AN ANALYSIS OF THE TRANSPORT AND INTERACTION OF OXYGEN AND CARBON DIOXIDE IN FISH.

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B.Sc. (Hon.) The University of British Columbia, 1988.

M.Sc. The University of British Columbia, 1991.

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Department of Zoology)

We accept this thesis as conforming

to the required standard

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THE UNIVERSITY OF BRITISH COLUMBIA

December, 1995

ABSTRACT

Many teleost fish haemoglobins (Hbs) exhibit a Root effect, a large Haldane effect and a low buffer capacity. This combination of characteristics influences the interaction between movements of oxygen (O_2) and carbon dioxide (CO_2) in the red cell, in the respiratory epithelium, and in the tissues. For example in rainbow trout, oxygenation of the blood at constant Pco_2 *in vitro*, induces a large acidosis (0.21 pH units) in the red cell. This acidosis results from the release of a large number of protons during Hb oxygenation (Haldane effect) in the presence of a Hb with a low buffer capacity. It can be hypothesized, that oxygen uptake in the absence of CO_2 removal, could limit oxygen binding to Hb at the gills by as much as 50% due to the presence of the Root effect.

Arapaima gigas is an obligate air breathing teleost fish from the Amazon. It possesses two respiratory surfaces for gas exchange: gills and a highly vascularized swimbladder which acts as an air-breathing organ (ABO). The movements of O_2 and CO_2 are spatially uncoupled in normoxia. That is, 78 % of the O_2 consumed was from the air and 85 % of the CO_2 excreted was into the water. Therefore, a large proportion of the oxygen uptake across the ABO occurred in the absence of CO_2 removal. The Hb in this species possessed a large Root effect and therefore, an acidosis induced by Hb oxygenation in the absence of CO_2 removal, could impair O_2 uptake as hypothesized above in rainbow trout. The Haldane effect in this Hb, however, was small preventing an acidosis during Hb oxygenation. Interestingly, the Hb buffer capacity was also low

relative to that in rainbow trout, seemingly maladaptive for CO_2 excretion. Thus, Hb characteristics appear to be modified to prevent impairment of O_2 uptake in the absence of CO_2 removal in *A. gigas*; however, the effect of these changes on CO_2 excretion is less clear.

A quantitative analysis of O_2 and CO_2 transport was conducted in resting and exercising rainbow trout, and these data were used to quantify the magnitude of the coupling between O_2 and CO_2 exchange, *in vivo*. In resting rainbow trout exposed to normoxia and two levels of hypoxia, or in fish during sustained exercise, 60% of the total CO_2 excreted was dependent upon HCO_3^-/Cl^- exchange during red cell transit through the gills. This is of significance to CO_2 excretion because HCO_3^-/Cl^- exchange is thought to be the rate limiting step. In both arterial and mixed-venous blood of trout, an acid-base disequilibrium was observed in resting fish exposed to normoxia and two levels of hypoxia, indicating that the blood pH and Pco_2 probably never reach equilibrium *in vivo*. However, inclusion of the acid-base disequlibrium in an analysis of partitioning of CO_2 excretion, did not result in a significant difference from a similar analysis using steady state values.

Oxygenation of whole blood from trout resulted in a non-linear release of protons (Bohr protons) over the Hb-O_2 equilibrium curve *in vitro*. That is, the majority of Bohr protons were released between 60 and 100% of Hb oxygen saturation (So_2). Rapid oxygenation of the blood over this region of the Hb-O_2 equilibrium curve elevated the HCO_3^- flux rate across the HCO_3^-/Cl^- exchanger on the red cell by about 30% during CO_2 excretion *in vitro*. Oxygenation of the Hb between 0 and 60% So_2 did not elevate

CO_2 excretion rate *in vitro*.

The non-linear release of Bohr protons over the Hb-O_2 equilibrium curve was also observed *in vivo*, in trout subjected to different levels of sustained exercise. At low swimming speeds, when venous blood O_2 content (C_vO_2) was high, there was a small acidosis as blood passed through the gills, indicating more protons were released during oxygenation of Hb than were consumed during HCO_3^- dehydration. At higher swimming speeds, when C_vO_2 was low, there was a significant alkalosis in the arterial blood relative to the venous blood, indicating fewer protons were released upon oxygenation than HCO_3^- ions were dehydrated to CO_2. Haldane coefficients (moles of protons released per mole of O_2 which binds to Hb), calculated from steady state arterial and mixed-venous parameters, revealed that under resting conditions 100% of CO_2 excreted was stoichiometrically related to O_2 uptake through the release of Bohr protons during Hb oxygenation. The magnitude of coupling between CO_2 excretion and O_2 uptake decreased from 100% to less than 50% at the maximal swimming velocity when the largest region of the Hb-O_2 equilibrium curve was used for gas exchange. The non-linear release of Bohr protons over the range of Hb-O_2 saturation in the blood limits HCO_3^- dehydration at the gills during greater work loads, conserving the HCO_3^- buffer capacity of the blood and tissues.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

First and foremost, I gratefully acknowledge the stimulating guidance, freedom to explore, and endless opportunities provided by my supervisor, David Randall.

I am thankful for the collaboration with Drs. Helgi Thorarensen and Pat Gallaugher in the work presented in Chapter 3. I am also indebted to Drs. Steve Perry and Katie Gilmour for the teaching of techniques and collaboration in the experiments presented in Chapters 1 and 2, and to the "Perry lab" for their hospitality during my brief stay in Ottawa. Dr. Adalberto Val collaborated in the work presented in Chapter 4 and I thank Drs. Adalberto and Vera Val and the members of their laboratories for everlasting memories and the eye opening experience of working in the Amazon.

The moral support and reality checks provided by present and past members of the lab, and from friends outside the lab, are also greatly appreciated. Finally, I thank my family who have always encouraged and supported me in all my endeavours, and of course Crystal Ballantyne; for everything!

Funding throughout my Ph.D. was provided from NSERC (PGS 3 and 4), a Killam pre-doctoral scholarship, an International Development Resource Council Young Canadian Researchers Award (which covered research expenses in Manaus, Brazil), a McLean Fraser Memorial Scholarship, a BC Science Council GREAT award and a Department of Zoology teaching assistantship.

LIST OF ABBREVIATIONS

$_a$: arterial blood (i.e.C_aO_2)

C: content

C_aO_2: oxygen content in arterial blood

CO_2: carbon dioxide

C_aCO_2: carbon dioxide content in arterial blood

Cl^-: chloride

$_e$: extracellular (plasma; i.e. pH_e)

Hb: haemoglobin

Hct: haematocrit

HCO_3^-: bicarbonate

$HCO_3^- \rightarrow CO_2$: bicarbonate dehydrated to CO_2

$_i$: intracellular (i.e. pH_i)

MetHb: Methaemoglobin

MCHC: mean cell haemoglobin concentration

mmHg: 0.1333 kPa

MS-222: tricaine methanesulphonate (anaesthetic)

pH_e: extracellular blood pH

pH_i: intracellular red blood cell pH

Po_2: partial pressure of O_2

$Pbco_2$: physically dissolved CO_2 in pre-branchial blood

Pco_2: partial pressure of CO_2

pK': apparent pK (dissociation constant) of carbonic acid in plasma

$\dot{M}o_2$: oxygen consumption rate ($mg \cdot kg \cdot h^{-1}$)

N_2: nitrogen

RE: respiratory exchange ratio

RE': modified respiratory exchange ratio

So_2: saturation of blood with oxygen

ppt: parts per thousand

$_v$: mixed-venous blood (i.e. P_vo_2)

Ucrit: critical swimming velocity

GENERAL INTRODUCTION

The classic study by Bohr, Hasselbalch and Krogh in 1904, was the first to demonstrate the influence of carbon dioxide (CO_2) on the affinity of blood for oxygen (O_2) in vertebrates (Bohr *et. al.*, 1904). As a consequence, the change in haemoglobin-oxygen (Hb-O_2) affinity with a change in either the partial pressure of CO_2 (Pco_2) or pH is now referred to as the Bohr effect. In 1914, Christiansen, Douglas and Haldane discovered that the CO_2 content of deoxygenated blood was greater than that of oxygenated blood at constant Pco_2, implicating a role for haemoglobin (Hb) in both CO_2 and O_2 transport. The greater CO_2 content in deoxygenated blood is now referred to as the Haldane effect and results from two phenomena: CO_2 binding to the deoxygenated Hb molecule elevating CO_2 content directly, and protons (Bohr protons) binding to the deoxygenated Hb elevating CO_2 content indirectly. The studies by Bohr *et. al.*, (1904) and Christiansen *et. al.*, (1914), among others, sparked investigations into the interaction between oxygen and carbon dioxide transport in the blood of vertebrates, which continues to the present, unabated. Most of these studies have been conducted *in vitro*, under non-physiological conditions. For example, the partial pressure of one gas is manipulated while the other is held constant, while *in vivo*, gas exchange normally involves simultaneous changes in both O_2 and CO_2. Thus, although the fact that an interaction exists between movements of O_2 and CO_2 is undisputed, the physiological importance remains largely unresolved, especially in lower vertebrates such as fish.

Gas exchange at the gills of fish can be loosely grouped into two components,

diffusion of gases between the environment and the blood, and processes which occur within the erythrocyte.

DIFFUSION OF GASES

The movement of a gas between the environment and the blood haemoglobin is complex. Some of the barriers which make this movement complex in fish consist of interlamellar water, the lamellar mucus layer, the apical and basal membranes and cytosol of the respiratory epithelial cells, the plasma, and the membrane and cytosol of the erythrocyte. Each barrier must be crossed by both O_2 and CO_2 and each barrier can potentially limit gas flux rate. For the purpose of this discussion; however, these barriers will be treated as one, the gills.

The flux rate of a gas across the gills can be mathematically represented by the Fick diffusion equation:

$$\textbf{Gas transfer rate} = \frac{\mathbf{D \times A \times (\Delta P)}}{\mathbf{t}}$$

where D is Krogh's constant of diffusion (a measure of diffusivity), A is the perfused gill surface area, t is the thickness of the diffusion path, and ΔP is the partial pressure difference of the gas across the gills. With the exception of diffusivity which is a physical constant, these parameters can be manipulated by the animal to maximize gas

exchange. During exercise, for example, when metabolic rate is elevated, the perfused area of the gills increases and diffusion path thickness decreases with an increase in ventral aortic blood pressure (Kiceniuk and Jones, 1977; Farrell *et. al.*, 1979; Randall and Daxboeck, 1981; Randall and Daxboeck, 1984). ΔP is also strongly influenced by changes in both ventilation volume and cardiac output. Fish at rest maintain a gill ventilation:blood perfusion ratio between 10 and 15 (Cameron and Davis, 1970; Kiceniuk and Jones, 1977; Jones and Randall, 1978), roughly equal to the ratio of O_2 solubility in water and blood.

The Fick diffusion equation implicitly assumes chemical equilibrium within the blood and the water, where the movement of gases is solely determined by the diffusive flux of the gas across the respiratory epithelium and the convective fluxes due to ventilation and perfusion of the gills. Recent evidence indicates that blood pH and Pco_2 may never reach equilibrium *in vivo* in fish (Gilmour *et. al.*, 1994). In addition, only a minor proportion of the total O_2 and CO_2 transported in the blood exists as physically dissolved molecules. Most of the CO_2 is carried as HCO_3^- in the plasma which must be dehydrated to CO_2 within the red cell (Perry *et. al.*, 1982) prior to diffusion into the ventilatory water, and the majority of O_2 taken up across the gills binds with Hb. Some of these reactions are considered to be rate limiting (Piiper, 1990). Thus, the diffusing capacity of the gills (gas transferred/unit partial pressure gradient) must be regarded as an "overall equilibration conductance" (Piiper, 1990) taking into account both the rate of gaseous diffusion and the rate of chemical reactions.

The control of features such as perfused area of the gills, diffusion path thickness

and gill ventilation:perfusion ratios undoubtedly have profound effects on gas exchange. The chemical reactions which occur within the red cell however will also greatly influence gas exchange and are described in the following section.

REACTIONS WITHIN THE RED CELL

CO_2 transport:

Most of the CO_2 excreted across the gills is transported as HCO_3^- in the plasma, but is released to the environment by way of diffusion of molecular CO_2 (Perry *et. al.*, 1982). The half time for bicarbonate dehydration:

$$HCO_3^- + H^+ \rightleftharpoons H_2CO_3 \rightleftharpoons CO_2 + H_2O$$

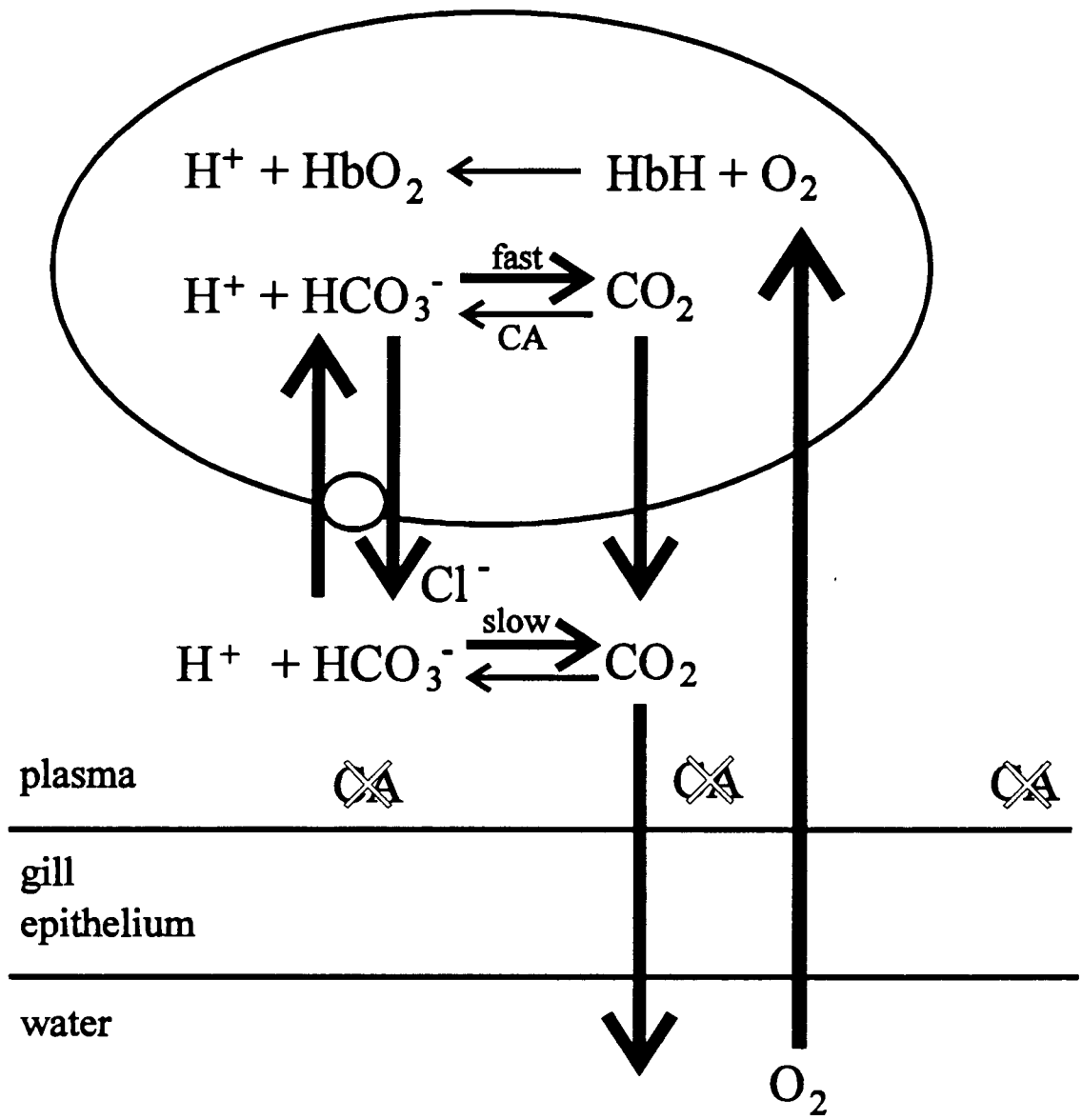
at the uncatalyzed rate, is 90 s for fish blood at 10 °C (Heming, 1984) and is only marginally faster at 37 °C (Swenson and Maren, 1978). As early as 1935, Roughton hypothesized that CO_2 excretion could not be achieved unless HCO_3^- dehydration occurred at the catalyzed rate. It is now well established that in both mammals and fish the enzyme which catalyzes HCO_3^- dehydration is carbonic anhydrase (CA). Unlike most air breathing vertebrates, the respiratory surface in trout and other freshwater teleost fishes lack plasma accessible carbonic anhydrase (Perry *et. al.*, 1982; Henry *et. al.*, 1988; Perry and Laurent, 1990) and therefore all HCO_3^- dehydration at the catalyzed rate

occurs within the red cell during gill blood transit.

Bicarbonate enters the red cell from the plasma in electro-neutral exchange with Cl^- by way of an exchanger in the band 3 protein of the erythrocyte membrane (Figure1; Romano and Passow, 1984). When examined independently of oxygen uptake, the limitation to CO_2 excretion is thought to lie at this HCO_3^-/Cl^- exchange site (Crandall and Bidani, 1981). Wieth *et. al.* (1982) determined *in vitro* that the contribution of red cell HCO_3^-/Cl^- exchanger to the total time course of CO_2 excretion in mammals is almost one-third and is the slowest reaction in CO_2 excretion. Under conditions of controlled blood flow in a spontaneously ventilating trout preparation, CO_2 excretion increased proportionately with haematocrit (Hct) at constant CO_2 content of the blood, and with plasma HCO_3^- concentration at constant Hct (Perry *et. al.*, 1982). In addition, CO_2 excretion was markedly reduced in the absence of red cells or in the presence of SITS (4-acetamido-4'-*iso*-thiocyanatostillbene-2,2' disulfonic acid), a HCO_3^-/Cl^- exchange blocker (Perry *et. al.*, 1982). Thus, there is evidence that the rate of HCO_3^- entry into the red cell limits CO_2 excretion in fish.

Not all CO_2 excreted is transported in the blood as HCO_3^-. In humans, oxylabile carbamate (CO_2 reversibly bound to Hb) accounts for approximately 13% of total CO_2 excreted at rest (Klocke, 1973; Klocke, 1987) and may increase to 20% during strenuous exercise (Swenson, 1990). The haemoglobins of fishes probably do not form much carbamate (Farmer, 1979; Heming *et. al.*, 1986). Carbamate is normally bound to the terminal amine groups of the α and β Hb sub-units. In fish, these groups on the α sub-units are acetylated and therefore unavailable for carbamate formation (Farmer, 1979,

FIGURE 1: A diagramatic representation of gas exchange at the gills. Oxygen diffuses into the red cell and binds to Hb releasing Bohr protons (H^+). The Bohr protons are consumed during HCO_3^- dehydration and CO_2 subsequently diffuses into the ventilatory water. The reverse scenario occurs in the tissues. CA- carbonic anhydrase, Hb- haemoglobin. Modified from Perry, 1986.


$H^+ + HbO_2 \longleftarrow HbH + O_2$
$H^+ + HCO_3^-$
fast
CA
CO_2
Cl^-
$H^+ + HCO_3^-$
slow
CO_2
plasma
CA
CA
CA
gill
epithelium
water
O_2

Riggs, 1979). The β sub-units are available but carbamates are in direct competition with organic phosphates which are preferentially bound (Weber and Lykkeboe, 1978). Thus, the lack of plasma accessible CA in the gills and reduced dependence upon carbamate during CO_2 excretion, will increase dependence upon HCO_3^- dehydration and red cell HCO_3^-/Cl^- exchange during CO_2 excretion relative to that in air-breathing vertebrates. The proportion of CO_2 excreted which is dependent upon HCO_3^-/Cl^- exchange in fish has not been measured.

The Haldane effect has long been implicated in CO_2 excretion. In fact in 1914, Christiansen, Douglas and Haldane, stated "The oxygenation of the blood in the lungs helps to drive out CO_2[,] and increases by about 50% or slightly more[,] the amount of CO_2 given off at each round of the circulation." In general, the Haldane effect is comprised of the oxylabile binding of both carbamate and protons which together give rise to the large difference in CO_2 content between oxygenated and deoxygenated blood *in vitro*, at constant P_{CO_2}. Although Hbs of teleost fishes do not appear to transport oxylabile carbamate, many (such as trout and carp) have very large Haldane effects relative to mammals, due to the large number of protons reversibly bound to Hb (Jensen and Weber, 1985; Weber and Jensen, 1988; Jensen, 1989; Jensen, 1991). Thus, transport and excretion of CO_2 in the blood of teleost fishes is very dependent upon the oxygenation of Hb (figure 1). The magnitude and importance of this interaction *in vivo* has not been quantified.

