adrenaline values. The statistical significance of differences in initial absolute values among treatments was assessed by one-way analysis of variance (ANOVA) or unpaired Student's *t*-tests, as appropriate. Two-way repeated measures ANOVA followed by the Bonferroni post-hoc multiple comparisons test, as appropriate, was used to assess the

statistical significance of differences in blood gases, pH, ventilatory variables and blood flow with time and treatment. The fiducial level of significance was considered to be 5% and a commercial software package (Sigmastat v2.03) was used to perform all statistical analyses. The sigmoidal curve in Figure 3-6A was fitted to the data using a curve fitting option in a commercial software package (SigmaPlot 2000; SPSS).

Results

Absolute values for cardiorespiratory variables prior to the modification of $\dot{V}b$ are presented in Table 3-1. Apart from the expected differences between venous and arterial blood, the only significant difference among the groups was a higher initial PaO_2 in fish that were later subjected to blood withdrawal than in those that were subsequently volume loaded. The effectiveness of volume manipulations in eliciting changes in $\dot{V}b$ is illustrated in Figs 3-1A and 3-2A. Addition to the circulation of 10 mL kg^{-1} saline containing 3% BSA increased $\dot{V}b$ by, on average, 50% over an untreated control group whereas plasma removal was associated with a 52% decrease in $\dot{V}b$. These changes in $\dot{V}b$, in turn, were accompanied by significant changes in $PaCO_2$. The injection of saline resulted in a significant elevation of $PaCO_2$ (maximum increase = 0.28 ± 0.03 Torr; Fig 3-1B), accompanied, as expected, by a significant drop in pHa (Fig 3-1D), while PaO_2 was not affected (Fig 3-1C). Although not statistically significant, saline injection tended to decrease the haematocrit (from 22.3 ± 1.7 to 18.8 ± 1.3 %; N = 10). The removal of plasma resulted in a significant decrease in $PaCO_2$ (maximum change = -0.40 ± 0.08

Torr; Fig 3-2B). In this case, a small but significant fall in PaO_2 occurred (Fig 3-2C). Unexpectedly, pHa also decreased significantly (Fig 3-2D). All measured variables remained constant throughout the experimental period in the untreated control fish.

To ensure that the increase in $PaCO_2$ in volume-loaded fish was not a consequence of changes in tissue CO_2 production, the effects on arterial blood gases of increasing $\dot{V}b$ were compared to those on venous blood gases. Although volume loading induced similar increases in $\dot{V}b$ in both groups of fish, venous PCO_2 was unaffected (Fig 3-3).

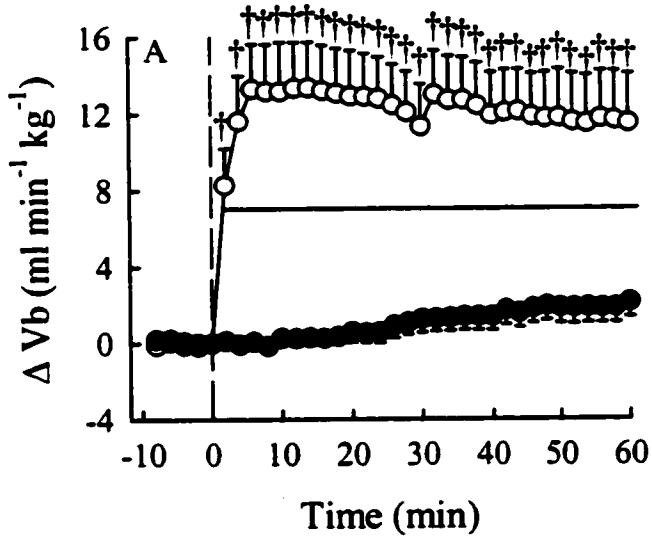
Except for pH, absolute values for $\dot{V}b$, blood gases, and ventilation parameters were similar prior to the elevation of blood volume for control and CA-treated fish (Table 3-2). Despite $\dot{V}b$ being elevated to the same extent as in the control group (Fig 3-4A), pre-treatment of fish with CA prevented the increase in $PaCO_2$ normally associated with volume loading (Fig 3-4B). A significant but transient fall in PaO_2 immediately following volume loading was observed for both groups and was significantly larger in control fish than in CA-treated fish (Fig 3-4C). Arterial pH declined significantly, in a similar fashion in both groups (Fig 3-4D).

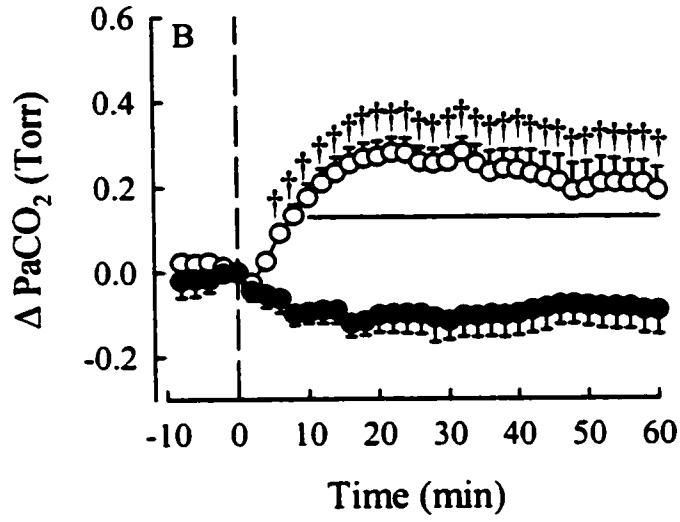
Volume loading was accompanied by significant increases in ventilation (Fig 3-5). In control fish, both frequency and amplitude were increased whereas ventilation amplitude only was increased in the CA-treated fish. Further, the elevation of ventilation amplitude was significantly greater in the control fish than in the CA-treated fish (Fig 3-5B).

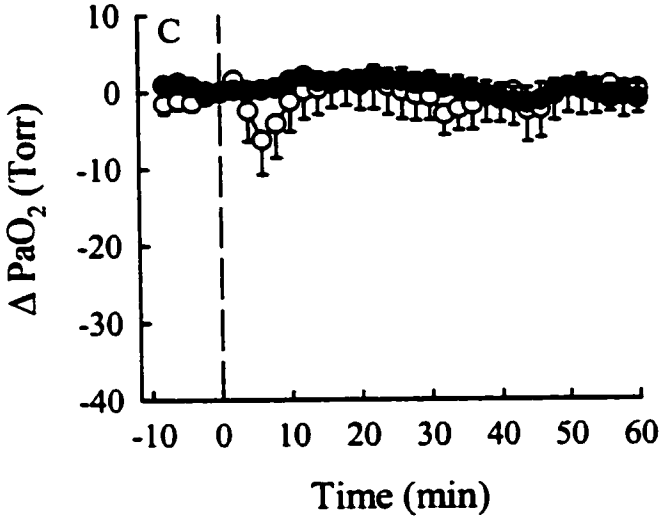
Fig 3-6 depicts the relationship between changes in $\dot{V}b$ and arterial blood gas tensions for all fish subjected to volume loading or blood withdrawal except those pre-treated with CA. Over the entire range of blood flows obtained, a significant sigmoidal relationship between $\Delta PaCO_2$ and $\Delta\dot{V}b$ ($r^2 = 0.75$; $P < 0.05$) was observed (Fig 3-6A). Furthermore, $\Delta PaCO_2$ demonstrated a significant linear relationship with $\Delta\dot{V}b$ ($r^2 = 0.72$; $P < 0.05$) within the narrower range of $\dot{V}b$ changes of -3 to 11 mL min^{-1} kg^{-1} as illustrated by the insert of Fig 3-6A. Fig 3-6B presents the relationship between changes in $\dot{V}b$ and the corresponding changes in PaO_2; no significant correlation was observed ($r^2 = 0.07$; $P > 0.05$). Among fish treated with CA, no significant correlation between $\Delta PaCO_2$ and $\dot{V}b$ was detected ($r^2 = 0.24$; $P > 0.05$).