O_2 transport:

In addition to the Bohr effect, the Hbs in many teleost fish possess a Root effect (Root, 1931; Brittain, 1987), whereby an acidification of the red cell reduces the maximal O_2 carrying capacity of the blood, even in the presence of 100 atmospheres of pure O_2 (Scholander and Van Dam, 1954). The Root effect is found only in fish (Brittain, 1987) and has been correlated with the presence of a swimbladder and more strongly correlated with the presence of a choroid rete (Farmer *et. al.*, 1979). In the rete a localized acidosis in the blood reduces the O_2 carrying capacity of the blood ("Root off" effect), subsequently elevating blood Po_2 and off-loading O_2 to the swimbladder or the retina (Pelster and Scheid, 1992). In the swim bladder of the eel, acidification of the blood to pH values as low as 6.6 has been reported (Kobayashi *et. al.*, 1990). However, much smaller changes in blood pH can induce the "Root off" effect in the blood of many fishes (Nikinmaa, 1990). Therefore, the Root effect may be of general importance to O_2 delivery in the tissues, where CO_2 movement into the blood results in small changes in blood pH. The half time ($T_{1/2}$) for the "Root off" effect in the blood of eels is 44.8 ms (Pelster *et. al.*, 1992), while blood transit through the capillaries of the tissues and gills may be as long as several seconds (Honig *et. al.*, 1977; Randall, 1982; Bhargava *et. al.*, 1992). Therefore, the "Root off" effect is sufficiently rapid to be exploited during blood transit through the capillaries.

Teleost fishes generally possess Hbs with large Haldane effects and a low buffer capacity in comparison with other vertebrate Hbs (Jensen, 1989). Thus, during O_2 uptake

at the gills, a large number of Bohr protons are released from Hb, which could acidify the contents of the red cell. In whole blood, maintained at constant Pco_2 *in vitro*, the difference in red cell pH of oxygenated and deoxygenated blood is 0.22 units (Table 1). This pH difference illustrates the magnitude of the acidosis due to the release of Bohr protons, in the absence of CO_2 removal. A decrease in red cell pH of 0.22 units could potentially reduce O_2-carrying capacity of the blood of trout by between 24 and 49% (Table 1) due to the presence of the Root shift. Normally; however, protons released during Hb oxygenation are consumed during HCO_3^- dehydration and subsequent CO_2 removal. Thus, a large proportion of O_2 uptake at the gills is dependent upon the removal of the Bohr protons released from Hb during oxygenation. That is, O_2 uptake at the gills may be strongly influenced by HCO_3^- dehydration within the red cell, due to the magnitude of the Haldane and Root effects and there is a tight coupling between O_2 and CO_2 transfer in teleost fish blood.

In summary, Hb-oxygenation releases protons and HCO_3^- dehydration consumes protons, thus, there is an extensive interaction between oxygen and carbon dioxide transfer in teleost fish. This interaction occurs in the blood, centred on the red blood cell, in both the tissues and the respiratory epithelium. The movement of oxylabile Bohr protons in the blood drives HCO_3^- dehydration in the gills and CO_2 hydration in the tissues. Acidification of the blood affects oxygen binding to Hb, via the Bohr effect and the Root shift, and it has been estimated that oxygen uptake may be severely limited in the blood of trout in the absence of CO_2 removal (Table 1).

I hypothesize that the combination of a large Root and Haldane effect and low Hb

buffer capacity requires tight coupling of O_2 and CO_2 transfer in the erythrocyte of teleost fishes, without which gas transfer is impaired. The research objectives of this thesis are: 1) to conduct a quantitative analysis of O_2 and CO_2 transport in resting and exercising trout and 2) to quantify the magnitude of the coupling between O_2 and CO_2 exchange *in vivo*.

TABLE 1: The effect of Bohr proton accumulation in the red cell on O_2 carrying capacity of the blood in rainbow trout.

Authors	Reported Arterial pH_e	Reported Arterial pH_i	Calculated pH_i with Bohr proton accumulation	Calculated decrease in So_2
Primmet *et. al.* (1986)	7.83	7.29	7.07	46%
Lessard *et. al.* (1995)	7.98	7.27	7.05	48%
Boutilier *et. al.* (1986)	7.95	7.41	7.19	24%
Steffensen *et. al.* (1987)	----	7.39	7.17	28%

- "Calculated pH_i with Bohr proton accumulation" is determined by subtracting 0.22 pH units from the reported arterial pH_i. The value of 0.22 pH units represents the difference in red cell pH between oxygenated and deoxygenated blood (Hct=20%) incubated at constant Pco_2 (taken from Perry and Gilmour, 1993).
- "Calculated decrease in So_2" is calculated as follows:

$$[(So_2 \text{ at reported arterial } pH_i - So_2 \text{ at calculated } pH_i)/S0_2 \text{ at reported arterial } pH_i] \times 100$$

where So_2 values at respective pH_i are derived from the relationship between So_2 and pH_i for trout blood, *in vitro* (from the data of Salama and Nikinmaa, unpublished data in Nikinmaa (1990)).

GENERAL MATERIALS AND METHODS

Experimental animals:

The source and species of fish, and the specific conditions in which the animals were maintained are described within each chapter.

Surgery and Handling:

In fish requiring cannulation, the fish was anaesthetized in a 1:10 000 solution of tricaine methanesulphonate (MS-222) in dechlorinated city water, adjusted to pH 7.5 with $NaHCO_3^-$ and bubbled with oxygen. The fish was placed on a surgery table similar to that of Smith and Bell (1967) and the gills were continually irrigated with a more dilute anaesthetic solution (1:30 000 MS-222 in water). The dorsal aorta was cannulated with polyethylene tubing (Clay-Adams PE-50; internal diameter = 0.580 mm; outer diameter = 0.965mm) according to Soivio *et. al.*, (1975). In some instances further surgery was required which is described in the materials and methods section of the respective chapters. Following surgery, the fish was revived by irrigating the gills with aerated water and transferred to an opaque acrylic box, and left to recover for 24-48h. Cannulae were flushed daily with heparinized (10 i.u. ml^{-1} ammonium heparin; Sigma), teleost ringer solution (Wolf, 1963).

Analytical procedures:

Haematocrit was determined after centrifuging 60 μl of blood in heparinized

micro-haematocrit tubes at 12 000 rpm for 5 min. Blood Hb concentration was determined spectrophotometrically on 20 μl of blood using a Sigma total haemoglobin (525-A) assay kit. The mean cellular [Hb] was calculated as ([Hb]/Hct)100. Methaemoglobin was determined on 50 μl of blood using the method of Bartlett *et. al.* (1987) modified by Brauner *et. al.* (1993). Whole blood or plasma pH (pH_e) and red cell pH (pH_i) were measured using a Radiometer micro-capillary pH electrode (G299A) using a Radiometer BMS3 Mk2 blood micro-system maintained at the temperature to which the fish was exposed. pH_i was measured according to the freeze thaw method of Zeidler and Kim (1977). Blood Po_2 was measured with a Radiometer Po_2 (E-5046) electrode, thermostatted in a D616 cell, in conjunction with a Radiometer PHM 71 acid-base analyzer. O_2 content of whole blood was measured according to Tucker (1967). Plasma and whole blood total CO_2 (Cco_2) were measured on 50 μl samples using a Corning model 965 CO_2 analyzer for data reported in chapters 2 and 4, or by using a gas chromatograph (Carle Instruments Inc., U.S.A., Model III), coupled to a chart recorder as described by Boutilier *et. al.* (1985). Plasma HCO_3^- and Pco_2 levels were calculated by re-arrangement of the Henderson-Hasselbalch equation:

$$1) \quad \text{Plasma } Pco_2 = \frac{\text{Plasma } Cco_2}{\alpha\, CO_2 \times [\text{antilog } (pHe - pK') + 1]}$$

$$2) \quad \text{Plasma } [HCO_3^-] = \text{Plasma } Cco_2 - (\alpha\, CO_2 \times Pco_2)$$

where pK' is the apparent pK of plasma and $\alpha\, CO_2$ is the solubility of CO_2 in plasma

taken from Boutilier *et. al.* (1984). The total CO_2 contained within the erythrocyte (Red cell Cco_2) was calculated as:

$$3) \quad \text{Red cell } Cco_2 = \frac{\text{Whole blood } Cco_2 - (\text{Plasma } Cco_2 \times (1\text{-Hct}))}{\text{Hct}}$$

The total red cell HCO_3^- concentration was calculated assuming Pco_2 was in equilibrium between the red cell and plasma, that the solubility of CO_2 in the erythrocyte was 0.86 of that in plasma (Van Slyke *et. al.*, 1928), and that no carbamate existed bound to trout Hb (see general introduction for clarification).

$$4) \quad \text{Red cell } [HCO_3^-] = \text{Red cell } Cco_2 - \text{Plasma } Pco_2 \times 0.86 \times \alpha\, CO_2$$

Plasma $[Cl^-]$ was measured on 10 μl aliquots using a coulometric Haake Buchler Instruments HBI digital Chloridometer. Plasma adrenaline and noradrenaline levels were measured on alumina extracted samples using HPLC in conjunction with electrochemical detection according to the basic protocol of Woodward, (1982).

Statistics:

All data are presented as mean $\pm$ one standard error of the mean. The specific statistical tests used to analyze data are described within each chapter for clarity.

CHAPTER 1: Blood gas disequilibria and CO_2 transport and excretion *in vivo*

INTRODUCTION

CO_2 transport and excretion in fish follows the typical mammalian pattern. That is the majority of CO_2 is transported in the blood as HCO_3^- but traverses the respiratory surface as molecular CO_2 (Perry *et. al.*, 1982). The Hbs of teleost fishes probably do not form much carbamate (Farmer, 1979) and therefore carbamate plays a minor role in CO_2 excretion in fishes. Thus, CO_2 which is excreted into the ventilatory water can exist in pre-branchial blood either as physically dissolved CO_2 ($Pbco_2$) or HCO_3^-. Unlike mammals; the respiratory surface in trout and other freshwater teleost fishes lack plasma accessible carbonic anhydrase (Henry *et. al.*, 1988; Perry and Laurent, 1990) and therefore all HCO_3^- dehydration at the catalyzed rate is restricted to the red cell during gill blood transit. The lack of plasma accessible CA and reduced dependence upon carbamate during CO_2 excretion will increase dependence upon HCO_3^- dehydration and red cell HCO_3^-/Cl^- exchange during CO_2 excretion.

Nearly all blood gas measurements are taken with all reactions in the blood at equilibrium. A steady state analysis of CO_2 transport and excretion is most simply conducted using equilibrium values for arterial and venous blood gases. However, if such an analysis is to be physiologically relevant, these values must be equivalent to those which exist *in vivo*. Recently, it has been demonstrated that blood pH and Pco_2 do not reach equilibrium during blood flow through the gills, and it has been suggested that they may never reach equilibrium *in vivo* (Gilmour *et. al.*, 1994).

Following the rapid removal of physically dissolved CO_2 across the respiratory

epithelium, CO_2 excretion is achieved by dehydration of HCO_3^- within the red cell at the catalyzed rate. Due to the large concentration of CA in the red cell, $[CO_2]$, $[HCO_3^-]$ and pH are virtually at equilibrium during blood transit through the gills. As intracellular HCO_3^- is depleted, HCO_3^- enters the red cell from the plasma in exchange for Cl^- but the pH in the plasma remains almost unchanged due to the lack of plasma accessible CA (Henry *et. al.*, 1988; Perry and Laurent, 1990) and the slow rate of proton flux across the red cell (Forster and Steen, 1969) relative to the rate of blood transit through the gills. Thus, when the blood leaves the gills, plasma $[HCO_3^-]$ and $[CO_2]$ have been reduced, but $[H^+]$ has not changed proportionately. Thus, HCO_3^- dehydration continues in post-branchial blood, elevating both Pco_2 and pH (Gilmour *et. al.*, 1994).

Exposure of fish to different environmental conditions such as hypoxia, hyperoxia and hypercapnia affects the respiratory status of the fish which in turn influences the magnitude of the disequilibria in post-branchial blood (Gilmour and Perry, 1994). The magnitude of the acid-base disequilibria in mixed-venous blood of trout has not been measured.

The objective of this study was to conduct a quantitative, steady state analysis of the partitioning of CO_2 excretion in resting trout and determine whether the acid-base disequilibria measured in arterial and venous blood, was of sufficient magnitude to influence the values obtained. Fish were subjected to a variety of experimental conditions to influence the magnitude of the blood gas disequilibria, and an extra-corporeal circulation in conjunction with stop-flow (Gilmour *et. al.*, 1994) was used to assess pre- and post-branchial changes in Pco_2, Po_2 and pH. The partitioning of CO_2 excretion was

calculated according to equations 1 to 3 in the Appendix, using blood gas values from flowing blood and equilibrium values obtained following stop flow.

MATERIALS AND METHODS

Experimental Animals:

Rainbow trout (*Oncorhynchus mykiss*; 750-1000g) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario) and transported to the University of Ottawa. Fish were acclimated to dechlorinated city water (10 °C) for at least 4 weeks prior to experiments. Trout were fed to satiation daily, but feeding was suspended 48 h prior to experimentation. A sub-group of rainbow trout was exposed to hyperoxia (Po_2= 330 ± 12 (SEM) mmHg) for 2 weeks prior to experimentation, to elevate blood total CO_2 levels (Wood and Jackson, 1980).

Surgery and Handling:

Following cannulation of the dorsal aorta, a small incision (2-3 cm) was made approximately 1 cm posterior to the right pectoral fin. The coeliac artery was teased away from the gall bladder and cannulated (PE 50) in the ortho- and retrograde directions (Thomas and Le Ruz, 1982). The first or second afferent branchial vessel was cannulated (PE 50) using the waggle technique in which the catheter was slowly advanced into the vessel while simultaneously being moved to and fro. The tubing was then sutured in place, thereby obstructing blood flow through that gill arch. Following surgery, fish were revived by irrigating the gills with aerated water. Fish were then transferred to individual opaque acrylic boxes, and left to recover for 24-48h at the water oxygen tension to which the fish were acclimated (ie. normoxic or hyperoxic water).

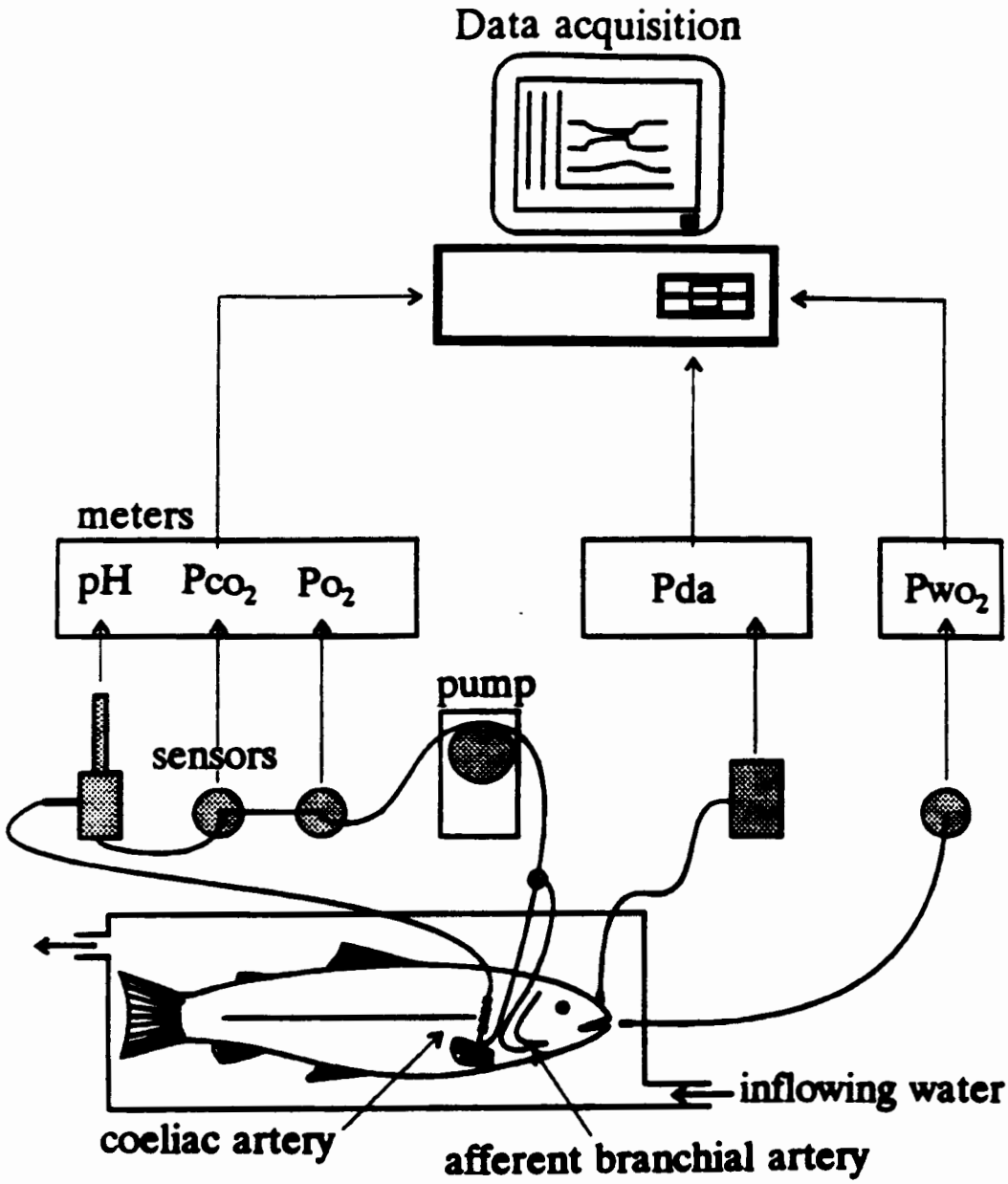
Cannulae were flushed daily with heparinized (10 i.u. ml^{-1} ammonium heparin; Sigma), teleost ringer solution (Wolf, 1963).

Extracorporeal circuit experimental procedure:

The arterial extracorporeal circuit was established by pumping blood from the coeliac artery through a series of thermostatted cuvettes containing Po_2, Pco_2 and pH electrodes. Blood was pumped at a constant rate of 0.55 ml min^{-1} and returned into the coeliac artery, via the orthograde facing cannula. The total volume of the circuit was 1.3 ml, comprising less than 5% of the total blood volume of the fish. To minimize chances of blood clotting, the circuit was rinsed with 10 ml of heparinized saline (1000 i.u. ml^{-1}) before initiating blood flow.

Blood pressure was monitored continuously in the dorsal aorta as a viability index of the preparation. If large changes in blood pressure were observed the experiment was terminated. Output from the Po_2, Pco_2 and pH electrodes was monitored until values stabilized and then all parameters were recorded (see figure 1.1). After a predetermined time period, the disequilibrium in arterial blood gases and pH was recorded by stopping the flow of blood for 8 min. Blood flow was then re-initiated and 200 μl of blood was collected immediately to measure Hct, Hb, whole blood total CO_2 and oxygen content of arterial blood. Arterial blood gases and pH quickly stabilized at pre-stopflow values which were recorded for several minutes. The pump was briefly stopped and blood input to the circuit was changed from the afferent branchial artery to that from the coeliac artery (arterial blood) and the pump turned back on. This permitted rapid measurement

FIGURE 1.1: Schematic representation of the extracorporeal circuit preparation. Blood flow through the extracorporeal circuit was maintained by a peristaltic pump. Arterial blood was drawn from the coeliac artery and mixed-venous blood was drawn from the afferent branchial artery. Only one blood source was sampled at a time. Regardless of the source, blood was returned into the coeliac artery, downstream from the arterial blood sampling site. The output from the blood and water electrodes and pressure transducer were recorded using a computerized data acquisition program. Pda: blood pressure in dorsal aorta, Pwo_2 : water Po_2 . (Figure modified from Gilmour *et al.*, 1994).


Data acquisition
meters
pH
Pco_2
Po_2
Pda
Pwo_2
pump
sensors
inflowing water
coeliac artery
afferent branchial artery

of arterial-venous differences in blood gases and pH in flowing blood. Following stabilization of these parameters, the disequilibrium in mixed venous blood was examined as described above and blood was collected to measure Hct, Hb, whole blood total CO_2 and oxygen content of venous blood. The coeliac artery was connected back to the extracorporeal circuit to ensure that blood gas and pH values of aortic blood had not changed and the fish was in steady-state. In several instances, stop-flow was repeated yielding similar degrees of disequilibria in blood gases and pH.

The Po_2 of water flowing through the fish chamber was continuously monitored by siphoning a small volume of water across a Radiometer Po_2 electrode. Following measurement of blood characteristics, the inflowing water to the opaque acrylic box was prevented and the water was slowly recirculated throughout the box with the aid of a pump. The decline in water Po_2 (which never exceeded about 20 mmHg) was monitored for 20 min to calculate oxygen consumption rate.

Experimental protocol:

The extracorporeal circuit experimental procedure described above was conducted initially in normoxia and the procedure described above was repeated using the same fish exposed to mild hypoxia (water $Po_2 = 95 \pm 12$ mmHg) and then moderate hypoxia (water $Po_2 = 65 \pm 5$ mmHg). The transition to different levels of hypoxia was achieved by gradually reducing the water Po_2 to the target value, over a 30 min period. The fish were left for a further 30 min period over which time blood Pco_2, Po_2 and pH stabilized. Some fish were acclimated to hyperoxia with the intention of inducing a respiratory

acidosis and elevating blood Cco_2 levels. In the hyperoxia acclimated fish, the extracorporeal circuit procedure was conducted one hour following exposure to normoxic water (hyperoxia-normoxia). In all cases, the extracorporeal circuit procedure described above was not initiated until stable blood gas values were obtained.

Analytical procedures:

A Metrohm combination glass pH electrode (model 6.0204.100(OC)) in conjunction with a Radiometer PHM 73 was used to measure blood pH. Blood Po_2 and Pco_2 were measured with Radiometer Po_2 (E-5046) and Pco_2 (E-5036) electrodes connected to a Radiometer PHM 73 analyzer. All three electrodes were thermostatted in cuvettes at ambient water temperature. The pH electrode was calibrated with Radiometer precision buffer solutions and blood gas electrodes with water equilibrated with appropriate gas mixtures (supplied by Wösthoff pumps).

Blood pressure was measured by connecting the dorsal aorta cannula to a pressure transducer (Bell and Howell, 4-327-I). Blood pressure was calibrated against a static column of water. Water Po_2 was measured with a Radiometer E-5946 Po_2 electrode in conjunction with a Radiometer PHM 72 Mk2 acid-base analyzer. Total CO_2 content of water was measured in quadruplicate using a Capnicon V CO_2 analyzer (Cameron Instruments).

The analog outputs from the pH, Po_2 and Pco_2 electrodes as well as that from the pressure transducer were transformed by an analog-digital interface (DT2801-DT707,

Data Translation Inc.). Data was acquired by customized software (written by P. Thoren: Göteborg, Sweden) and mean values for each variable were recorded at 5 s intervals.

Calculations:

Plasma [HCO_3^-] in arterial and venous blood were calculated from equilibrium values for pH_e and Pco_2 (ie. measured following 8 min of stopflow in the extracorporeal circuit) by rearrangement of the Henderson-Hasselbalch equation and using the appropriate constants from Boutilier *et. al.* (1984).

The proportion of total CO_2 excreted which was due to the movement of physically dissolved CO_2 in pre-branchial blood ($Pbco_2$) was calculated according to equation 1a in the Appendix. To account for the influence on $Pbco_2$ of blood-gas disequilibria which exists in arterial and mixed-venous blood in vivo, the calculation was conducted with three different values for P_vco_2 and P_aco_2. The first Pco_2 values used were at the end of the stop-flow period (8 min), the second were values measured in flowing blood in the extracorporeal circuit, and the third were estimated *in vivo* values. The *in vivo* values were estimated from a best fit regression to the arterial and venous blood stop-flow disequilibrium curve for each fish, extrapolated back to the time at which the blood left the fish.

Statistics:

Statitstically significant differences between treatment means were detected using

a repeated measures ANOVA , or Friedman repeated measures ANOVA on ranks, followed by a Dunnett's test. Comparisons between the hyperoxia-normoxia group and the normoxic controls were conducted using a students t-test. To determine whether the change in respiratory parameters during stopflow differed significantly from flowing blood, a students t-test was also employed. In all cases a probability level of 5% was chosen as the limit of statistical significance.

RESULTS

Blood parameters and changes in blood Po_2, Pco_2 and pH during stop flow:

Resting fish were exposed to different conditions to influence arterial and mixed-venous blood-gas and pH levels with the objective of altering the magnitude of the change in these parameters during stop flow. The blood-gas status of arterial and mixed venous blood at the end of the stop flow period is presented in Table 1.1. In fish acclimated to hyperoxia and then subsequently exposed to normoxia (hyperoxia-normoxia) there was a significant change in pH, Po_2, and Pco_2 of arterial blood, and a significant change in pH and Pco_2 of mixed venous blood, relative to fish exposed to normoxia. Exposure to mild and moderate hypoxia resulted in a significant change in Po_2 of arterial and mixed-venous blood relative to fish exposed to normoxia.

Traces of the temporal changes in Po_2, Pco_2 and pH during stop flow are presented for absolute values in arterial blood (Fig. 1.2). Due to the large variability in the absolute values, the mean normalized changes in the respiratory parameters during stop flow are also presented (Fig. 1.3). Data were mean normalized by subtracting the absolute value for each parameter at initiation of stopflow, from all subsequent values obtained during stopflow. The temporal changes in absolute and mean normalized values for respiratory parameters in mixed-venous blood are presented in figures 1.4 and 1.5 respectively.Traces for the other conditions to which fish were exposed are not shown, but a summary of the respiratory parameters prior to stop flow, and the magnitude to

TABLE 1.1: Equilibrium values of arterial and venous blood parameters following 8 min of stop flow during extracorporeal circulation.

Condition	Hct_a	pH_a	pH_v	P_aO_2 (mmHg)	P_vO_2 (mmHg)	P_aCO_2 (mmHg)	P_vCO_2 (mmHg)	Plasma $[HCO_3^-]_a$	Plasma $[HCO_3^-]_v$	$C_{a-v}O_2$
Normoxia	27.8 (3.6) 7	7.84 (0.02) 7	7.83 (0.02) 7	78.4 (5.9) 7	24.4 (2.1) 7	2.71 (0.24) 7	3.28 (0.21) 7	7.07 (0.65) 7	8.74 (0.69) 7	1.62 (0.20) 7
Mild Hypoxia	28.4 (2.6) 7	7.89 (0.03) 7	7.85 (0.03) 7	32.4* (1.7) 7	16.6* (1.1) 7	2.55 (0.36) 7	3.59 (0.42) 7	7.42 (0.90) 7	9.81 (1.01) 7	1.91 (0.17) 7
Moderate Hypoxia	29.5 (2.2) 6	7.84 (0.05) 6	7.79 (0.05) 6	19.5* (1.2) 6	11.8* (1.4) 6	2.09 (0.19) 6	2.87 (0.25) 6	5.64 (0.75) 6	6.85 (0.83) 6	1.50 (0.20) 6
Hyperoxia-Normoxia	29.6 (3.7) 7	7.67* (0.07) 6	7.64* (0.07) 6	45.2* (7.6) 7	23.0 (5.4) 6	5.09* (0.43) 7	6.43* (0.84) 6	8.24 (1.15) 7	10.58 (1.38) 6	1.28 (0.41) 6

Water Po_2 was 155 $\pm$ 0.5 mmHg in normoxia, 95 $\pm$ 12 in moderate hypoxia, 65$\pm$ 5 in moderate hypoxia, 163 $\pm$ 2 in hyperoxia-normoxia (fish initially exposed to hyperoxia but returned to normoxia). Values represent mean with s.e.m. in brackets and "n" beneath.* indicates statistically significant difference relative to normoxia.

FIGURE 1.2: Arterial blood Po_2, Pco_2 and pH during blood flow through the extracorporeal loop, and during stopflow, in fish exposed to normoxia. Stop flow was initiated at 3 min and terminated at 11 min (indicated by the vertical dashed line). Error bars represent S.E.M. and are shown every 2 min for clarity. The trace represents the mean value for the respective parameter, recorded at 5 s intervals for seven fish (n=7).

NORMOXIC ARTERIAL BLOOD

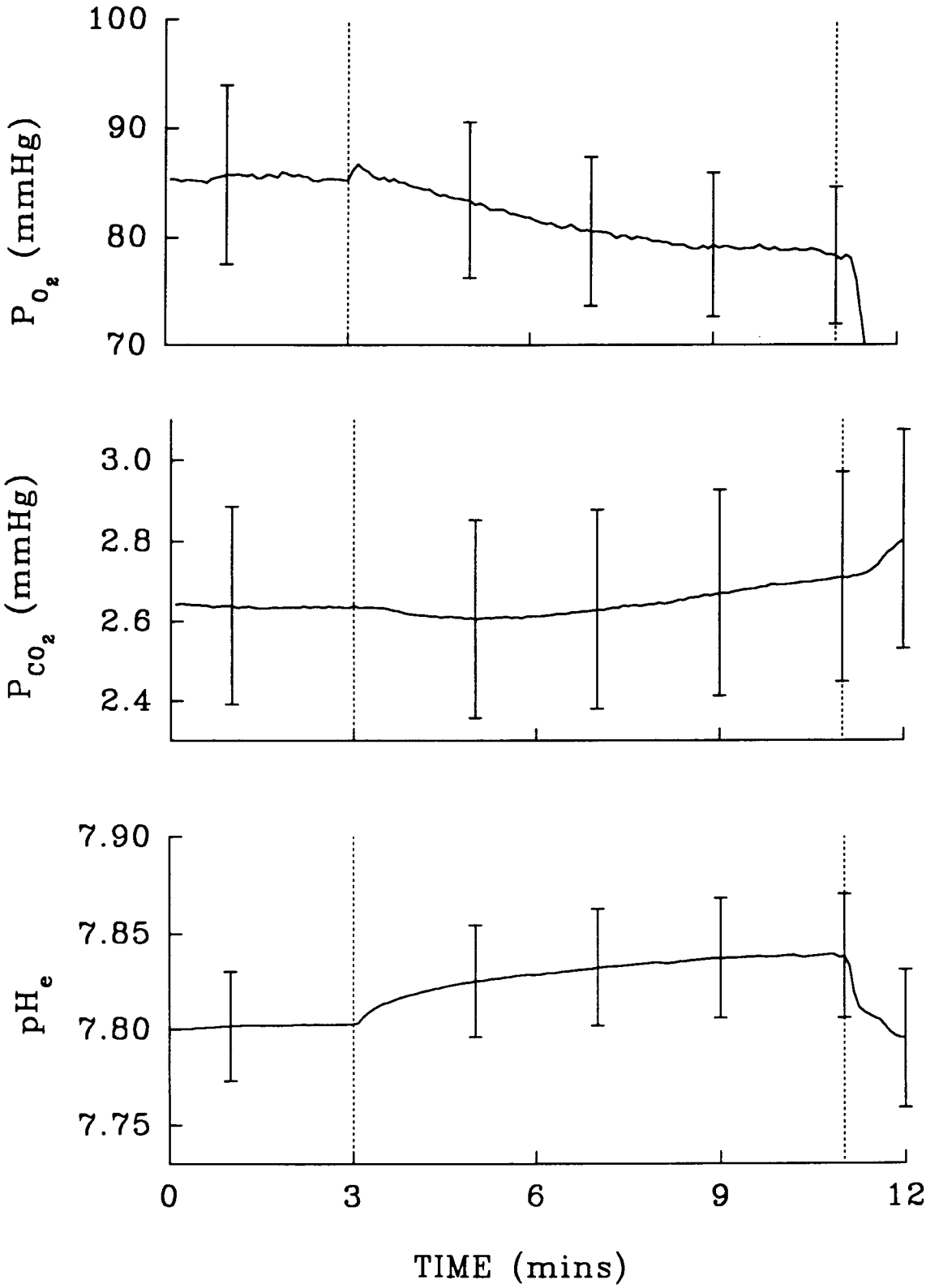


FIGURE 1.3: Mean normalized values for arterial blood Po_2, Pco_2 and pH during blood flow through the extracorporeal loop and during stopflow in fish exposed to normoxia. To normalize data, the absoute value for each parameter at initiation of stopflow was subtracted from all subsequent values obtained during stopflow. See legend for fig. 1.2 for further details.

NORMOXIC ARTERIAL BLOOD

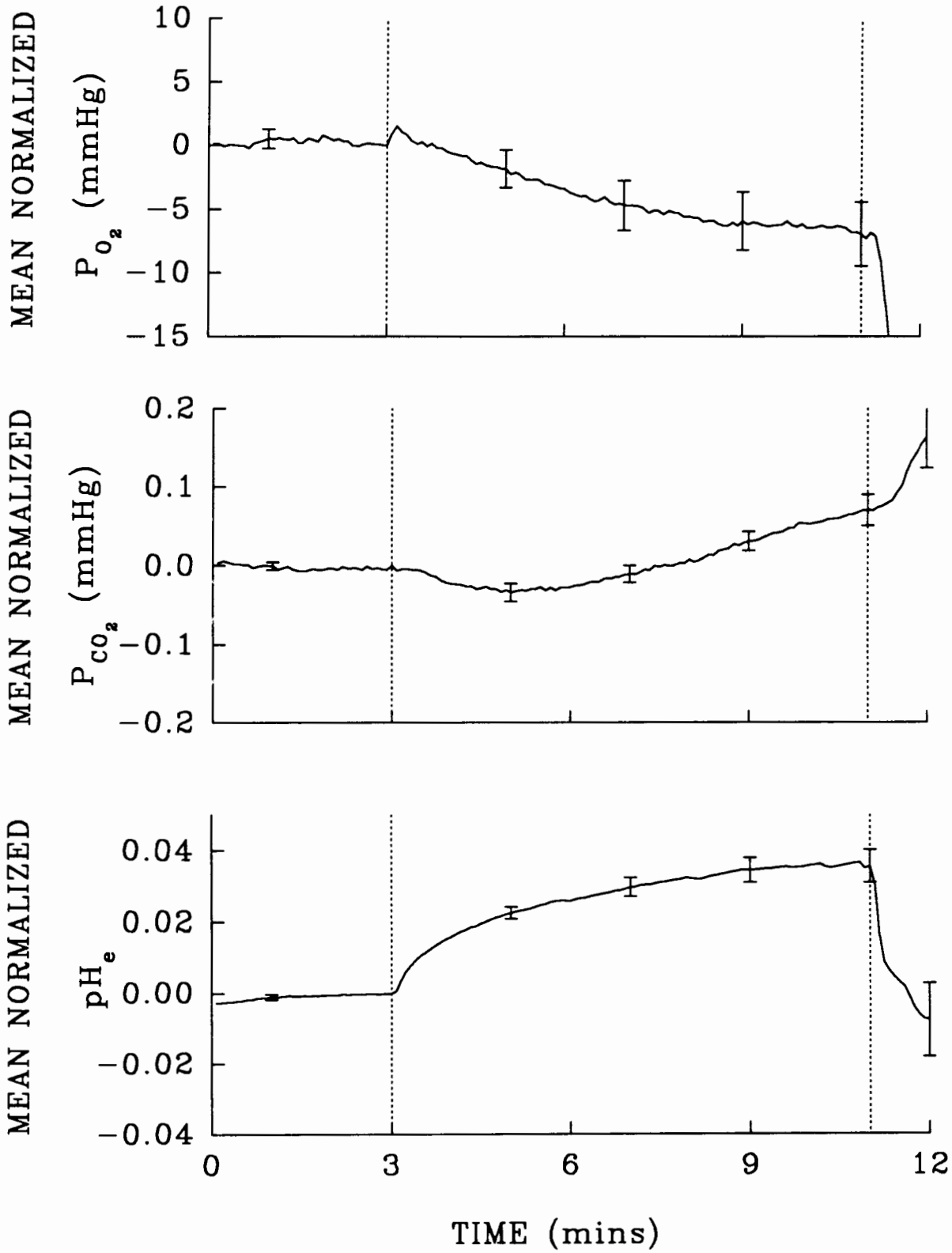


FIGURE 1.4: Mixed-venous blood Po_2, Pco_2 and pH during blood flow through the extracorporeal loop and during stopflow in fish exposed to normoxia. See legend for fig. 1.2 for further details.

NORMOXIC VENOUS BLOOD

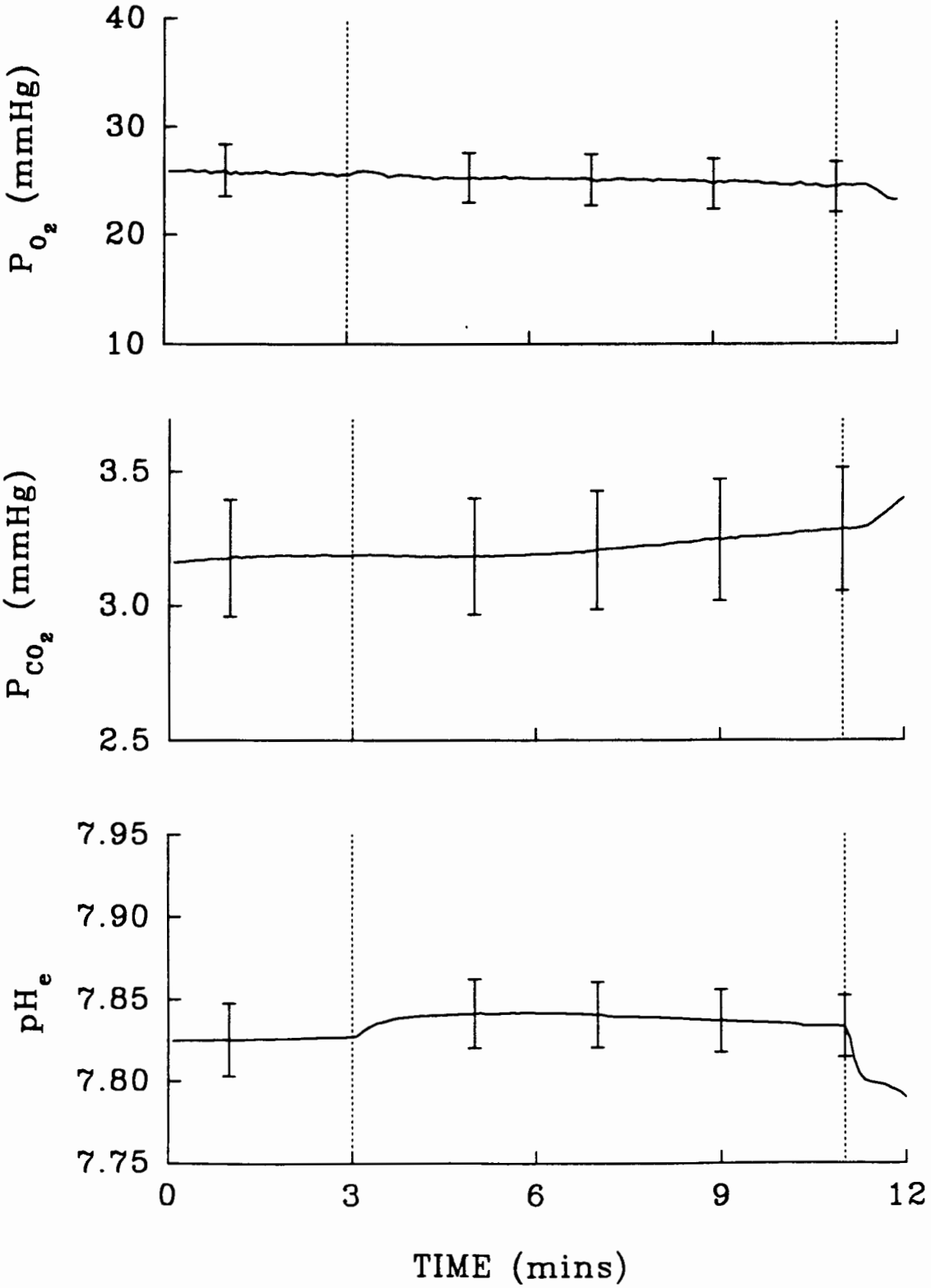
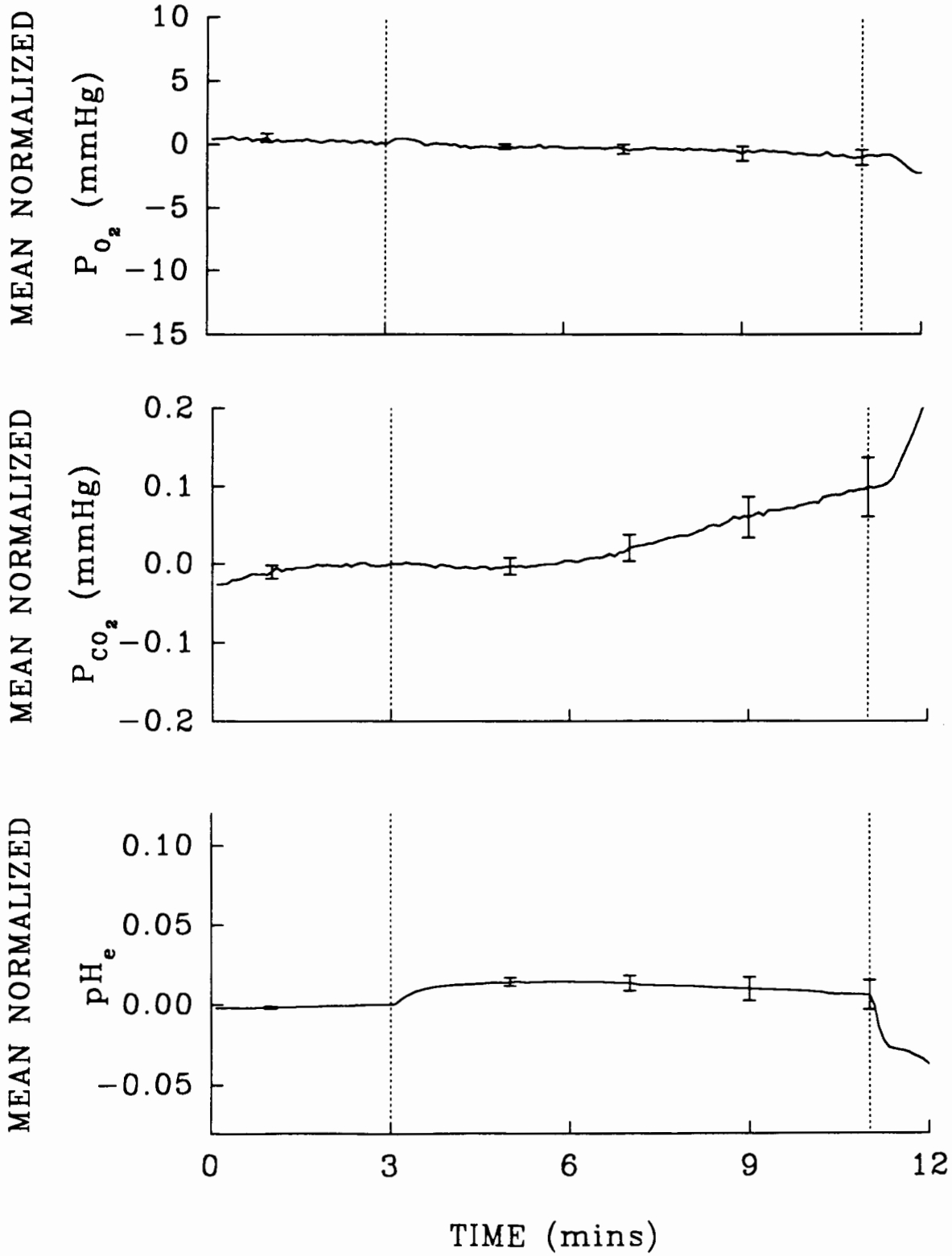


FIGURE 1.5: Mean normalized values for mixed-venous blood Po_2, Pco_2 and pH during blood flow through the extracorporeal loop and during stopflow in fish exposed to normoxia. See legends for figures 1.2 and 1.2 for further details.