To test the possibility of adrenergic stimulation of the red cell Na^+/H^+ exchanger contributing to pH changes in the blood, circulating catecholamine concentrations were measured in untreated and volume loaded fish and were found to increase significantly from pre-treatment levels of 4.7 nmol L^{-1} to 21.1 nmol L^{-1}). However, this increase could not account for the fall in pHa in volume-loaded CA-treated fish because treatment of such fish with the β-adrenoreceptor antagonist sotalol had no significant effect on the content of the pH drop following volume loading (ΔpHa = - 0.062 in CA-treated fish and –0.100 in CA-treated fish to which sotalol was administered).

Figure 3-1. The effects of vascular volume loading (10 mL kg^{-1}; unfilled symbols, N = 11) on changes in (A) cardiac output ($\Delta\dot{V}b$), (B) arterial PCO_2 (Δ $PaCO_2$), (C) arterial PO_2 (Δ PaO_2) and (D) arterial pH (pHa) in rainbow trout (*Oncorhynchus mykiss*). Control fish were not treated (filled symbols, N = 6). The dashed vertical line at time = 0 min indicates the point of saline injection. Significant differences ($P < 0.05$) from the final pre-injection values (time = 0 min) are indicated by solid horizontal lines. Significant differences ($P < 0.05$) from the corresponding value in the control group are denoted by daggers. Data are presented as mean values ± 1 SEM.


A
16
12
8
4
0
-4
Δ Vb (ml min-1 kg-1)
-10 0 10 20 30 40 50 60
Time (min)


B
0.6
0.4
0.2
0.0
-0.2
Δ PaCO2 (Torr)
-10 0 10 20 30 40 50 60
Time (min)


C
10
0
-10
-20
-30
-40
Δ PaO2 (Torr)
-10 0 10 20 30 40 50 60
Time (min)

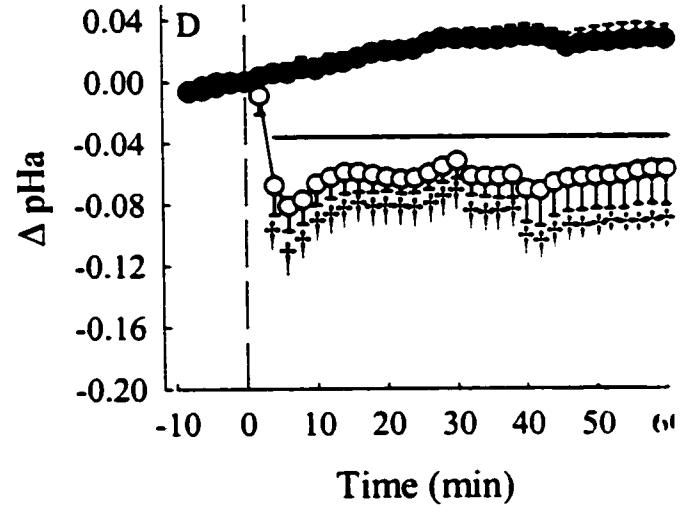
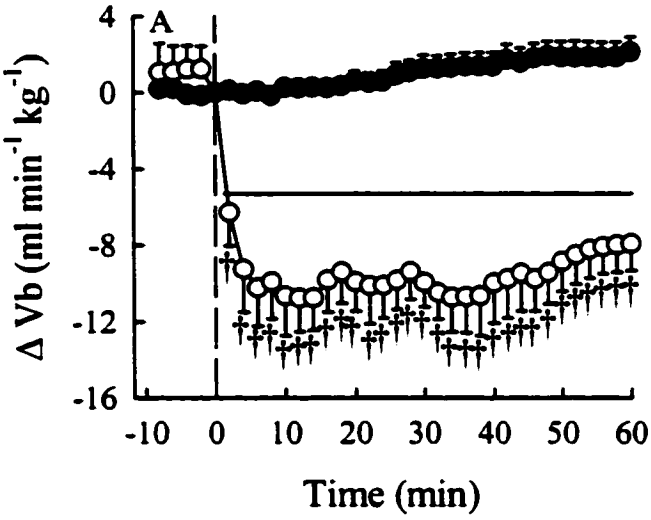
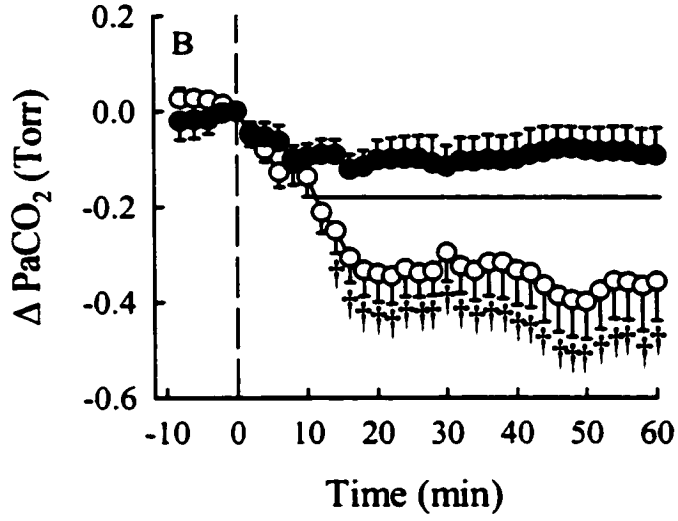
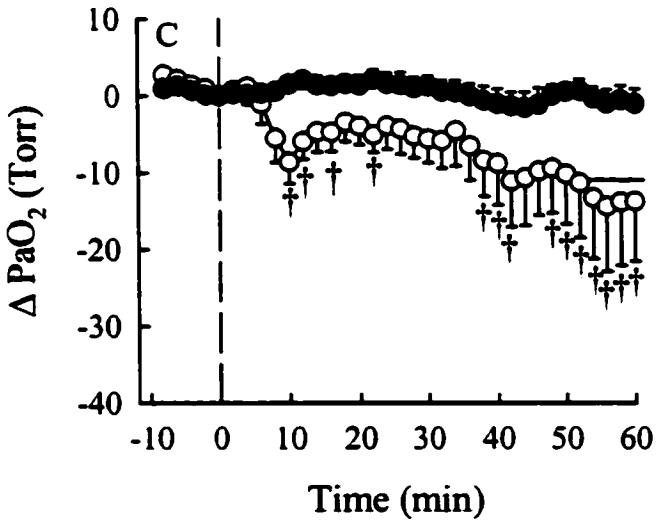

D
0.04
0.00
-0.04
-0.08
-0.12
-0.16
-0.20
Δ pHa
-10 0 10 20 30 40 50
Time (min)

Figure 3-2. The effects of blood plasma removal (8-12 mL kg^{-1}; unfilled symbols, N = 7) on changes in (A) cardiac output ($\Delta\dot{V}b$), (B) arterial PCO_2 (Δ $PaCO_2$), (C) arterial PO_2 (Δ PaO_2) and (D) arterial pH (pHa) in rainbow trout (*Oncorhynchus mykiss*). Control fish (same data as in Fig 3-1) were untreated (filled symbols, N = 6). The dashed vertical line at time = 0 min indicates the beginning of blood removal. Significant differences ($P < 0.05$) from the final pre-removal values (time = 0 min) are indicated by solid horizontal lines. Significant differences ($P < 0.05$) from the corresponding value in the control group are denoted by daggers. Data are presented as mean values ± 1 SEM.