NORMOXIC VENOUS BLOOD



which they changed during stop flow, are presented in tables 1.2 (arterial blood) and 1.3 (mixed-venous blood). In almost all conditions, there was a significant change in Po_2, Pco_2 and pH during stopflow in both arterial and mixed-venous blood. The only parameter which changed during stop flow (Δ) relative to that measured in normoxic fish, was Pco_2. This was only observed in the hyperoxia-normoxia group.

Partitioning of CO_2 excretion in vivo:

The partitioning of CO_2 excretion was calculated according to equations 1 to 3 (Appendix). CO_2 excretion was assumed to occur either by movement of physically dissolved CO_2 which existed in pre-branchial blood prior to gill blood transit ($Pbco_2$), or by HCO_3^- dehydration when the blood entered the gills (see discussion for assumptions). The contribution of $Pbco_2$ to total CO_2 excretion was calculated using stop flow Pco_2 values (following 8 min of stop flow), values obtained just prior to stop flow (flowing blood), or using estimates of *in vivo* Pco_2 values based upon the shape of the stop flow disequlibrium curve and the blood transit time from the gills to the Pco_2 electrodes. These calculations were conducted for fish exposed to normoxia, mild hypoxia and moderate hypoxia and in fish acclimated to hyperoxia and subjected to normoxia (hyperoxia-normoxia). In all cases, there was no significant difference in the proportion of total CO_2 excreted which was dependent upon $Pbco_2$, which was generally between 2 and 3% (Table 1.4). The remaining CO_2 excreted was assumed to be either HCO_3^- which resided within the red cell prior to gill entry or HCO_3^- which entered the red cell via the

TABLE 1.2: Arterial blood parameters at the beginning of the stopflow period and the change (Δ) in the respective parameter following 8 min of stop flow.

Condition	P_aO_2 (mmHg)	ΔP_aO_2	P_aCO_2 (mmHg)	ΔP_aCO_2	pH_a	ΔpH_a
Normoxia	85.3 (7.5) 7	-6.8+ (2.5) 7	2.64 (0.23) 7	0.07+ (0.02) 7	7.80 (0.03) 7	0.04+ (0.01) 7
Mild Hypoxia	34.0* (1.7) 7	-1.7+ (0.5) 7	2.45 (0.36) 7	0.10+ (0.03) 7	7.83 (0.03) 7	0.05+ (0.01) 7
Moderate Hypoxia	21.0* (1.0) 6	-1.5+ (0.4) 6	1.91 (0.16) 6	0.18+ (0.06) 6	7.78 (0.06) 6	0.06+ (0.01) 6
Hyperoxia-Normoxia	56.1* (7.7) 7	-10.8+ (2.7) 7	3.95* (0.39) 7	1.14*+ (0.23) 7	7.55* (0.07) 7	0.07+ (0.02) 7

See legend for Table 1.1 for details. + indicates values differ significantly from zero.

TABLE 1.3: Venous blood parameters at the beginning of the stopflow period and the change (Δ) in the respective parameter following 8 min of stop flow.

Condition	P_vO_2 (mmHg)	ΔP_vO_2	P_vCO_2 (mmHg)	ΔP_vCO_2	pH_v	ΔpH_v
Normoxia	25.5 (2.2) 7	-1.2+ (0.6) 7	3.19 (0.20) 7	0.10+ (0.04) 7	7.83 (0.02) 7	0.01 (0.01) 7
Mild Hypoxia	17.6* (1.4) 7	-1.0 (0.8) 7	3.43 (0.43) 7	0.16+ (0.05) 7	7.83 (0.03) 7	0.02+ (0.01) 7
Moderate Hypoxia	13.5* (1.2) 6	-1.7+ (0.3) 6	2.58 (0.22) 6	0.28+ (0.08) 6	7.75 (0.05) 6	0.04+ (0.01) 6
Hyperoxia-Normoxia	24.0 (5.3) 6	-1.0+ (0.4) 6	5.17* (0.50) 6	1.26*+ (0.38) 6	7.59* (0.07) 6	0.03+ (0.03) 6

See legend of Table 1.1 for details. + indicates values differ significantly from zero.

TABLE 1.4: Percent of total CO_2 excreted *in vivo*, due to the movement of physically dissolved CO_2 in pre-branchial blood ($Pbco_2$) in fish exposed to different treatments.

	Calculated % $Pbco_2$ using arterial and venous Pco_2 values obtained from:		
Treatment	Estimated *in vivo* Pco_2	Time 0 stop flow Pco_2	Time 8 min stop flow Pco_2
Normoxia	2.17 (1.55)	2.04 (1.52)	2.10 (1.67)
Hypoxia (mild)	3.46 (1.24)	3.3 (1.16)	3.50 (1.21)
Hypoxia (moderate)	3.21 (1.04)	3.03 (0.97)	3.57 (1.05)
Hyperoxia-Normoxia	8.14 (6.35)	8.08 (5.67)	12.0 (10.9)

%$Pbco_2$ was calculated according to equation 1 in the Appendix. Different values for %$Pbco_2$ within each treatment are based upon the different sources for arterial and venous blood Pco_2 values indicated in the column head. Values represent mean $\pm$ (SEM), n=7 for normoxia and hypoxia (mild), and n=6 for remaining treatments.

HCO_3^-/Cl^- exchanger. Because there were no significant differences in the relative proportion in either pathway for HCO_3^- between the treatments of normoxia, mild or moderate hypoxia, the values obtained were pooled. The majority of total CO_2 excreted involved HCO_3^-/Cl^- exchange (59%) while 38% of the HCO_3^- dehydrated consisted of HCO_3^- which existed within the red cell in pre-branchial blood, prior to gill entry (Table 1.5).

TABLE 1.5: Partitioning of CO_2 excretion during blood passage through the gills in fish exposed to normoxia, mild hypoxia and moderate hypoxia.

% $Pbco_2$	% HCO_3^-/Cl^-	% red cell HCO_3^-
2.8 (0.3)	59.1 (4.8)	38.1 (4.8)

Values were pooled because no significant differences were observed between groups. $Pbco_2$ refers to the excretion of CO_2 which existed as physically dissolved CO_2 in pre-branchial blood, (calculated from the arterial-venous Pco_2 difference and equation 1, Appendix). % HCO_3^-/Cl^- refers to the proportion of total CO_2 excreted which involved HCO_3^-/Cl^- exchange (equation 2, Appendix). % red cell HCO_3^- refers to the proportion of total CO_2 excreted due to dehydration of HCO_3^- which resided within the red cell prior to gill entry (equation 3, Appendix). All values represent the mean for normoxia, mild and moderate hypoxia grouped together. n=18.

DISCUSSION

The experimental condition to which fish were exposed influenced the magnitude of the change in blood Pco_2 during stop flow but did not influence the calculated proportion of total CO_2 excreted which was dependent upon physically dissolved CO_2 in pre-branchial blood ($Pbco_2$). In fish exposed to normoxia and two levels of hypoxia, the contribution of $Pbco_2$ to total CO_2 excreted was 3%. The remaining CO_2 excreted was assumed to be a result of HCO_3^- dehydration, where 59% of the CO_2 excreted was dependent upon HCO_3^-/Cl^- exchange and the remaining 38% was dependent upon HCO_3^- which existed within the red cell before blood entered the gills.

When blood first enters the gills, any physically dissolved CO_2 existing in the pre-branchial blood will rapidly diffuse across the gills into the ventilatory water. The rapid removal of dissolved CO_2 in the blood will create conditions for HCO_3^- dehydration, resulting in continued CO_2 excretion during gill blood transit.
For the intent of this anaysis, it was assumed that CO_2 excretion during blood transit through the gills was achieved either through the rapid movement of physically dissolved CO_2 which existed in pre-branchial blood ($Pbco_2$) or by HCO_3^- dehydrated to CO_2 ($HCO_3^- \rightarrow CO_2$) within the red cell during gill blood transit. The relative role of each to CO_2 excretion in trout was calculated according to equations 1 to 3 in the Appendix.

In fish exposed to different experimental conditions, the contribution of $Pbco_2$ to total CO_2 excreted reached a maximum of 8% (Table 1.4) in fish acclimated to hyperoxia and returned to normoxia (hyperoxia-normoxia). The total contribution was between 2

and 4% in fish subjected to normoxia or 2 different levels of hypoxia but none of these values differed significantly from that calculated in normoxia. The role of $Pbco_2$ to total CO_2 excreted was calculated according to equation 1 (appendix) where it was assumed that arterial and mixed-venous blood Pco_2 values represent *in vivo* values. It is apparent that this was not the case (Tables 1.2 and 1.3; Gilmour *et. al.*, 1994). The changes in Pco_2 and pH in arterial blood during stopflow were significant and were similar in magnitude to that which has been observed by others in normoxia (Gilmour *et. al.*, 1994) and hypoxia (Gilmour and Perry, 1994). The change in pH during stopflow in mixed-venous blood was slightly lower than that measured in arterial blood, while the change in Pco_2 was similar to that measured in arterial blood (Table 1.3). Changes in Pco_2 and pH have not previously been measured in mixed venous blood of fish.

Although the equilibrium values for Pco_2 in arterial and mixed-venous blood do not represent *in vivo* values, they may represent *in vivo* arterial-venous differences because the magnitude of the change in Pco_2 during stop flow is similar in arterial and mixed-venous blood in fish at rest. Indeed, the $\%Pbco_2$ calculated from Pco_2 values measured before stop flow did not differ statistically from Pco_2 values measured 8 min following stop flow (Table 1.4). Furthermore, extrapolation back to estimated *in vivo* Pco_2 values did not significantly affect the calculation. Thus, in this study, accounting for the magnitude of acid-base disequililbria in arterial and mixed-venous blood did not result in a significant difference from a steady state analysis (using equilibrium values) of the proportion of total CO_2 excreted which was dependent upon $Pbco_2$.

The remaining CO_2 excreted during gill blood transit was assumed to be due to

HCO_3^- dehydration confined to the red cell. This rationalization is based upon the lack of oxylabile carbamate formation in fish (Farmer, 1979; Heming, 1984), the lack of plasma accessible CA in the gills of fish (Perry and Laurent, 1990) and the slow uncatalyzed rate of HCO_3^- dehydration in the plasma relative to gill blood transit time (Cameron and Polhemus, 1974). HCO_3^- can exist within the red cell pre-branchially, or enter the red cell via HCO_3^-/Cl^- exchange during gill blood transit. The contribution of the latter to total CO_2 excretion can be calculated from the arterial-venous difference in plasma $[HCO_3^-]$ (see equation 2, Appendix). In fish exposed to normoxia and 2 levels of hypoxia, no significant difference was detected in the role of HCO_3^-/Cl^- exchange to total CO_2 excreted and consequently, the values calculated for these treatments were pooled yeilding a mean value of 59.1 $\pm$ 4.8% (Table 1.5).

The equation used to calculate HCO_3^-/Cl^- exchange is based upon the assumption that the calculated equilibrium values for plasma $[HCO_3^-]$ are representative of *in vivo* values. Although this may not be entirely correct, the magnitude to which it will affect the calculation is minor. In arterial blood of resting normoxic fish, HCO_3^-/Cl^- exchange following gill blood transit is most likely complete (Gilmour *et. al.*, 1994). The increase in CO_2 and pH during stop flow in both arterial and pre-branchial blood, indicated that HCO_3^- was being dehydrated in the plasma. This resulted in a small reduction in $[HCO_3^-]$ at equilibrium in both arterial and pre-branchial blood relative to the situation *in vivo*. However, the changes in Pco_2, and therefore $[HCO_3^-]$ in arterial and pre-branchial blood were similar in both direction and magnitude, reducing the error associated with the violation of this assumption.

In conclusion, the acid-base disequilibrium which existed in both arterial and mixed-venous blood in resting fish did not significantly affect the calculation of the contribution of $Pbco_2$ to CO_2 excretion. Of the total CO_2 excreted across the gills, $Pbco_2$ accounted for between 2 and 4% in fish exposed to normoxia and hypoxia, and about 8% in fish acclimated to hyperoxia and returned to normoxia (hyperoxia-normoxia). Approximately 60% of the total CO_2 excreted in resting rainbow trout *in vivo* was dependent upon HCO_3^-/Cl^- exchange across the erythrocyte membrane which is of significance to gas exchange because this is thought to be the rate limiting step in CO_2 excretion (Perry *et. al.*, 1982; Weith *et. al.*, 1982; Perry, 1986).

SUMMARY

1) The magnitude of the acid-base disequlibrium in arterial and venous blood of trout was significantly influenced by the condition to which fish were exposed. Incorporation of the changes in Pco_2 during stop flow into an analysis of the partitioning of CO_2 excretion, did not result in a significant difference from a similar analysis using equilibrium Pco_2 values.

2) In fish exposed to normoxia and two levels of hypoxia, $Pbco_2$ comprised 3% of the CO_2 excreted. The remaining CO_2 excreted was assumed to be a result of HCO_3^- dehydration where about 60% of the total CO_2 excreted involved HCO_3^-/Cl^- exchange.

CHAPTER 2: The influence of O_2 uptake on Bohr proton release and CO_2 excretion *in vitro*

INTRODUCTION

Many teleost fishes possess haemoglobins with a large Haldane effect and low buffer capacity (Jensen, 1989). The large Haldane effect is important to CO_2 transport and excretion across the gills (Jensen, 1991). Recently, a radioisotopic assay has been used to quantify the contribution of the Haldane effect to CO_2 excretion *in vitro* (Perry and Gilmour, 1993; Perry *et. al.*, 1994). The "boost" to CO_2 excretion associated with rapid oxygenation of deoxygenated blood was correlated with the magnitude of the Haldane effect exhibited in whole blood (Perry *et. al.*, 1994). In rainbow trout, rapid oxygenation of the blood *in vitro*, provides a 40% "boost" to CO_2 excretion rate relative to that measured at constant oxygenation status (Perry and Gilmour, 1993). Venous blood never becomes completely deoxygenated *in vivo* and therefore arterial-venous differences in So_2 of 100% will never be realized in living trout. In tench (*Tinca tinca*), the majority of Bohr protons are released between about 50 and 100% So_2 (Jensen, 1986), indicating that the entire Haldane effect may be exploited within the normal physiological range of arterial and venous So_2. It is not known if the same relationship exists in trout. The main objectives of this study were to examine the region of the Hb-O_2 equilibrium curve over which Bohr protons were released and quantify the influence of Bohr proton release on CO_2 excretion rate, *in vitro*. Bohr proton release was quantified by measuring the changes in red cell pH of blood incubated at 0, 20, 60 and 100% So_2 and constant Pco_2. CO_2 excretion rates were measured in the presence and absence of rapid oxygenation, from initial levels of 0, 60 and 100% So_2, using the *in vitro* assay of Wood and Perry (1991).

MATERIALS AND METHODS

Experimental Animals:

Rainbow trout (*Oncorhynchus mykiss*; 200-400g) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario) and transported to the University of Ottawa. Fish were acclimated to dechlorinated city water (10 °C) for at least 4 weeks prior to experiments. Trout were fed to satiation daily, but feeding was withheld 48 h prior to experimentation.

Blood sampling:

Blood was withdrawn from the dorsal aorta and placed in a heparinized, round bottom glass flask kept on ice. Blood was pooled if the volume obtained from one fish was not sufficient for the entire experimental run (4.0 ml of blood and 0.5 ml of plasma). Blood was centrifuged (5900g at 4 °C) and haematocrit was adjusted to 25% by addition or withdrawal of separated plasma. Haemoglobin concentration was measured and recorded.

CO_2 excretion assay:

In vitro blood CO_2 excretion rates were measured using the radioisotopic assay of Wood and Perry (1991) as modified by Perry and Gilmour (1993) for flow through experiments. This assay measures the accumulation of [^{14}C] labelled CO_2, 2.5 min

following the addition of [^{14}C]bicarbonate to incubated whole blood. Briefly, 0.5 ml of blood or plasma was placed in a 20 ml scintillation vial which was stoppered and equilibrated with the desired mixture of CO_2, O_2 and N_2 for 2 h, at 15 °C, in a shaker water bath. Following incubation, 74 kBq (10 μl of 7400kBq ml^{-1}) of sodium [^{14}C]bicarbonate (in teleost Ringer; Wolf, 1963) was added to the plasma or blood in the scintillation vial. The vial was immediately sealed with a rubber septum equipped with a plastic well containing filter paper (Whatman GF/A 2.4 cm glass microfibre filter). 150 μl of hyamine hydroxide (a CO_2 absorbing solution) was injected through the septum into the filter paper and a second scintillation vial containing 1.5 ml of a CO_2 absorbing solution (Carbo-Trap 2;Baker) was placed in series with the first vial. The first vial was gassed with the desired mixture of O_2 and N_2 at a flow rate of 120 ml·min^{-1} resulting in an open system for the measurement of CO_2 evolution (Perry and Gilmour, 1993). The vials were placed back in the shaker for 2.5 min. Immediately thereafter, the carbo-trap solution and filter paper were removed and assayed for ^{14}C activity, and whole blood and plasma total CO_2 content were measured. The CO_2 excretion rate for each assay vial was calculated by dividing the sum of the filter paper and carbo-trap ^{14}C activity by the specific activity of plasma and time of the assay.

Experimental protocol:

The objective of this series was to examine the elevation in CO_2 excretion rate due to haemoglobin-oxygenation, at three different initial levels of So_2: 0, 60 and 100%. All measurements were obtained from each pool of blood (n=1); 12 pools in total were

examined. Blood was equilibrated in 0.5 ml aliquots to one of three gas mixtures supplied by Wösthoff pumps: a deoxygenated mixture (0.4% CO_2 and 99.6% N_2), a partially deoxygenated mixture (0.4% CO_2, 5.9% O_2, and the remainder N_2) which resulted in an So_2 of about 60%, and an oxygenated mixture (0.4% CO_2 and 99.6% O_2). Following the 2 h equilibration period, the following parameters were measured from blood equilibrated with each gas mixture: oxygen content, pH_e, pH_i and total CO_2 content of both whole blood and plasma. CO_2 excretion rates were measured in blood equilibrated to each gas mixture with and without rapid oxygenation. One group of vials was gassed with the same Po_2 to which the blood was incubated (deoxygenated, partial deoxygenated, and oxygenated), while the remaining vials (deoxygenated and partially deoxygenated blood) were oxygenated rapidly with 100% O_2. In all cases, CO_2 was omitted from these gas mixtures supplying the vials resulting in a large Pco_2 gradient for CO_2 removal, and gas flow rate was 120 $ml \cdot min^{-1}$ (Perry and Gilmour, 1993). Preliminary experiments revealed that during rapid oxygenation of the blood, Hb oxygenation was complete within 60 s of the beginning of the assay. In addition, the absolute "boost" to CO_2 excretion associated with rapid oxygenation was not increased with assay durations longer than 2.5 min. The CO_2 excretion rate in plasma was measured only in the high oxygen gas mixture (0.4% CO_2 and 99.6% O_2) and served as a control to ensure that no red cell lysis had occurred during sampling and storage.

In addition to the three gas mixtures to which blood was equilibrated for 2 h, one more group of vials of blood was equilibrated with a mixture of 0.4% CO_2, 3.8% O_2, and the remainder N_2. Following a 2 h incubation period, oxygen content, pH_e, pH_i and

total CO_2 content of whole blood and plasma were measured. These data were grouped with values measured in blood prior to the CO_2 excretion assay to derive the relationship between pH_i and So_2 at constant Pco_2.

Analytical procedures

^{14}C activity was measured by liquid scintillation counting (Packard TR 2500) automatically corrected for quenching. Plasma (50 ul) and filter paper ^{14}C activity were counted in 10 ml of ACS II (Amersham), and Carbo-trap (1.5 ml) was counted in 18 ml of OCS II.

Statistics:

Statistical differences between CO_2 excretion rates at constant initial So_2 were determined using a paired, students t-test. A level of 5% was chosen as the fiducial limit of statistical significance. Regression analysis was conducted by least squares regression.

RESULTS

Following a 2h incubation period at constant Pco_2 (0.4%, 3.0 mmHg), large differences in pH_i were observed at the different oxygen levels. In completely oxygenated blood, pH_i was 0.21 units lower than that in deoxygenated blood (Fig. 2.1) and the majority of the pH_i change occurred when So_2 was between approximately 60 and 100%. Deoxygenated blood pH_e was 7.86 $\pm$ 0.01 and was not significantly different at 60 or 100% So_2. These data indicate that at constant Pco_2 and pH_e, the majority of Bohr protons were released in the upper region of the Hb-O_2 equilibrium curve (60-100% So_2).

The *in vitro* CO_2 excretion rate of whole blood at constant Po_2, was not affected by So_2 (filled bars in figure 2.2) but increased significantly during rapid oxygenation of the blood (hatched bars in figure 2.2). The relative increase in CO_2 excretion rate following rapid oxygenation of the blood will be referred to as the "boost" to CO_2 excretion from this point onwards. The boost to CO_2 excretion was 32% in blood which was initially completely deoxygenated, and 30% in blood which was initially partially oxygenated (60% So_2). No significant difference in the boost to CO_2 excretion was detected between these incubation conditions. Thus, oxygenation of Hb between 60 and 100% saturation resulted in the boost to CO_2 excretion, consistent with the range over which Bohr protons were released (Fig. 2.1).

FIGURE 2.1: Red cell pH (pH_i) as a function of % saturation of haemoglobin (Hb) in whole blood (Hct=25%) of rainbow trout, incubated at constant Pco_2. Data points represent individual measurements and are fit with a second order regression ($r^2=0.76$).

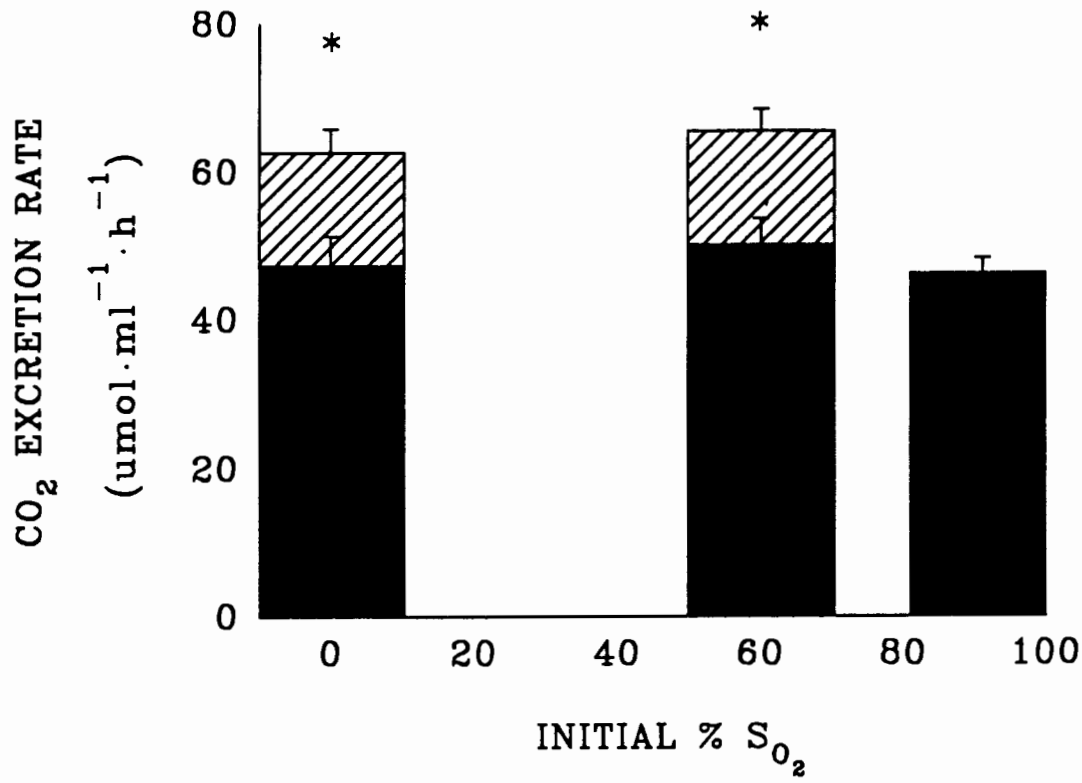
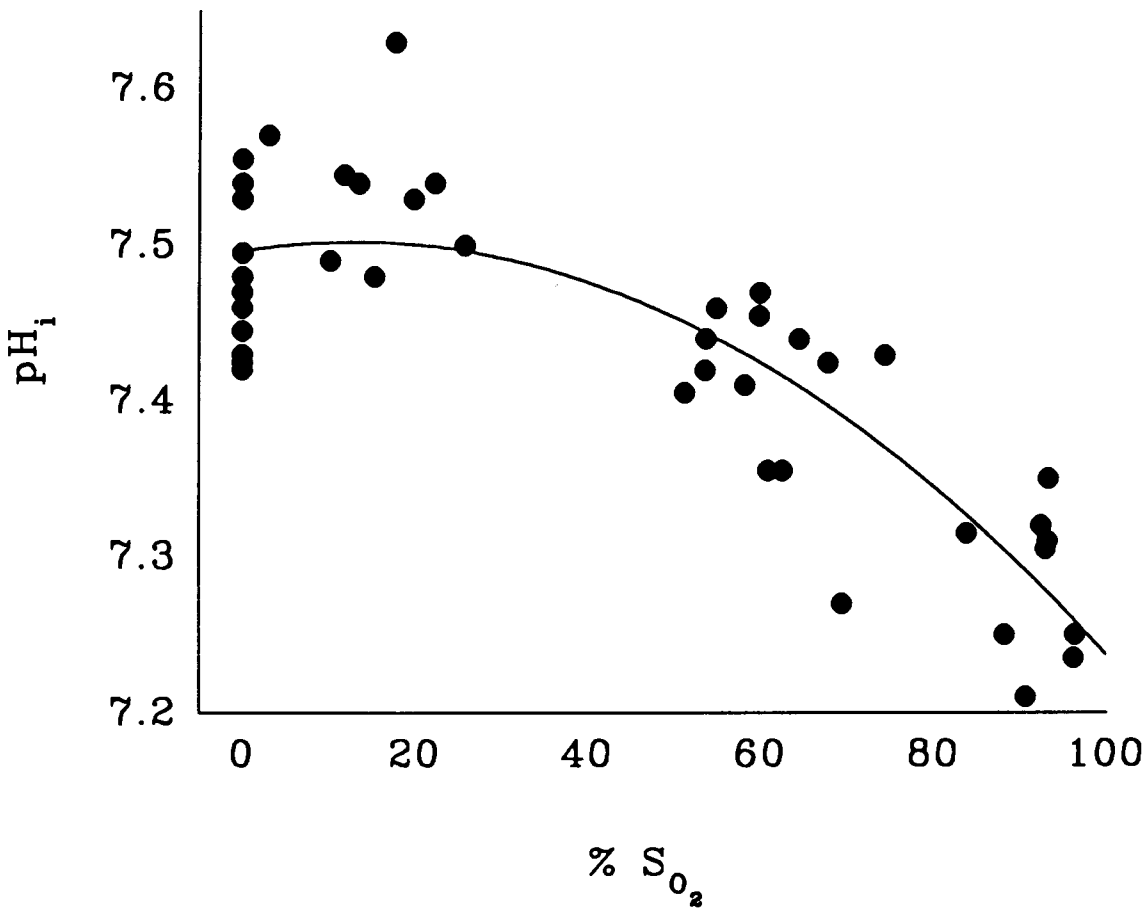

80
60
40
20
0
*
*
0
20
40
60
80
100
CO_2 EXCRETION RATE
(umol·ml^{-1}·h^{-1})
INITIAL % S_{O_2}

FIGURE 2.2: Whole blood CO_2 excretion rate, *in vitro*, with and without rapid oxygenation of the blood. Rainbow trout blood was incubated at different initial % So_2 before the assay was conducted. Solid bars represent rates measured with no change in oxygen level during the assay, while cross hatches represent the boost associated with rapid oxygenation during assay conditions. * indicates statistically significant difference between solid and cross-hatch bars at respective initial % So_2. (n=12).


7.6
7.5
7.4
7.3
7.2
pH_i
0
20
40
60
80
100
% S_{O_2}

(Cameron and Polhemus, 1974). Under conditions where the lower reaches of the Hb-O_2 equilibrium curve are utilized, such as during hypoxia and exercise, the magnitude of the Haldane effect which is exploited during gill blood transit will be reduced affecting CO_2 excretion accordingly.

In conclusion, whole blood in trout displays a non-linear release of Bohr protons over the Hb-O_2 equilibrium curve, where the majority of Bohr protons were released between 60 and 100% So_2. Rapid oxygenation of the blood over this region of the Hb-O_2 equilibrium curve elevated HCO_3^- flux rate through the red cell by about 30% during CO_2 excretion *in vitro*.

SUMMARY

1) The majority of Bohr protons were released from the blood of trout between 60 and 100% of So_2 *in vitro*.

2) Rapid oxygenation of the blood elevated *in vitro* CO_2 excretion rates by 30% relative to conditions where Po_2 was held constant. The boost in CO_2 excretion rate associated with rapid oxygenation of the blood occurred between 60 and 100% of So_2, consistent with the region over which the majority of Bohr protons were released.

CHAPTER 3: Gas transport and the interaction between O_2 and CO_2 during graded sustained exercise.

PREFACE

This chapter is adapted from a paper published by Brauner,CJ and Randall,DJ, 1995. *Comp. Biochem. Physiol.* (In press) and a paper by Brauner,CJ, Thorarensen, TH, Gallaugher, P, Farrell, AP, and DJ Randall, 1994. In: *High Performance Fish:* Proceedings of an International Fish Physiology Symposium held at the University of British Columbia, Vancouver, Canada, July 16-21, 1994, pp.500-505. Some of the data were obtained in collaboration with P. Gallaugher and T.H. Thorarensen.All other aspects of collection of data and presentation of results were performed by myself.

INTRODUCTION

Exercise can be used as a tool to 1) examine the limitations to gas exchange by accelerating the rate at which the components of the respiratory system must function and 2) influence the region of the Hb-O_2 equilibrium curve used for gas transport. In fish swimming at maximal levels of exercise, oxygen consumption rate can increase by 12-20 fold from resting levels (Brett, 1964; Brett and Glass, 1973; Puckett and Dill 1984). There are a suite of physiological adjustments during exercise which ensure oxygen delivery to the active tissues such as an increase in arterial-venous oxygen content difference (Kiceniuk and Jones, 1977) and an elevation in cardiac output, primarily due to an increase in stroke volume (Kiceniuk and Jones, 1977; Randall, 1982). Haematocrit and oxygen carrying capacity of the blood have been shown to increase at fatigue (Jones

and Randall, 1978; Thomas *et. al.*, 1987) and there is evidence of a graded release of red cells from the spleen with increasing swimming velocity (Gallaugher *et. al.*, 1992).

The elevated capacity for gas transport in the blood is matched with adjustments at the gills. Kiceniuk and Jones (1977) measured an increase in the gill ventilation: perfusion ratio from 12 at rest to 32 during maximal exercise indicating that ventilation volume is elevated disproportionately relative to changes in cardiac output. The increase in ventral aortic pressure during swimming (Kiceniuk and Jones, 1977) elevates the proportion of secondary lamellae which are perfused (Randall and Daxboeck, 1984) and reduces epithelial thickness (Farrell *et. al.*, 1979; Randall and Daxboeck 1981; Randall and Daxboeck 1984) increasing the diffusing capacity of the gills.. Despite the increased blood volume of the gills, the elevation in cardiac output reduces blood transit time through the gills from 3s to 1s (Randall, 1982) limiting the time available for the processes involved in gas exchange. All of these modifications to the cardio-respiratory system are crucial for the maintenance of elevated gas flux across the gills. However, diffusing capacity of the gills is also dependent upon the reactions which occur within the red cell.

Most of the studies to date examining exercise in fish, have concentrated specifically on oxygen transport. Respiratory exchange ratios (RE; CO_2 excretion rate/O_2 consumption rate) have been measured during exercise in fish and they confirm that CO_2 excretion rate is elevated in accordance with oxygen consumption rate (Kutty, 1968; Van de Thillart *et. al.*, 1983). In general, however, there have been few reports of CO_2 transport during sustained exercise in fish (Wood and Perry, 1985). The first objective of

this experiment was to conduct a quantitative analysis of CO_2 transport during different levels of sustained exercise in rainbow trout.

With increased intensity of sustained exercise, there is a marked increase in the arterial-venous oxygen content difference of blood (Kiceniuk and Jones, 1977). Thus, the region of the Hb-O_2 equilibrium curve used for gas transport increases with exercise intensity. As demonstrated *in vitro*, the release of Bohr protons was non-linear with Hb oxygenation; the majority of Bohr protons being released between 60 and 100% saturation (Chapter 2). In addition, CO_2 excretion *in vitro* was strongly influenced by the release of Bohr protons during Hb oxygenation. Thus, the second objective of this experiment was to determine whether the Haldane effect varied over the region of the Hb-O_2 equilibrium curve used *in vivo*, and to evaluate its impact on the linkage between O_2 and CO_2 exchange at different levels of exercise intensity.

MATERIALS AND METHODS

Experimental animals:

Rainbow trout (*Oncorhynchus mykiss,* weight=649 ± 13g; length=36 ± 0.4cm) were purchased from a local supplier (West Creek Trout Ponds, Aldergrove, BC) and acclimated to sea water [29 parts per thousand (ppt), 9 °C] for at least one month prior to experiments. Fish were housed and experiments were conducted at the Department of Fisheries and Oceans, West Vancouver. Fish were fed to satiation bi-weekly and starved for 2 days prior to surgery. In all conditions, fish were maintained in 29 ppt sea water.

Surgery and Handling:

Fish were anaesthetized before surgery using tricaine methanesulphonate (MS-222) dissolved in sea water and the dorsal aorta was cannulated as described in the general materials and methods. In some fish, the pre-branchial artery of the first gill arch was cannulated (PE-50) to sample mixed venous blood, in which case the entire cannulated gill arch was tied off to secure the tubing. Fish which had both the arterial and venous systems cannulated will be referred to as "Series I" and those with only the dorsal aorta cannulated will be referred to as "Series II". Following surgery, weight and fork length were recorded and the fish was left to recover for 24 to 48 h in a black perspex box and placed in a Brett-type swim tube respirometer the night before swimming experiments. During acclimation to the swim tunnel, water flow velocity was maintained at 11 cm/s and water was continually replaced to prevent build up of

metabolic waste products.

Experimental Procedure:

Blood parameters were measured in rainbow trout at four swimming velocities: the velocity to which fish were acclimated over night, two intermediate swimming speeds, and finally, at the maximal critical swimming velocity (Ucrit). At the start of each swimming trial, the swim tunnel was sealed and the rate of oxygen depletion was recorded over a 10 minute period for calculation of oxygen consumption rate. Water Po_2 was recorded by computerized data acquisition described below. After recording these data, the blood was sampled.

In Series I fish, 0.6 ml of blood was withdrawn from the dorsal aorta and pre-branchial artery for measurement of Co_2 and Po_2, Cco_2 of whole blood and plasma, pH_e, pH_i, Hct, [Hb], [MetHb] and plasma [Cl^-]. Plasma Pco_2 and [HCO_3^-] were calculated from plasma Cco_2 and pH by re-arrangement of the Henderson-Hasselbalch equation as described in the General Materials and Methods section. There was a 5 min interval between sampling of arterial and mixed venous blood and the first source of blood sampled was chosen at random. Following blood removal, 1.2 ml of blood from a resting donor fish was injected into the swimming fish to restore blood volume to pre-sample levels. In Series II fish, the above procedure was conducted only on arterial blood but the remaining procedures were the same for both groups of fish.

Upon completion of this procedure, water velocity was gradually elevated by 0.66 body length per second ($Bl \cdot s^{-1}$) over a 10 min period. The fish were left at the new

velocity for 30 min before the sampling procedure described above was repeated. Thirty minutes is sufficient time for blood-gas and acid-base parameters to stabilize following a change in water velocity (Kiceniuk and Jones, 1977; Thomas *et. al.*, 1987). The change in water velocity was increased by 0.66 $Bl \cdot s^{-1}$ increments followed by the sampling regime until the fish could no longer maintain the swimming velocity. This water velocity and time to fatigue were noted for the calculation of Ucrit as described by Brett, (1964) taking into consideration the solid blocking effects of the fish as described by Bell and Terhune (1970). The water velocity was then reduced to a level in which the fish could sustain swimming, and a final sampling procedure was conducted where an additional 200 μl of blood was removed for measurement of catecholamines.

Analytical techniques:

Fish oxygen consumption rate was calculated from the change in the partial pressure of oxygen (Po_2) in water over the duration that the respirometer was sealed, taking into account the solubility of O_2 in sea water at 10 °C (Boutilier *et. al.*, 1984) and the volume of the swim tunnel according to Kiceniuk and Jones (1977). Water Po_2 was measured with Radiometer Po_2 (E-5046) electrodes, thermostatted in D616 cells at the respirometer temperature, and displayed on a Radiometer PHM 71 acid-base analyzer. The electrode was calibrated with air saturated water daily and checked regularly. The analog output from the PHM 71 meter was sampled at 1 Hz by the analog-to-digital converter of the data acquisition card (DT 2801) installed in the computer. LABTECH

NOTEBOOK was used for data acquisition and the data was stored in a Lotus 123 file for future data analysis.

Statistics:

Statistically significant differences between mean values measured at different swimming velocities were detected using a repeated measures ANOVA , or Friedman repeated measures ANOVA on ranks, followed by a Dunnett's test. Comparisons between arterial and venous parameters were conducted using a paired t-test. In all cases a probability level of 5% was chosen as the limit of statistical significance. Regression coefficients were calculated using least squares regression.

RESULTS

O_2 transport and uptake during exercise:

Oxygen uptake increased approximately 5 fold over resting levels during sustained exercise (Table 3.1). Although C_ao_2 and S_ao_2 did not change significantly during exercise there was a marked reduction in P_ao_2 at greater swimming velocities (Table 3.2). In mixed-venous blood, C_vo_2, S_vo_2 and P_vo_2 decreased significantly during exercise and differed significantly from arterial values at all swimming velocities (Table 3.2). The reduction in P_ao_2 and the increased arterial-venous difference in CO_2 content ($C_{a-v}o_2$, Table 3.2) resulted in a significant increase in the proportion of oxygen taken up across the gills which was transported away in the blood bound to Hb. In resting fish, 91% of the O_2 uptake across the gills was bound to Hb while this increased significantly to 97.5% at the maximum swimming velocity (Table 3.2).

CO_2 transport and excretion during exercise:

Arterial Pco_2 and Cco_2 levels increased significantly with swimming velocity (Table 3.3) while no significant changes were observed in arterial pH (Table 3.2). A pH/HCO_3^- plot of these data reveal that the elevation in blood CO_2 levels was associated with net HCO_3^- retention (Figure 3.1). In this group of fish (Series I), the first afferent

TABLE 3.1: Oxygen consumption rate and blood parameters during different levels of sustained exercise in rainbow trout (Series I).

% Ucrit	Velocity ($cm \cdot s^{-1}$)	Mo_2 ($mg \cdot kg^{-1} \cdot h^{-1}$)	Hct_a	Hct_v	$[Hb_a]$ ($g \cdot dl^{-1}$)	$[Hb_v]$ ($g \cdot dl^{-1}$)	Plasma [Cl^-] ($meq \cdot l^{-1}$)	Adr (nM)	Noradr (nM)	Lactate (mM)
15.8 (1.7) 9	9.2 (1.0) 9	49.3 (4.0) 9	23.8 (1.1) 9	23.4 (1.3) 9	8.5 (0.4) 9	8.6 (0.5) 9	155.8 (3.8) 9	-	-	0.31 (0.04) 8
55.4 (1.5) 9	32.1 (1.0) 9	94.2 (12.9) 9	24.4 (1.1) 9	24.1 (1.3) 9	8.8 (0.4) 9	8.6 (0.4) 9	154.2 (2.8) 9	-	-	-
90.9 (1.2) 8	53.1 (1.1) 8	218.0* (13.8) 8	27.7* (0.9) 8	28.0* (0.9) 8	9.6 (0.4) 8	9.7 (0.5) 8	155.5 (2.8) 8	-	-	-
98.8 (0.9) 9	57.1 (1.1) 9	229.2* (15.6) 9	25.5 (0.8) 9	26.6 (0.7) 9	9.0 (0.5) 9	8.7 (0.5) 9	161.8 (3.1) 9	15.4 (6.5) 6	7.4 (3.1) 6	1.97* (0.29) 8

Where Adr is adrenaline, Noradr is noradrenaline. Plasma[Cl^-], Adr, Noradr, and Lactate levels were only measured in arterial blood. Values represent mean with s.e.m. in brackets and "n" beneath. * signifies statistically different from the lowest swimming velocity.