A
Δ Vb (ml min-1 kg-1)
4
0
-4
-8
-12
-16
-10 0 10 20 30 40 50 60
Time (min)


B
Δ PaCO2 (Torr)
0.2
0.0
-0.2
-0.4
-0.6
-10 0 10 20 30 40 50 60
Time (min)


C
Δ PaO2 (Torr)
10
0
-10
-20
-30
-40
-10 0 10 20 30 40 50 60
Time (min)

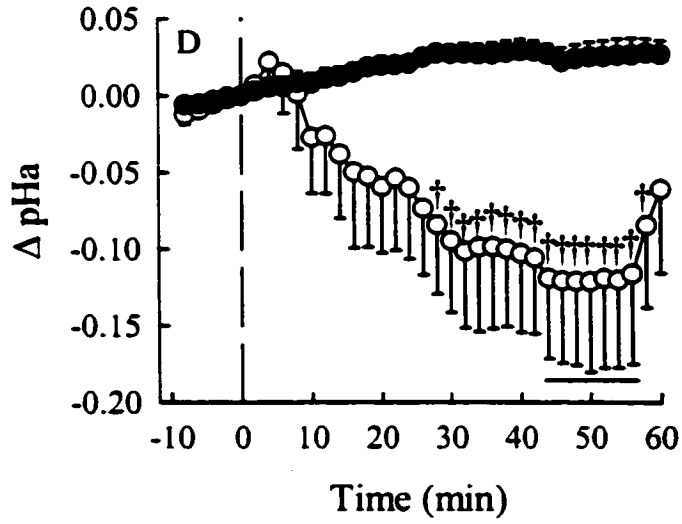

D
Δ pHa
0.05
0.00
-0.05
-0.10
-0.15
-0.20
-10 0 10 20 30 40 50 60
Time (min)

Figure 3-3. The effects of vascular volume loading (10 mL kg^{-1}) on changes in (A) cardiac output ($\Delta\dot{V}b$) and (B) arterial (unfilled symbols, N = 11) or venous (filled symbols, N = 6) PCO_2 (Δ PCO_2) in rainbow trout (*Oncorhynchus mykiss*). Data for fish in which arterial blood was monitored (unfilled symbols) are re-plotted from Fig 3-1. The dashed vertical line at time = 0 min indicates the point of saline injection. Significant differences ($P < 0.05$) from the final pre-injection values (time = 0 min) are indicated by asterisks or a horizontal line. Significant differences ($P < 0.05$) from the corresponding value in the control group (in which arterial blood was monitored) are denoted by daggers. Data are presented as mean values ± 1 SEM.

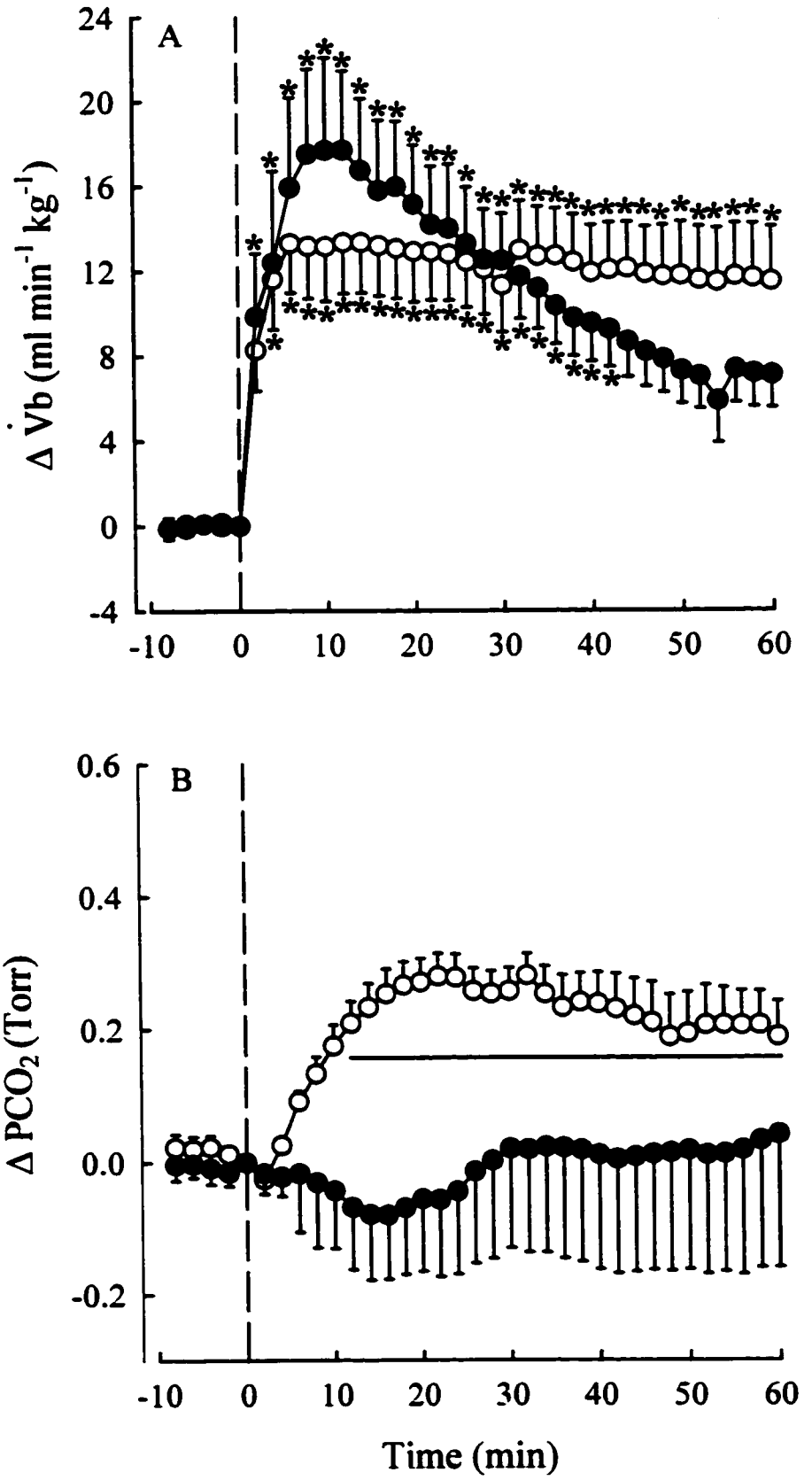
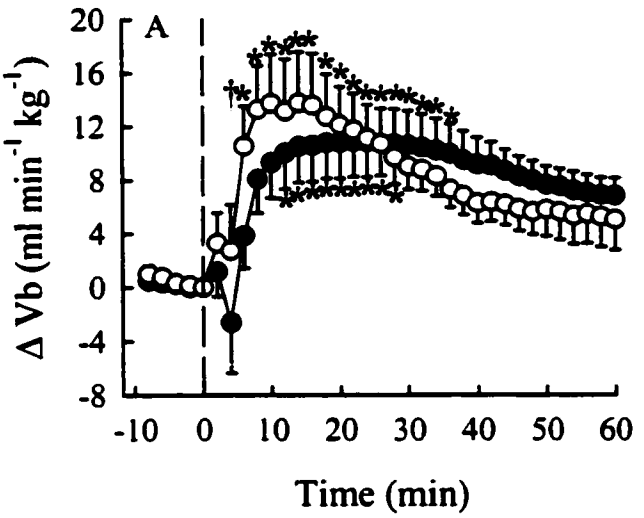
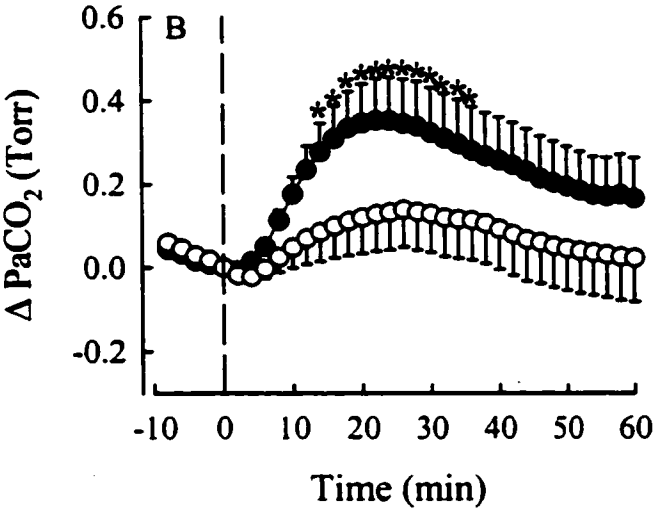
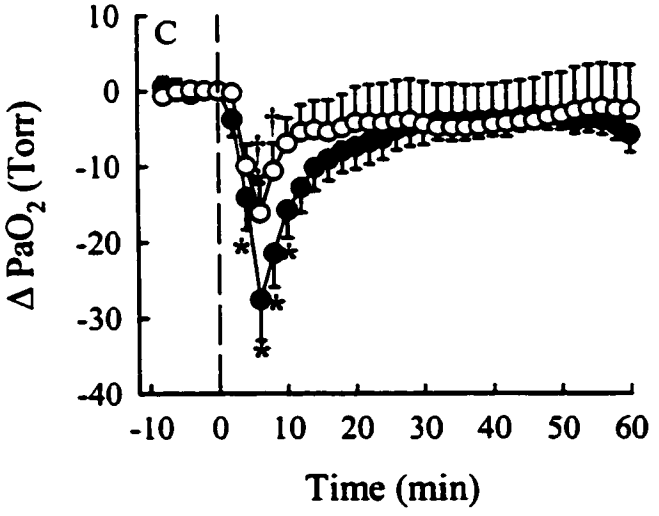

A
B
Δ Vb (ml min-1 kg-1)
Δ PCO2 (Torr)
Time (min)
24
20
16
12
8
4
0
-4
0.6
0.4
0.2
0.0
-0.2
-10
0
10
20
30
40
50
60

Figure 3-4. The effects of vascular volume loading (10 mL kg^{-1}) on changes in (A) cardiac output ($\Delta\dot{V}b$), (B) arterial PCO_2 (Δ $PaCO_2$), (C) arterial PO_2 (Δ PaO_2) and (D) arterial pH (pHa) in rainbow trout (*Oncorhynchus mykiss*) pre-treated with bovine carbonic anhydrase (10 mg kg^{-1}) (unfilled symbols, N = 9) or saline vehicle alone (control; filled symbols, N = 9). The dashed vertical line at time = 0 min indicates the beginning of volume loading. Significant differences ($P < 0.05$) from the final pre-loading values (time = 0 min) are indicated by asterisks. Significant differences ($P < 0.05$) from the corresponding value in the control group are denoted by daggers. Data are presented as mean values ± 1 SEM.


A
Δ Vb (ml min-1 kg-1)
20
16
12
8
4
0
-4
-8
-10 0 10 20 30 40 50 60
Time (min)


B
Δ PaCO2 (Torr)
0.6
0.4
0.2
0.0
-0.2
-10 0 10 20 30 40 50 60
Time (min)


C
Δ PaO2 (Torr)
10
0
-10
-20
-30
-40
-10 0 10 20 30 40 50 60
Time (min)

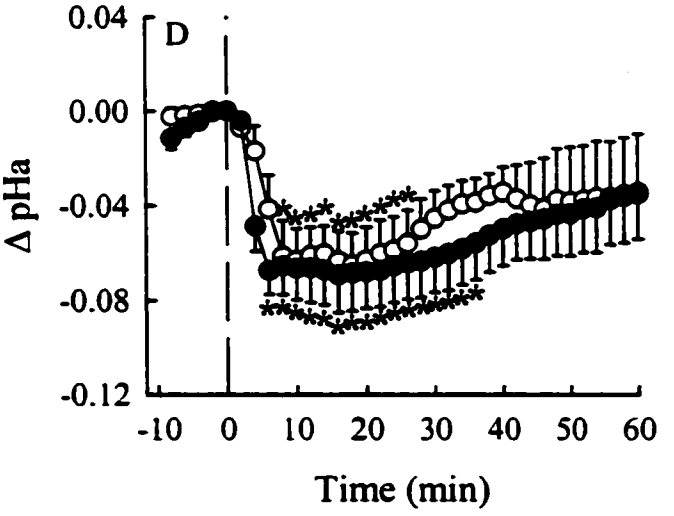

D
Δ pHa
0.04
0.00
-0.04
-0.08
-0.12
-10 0 10 20 30 40 50 60
Time (min)

Figure 3-5. The effects of vascular volume loading (10 mL kg^{-1}) on changes in (A) ventilation frequency (ΔV_f) and (B) ventilation amplitude (ΔV_{AMP}) in rainbow trout (*Oncorhynchus mykiss*) pre-treated with bovine carbonic anhydrase (10 mg kg^{-1}) (unfilled symbols, N = 9) or saline vehicle alone (control; filled symbols, N = 9). The dashed vertical line at time = 0 min indicates the beginning of volume loading. Significant differences ($P < 0.05$) from the final pre-loading values (time = 0 min) are indicated by asterisks. Significant differences ($P < 0.05$) from the corresponding value in the control group are denoted by daggers. Data are presented as mean values ± 1 SEM.

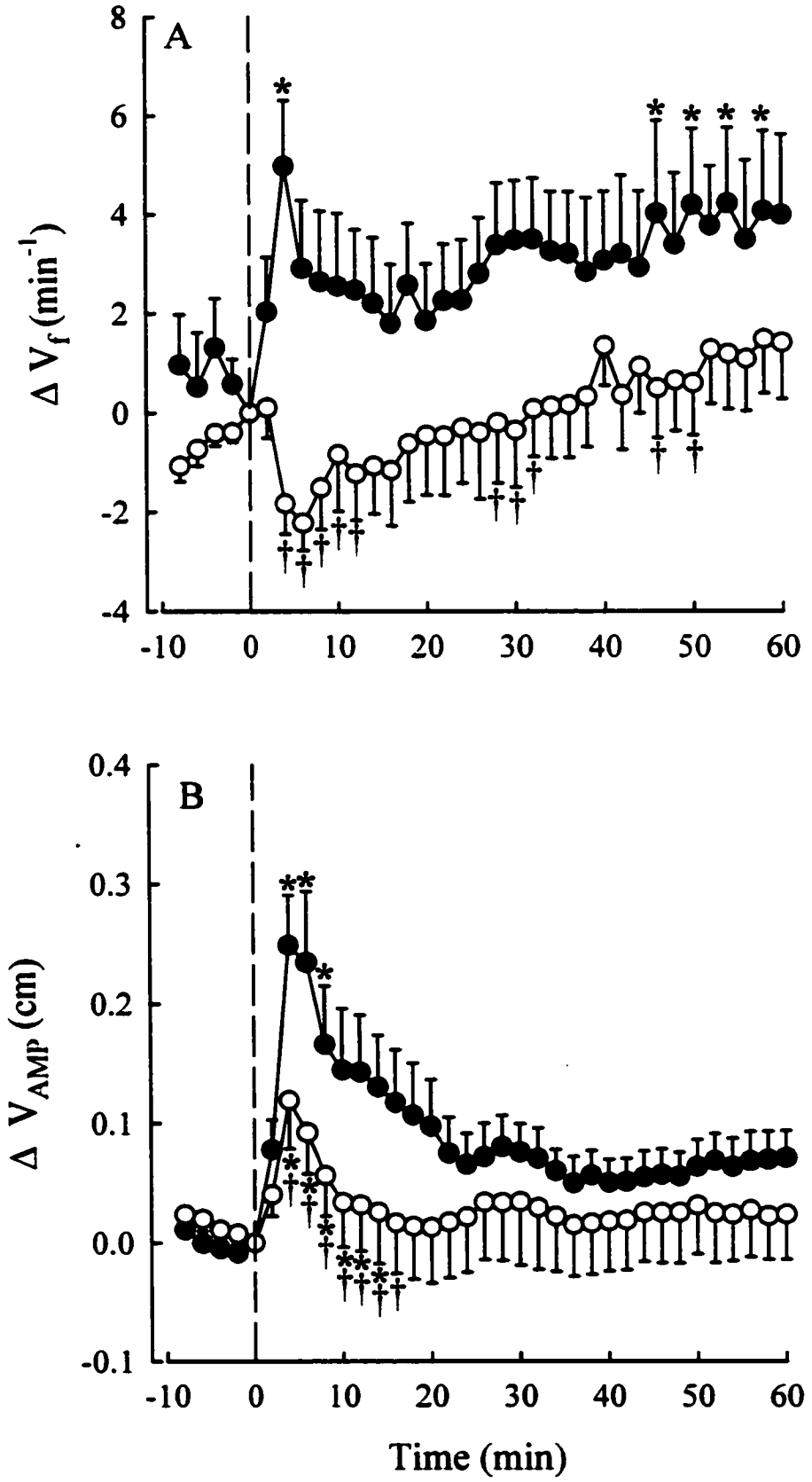
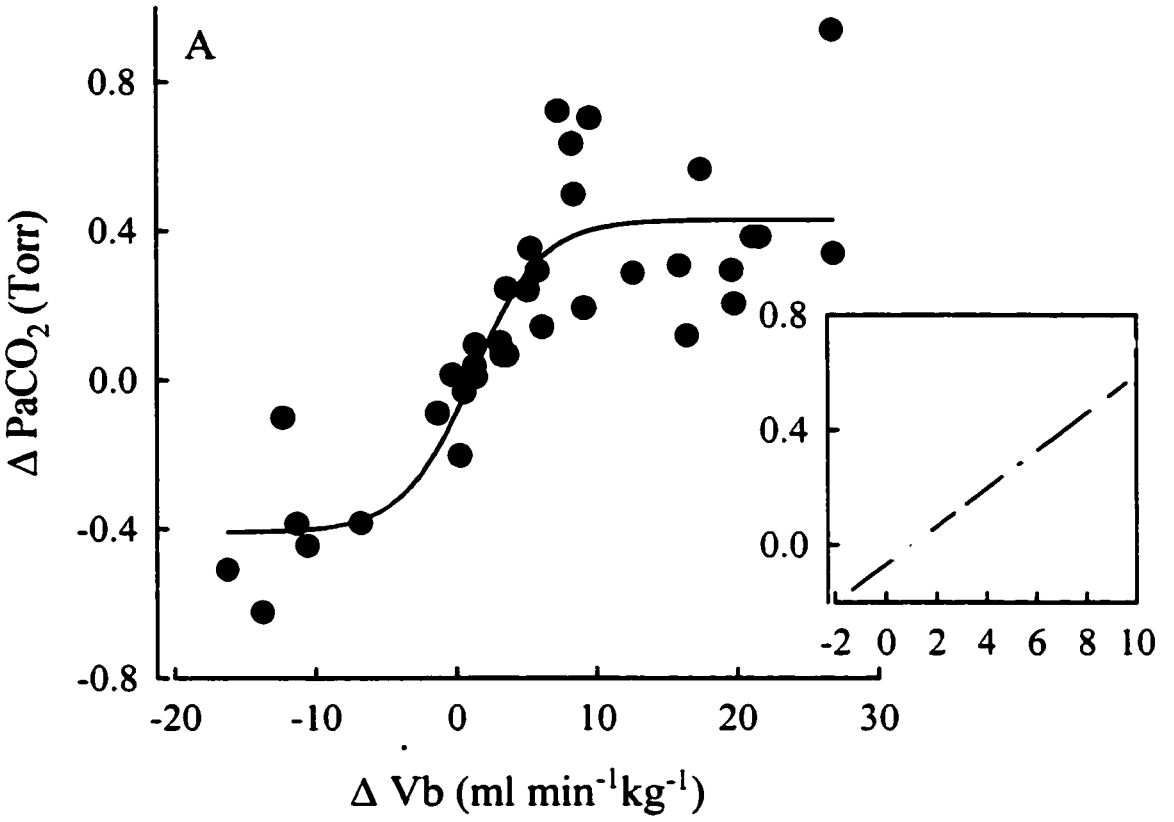