TABLE 3.2: Blood pH and blood oxygen transport parameters in rainbow trout at different levels of sustained exercise (Series I).

% Ucrit	pH_{ea}	pH_{ev}	pH_{ia}	pH_{iv}	P_aO_2 (mmHg)	P_vO_2 (mmHg)	C_aO_2 (mM)	C_vO_2 (mM)	S_aO_2	S_vO_2	$C_{a-v}O_2$ (mM)	% $C_{a-v}O_2$ as Hb-O_2
15.8 (1.7) 9	7.96 (0.01) 9	7.97 (0.02) 9	7.56 (0.01) 6	7.58 (0.01) 6	101.3 (8.7) 9	30.4 § (1.5) 9	4.75 (0.21) 9	2.88 § (0.30) 9	0.90 (0.05) 9	0.55 § (0.06) 9	1.87 (0.2) 9	91.0 (1.8) 9
55.4 (1.5) 9	7.95 (0.01) 9	7.95 (0.02) 9	7.55 (0.01) 6	7.60 (0.03) 5	95.9 (7.6) 9	23.0 *§ (1.3) 9	4.66 (0.29) 9	2.23 § (0.26) 9	0.85 (0.05) 9	0.42 § (0.05) 9	2.43 (0.24) 9	93.5 (0.9) 9
90.9 (1.2) 8	7.97 (0.02) 8	7.83 *§ (0.04) 8	7.61 * (0.03) 5	7.62 (0.02) 5	64.3 * (7.5) 8	15.3 *§ (0.4) 8	5.09 (0.21) 8	1.63 *§ (0.27) 8	0.87 (0.06) 8	0.29 *§ (0.06) 8	3.46 * (0.21) 8	97.0 * (0.5) 8
98.8 (0.9) 9	7.94 (0.02) 9	7.79 *§ (0.03) 9	7.63 * (0.01) 7	7.64 (0.01) 7	49.8 * (7.3) 9	13.1 *§ (0.7) 9	4.55 (0.24) 8	1.06 *§ (0.15) 8	0.83 (0.05) 8	0.20 *§ (0.03) 8	3.49 * (0.21) 8	97.5 * (0.5) 8

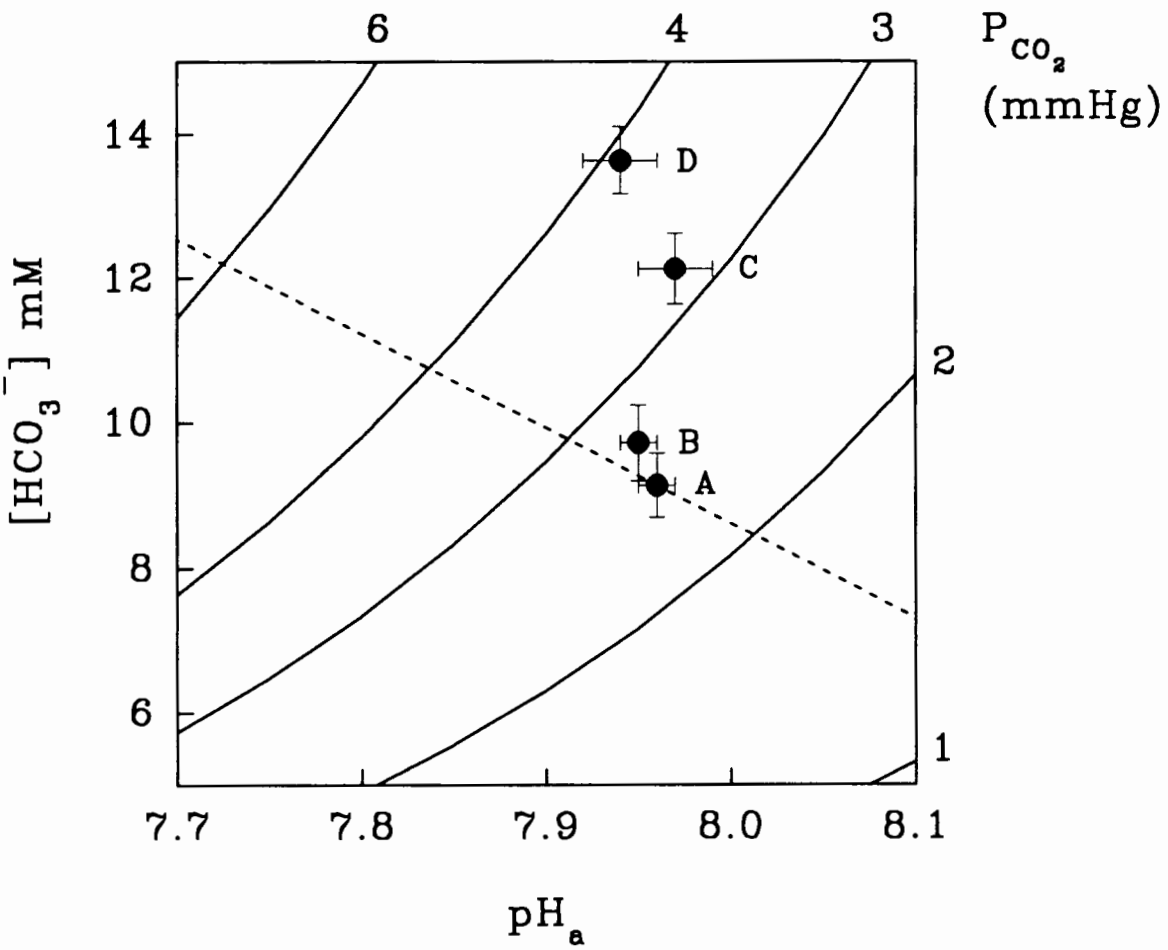
Where $\%C_{a-v}O_2$ as Hb-O_2 refers to the proportion of oxygen taken up across the gills, transported by Hb (ie. dissolved O_2 removed). Values represent mean values with s.e.m. in brackets and "n" beneath. * signifies statistically different from the lowest swimming velocity. § signifies statistically different from respective arterial value.

TABLE 3.3: Blood CO_2 transport parameters and partitioning of CO_2 excretion in rainbow trout at different levels of sustained exercise (Series I).

% Ucrit	P_aco_2 (mmHg)	P_vco_2 (mmHg)	Plasma $[HCO_3^-]_a$	Plasma $[HCO_3^-]_v$	Red cell $[HCO_3^-]_a$	Red cell $[HCO_3^-]_v$	$C_{a-v}co_2$ (mM)	% $Pbco_2$	% $HCO_3^- \rightarrow CO_2$	Hald. coeff.	$\%MCO_2:MO_2$
15.8 (1.7) 9	3.10 (0.19) 9	3.26 (0.23) 9	9.14 (0.44) 9	9.93 § (0.53) 9	3.56 (0.63) 7	5.90 (0.74) 7	1.28 (0.14) 7	0.7 (1.1) 7	99.3 (1.1) 7	0.99 (0.17) 7	115.9 (17.2) 7
55.4 (1.5) 9	3.40 (0.18) 9	3.87 (0.34) 9	9.72 (0.52) 9	11.09 § (0.64) 9	3.87 (0.60) 8	6.14 (0.72) 8	1.67 (0.16) 8	2.0 (0.8) 8	98.0 (0.77) 8	0.67 (0.08) 7	95.3 (9.4) 7
90.9 (1.2) 8	4.01 *§ (0.27) 8	6.69 *§ (0.69) 8	12.12 * (0.49) 8	13.85 *§ (0.66) 8	5.79 * (0.76) 7	9.85 *§ (1.66) 7	2.58 * (0.15) 7	6.5 * (1.7) 7	93.5* (1.69) 7	0.51 (0.20) 6	53.2 * (19.2) 6
98.8 (0.9) 9	4.85 *§ (0.26) 9	8.39 *§ (0.54) 9	13.63 * (0.47) 9	15.79 *§ (0.57) 9	6.95 * (0.83) 7	8.78 (0.61) 7	2.47 * (0.26) 7	11.2 * (2.0) 7	88.8* (2.0) 7	0.42* (0.13) 5	44.1* (9.5) 5

Where % $Pbco_2$ represents the proportion of CO_2 excreted due to dissolved CO_2 which existed in pre-branchial blood (equation 1 a, Appendix), % $HCO_3^- \rightarrow CO_2$ refers to the proportion of CO_2 excreted due to HCO_3^- dehydrated to CO_2 during gill blood transit (equation 1 b, Appendix). Hald. coeff. refers to the Haldane coefficient (moles of protons released per mole of O_2 which binds to Hb) calculated over the region of the Hb-O_2 equilibrium curve used for gas exchange (equation 4, Appendix). $\%MCO_2:MO_2$ refers to the maximum proportion of CO_2 excretion linked with O_2 uptake via the Haldane effect (equation 5, Appendix).
See Table 3.2 legend for further details.

FIGURE 3.1: A pH/HCO_3^- plot of changes in blood acid-base status of rainbow trout during different levels of sustained exercise (Series I). The data points represent mean values (error bars represent S.E.M.) for arterial pH_e and plasma [HCO_3^-] from fish swimming at different velocities (16 (A), 55 (B), 91 (C) and 99 (D) % of Ucrit, Tables 3.2 and 3.3). The buffer line (dotted line) was calculated from the regression equation for β as a function of [Hb] derived by Wood *et. al.* (1982) and a Hb concentration of 8.5 $g \cdot dl^{-1}$ (the value measured at the lowest swimming velocity (16% Ucrit, Table 3.1).


6
4
3
P_{CO_2}
(mmHg)
14
12
10
8
6
$[HCO_3^-]$ mM
D
C
B
A
2
1
7.7
7.8
7.9
8.0
8.1
pH_a

branchial artery was tied off; however the same trends in dorsal aortic blood CO_2 were observed in fish which did not have the afferent branchial artery cannulated (Series II, Table 3.4, Figure 3.2). There was a significant relationship between arterial Po_2 and arterial CO_2 levels during exercise. The lower the P_ao_2, the greater the P_aco_2 and Cco_2 levels in arterial blood (Fig. 3.3).

In mixed-venous blood, the increase in total blood CO_2 and Pco_2 during exercise was more pronounced than that in arterial blood (Table 3.3). Thus, with an increase in exercise intensity, there was an increased arterial-venous difference in Pco_2 and total blood CO_2 (Table 3.3). The arterial-venous difference in Pco_2 was used to calculate the proportion of CO_2 excreted which did not depend on HCO_3^- dehydration within the red cell during blood transit through the gills (see Appendix, equation 1 and discussion for elaboration). The increased arterial-venous Pco_2 difference, led to a significant increase in the contribution of physically dissolved CO_2 in pre-branchial blood to total CO_2 excretion (Table 3.5). The remaining CO_2 excreted was assumed to be due to HCO_3^- dehydration within the red cell (see discussion for elaboration) which decreased significantly from 99.4% at rest to 91% at the maximum swimming velocity. The proportion of total CO_2 excreted which involved HCO_3^-/Cl^- exchange across the red cell did not change significantly during exercise and a mean value of 62% was calculated for all swimming velocities (Table 3.5).

The partitioning of CO_2 transport between the plasma and red cells did not change significantly during exercise in arterial or venous blood. Consequently, the values calculated at different levels of exercise intensity were pooled. The proportion of

TABLE 3.4: Oxygen consumption rate and arterial blood parameters during sustained exercise in rainbow trout without the afferent branchial artery cannulated (Series II).

% Ucrit	Velocity ($cm \cdot s^{-1}$)	$\dot{M}o_2$	Hct_a	Hb ($g \cdot dl^{-1}$)	Plasma [Cl^-]	pH_e	pH_i	Po_2 (mmHg)	C_aO_2 (mM)	P_aCO_2 (mmHg)	Plasma [HCO_3^-]
18.4 (1.0) 7	11.0 (0.4) 7	58.4 (12.3) 7	29.4 (1.1) 7	10.2 (0.8) 7	166 (11.2) 4	7.97 (0.03) 7	7.54 (0.03) 4	102.0 (8.6) 7	5.98 (0.57) 7	2.54 (0.20) 5	9.6 (0.9) 5
63.8 (6.1) 7	36.7 (2.4) 7	85.8 (12.4) 7	29.2 (0.9) 7	10.5 (0.6) 7	166 (10.3) 5	7.95 (0.02) 7	7.56 (0.03) 5	87.0 (12.3) 7	6.19 (0.32) 7	2.78 (0.17) 5	10.3 (1.0) 5
81.3 (4.5) 9	50.7 (2.6) 9	179.0 * (18.2) 9	28.8 (1.3) 7	9.9 (0.4) 8	147 (3.9) 7	7.95 (0.01) 9	7.49 (0.02) 7	77.8 (4.0) 9	5.3 (0.38) 9	3.12 * (0.15) 7	10.8 (0.6) 7
95.2 (1.4) 7	60.9 (1.0) 7	249.0 * (24.5) 7	27.4 (1.8) 4	9.5 (0.2) 7	149 (3.8) 5	7.94 (0.02) 7	7.51 (0.02) 5	55.0 * (4.5) 7	5.51 (0.38) 7	3.44 * (0.05) 5	11.2 (0.6) 5

Where $\dot{M}o_2$ is measured in $mg \cdot kg^{-1} \cdot h^{-1}$. See Table 3.2 legend for further details.

FIGURE 3.2: A pH/HCO_3^- plot of changes in blood acid-base status of rainbow trout during different levels of sustained exercise (Series II). See legend for figure 3.1 for further details.

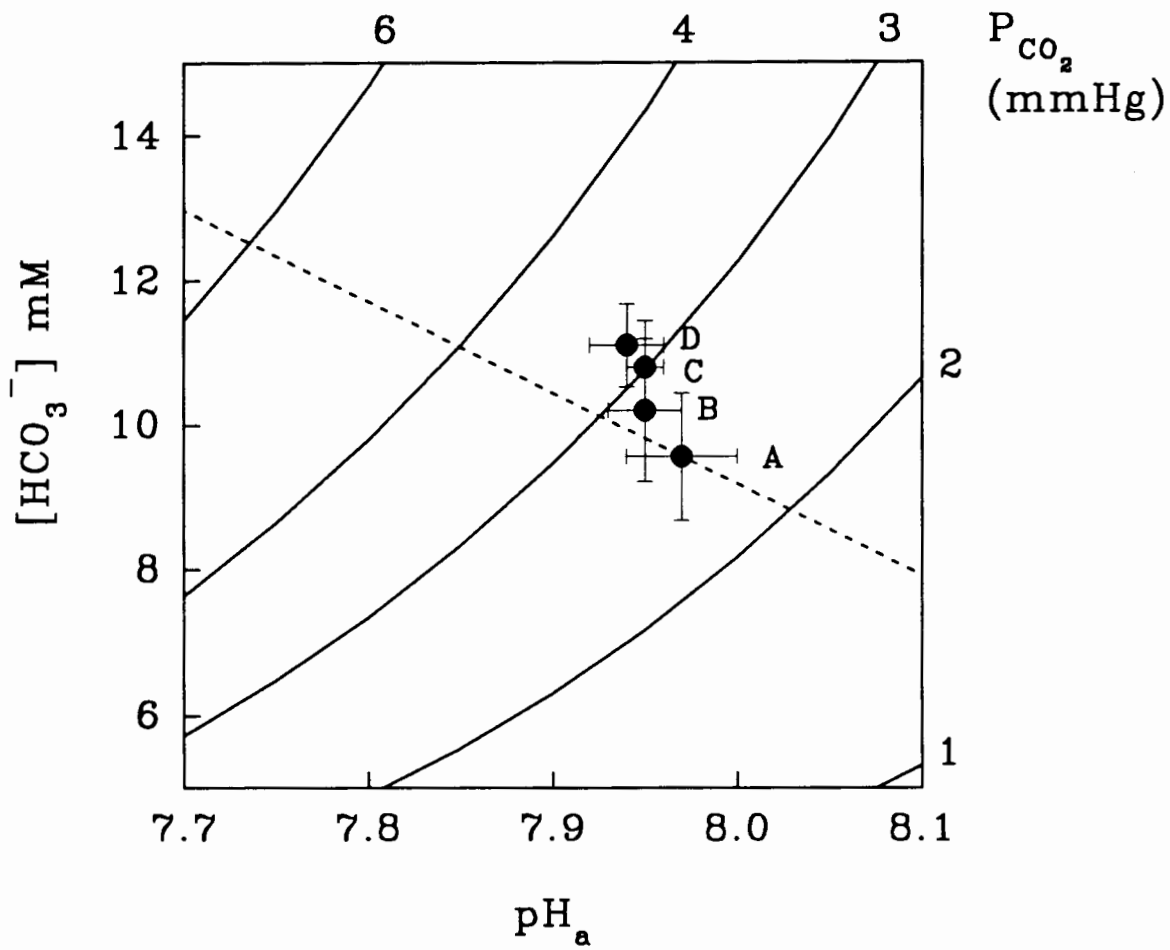

6
4
3
P_{CO_2} (mmHg)
14
12
10
8
6
$[HCO_3^-]$ mM
2
1
D
C
B
A
7.7
7.8
7.9
8.0
8.1
pH_a

FIGURE 3.3: The relationship between venous-arterial ΔpH_e and arterial-venous O_2 content ($C_{a\text{-}v}O_2$) in rainbow trout during different levels of sustained exercise (Series I). Cross-hatches at the top of the figure indicate the region of net acidosis in the blood following blood transit through the gills. Data points represent individual measurements ($r^2 = 0.66$). Note that low $C_{a\text{-}v}O_2$ values indicate high venous O_2 content and low swimming velocity, while high values indicate the reverse.

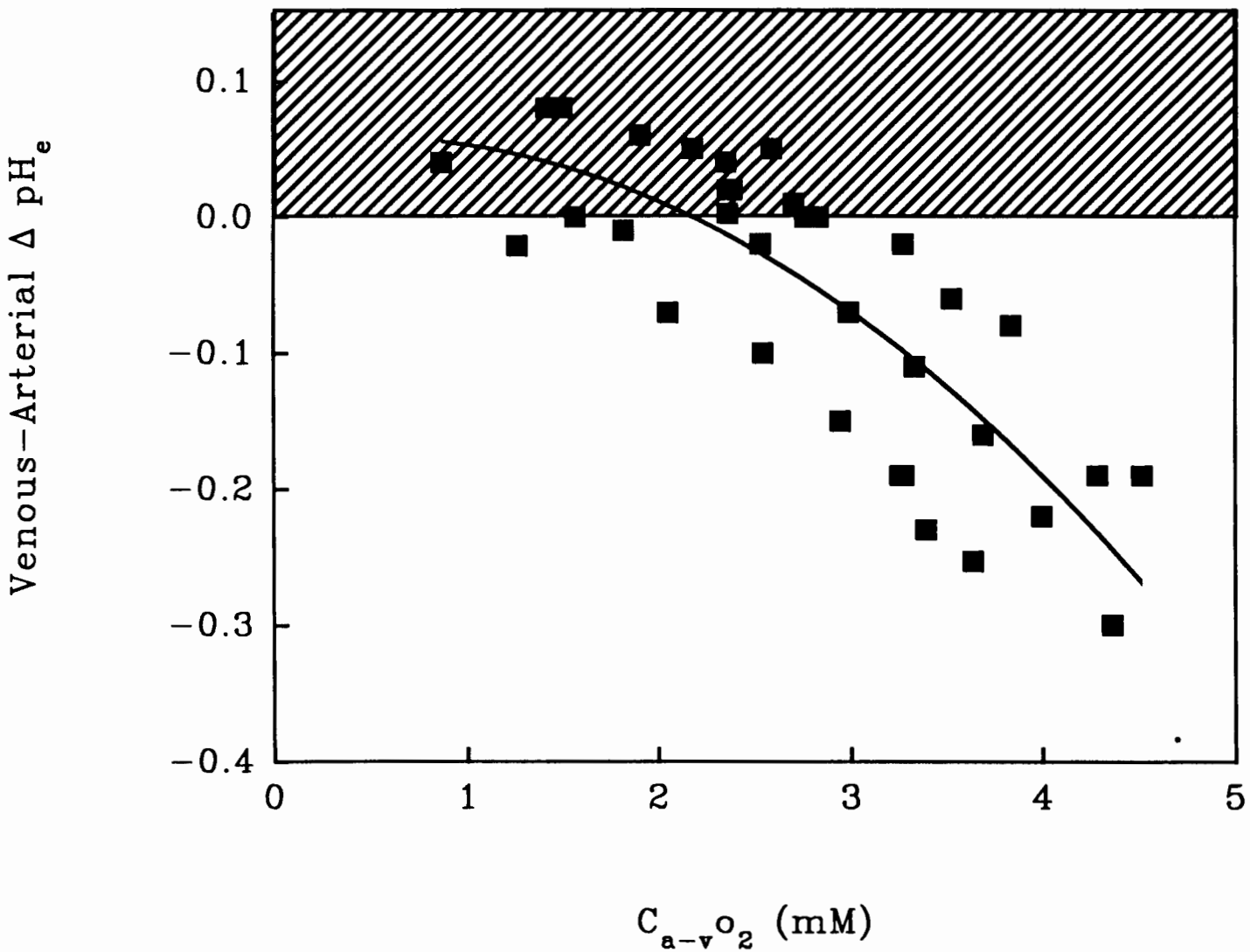

Venous−Arterial Δ pH$_e$
0.1
0.0
−0.1
−0.2
−0.3
−0.4
0
1
2
3
4
5
$C_{a-v}O_2$ (mM)

TABLE 3.5: Respiratory exchange ratios and partitioning of CO_2 transport and excretion during exercise in rainbow trout (Series I).