A
B
8
6
4
2
0
-2
-4
0.4
0.3
0.2
0.1
0.0
-0.1
-10
0
10
20
30
40
50
60
Δ V_f (min^-1)
Δ V_AMP (cm)
Time (min)

Figure 3-6. The relationship between the changes in cardiac output ($\Delta \dot{V}b$) and the changes in (A) arterial PCO_2 ($\Delta PaCO_2$; N = 36) and (B) arterial PO_2 (ΔPaO_2; N = 33) in rainbow trout (*Oncorhynchus mykiss*). The changes in $\dot{V}b$ were elicited by vascular volume loading or plasma removal. Note the significant sigmoidal correlation ($r^2 = 0.75$; $P < 0.05$) between $\dot{V}b$ and $PaCO_2$. The inset in panel (A) represents the linear portion of the curve ($r^2 = 0.72$; N = 20) shaded in grey. $\dot{V}b$ and PaO_2 were not significantly correlated ($r^2 = 0.07$).


A
0.8
0.4
0.0
-0.4
-0.8
Δ PaCO2 (Torr)
-20
-10
0
10
20
30
Δ Vb (ml min-1kg-1)
0.8
0.4
0.0
-2 0 2 4 6 8 10

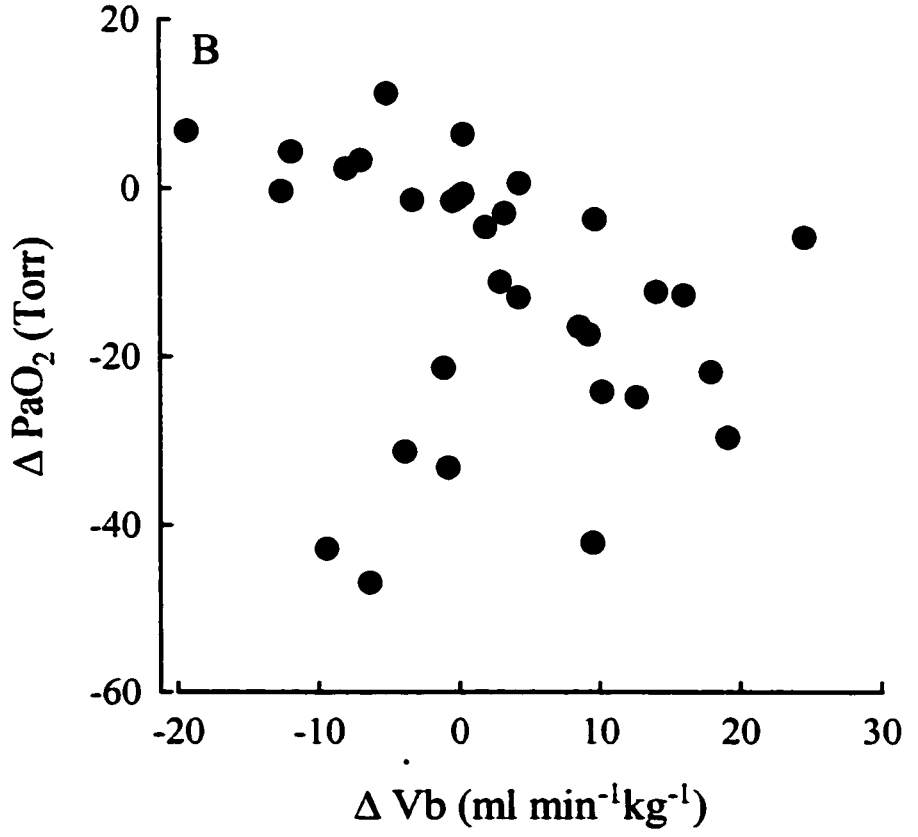

B
20
0
-20
-40
-60
Δ PaO2 (Torr)
-20
-10
0
10
20
30
Δ Vb (ml min-1kg-1)

Table 3-1. Absolute values for cardiac output ($\dot{V}b$), arterial or venous blood gases (PCO_2 and PO_2) and pH in rainbow trout (*Oncorhynchus mykiss*) prior to the manipulation of blood flow. Values presented are means ± 1 SEM; N numbers are indicated in parentheses. * denotes a statistically significant difference from all other treatments. For blood gases and pH, only those groups in which arterial blood was monitored were included in the analysis.

	Control	Volume-loaded (increased $\dot{V}b$)		Volume withdrawal (decreased $\dot{V}b$)
	Arterial blood (6)	Arterial blood (11)	Venous blood (6)	Arterial blood (6)
$\dot{V}b$ (ml min^{-1} kg^{-1})	22.2 ± 2.3	25.5 ± 2.1	22.3 ± 5.1	20.6 ± 3.2
PCO_2 (Torr)	1.58 ± 0.21	1.77 ± 0.22	3.27 ± 0.90	1.42 ± 0.16
PO_2 (Torr)	123.7 ± 9.9	102.1 ± 5.2 *	20.0 ± 5.0	130.3 ± 6.0
pH	7.97 ± 0.03	7.99 ± 0.06	8.03 ± 0.06	7.90 ± 0.07

Table 3-2. Absolute values for cardiac output ($\dot{V}b$), arterial blood gases (PCO_2 and PO_2), pH and ventilation parameters (frequency and amplitude), prior to volume loading in rainbow trout previously treated with either saline (control) or bovine carbonic anhydrase (10 mg kg^{-1}). Values presented are means ± 1 SEM; N numbers are indicated in parentheses. * denotes a statistically significant difference ($P < 0.05$) between the two treatments (unpaired Student's *t*-test).

	CA-treated (9)	**Control (9)**
$\dot{V}b$ (ml min^{-1} kg^{-1})	43.1 ± 6.8	35.7 ± 5.3
V_f (min^{-1})	87.2 ± 3.4	96.9 ± 4.1
V_{amp} (cm)	0.65 ± 0.04	0.61 ± 0.07
$PaCO_2$ (Torr)	1.77 ± 0.16	2.01 ± 0.16
PaO_2 (Torr)	106.5 ± 5.5	108.4 ± 5.3
pHa	8.04 ± 0.03 *	7.79 ± 0.05

Discussion

The results of the present study constitute the first direct *in vivo* evidence that $PaCO_2$ is sensitive to changes in $\dot{V}b$ over the physiological range. Because the sensitivity of $PaCO_2$ (and hence CO_2 transfer efficiency) to changes in $\dot{V}b$ is a hallmark of a diffusion-limited system (Wagner 1977), the current results confirm the indirect findings of previous studies, i.e. that CO_2 excretion in teleost fish behaves as a diffusion-limited process. The observation that the transit time limitations on $PaCO_2$ were relieved by providing plasma CO_2 reactions with access to CA activity speaks to the key role played by chemical equilibrium limitations and reveals that diffusion, per se, does not constrain CO_2 excretion. These data were collected for rainbow trout, a teleost fish, chosen for the present study because apparently uniquely among vertebrates, teleost fish lack plasma accessible CA at the gas exchange surface and are therefore ideal model organisms in which to conduct experiments involving the manipulation of CA availability (Henry and Swenson 2000).

Owing to its high capacitance in water and tissue, the permeation coefficient of CO_2 is approximately 30-fold greater than that for O_2. Previous studies, however, have suggested that CO_2 excretion in fish behaves as a diffusion-limited system whereas O_2 uptake is perfusion-limited (Daxboeck et al. 1982; Pärt et al. 1984; Malte and Weber 1985; Piiper 1989; Swenson 1990; Brauner et al. 2000). In a diffusion-limited system, gas transfer efficiency is inversely related to cardiac output because transit time through the respiratory structure is largely set by cardiac output. However, capillary recruitment and/or distension, both of which would tend to decrease blood flow velocity, likely lessen the impact of increased cardiac output on transit time (Presson et al. 1995; Hopkins et al.

1996). In contrast, gas transfer efficiency in a perfusion-limited system remains constant during changes in transit time within the physiological range because of the rapid rate of equilibration of gases between the respiratory medium and the blood. A perfusion-limited system could, however, become diffusion-limited if the residence time of the blood in the gas exchange surface was decreased so as to approach the diffusion equilibration time of the respiratory gas. Because the partial pressures of gases in the blood leaving the exchange surface provide an index of transfer efficiency (provided that as in the present study, inspired and venous gas tensions remain constant), changes in arterial blood gas tensions with changes in $\dot{V}b$ (hence transit time), can readily demonstrate diffusion limitations. This approach was used in the present study. The teleost gill lacks plasma-accessible CA (Rahim et al. 1988) and therefore blood exiting the gill is in a state of acid-base disequilibrium (reviewed by Gilmour 1998a). Consequently, there are downstream increases in PCO_2 in the arterial blood owing to the continuing uncatalysed dehydration of plasma HCO_3^- (Gilmour et al. 1994). Fortunately, the magnitude of these changes is negligible (i.e. $\Delta PCO_2 = 0.04$ mm Hg) and thus the measurement of $PaCO_2$ can be used as an accurate estimate of post-branchial PCO_2. The abolishment of this acid-base disequilibrium is responsible for the higher starting point of pHa in the CA-treated fish as observed by Desforges et al. (2001; chapter 2)

In the present study, O_2 uptake fit the criterion of a perfusion-limited system in that PaO_2 was largely constant despite significant changes in $\dot{V}b$ and the presumed concomitant changes in the transit time of blood through the gills. In some instances, PaO_2 declined immediately after the volume loading. Frequently, saline injections into

the venous circulation induced a sudden and profound apparent reflex decrease in $\dot{V}b$ lasting 10 - 40 sec (P.R. Desforges; unpublished observations; see Fig 3-4). It is possible that the immediate reduction in PaO_2 in volume-loaded fish may have been related to this injection artefact. Importantly, however, the reductions in PaO_2 (unlike the increases in $PaCO_2$) were transient and not synchronised with the long lasting increases in $\dot{V}b$. Moreover, correlation analysis revealed that there was no significant relationship between PaO_2 and $\dot{V}b$ (Fig 3-6B). Thus, despite the unexplained transient decline in PaO_2 in volume-loaded fish, we are confident that the overall results of the current study support the notion of perfusion-limited O_2 transfer.

Whereas O_2 uptake is perfusion-limited, the significant relationship between $\Delta PaCO_2$ and $\Delta\dot{V}b$ indicated that CO_2 excretion was effectively diffusion-limited. Several additional lines of evidence from recent *in vivo* studies support this contention. Julio et al. (2000) observed a significant increase in $PaCO_2$ (PaO_2 was unchanged) in trout experiencing a 30% reduction in gill surface area as a result of selective gill arch ligation. Similarly, hormonally induced thickening of the blood-to-water diffusion barrier also caused a specific impairment of CO_2 transfer (Bindon et al. 1994). Unlike the present investigation, however, these previous studies did not focus specifically on the assessment of diffusion/perfusion limitations. Thus, their interpretation was confounded by uncontrolled changes to ventilation and the absence of blood flow measurements.

The increase in $PaCO_2$ that was observed in the present study during volume loading could not be explained by changes in pre-branchial PCO_2 because venous PCO_2 was unaltered in the volume-loaded fish. Similarly, although volume loading was

associated with an increase in ventilation, such a response would be expected to lower $PaCO_2$ (Iwama et al. 1987). Interestingly, the fish that were pre-treated with CA exhibited a smaller increase in ventilation after volume loading. Thus, ventilatory adjustments clearly did not contribute to the changes in $PaCO_2$ that accompanied the variations in $\dot{V}b$, nor could they explain the effect of CA on these changes. Finally, dilution of the blood after volume loading may have reduced the circulating concentration of RBC Cl^-/HCO_3^- exchangers and thus impeded overall CO_2 excretion. Given the small change in haematocrit (from 22.3 to 18.8%) that accompanied volume loading, this possibility seems unlikely. Indeed, such a variation in haematocrit is both common within trout populations and without any apparent impact on gas transfer. Much larger reductions in haematocrit (to approximately 5%) are required to impair CO_2 excretion (Wood et al. 1982).

Apparently anomalous pH decreases were observed with blood removal (where $PaCO_2$ also fell) and volume loading in CA-treated fish (where $PaCO_2$ remained constant). A possible explanation for these unexpected results is that changes in blood volume stimulated catecholamine release, which in turn triggered RBC β-activated Na^+/H^+ exchange to evoke metabolic acidosis within the plasma. However, the adrenergic activation of the red cell βNHE could not account for the fall in pHa because pHa also fell in fish having received the β-adrenoreceptor antagonist sotalol.

The present study has demonstrated that the overall process of chemical conversion of HCO_3^- to CO_2 is responsible for the apparent diffusion limitations on CO_2 excretion and its sensitivity to transit time changes. Although HCO_3^- is dehydrated rapidly at the catalysed rate by RBC CA, the RBC Cl^-/HCO_3^- exchanger (Hubner et al.

1992) by providing the pathway for HCO_3^- entry into the RBC, sets the rate at which catalysed HCO_3^- dehydration occurs (Cameron 1978; Obaid et al. 1979; Perry et al. 1982; Perry and Gilmour 1993; Jensen and Brahm 1995). As the slowest step in the cascade of events comprising CO_2 excretion, Cl^-/HCO_3^- exchange is also believed to be the rate-limiting step in this process (Perry 1986). Experimental support for this hypothesis was recently provided by Desforges et al. (2001; chapter 2) who demonstrated that addition of CA to the plasma of trout, *in vivo*, caused a significant and rapid lowering of $PaCO_2$. Thus, it is not an intrinsically low activity of RBC CA, per se, that is the basis for CO_2 chemical equilibrium limitations in teleosts. Rather, CO_2 excretion is constrained by the relatively slow delivery of substrate (HCO_3^-).