RE	RE'	$\%Cco_{2a}$ in plasma	$\%Cco_{2v}$ in plasma	$\%Cco_{2a}$ in red cells	$\%Cco_{2v}$ in red cells	% HCO_3^- /Cl^-	% red cell HCO_3^-
0.76	0.78	86.7	82.7 §	13.3	17.3 §	62.0	33.1
(0.04)	(0.04)	(0.9)	(1.2)	(0.9)	(1.2)	(8.3)	(8.3)
28	25	29	29	29	29	29	29

Where RE is the respiratory exchange ration and RE' is a modified respiratory exchange ratio (equation 4 in Appendix). $\%Cco_{2a}$ in plasma, refers to the proportion of the total CO_2 in arterial blood transported in the plasma. % HCO_3^-/Cl^- refers to the proportion of total CO_2 excreted which involved HCO_3^-/Cl^- exchange (equation 2, Appendix). % red cell HCO_3^- refers to the proportion of total CO_2 excreted due to dehydration of HCO_3^- which resided within the red cell prior to gill entry (equation 3, Appendix). All values are the mean for all swimming velocities grouped together.

total CO_2 carried in red cells of venous blood was significantly greater than that in arterial blood while the reverse was observed for CO_2 in the plasma compartment (Table 3.5).

Respiratory exchange ratios:

The respiratory exchange ratio in rainbow trout was not significantly different among swimming velocities. Consequently values at all exercise intensities were pooled to yield a value of 0.76 (Table 3.5). A modified respiratory exchange ratio (RE') was calculated by dividing the amount of HCO_3^- dehydrated to CO_2 by the amount of oxygen which bound to Hb during gas exchange across the gills (see Appendix, equation 4). Again no significant differences were observed among swimming velocities and the data were pooled to yield an RE' of 0.78 (Table 3.5).

Blood pH and haematological parameters during exercise:

While arterial blood pH remained constant during exercise, venous pH_e was significantly reduced at the two highest swimming velocities, resulting in a significant arterial-venous difference in pH_e (Table 3.2). Red cell pH increased significantly in arterial blood during exercise but there were no significant differences between arterial and venous pH_i (Table 3.2). The venous-arterial pH_e difference was regressed against the arterial-venous difference blood O_2 content measured in fish at different levels of

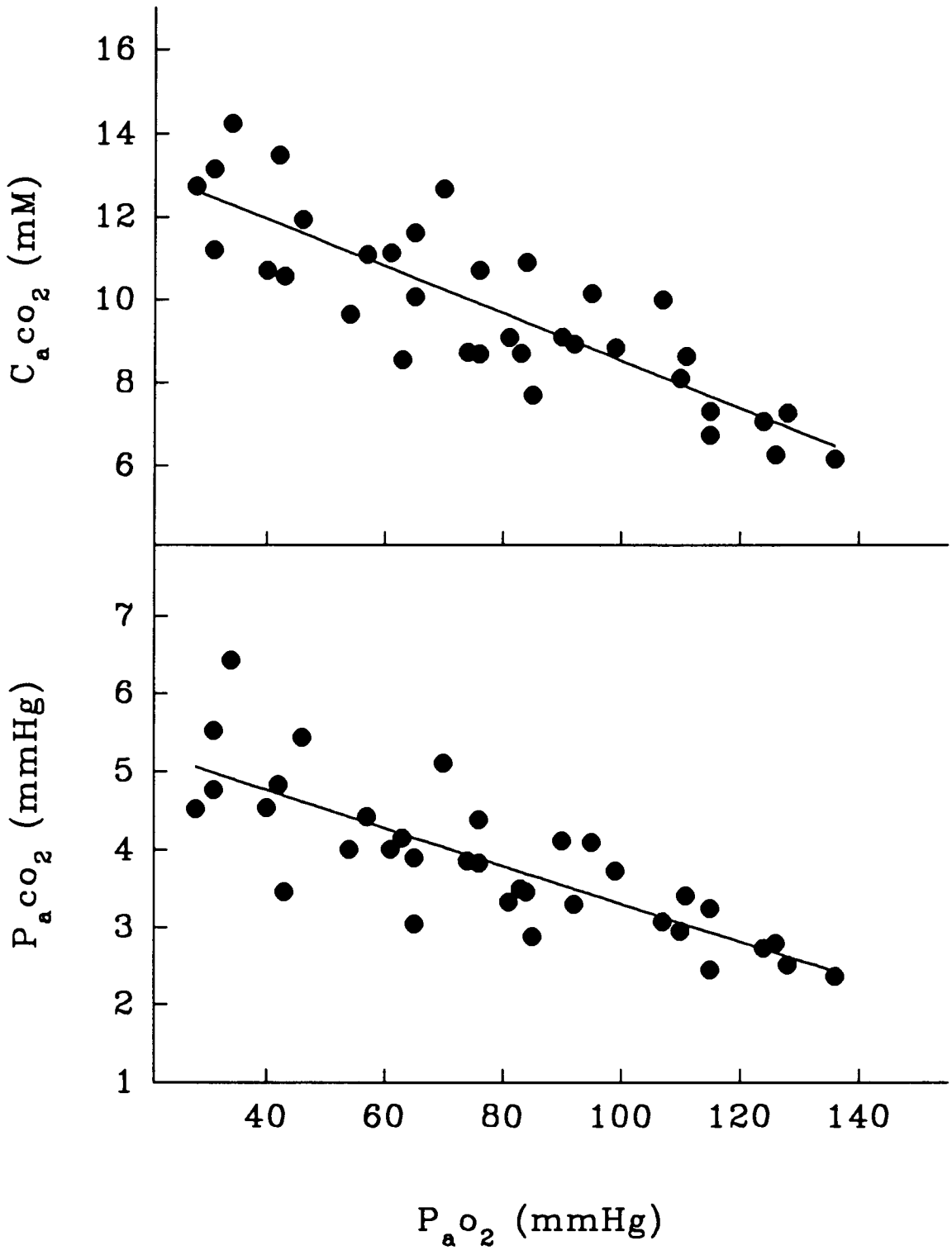
sustained exercise (Fig. 3.3). When $C_{a-v}O_2$ was low, venous-arterial pH_e was positive in many fish, indicating that arterial pH_e was more acidic than venous pH_e. As $C_{a-v}O_2$ increased, arterial pH_e became increasingly more alkaline relative to venous blood.

There was no significant difference in Hct between arterial and venous blood; however, both increased significantly during exercise (Table 3.2). No significant changes in MCHC or plasma [Cl^-] were observed in this study. Methaemoglobin levels were low ($1.5 \pm 0.24\%$) under all conditions and did not change significantly during exercise. Plasma adrenaline and noradrenaline concentrations were only measured in arterial blood at Ucrit and were 15.4 ± 6.5 and 7.4 ± 3.1 nM, respectively.

Haldane coefficient:

Haldane coefficients (moles of protons released per mole of O_2 which binds to Hb) were calculated from *in vivo* data according to equation 5 in the Appendix. The Haldane coefficient was determined over the region of the Hb-O_2 equilibrium curve used for gas exchange by the fish during exercise. The Haldane coefficient was 0.96 in slowly swimming fish, and decreased significantly with exercise as the arterial-venous So_2 difference increased (Table 3.3). The lowest value of 0.42 was obtained in fish swimming at Ucrit. The ratio of protons released during oxygenation at the gills to total CO_2 excreted across the gills (equation 6 in the Appendix) indicates that at the lowest swimming velocity all of the CO_2 excreted may have been linked with oxygenation of the Hb but this decreased to 44% at maximal swimming velocity.

FIGURE 3.4: The relationship between arterial blood Po_2 and a) arterial total CO_2 content and b) arterial blood Pco_2 in rainbow trout during different levels of sustained exercise (Series I). The regression equations are a) $Y=14.22-0.057X$, $r^2 = 0.71$ and b) $Y=5.736-0.02432X$, $r^2 = 0.65$. Data points represent individual measurements.


16
14
12
10
8
6
C_aCO_2 (mM)
7
6
5
4
3
2
1
P_aCO_2 (mmHg)
40
60
80
100
120
140
P_aO_2 (mmHg)

DISCUSSION

Although the oxygen transport capacity during sustained exercise in fish has been investigated in detail (Kiceniuk and Jones, 1977; Primmett *et. al.*, 1986), relatively little is known about CO_2 transport during exercise. As the sustained swimming velocity in rainbow trout was increased, there was a graded increase in both Cco_2 and Pco_2 of arterial and venous blood (Tables 3.3 and 3.5 and Figs. 3.1 and 3.2). Of the total CO_2 excreted across the gills, approximately 62% involved HCO_3^-/Cl^- exchange across the red cell prior to dehydration to CO_2. This value did not change significantly with exercise intensity (Table 3.5). A Haldane coefficient calculated from data obtained *in vivo*, indicated that the release of Bohr protons was greatest in the upper region of the Hb-O_2 equilibrium curve. The Haldane coefficient decreased as the arterial-venous difference in blood gas content increased during exercise (Table 3.3). The non-linear release of Bohr protons over the region of the Hb-O_2 equilibrium curve affects CO_2 elimination at the gills and uptake from the tissues.

O_2 and CO_2 transport during exercise:

In general, the physiological adjustments to exercise in rainbow trout were sufficient to maintain conditions for oxygen uptake and delivery, as was observed by Kiceniuk and Jones (1977) and no significant changes in C_aO_2 were observed at any swimming velocity (Tables 3.2 and 3.4). Metabolic rate was elevated nearly five fold

between low and maximal swimming velocity (Tables 3.1 and 3.4), consistent with the data reported by Stevens and Randall (1967), but slightly less than that observed by Kiceniuk and Jones (1977). In contrast to the findings of Kiceniuk and Jones (1977) and Stevens and Randall (1967), there was a significant and pronounced reduction in arterial Po_2 at the higher swimming velocities in fish from Series I (Table 3.2) and Series II (Table 3.4), indicating that there may have been a diffusion limitation for oxygen uptake. A large reduction in Po_2 has been observed by other investigators during sustained exercise in trout (Thomas *et. al.*, 1987; Gallaugher *et. al.*, 1992). The maintenance of C_aO_2 despite the large reduction in Po_2 was partly achieved through a significant increase in Hct (Table 3.1). The increase in Hct may have been mediated in part through the graded release of red cells from the spleen (Yamamoto *et. al.*, 1980; Gallaugher *et. al.*, 1992) and an elevation in circulating levels of catecholamines (Nilsson and Grove, 1974). Catecholamine concentrations were only measured in fish near Ucrit (Table 3.1) but these values were elevated relative to those measured by others in resting fish (Perry and Reid, 1992) and similar to those measured in fish during maximal exercise (Ristori and Laurent, 1985; Gallaugher *et. al.*, 1992). In many teleost fishes, catecholamines activate the Na^+/H^+ exchanger on the red cell which subsequently elevates red cell pH (Baroin *et. al.*, 1984; Cossins and Richardson, 1985; Primmett *et. al.*, 1986, and see Nikinmaa, 1990 for a review). The significant increase in red cell pH at swimming speeds above 90% Ucrit may have been adrenergically mediated influencing O_2 binding to Hb through the Bohr and Root effects. Interestingly, the characteristic decrease in MCHC associated with adrenergic stimulation of red cells (Nikinmaa, 1990) was not

observed in this study.

The absence of a metabolic acidosis (Table 3.2) and the relatively low levels of lactate in the plasma near Ucrit (Table 3.1) indicate that metabolism was predominantly aerobic during exercise in this study. Although only minor changes with respect to O_2 transport in fish were observed in this study, large changes in CO_2 transport were observed. Blood Pco_2 and Cco_2 levels increased in arterial blood of trout during exercise indicating that CO_2 released from the tissues was not matched by CO_2 removal at the gills (Tables 3.2 and 3.4). In fact, plasma HCO_3^- levels increased during exercise by as much as 50% at the maximum swimming velocity (Table 3.3) in fish which had one afferent branchial artery tied off (Series I). This increase was larger than that observed in Series II indicating that there was an effect on CO_2 transport resulting from eliminating blood flow through one of the gill arches. Interestingly, the elevation in blood Pco_2 and total CO_2 did not result in an acidosis in arterial blood (Figures 3.1 and 3.2). This could be achieved either through acid excretion (coupled with HCO_3^- retention) at the level of the gills or kidney, or through HCO_3^- uptake across the gills.

Partitioning of CO_2 excretion during exercise:

For the intent of this analysis, it was assumed that CO_2 excretion during blood transit through the gills was achieved either through the rapid movement of physically dissolved CO_2 which existed in pre-branchial blood ($Pbco_2$) or by HCO_3^- dehydrated to CO_2 ($HCO_3^- \rightarrow CO_2$) within the red cell during gill blood transit (see discussion of

chapter 1 for an elaboration). The relative role of each to CO_2 excretion in trout was calculated according to equations 1 to 3 in the Appendix.

In fish swimming at 16% Ucrit, the arterial-venous difference in Pco_2 was very small and the contribution of $Pbco_2$ to total CO_2 excretion was negligible (Table 3.3). However, as exercise intensity was increased, this route for CO_2 excretion reached a maximum of 11%, comparable to that in resting humans (Comroe, 1974). These calculations are based upon Pco_2 values measured in blood at equilibrium where it is assumed that these parameters are at equilibrium in the fish. In actuality, equilibrium conditions may never be achieved *in vivo* (Chapter 1, Gilmour *et. al.*, 1994). The absence of plasma accessible CA in the gills of fresh water teleost fishes gives rise to a post branchial blood disequilibrium. As blood flows away from the gills, arterial blood pH slowly increases as the plasma HCO_3^- is titrated to CO_2 at the uncatalyzed rate, resulting in an elevation in plasma Pco_2. In mixed venous blood (from the afferent branchial artery) pH and Pco_2 also increase during stop flow (chapter 1). The elevation in Pco_2 during stopflow in arterial and mixed venous blood in trout are approximately equal in resting fish exposed to normoxia and different levels of hypoxia (chapter 1). Although the absolute Pco_2 values reported in Table 3.3 do not likely represent *in vivo* values, the arterial-venous Pco_2 difference *in vivo* and at equilibrium may be similar. Thus, the role of $Pbco_2$ to total CO_2 excretion described above may be accurate, at least at the lower swimming velocities. The magnitude of the disequilibria in arterial and mixed-venous blood in exercising fishes has not been measured and the degree to which the disequilibria will affect the above calculations are unknown.

As described above it was assumed that all remaining CO_2 excreted consisted of HCO_3^- dehydrated to CO_2 ($HCO_3^- \rightarrow CO_2$) within the red cell during gill blood transit. HCO_3^- dehydration comprised about 99% of total CO_2 excretion in resting fish and reached a minimum of 89% of the total CO_2 excreted at the maximal swimming velocity. Some HCO_3^- resided within the red cell before the blood entered the gills, while the remainder traversed the red cell via the HCO_3^-/Cl^- exchanger. No significant differences were observed in the relative proportion of either pathway during exercise, resulting in 62% of the total CO_2 excreted being dependent upon HCO_3^-/Cl^- exchange. This value is in close agreement with that obtained in resting fish exposed to normoxia and 2 levels of hypoxia (Chapter 1). This is of significance to CO_2 transport in fish because HCO_3^- entry into the red cell by way of HCO_3^-/Cl^- exchange is thought to be the rate limiting step in CO_2 excretion (Perry *et. al.*, 1982).

In resting fish, HCO_3^-/Cl^- exchange is thought to be complete during CO_2 excretion across the gills (Gilmour *et. al.*, 1994). During exercise when the residence time of red cells in the gill lamellae are greatly reduced, HCO_3^-/Cl^- exchange may not be complete in post-branchial blood and this could result in an overestimation of HCO_3^-/Cl^- exchange during CO_2 excretion according to these equations.

CO_2 partitioning between plasma and red cells during exercise:

The majority of CO_2 transported in the blood was carried within the plasma compartment with 13 and 17% transported by the red cells in arterial and venous blood

respectively (Table 3.5). These values are consistent with those measured by Heming (1984). No significant changes were observed during exercise (Table 3.5). In resting fish, Currie and Tufts (1993) measured 2% of Cco_2 in the erythrocytes in arterial blood and 9% in venous blood. Following exhaustive exercise, this increased significantly to 13.5 and 20% in arterial and venous blood, respectively (Currie and Tufts, 1993). They attributed the increased proportion of CO_2 within the red cells to the increase in Hct and the effects of catecholamines on red cell pH and the subsequent distribution of CO_2 between the red cells and the plasma. The changes in both red cell and plasma pH during sustained exercise in this study were small in comparison with those of Currie and Tufts (1993). Although, the proportion of CO_2 transported within the red cells did not change, the absolute total CO_2 levels increased significantly during exercise (Table 3.3). This resulted in a constant proportion of total CO_2 excreted due to HCO_3^- which resided within the red cell prior to gill entry and that dependent upon HCO_3^-/Cl^- exchange (Table 3.5).

Interaction between O_2 and CO_2 exchange during exercise:

Catalyzed HCO_3^- dehydration must occur within the red cell during blood transit through the gills. Bicarbonate dehydration consumes a proton and thus CO_2 excretion will be dependent upon both HCO_3^- and proton availability within the red cell. The half time for proton flux across the red cells of eel at 24 °C is about 10 s, considerably slower than the 0.5 to 2.5 s transit time of red cells through the gills of fish (Cameron

and Polhemus, 1974). Protons can be supplied within the red cell either by the Hb buffer capacity or via the release of Bohr protons during Hb oxygenation. The Hb of rainbow trout possess a low buffer capacity but a large Haldane effect relative to air breathing vertebrates (Jensen, 1989) and therefore, CO_2 excretion at the gills in trout is dependent upon the protons released by Hb oxygenation, and therefore, oxygen uptake.

The modified respiratory exchange ratio (RE') relates the quantity of HCO_3^- dehydrated to CO_2 relative to the amount of oxygen which bound to Hb during gill blood transit. A mean value of 0.78 was derived for all swimming speeds combined (Table 3.5). This value is a ratio of proton consuming and potential proton donating reactions. That is, if 0.78 protons were released per mole of oxygen bound to Hb over the entire range of arterial-venous Hb oxygen saturations used *in vivo*, there would be no net arterial-venous difference in blood pH during gas exchange. It is apparent; however, that this was not the case (Fig. 3.3) and there were arterial-venous differences in blood pH.

The changes in arterial-venous difference in blood oxygen content ($C_{a-v}O_2$) during exercise were predominantly due to changes in venous O_2 content because at all swimming velocities there were no significant changes in C_aO_2 (Table 3.2). In addition, there were no statistically significant differences in red cell pH between arterial and venous blood (Table 3.2) and consequently, changes in blood pH during gill transit reflect net proton difference between production, due to Hb oxygenation, and removal, due to HCO_3^- dehydration. When the arterial-venous difference in oxygen content was low, corresponding to a low swimming velocity, there was a net acidosis during gill blood transit, indicating that more protons were released during oxygenation of the Hb

than were consumed during HCO_3^- dehydration. With an increase in $C_{a-v}O_2$ due to increased exercise intensity the opposite was observed. Given that RE did not change significantly during exercise, these data indicate a non-linear release of Bohr protons over the Hb-O_2 equilibrium curve *in vivo*. This is consistent with those data obtained *in vitro* (chapter 2).

In order to interpret the data more quantitatively a Haldane coefficient was derived from the *in vivo* arterial-venous differences in plasma and red cell [HCO_3^-], pH, O_2 content and Po_2 taking into account [Hb], Hct, and non-bicarbonate buffering capacity of the blood (see Appendix, equation 4). The analysis reveals that the derived Haldane coefficient was significantly lower in fish swimming at Ucrit, where large regions of the Hb-O_2 equilibrium curve were utilized, than in fish swimming at the lowest velocity (Table 3.3). Thus, the release of Bohr protons is non-linear over the region of the Hb-O_2 equilibrium curve used *in vivo*, consistent with the data obtained *in vitro* (chapter 2), and the majority of the Haldane effect may be exploited under routine conditions.

The calculation used to derive the Haldane coefficient is based upon a number of assumptions, some of which are described here and others in the Appendix (equation 4). The first assumption is that there was no H^+ excretion or HCO_3^- uptake across the gills, independent of CO_2 excretion during exercise. From figure 1, it is apparent that during exercise there was a net H^+extrusion or HCO_3^- uptake which could have occurred at the level of the kidney or the gills. If this compensation occurred at the gills, the result would be an underestimation of the Haldane coefficient; however, the magnitude of this error would be minor.

In addition, it was assumed that there was no influence of Hb oxygenation status on whole blood buffer capacity. Although oxygenation status influences the buffer capacity of whole blood *in vitro* (Albers *et. al.*, 1983), the difference in buffer capacity between oxygenated and deoxygenated blood in rainbow trout is small (Eddy, 1974). The magnitude to which the estimate of the Haldane coefficients in this study were influenced by violation of these assumptions is probably minor.