The dehydration of plasma HCO_3^- requires equimolar quantities of H^+ that are largely derived from non-HCO_3^- buffers. Despite the low buffering capacity (ß) of trout plasma (~ -3 mmol l^{-1} pH $unit^{-1}$;Wood et al. 1982), the addition of extracellular CA fully relieved the transit-time limitations imposed by the elevated $\dot{V}b$. This finding suggests that inadequate buffering did not limit the catalysed dehydration of HCO_3^- in the plasma, at least at the rates required to eliminate the transit time limitations. Similarly, Desforges et al. (2001; chapter 2) demonstrated that the capacity of injected exogenous CA to lower $PaCO_2$ in trout was unrelated to ß within the range -3.9 to -12.1 mmol L^{-1} pH $unit^{-1}$. Thus, unlike in mammals, where low plasma ß is believed to restrict the contribution of pulmonary endothelial CA to less than 10% of total CO_2 excretion (reviewed by Bidani and Crandall 1988; Swenson 2000), the lower rates of CO_2 excretion in fish may allow for a greater contribution of extracellular HCO_3^- dehydration when CA is added to the plasma. Indeed, in dogfish, a species that is known to posses plasma-accessible gill

membrane-bound CA (Henry et al. 1997; Gilmour et al. 1997), the extracellular dehydration of HCO_3^- contributes significantly to overall CO_2 excretion (Gilmour et al. 2001).

During exercise in trout, $\dot{V}b$ can rise by as much as 4-fold and this increase is associated with an estimated reduction in gill transit time from 3 to 1 sec (Randall 1982). Based on the results of the present study, such changes in $\dot{V}b$ during exercise would be expected to impose transit time limitations on CO_2 transfer, and hence cause an elevation of $PaCO_2$ (independently of the potential impact of elevated CO_2 production). Indeed, in most fish species that have been examined, exercise results in a marked elevation of $PaCO_2$ without any concomitant reduction in PaO_2 (see Wood 1991). The possible cause(s) of the post-exercise respiratory acidosis have been debated previously (Wood 1991) and only recently has diffusion limitation been suggested to be a causative factor (Brauner et al. 2001). Given the results of the present study, the simplest explanation is that CO_2 transfer, by behaving as a diffusion-limited system, is constrained by the reduced transit time such that $PaCO_2$ rises. PaO_2 is unaffected by the transit time reductions (as in the present study) because O_2 transfer behaves as a perfusion-limited system. It is less clear to what extent transit time reductions would affect $PaCO_2$ in exercising mammals. It has been argued that during intense exercise, pulmonary transit time may fall below the time required to complete RBC Cl^-/HCO_3^- exchange (Bidani and Crandall 1988) and thus may impede CO_2 excretion. Whether the activity of pulmonary endothelial CA could supplement CO_2 excretion under such conditions, to enable $PaCO_2$ to be maintained is uncertain. Indeed, the contribution made to CO_2 excretion by pulmonary endothelial CA IV remains unclear, with estimates ranging from <10% to

>40% (Bidani 1991; Klocke 1997; reviewed by Swenson 2000), although the consensus appears to be that its contribution is limited to <10% *in vivo* owing to limited H^+ availability in the plasma (Henry and Swenson 2000).

In conclusion, the present study utilised experimental manipulation of the blood transit time through the gill *in vivo* to demonstrate apparent diffusion limitations on CO_2 excretion but not O_2 uptake. Because CA eliminated the apparent diffusion limitation on CO_2 excretion, the results indicate that, rather than a true diffusion limitation, CO_2 excretion is constrained by a chemical equilibrium limitation.

CHAPTER 4

GENERAL DISCUSSION

Discussion

The work of chapter 2 is the first *in vivo* evidence to support the hypothesis that the chloride shift is the rate limiting step in CO_2 excretion in teleost fish under resting conditions, thus suggesting that a chemical equilibrium limitation exists. Although many previous experiments had investigated the indirect (Wood et al. 1982; Heming and Randall 1982) or direct (Cameron 1978; Obaid et al. 1979; Perry et al. 1982, 1991, 1996; Tufts et al. 1988; Perry and Gilmour 1993; Jensen and Brahm 1995; Gervais and Tufts 1999) implication of Cl^-/HCO_3^- exchange in limiting CO_2 excretion, no study was able to demonstrate this phenomenon *in vivo* under steady state conditions. In numerous studies, bovine carbonic anhydrase (CA) was injected into the circulation of resting rainbow trout, but the investigators failed to find a significant decrease in arterial CO_2 tension ($PaCO_2$) or blood total CO_2 content (Gilmour et al. 1994; Wood and Munger 1994; Lessard et al. 1995). Following the findings described in chapter 2 and those of Julio et al. (2000), the sensitivity of $PaCO_2$ (as an index of CO_2 excretion) to changes in blood flow ($\dot{V}b$) was tested. Again, the results presented in chapter 3 were the first *in vivo* experimental data confirming the sensitivity of $PaCO_2$ to changes in $\dot{V}b$ in teleost fish under resting conditions, and hence providing direct evidence of the apparent diffusion limitations on CO_2 excretion in teleost fish. Furthermore, the administration of CA relieved the diffusion limitation, exposing the key role of chemical equilibrium limitations in accounting for the apparent diffusion limitation of CO_2 excretion. Therefore, the results presented in this thesis reaffirm the well known gas transfer models for teleost fish, in which perfusion limitations exist on oxygen uptake and diffusion limitations exist on CO_2 excretion (Daxboeck et al. 1982; Pärt et al. 1984; Malte and

Weber 1986; Piiper 1989; Swenson 1990; Brauner et al. 2000), and in addition, provide an explanation for the apparent diffusion limitations on CO_2 movement across the respiratory epithelium.

The significance of the experimental methods employed

In this thesis, results that contradict the findings of previous studies are presented, particularly in chapter 2, and we suspect that our experimental methods are responsible for these differences. The extracorporeal blood shunt enables the continuous measurements of blood gases (Thomas 1994), therefore allowing the detection of even subtle changes in the monitored gases. The administration of bovine CA to the circulation of resting rainbow trout resulted in a small decrease in $PaCO_2$ over a long period of time, a change that would be difficult to detect with standard sampling techniques. The response to CA injection in chapter 2 was monitored over an extended period (90 min), unlike previous studies in which the experimental period was typically 12-60 min (Gilmour et al. 1994; Currie et al. 1995; Lessard et al. 1995). Notably, both Wood and Munger (1994) and Currie et al. (1995) reported effects of exogenous CA injection under conditions of enhanced CO_2 production (resulting from exhaustive exercise), when the impact of extracellular CA in enhancing CO_2 excretion would be expected to be larger and therefore more easily detected. Similarly, Lessard et al. (1995) calculated that $PaCO_2$ would be decreased significantly by CA infusion during hypoxia, when CO_2 excretion is elevated by the hypoxia-induced hyperventilation.

Furthermore, only a few studies have examined the effects of changes in $\dot{V}b$ on CO_2 excretion. Perry (1986), using data collected by Perry et al. (1982), reported a

correlation between $\dot{V}b$ and CO_2 excretion using a spontaneously ventilating blood perfused trout preparation. Importantly, however, $PaCO_2$ also increased significantly with increasing $\dot{V}b$, providing evidence for a diffusion-limited system (Perry, 1986). According to Davie et al. (1982), this preparation consisted of a catheter implanted in the ventral aorta, and a venous return catheter in the bulbus arteriosus. Utilising a cardiac pump to manipulate the flow, blood was perfused through the entire body via the ventral aortic catheter, and returned to their blood pool tonometer via the venous return catheter. Although this preparation allowed complete control of $\dot{V}b$, it suffered from several drawbacks. First, the cardiovascular system of the experimental fish was flushed with saline during surgery so as to be immediately perfused with blood provided from donor fish after surgery (Davie et al. 1982). Severe anaemia elicits release of the stress hormones adrenaline and noradrenaline (catecholamines) in rainbow trout (Iwama et al. 1987; Perry et al. 1989). Also, the experiments began 3-4 hours after the heart bypass surgery, just long enough to allow the stabilisation of ventilation. It is known that fish experience elevated stress levels after surgery, and replacement of the fish's blood with donor blood probably contributed to increasing the stress level. The release of catecholamine during those experiments may have inhibited CO_2 excretion according to the CO_2 retention theory.

Brauner et al. (2000) observed an elevation in $PaCO_2$ when $\dot{V}b$ was increased during sustained exercise. Again, while this study provided support for the presence of diffusion limitations on CO_2 excretion, several factors may have confounding effects. Although $\dot{V}b$ increases during exercise, many other factors also change and could

potentially influence $PaCO_2$; for instance, CO_2 production increases, $\dot{V}w$ is elevated and the stress level of the fish rises. The potential role of catecholamines in affecting CO_2 excretion is still debated (Perry and Vermette 1987; Steffensen et al. 1987; Tufts et al. 1988; Vermette and Perry 1988; Playle et al. 1990; Wood and Perry 1991; Perry et al. 1991; Thomas and Perry 1992). According to the 'CO_2 retention theory' (Wood and Perry 1985; Perry and Wood, 1989), CO_2 excretion may be inhibited by catecholamine release because catecholamines activate via a β-adrenoreceptor the Na^+/H^+ exchanger on the RBC membrane, which extrudes H^+ ions from the erythrocyte to the plasma. This efflux reduces the number of H^+ available in the RBC, driving CO_2 reactions towards the hydration of CO_2 into HCO_3^-, and therefore impeding its excretion. This theory is difficult to test during exercise since it is not clear if the increase in $PaCO_2$ is caused by an increased production of CO_2 at the tissue level, or by an inhibition of CO_2 elimination at the gills. In contrast, the manipulation of $\dot{V}b$ under steady state conditions, by volume loading or unloading, as utilised in the experiments described in chapter 3, allowed modification of the transit time without either significantly changing haematocrit or stressing the fish. By eliminating confounding factors through the use of a controlled experimental design, we feel confident that the effects observed were the result of the treatment imposed.

Chemical equilibrium limitations

Owing to the low solubility but relatively high capacitance of plasma for CO_2, CO_2 is transported in the plasma primarily as HCO_3^- ions (Boutilier et al. 1984). However, it is essential that HCO_3^- be converted back to molecular CO_2 to be excreted by

diffusion across the gill, since the branchial epithelium has a very low permeability to HCO_3^- ions (Perry et al. 1982, 1984). The dehydration of HCO_3^- is catalysed by CA, which in teleost fish is available only in the RBC for CO_2 excretion (Henry and Swenson 2000); gill epithelial cell intracellular CA serves to hydrate CO_2 to provide H^+ and HCO_3^- for gill ion transport proteins. This thesis demonstrated, through the addition of bovine CA to the plasma and resultant decrease in $PaCO_2$, that CO_2 elimination is limited by the chemical equilibration of HCO_3^- and CO_2.

The CA activity in the erythrocyte is believed to be greatly in excess of what is required for the dehydration of HCO_3^- (Henry and Swenson 2000), and therefore, should not limit the chemical equilibration of CO_2/HCO_3^-. Buffering capacity also plays a role in the chemical equilibration reactions by providing the protons necessary for the dehydration of HCO_3^-. As presented in chapter 2, increasing the buffering capacity of rainbow trout plasma by 3-fold *in vivo* did not significantly enhance CO_2 excretion in the presence of exogenous (plasma) CA, probably because the experimentally-elevated plasma buffering was still swamped by the high degree of buffering provided by haemoglobin and also the protons provided by the Haldane effect. Thus, proton availability in the RBC appears to be sufficient for the dehydration of HCO_3^-. Since effective HCO_3^- dehydration occurs in the RBC, the entry of HCO_3^- in the erythrocyte is crucial. It was believed (Cameron 1978; Perry 1986) that HCO_3^- transfer via the chloride shift was a slower process than the catalysed dehydration of HCO_3^-, making it the limiting step in the process of CO_2 excretion. We circumvented the Cl^-/HCO_3^- exchanger by administering bovine CA to the circulation of rainbow trout, and thereby showed that the essential entry of HCO_3^- in the RBC was responsible for limiting the chemical

equilibration of HCO_3^- and CO_2, confirming this belief. Furthermore, in chapter 3, we demonstrated that the chemical equilibration limitation originating from the chloride shift was responsible for the apparent diffusion limitation of CO_2 excretion.

Future directions

The findings of this thesis have raised several interesting questions that warrant further investigation. Notably, three different directions could be pursued; the direct inhibition of the Cl^-/HCO_3^- exchanger *in vivo* in fish, the comparison of teleost and elasmobranch fish with regard to apparent diffusion limitations on CO_2 excretion, and finally, the physiological significance and/or role of the apparent diffusion limitations on CO_2 excretion. First, although some studies have directly examined the role of the Cl^-/HCO_3^- exchanger in CO_2 excretion in mammals (Swenson et al. 1993), in elasmobranchs (Gilmour et al. 1997; 2001) and in teleost fish *in vitro* (Gilmour and Perry 1993), to our knowledge, no studies have used stilbenedisulfonates to directly inhibit the chloride shift in teleost fish *in vivo*. The use of stilbenedisulfonate inhibitors in teleost fish *in vivo* to inhibit the chloride shift, coupled with measurements of CO_2 excretion, could directly confirm the limiting role of the chloride shift in CO_2 excretion in fish. By inhibiting the Cl^-/HCO_3^- exchange, we would expect a rise in $PaCO_2$ since the only CA activity in the RBC would not be available for the dehydration of HCO_3^-. Furthermore, because of the elevation in $PaCO_2$ in the absence of the chloride shift, the high partial pressure gradient between the blood and the water will make CO_2 exchange rely only on diffusion without any chemical equilibrium limitations thus, the exchange of CO_2 will behave as a perfusion limited system. Therefore, the blockage of the chloride shift could confirm the

existence of the apparent diffusion limitations by totally eliminating the relationship between cardiac output and CO_2 excretion.

Secondly, our conclusions concerning the chemical equilibrium limitations on CO_2 excretion give rise to interesting questions regarding potential differences among different fish groups. In elasmobranchs, extracellular CA activity is available in two locations to dehydrate HCO_3^- in the plasma, it is present in the plasma and bound to gill epithelial cell membranes with an extracellular orientation (Henry et al. 1997; Gilmour et al. 1997; 2001, Wilson et al. 2000). This situation differs from that in teleost fish, which lack plasma-accessible CA activity (Gilmour 1998). In addition, recent work suggests that HCO_3^- dehydration in the plasma of dogfish makes a substantial contribution to CO_2 excretion *in vivo* (Gilmour et al. 2001). If in fact, the plasma-accessible CA does make a substantial contribution to CO_2 excretion, no chemical equilibrium limitations would be observed resulting from the slow rate of the chloride shift in elasmobranchs. Consequently, CO_2 excretion should behave as a perfusion limited system in elasmobranchs as observed in trout after the administration of exogenous CA (as seen in chapter 3). Therefore, more work is needed to investigate whether an apparent diffusion limitation for CO_2 excretion is present in elasmobranchs, and if it is present, to compare it with that observed in teleost fish.

Finally, the results of the present study suggest that, owing to the apparent diffusion limitations on CO_2 excretion in teleost fish, $PaCO_2$ is closely related to $\dot{V}b$. Further investigations are necessary to examine the significance of this relationship and to determine if fish use the manipulation of cardiac output as a strategy to control $PaCO_2$

as well as acid-base balance. Also, if $\dot{V}b$ is regulated in this regard, additional studies are needed to understand the conditions under which this strategy is employed.

Conclusions

In conclusion, this thesis demonstrated experimentally that the widely held view that CO_2 excretion is limited by the slow entry rate of HCO_3^- into the RBC via the chloride shift in teleost fish *in vivo* is valid. The low buffering capacity of rainbow trout plasma does not appear to be a limiting factor for the dehydration of HCO_3^- in the plasma in the presence of exogenous CA *in vivo*, but does seem to be limiting *in vitro*, probably because *in vivo*, small changes in plasma buffering are swamped by the much larger buffer capacity in the RBC. Furthermore, we demonstrated directly *in vivo* the apparent diffusion limitations on CO_2 excretion and perfusion limitations on O_2 uptake by manipulating the blood transit time through the gills; the arterial CO_2 tension was sensitive to changes in cardiac output but the arterial O_2 tension was not. Moreover, the administration of CA to the plasma relieved the apparent diffusion limitation on CO_2 excretion, suggesting that it arises from a chemical equilibrium limitation between HCO_3^-, H^+ and CO_2 originating from the slow rate of the chloride shift. Therefore, the slow entry of HCO_3^- into the RBC is responsible for the apparent diffusion limitations. These results provide a logical explanation for the apparent discrepancies between the existence of diffusion limitations on CO_2 excretion but not O_2 uptake and for the higher permeation coefficient for CO_2 over O_2 for movement across a biological membrane.

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