Significance of disproportionate release of Bohr protons to CO_2 transport and excretion during exercise:

When blood first enters the gills any dissolved CO_2 in the blood will rapidly diffuse out into the ventilatory water. At the same time O_2 will diffuse into the red cell and bind to Hb. The rapid reduction in dissolved CO_2 in the blood will create conditions for HCO_3^- dehydration in the red cell, depleting both HCO_3^- and protons. The rate of CO_2 excretion will then be determined by the rate at which HCO_3^- and protons can be replenished (Perry and Gilmour, 1993). HCO_3^- enters the red cell in exchange for chloride by the HCO_3^-/Cl^- exchanger in the band 3 protein on the red cell membrane. This is thought to be the rate limiting step in CO_2 excretion (Cameron and Polhemus, 1974; Perry *et. al.*, 1982; Perry, 1986; Jensen and Brahm, 1995).

The HCO_3^- gradient across the red cell is determined by the difference in pH between the plasma and the red cell. Consequently, HCO_3^- flux across the red cell, and therefore CO_2 excretion, will be influenced by the release of Bohr protons during Hb

oxygenation (Figure 1). Rapid oxygenation of Hb *in vitro* elevates CO_2 excretion rates over the region of the Hb-O_2 equilibrium curve that Bohr protons are released (Chapter 2). In addition, a relationship exists between the oxygenation induced boost in CO_2 excretion rate, measured *in vitro*, and the magnitude of the Haldane effect in four species of fishes (Perry *et. al.*, 1995).

In exercising fish, the disproportionate release of Bohr protons during Hb oxygenation will influence CO_2 excretion across the gills depending upon the region of the Hb-O_2 equilibrium curve used for gas exchange. At low swimming velocities, C_vO_2 was high resulting in a relatively large Bohr proton release relative to O_2 bound to Hb. These conditions favoured HCO_3^- flux across the red cell and CO_2 excretion and the ratio between Bohr protons released during Hb oxygenation and CO_2 excreted across the gills was greater than 100% (Table 3.5), indicating that more protons were released during oxygenation of the blood than CO_2 was excreted. Thus, stoichiometrically all CO_2 excretion was linked with O_2 uptake.

As swimming velocity was increased, C_vO_2 was reduced and relatively fewer Bohr protons were released during oxygenation of the blood. Thus HCO_3^- dehydration during CO_2 excretion at the gills resulted in titration of protons from the weakly buffered Hb within the red cell which elevated red cell pH which reduced the pH and HCO_3^- gradient across the red cell, affecting CO_2 excretion accordingly. This continued until the Hb reached approximately 50% saturation, following which Hb oxygenation was associated with Bohr proton release. At Ucrit, the ratio between Bohr proton release and Hb oxygenation was reduced to 46%. Thus, stoichiometrically, less than 50% of CO_2

excretion was linked with O_2 uptake. This likely contributed to the increase in total CO_2 levels in the blood during moderate and maximal levels of sustained exercise, altering the pattern of CO_2 excretion at the gills.

At higher swimming velocities, there appeared to be a diffusion limitation to O_2 uptake as indicated by the low arterial Po_2. Any limitation to O_2 uptake will influence CO_2 excretion by reducing the total release of Bohr protons during gill blood transit. This may partly explain the relationship obtained between Po_2 and CO_2 levels of arterial blood over a range of exercise intensities in Series I (Fig. 3.4). However, the removal of blood flow through one gill arch must also have contributed to the relationship because the changes in CO_2 levels during exercise were not as great in Series II fish as they were in Series I fish.

The release of Bohr protons during Hb oxygenation is reversible. Therefore, the disproportionate binding of Bohr protons during oxygen delivery to the tissues will influence CO_2 movement from the tissues. During exercise, as S_vo_2 dropped below 50%, the "effective" buffering capacity of the blood was reduced. At constant tissue respiratory quotient (CO_2 production/O_2 consumption), P_vco_2 would be expected to increase as S_vo_2 dropped below 50%. This was observed in exercising fish (Table 3.3). It should be noted that Pco_2 was measured in mixed venous blood not in blood leaving the muscle. Assuming that mixed-venous blood is indicative of that leaving the muscle, an increase in venous Pco_2 reflects an increase in total CO_2 content of the muscle during sustained aerobic exercise. An elevation in blood and muscle total CO_2 levels may be important in elevating buffer capacity in preparation for a metabolic acidosis associated

Influence of CO_2 transport on O_2 delivery during exercise:

In addition to implications for CO_2 transport, the non-linear Haldane effect will also influence conditions for O_2 transport. In the tissues, metabolically produced CO_2 acidifies the blood during capillary transit. This acidosis induces a rightward shift of the Hb-O_2 equilibrium curve (Bohr effect) enhancing oxygen delivery to the tissues at a given blood Po_2. The Haldane effect acts to reduce the arterial-venous change in Pco_2 and pH due to proton binding during Hb deoxygenation, reducing the extent of the Bohr shift. Thus, it has long been argued that a large Bohr effect can not be exploited in the tissues due to the associated large Haldane effect which minimizes any arterial-venous pH changes. This arguement is based upon the theory of linked functions where, thermodynamically, the Bohr and Haldane coefficients are equivalent (Wyman, 1973).

The theory of linked functions is dependent upon a number of assumptions (Wyman, 1973) which are violated in the Hbs of many fish species. One assumption is that the shape of the Hb-O_2 equilibrium curve is independent of pH. Although this is true for many Hbs, it is not true for fish Hbs which possess a Root effect. In fact, one of the defining characteristics of a Root shift is the low cooperativity in oxygen binding to Hb (n) at low relative to high pH (Brittain, 1987; Riggs, 1988). A second assumption is that there is a linear relationship between oxygen binding and proton release. While this assumption is not strongly violated in mammalian Hbs (Baumann *et. al.*, 1987), in the

Hbs of trout (Chapter 2, Table 3.3) and tench (Jensen, 1986) the majority of Bohr protons are released between 50 and 100% So_2.

A Hb which possesses a large Root and Bohr effect coupled with a disproportionate release of Bohr protons may permit the Bohr/Haldane effect to be optimized for both O_2 and CO_2 transport, depending upon the region of the Hb-O_2 equilibrium curve used for gas transport. Under routine conditions (S_vo_2 50% or greater), the binding of Bohr protons during capillary blood transit resulted in venous blood being alkaline relative to arterial blood (Table 3.2). Under these conditions, a reverse Bohr and Root shift would occur, reducing tissue Po_2. Although, seemingly maladaptive for O_2 delivery, a reduction in Po_2 due to an alkalosis during capillary blood transit would only occur under conditions when the animal was at rest. During exercise, the reduction in S_vo_2 and reduced uptake of Bohr protons during oxygen delivery resulted in an acidification of venous relative to arterial blood (Table 3.2). The acidification of blood during capillary transit will elevate blood Po_2 via the Root and Bohr effect enhancing oxygen delivery to the tissues when metabolism is elevated. Thus, the characteristics of the Root effect may permit an elevation in blood Po_2 when S_vo_2 drops to low levels; however clearly more work is needed to clarify this point.

In conclusion, the suite of physiological adjustments during exercise were sufficient to maintain O_2 uptake despite a reduction in P_ao_2 with an increase in swimming velocity. The release of Bohr protons was greatest in the upper reaches of the Hb-O_2 equilibrium curve consistent with the *in vitro* data presented in Chapter 2. In fact, in slowly swimming fish, all CO_2 excretion was stoichiometrically linked with O_2 uptake

via the Haldane effect. At maximal exercise; however, less than 50% of CO_2 excretion was linked with O_2 uptake. The release of Bohr protons facilitated CO_2 excretion at the gills and the binding of protons promoted CO_2 hydration at the tissues. The non-linear release of Bohr protons influenced CO_2 transport and altered the pattern of CO_2 excretion. CO_2 levels in arterial blood increased during exercise and the proportion of total CO_2 excreted which could be attributed to the movement of $Pbco_2$ increased from almost zero in slowly swimming fish to 11% during exercise. The increased CO_2 levels in the blood were distributed equally between the red cells and the plasma and therefore, the role of HCO_3^-/Cl^- exchange to total CO_2 excretion did not change significantly during exercise, and comprised 62% of all CO_2 excreted.

SUMMARY

1) Pco_2 and Cco_2 levels increased in arterial and mixed venous blood of trout during exercise. 62% of total CO_2 excreted was due to HCO_3^-/Cl^- exchange, which did not change significantly during exercise. However, the role of $Pbco_2$ to total CO_2 excretion increased from almost zero to 11% at the maximum swimming speed.

2) Most of the Bohr protons were released in the upper region of the Hb-O_2 equilibrium curve. The Haldane coefficient, calculated from *in vivo* data, was greatest (0.99 ± 0.17) in slowly swimming fish where all CO_2 excreted was stoichiometrically linked with O_2 uptake. As swimming velocity (and therefore $C_{a-v}O_2$) increased, the Haldane coefficient decreased reaching a minimum of 0.42 ± 0.13, at Ucrit. At this swimming speed less than 50% of CO_2 removal was linked with O_2 uptake.

3) The non-linear release of Bohr protons affects CO_2 transport and alters the pattern of CO_2 excretion at the gills.

CHAPTER 4: Air-breathing in *Arapaima gigas*: uncoupled movements of O_2 and CO_2

PREFACE

This chapter is adapted from a paper published by Brauner, C.J. and Val, A.L., 1995. Proceedings of an International symposium on *The Physiology and Biochemistry of Fishes of the Amazon*, held in Manaus, Amazonas, Brazil, Aug. 30- Sept. 2, 1994. (In Press). A.L. Val provided laboratory facilities, technical support, and critical review of the manuscript.

INTRODUCTION

In most animals, the movements of O_2 and CO_2 at the respiratory exchange surface are coupled. This is especially the case in many teleost fishes, where the Hb's are characterized by large Root and Haldane effects and a low buffer capacity (Jensen, 1989). For example, oxygenation of trout blood in the absence of CO_2 removal may reduce oxygen carrying capacity of the blood by as much as 48% (Table 1). In addition, CO_2 excretion rate in the absence of Hb oxygenation was reduced by about 25% *in vitro* (Chapter 2) and in slowly swimming fish virtually all CO_2 excreted *in vivo* is stoichiometrically related to O_2 uptake through the Haldane effect (Chapter 3). Thus, CO_2 excretion may be reduced in the absence of O_2 uptake and O_2 uptake may be reduced in the absence of CO_2 excretion.

In most fish, O_2 uptake and CO_2 excretion occurs predominantly at the gills (there is some gas exchange across the cutaneous surface) and the movement of O_2 and CO_2 are both spatially and functionally coupled. This, however, is not the case in many bimodal

breathers, where gas exchange can occur across two or more respiratory organs (Johansen, 1970). This is the case in *A. gigas*, an obligate air breathing teleost fish from the Amazon which possesses both gills and a highly vascularized swim bladder which acts as an air breathing organ. The majority of O_2 uptake (78%) occurs from the air bladder in the absence of CO_2 removal and the majority of CO_2 excretion (63%) occurs across the gills in the absence of oxygen uptake (Randall *et al.* 1978). If the Hb in *A. gigas* possesses a large Haldane and Bohr effect and low buffer capacity, as do the Hb's of many teleost fishes (Jensen, 1989), this mode of gas exchange would be hypothesized to compromise both O_2 uptake and CO_2 excretion.

The main objectives of this study were: 1) to confirm the observations made by Randall *et al.* (1978) with respect to the partitioning of O_2 and CO_2 exchange between water and air in *A. gigas*, and 2) to determine whether the Hb characteristics of *A. gigas* differ from trout with respect to the magnitude of the Root effect, Haldane effect and buffer capacity.

MATERIALS AND METHODS

Experimental animals:

Arapaima gigas (1.7 ± 0.37 kg) were purchased from an aquaculture facility in Itacoartiara, 200 km East of Manaus, Amazonas, Brazil. Fish were fed live fish and held for at least 2 weeks in the aquaculture department at the Instituto Nacional de Pesquisas da Amazonia (INPA) until experiments were performed.

Surgery and Handling:

Arapaima gigas were anaesthetized by immersing the fish in a 1:1000 solution of MS-222 in water, buffered with $NaHCO_3^-$. *Arapaima gigas* is an obligate air breather and dies within 10 minutes when denied access to air. With this in mind, immediately after the fish lost equilibrium a piece of polyethylene tubing (PE 190) was inserted through the pneumatic duct, accessing the air breathing organ, and permanently sutured in place as described by Farrell (1978) and Farrell and Randall (1978). Throughout surgery, the volume of air in the air bladder was replaced every 5 min. The gills were not ventilated during surgery, but the body and gills were kept moist at all times. The first afferent branchial artery was cannulated with PE-50 and tied off to secure the tubing. The urinary papilla was catheterized with PE-90 as described by Wood and Randall (1973). Following surgery, the fish was placed in a 25 l holding tank or in a respirometer and the air bladder was repeatedly ventilated until the fish recovered and could access the air unassisted. The tanks and respirometer were supplied with 60 l of

recirculated, aerated water (26 °C, pH= 6.9 ± 0.28, Pco_2=1.6 ± 0.3 mmHg) replenished periodically throughout the day. Each fish was permitted to recover from surgery for 24-48 h prior to experimentation.

In vivo experiments:

Respirometry:

The respirometer contained a water volume of 5.5 l and an air chamber of 1.3 l. To measure the rate of gas exchange in water and air, the respirometer was sealed and a pump slowly circulated the water throughout the system. Po_2 of water and air and changes in CO_2 content of water and Pco_2 of air were measured every 5 minutes for up to 30 minutes to calculate total O_2 uptake and CO_2 excretion by *A. gigas*. Background changes in O_2 and CO_2 were taken into account but were generally minor. The air and water chambers of the respirometer were in contact with one another and some diffusion between the compartments undoubtedly occurred. Preliminary experiments revealed that the influence on the partitioning of gas exchange between water and air was minor, consistent with the findings of Stevens and Holeton (1978) using a similar experimental set up.

During respirometry, urine was continuously collected in covered vials to determine urine flow rate. Samples were also collected anaerobically for CO_2 content and pH measurements. In addition, mixed venous blood was removed for measurement of Hct, pH_e and Cco_2.

In vitro experiments:

Blood from the caudal vein of *A.gigas* was taken from fish which were not used in respirometry studies. Red blood cells were separated from plasma by centrifugation at 5900g for 10 min at 4 °C in a Sorvall Instruments RC5C refrigerated centrifuge. The red cells were washed twice with cold 0.9% NaCl before they were lysed by addition of weakly buffered (5 mM Na-Hepes, pH 7.8) distilled water and repeatedly frozen and thawed (Jensen, 1989). The red cell debris was removed by refrigerated centrifugation and the haemolysates were repeatedly dialysed against distilled water at 4 °C. The haemolysates were repeatedly subjected to ion exchange resins and measurements indicated complete removal of Cl^- and organic phosphates. The stripped haemolysates were used immediately in the measurement of the Root effect or diluted to a Hb concentration of 0.3 to 0.5 mM in 0.1 M KCl and kept frozen at -24 °C for a maximum of 2 days.

Analytical Techniques:

Respirometry:

The Po_2 of water and air were measured from samples collected in gas tight syringes using a Radiometer Po_2 (E-5046) electrode. The Pco_2 in air was measured with a Radiometer Pco_2 (E-5036) electrode. The Po_2 and Pco_2 electrodes were maintained in a Radiometer BMS3 Mk2 blood microsystem, and the output simultaneously displayed on a Radiometer PHM 73 acid-base analyzer. The Po_2 electrode was calibrated with air saturated water, and the Pco_2 electrode was calibrated with appropriate CO_2 mixtures.

The calibration of these electrodes were checked routinely throughout the day. The CO_2 content of water, blood and urine was measured using gas chromatography as described in the general materials and methods. The Po_2 of water was converted to oxygen content by using the solubility coefficient reported in Boutilier *et. al.* (1984). A best fit linear regression was used to calculate the change in respective gas content over the duration of respirometry. Oxygen consumption and CO_2 excretion rates were calculated from the rate of change in gas content, the volume of the respective chambers (accounting for the volume of water displaced by the fish) and the fish mass.

Hb Characteristics:

The stripped haemolysates were used immediately after they were prepared to measure the magnitude of the Root effect which was measured spectrophotometrically according to Pelster and Weber (1990). Briefly, haemolysates from one fish were suspended in a pH 5.5 or pH 8.0 buffer at equal Hb concentrations. The haemolysates in pH 8 buffer was bubbled with 100% O_2 and the absorbance was read at 541, 555 and 577 nm. The absorbance of deoxygenated blood was measured at each wave length following the addition of a pinch of dithionite. This procedure was repeated for the haemolysates in pH 5.5 buffer. The percent reduction in saturation of the Hb due to the Root effect was calculated as:

$$[1- (\Delta OD_{pH\ 5.5\ (oxy\text{-}deoxy)} / \Delta OD_{pH\ 8.0\ (oxy\text{-}deoxy)})] \times 100$$

where $\Delta OD_{pH\ 5.5\ (oxy\text{-}deoxy)}$ and $\Delta OD_{pH\ 8.0\ (oxy\text{-}deoxy)}$ refer to the difference in optical density between oxygenated and deoxygenated blood at pH 5.5 and pH 8.0, respectively. This calculation was performed at each of the three wavelengths and were averaged for each blood sample.

Haemoglobin titration curves were conducted on oxygenated and deoxygenated haemolysates as described by Jensen (1989). Briefly, haemolysates from one fish were divided into four, 3 ml aliquots. Each aliquot was placed in a tonometer maintained at 27°C. Two tonometers were incubated with humidified O_2 and two with humidified N_2 for 1 h while the solutions were continually mixed. Following incubation, the pH was measured (zero net proton charge) and one pair of oxygenated and deoxygenated haemolysates were titrated with NaOH, the other with HCl. Throughout the entire titration, all tonometers were continually supplied with gas and the solutions were mixed. Freshly prepared and analyzed 0.1 M NaOH and HCl were injected by Hamilton syringe in 10 μl aliquots into each tonometer and the pH was recorded after 20 min. This procedure was repeated to obtain titrations over the pH range between 5 and 9. Haemolysate pH was measured with pH Microelectrodes in conjunction with Radiometer PHM 64 pH meters. Haemoglobin concentrations were determined spectrophotometrically. Six separate titration curves were conducted for blood from different fish, all yielding similar results.

RESULTS

Partitioning of gas exchange in A. gigas:

In *A. gigas*, 78.1% of the O_2 consumed was from the air (via the ABO) and 85.3% of the CO_2 was excreted into the water (Table 4.1). About 79% of the CO_2 excreted into the water was assumed to have diffused across the gills while the remaining 6.3% of the total CO_2 excreted entered the water via the kidney. About 37% of the movements of O_2 and CO_2 occurred simultaneously across the same respiratory surface (Table 4.1) while the remaining 63% occurred at different locations.

During these measurements, oxygen consumption rate was 81 mg kg^{-1} h^{-1} and the combined respiratory exchange ratio in air and water was 0.98 (Table 4.2). The Pco_2 of mixed venous blood was 25 mmHg and plasma HCO_3^- concentration was 31.8 mM (Table 4.2).

Haemoglobin characteristics:

The Hb in *A. gigas* possessed a large Root effect. Stripped haemolysates in a pH 5.5 buffer exhibited a 44.4 $\pm$ 0.95% reduction in oxygen saturation relative to haemolysates in a pH 8 buffer (Table 4.3). The haemoglobin in *A. gigas* possessed a small Haldane effect as indicated by the vertical distance between the haemoglobin titration curves of the oxygenated and deoxygenated stripped haemolysates (Fig. 4.1).

TABLE 4.1: Partitioning of O_2 uptake and CO_2 excretion between the air-breathing organ (A.B.O.), gills and kidney in *Arapaima gigas*.

	A.B.O.	GILLS	KIDNEY	TOTAL
O_2 UPTAKE	78.1 ±1.0%	21.9 ± 1.0%	----------	100%
CO_2 EXCRETION	14.7 ± 2.1%	79.0 ± 2.1%	6.3 ± 1.3%	100%
SPATIALLY COUPLED	14.7%	21.9%	----------	36.6%

Spatially coupled refers to the maximum proportion of gas exchanged involving simultaneous exchange of O_2 and CO_2 across the respective respiratory surface. (n=5).

TABLE 4.2: Metabolic rate and measurements in mixed venous blood and urine in *A. gigas* during respirometry.

Mo_2 (mg · kg · h^{-1})	Total RE	Blood pH_e	pH_i	Pco_2 (mmHg)	$[HCO_3^-]_e$ (mM)	Hct	Urine Flow (ml · kg · h^{-1})	Urine pH	Urine Cco_2 (mM)
81.0 (5.2)	0.98 (0.03)	7.64 (0.03)	7.21 (0.01)	25.0 (1.8)	31.8 (1.0)	21.6 (2.1)	5.8 (0.8)	7.75 (0.07)	28.7 (2.8)

All blood parameters were measured 1 min following an air-breath in mixed venous blood from the afferent branchial artery. The remaining parameters were measured over the entire duration of respirometry. Total RE refers to the respiratory exchange ratio calculated from gas exchange in both water and air. n=5 and values in brackets are s.e.m. of the mean.

TABLE 4.3: Haemoglobin characteristics of *Arapaima gigas* and *Oncorhynchus mykiss*

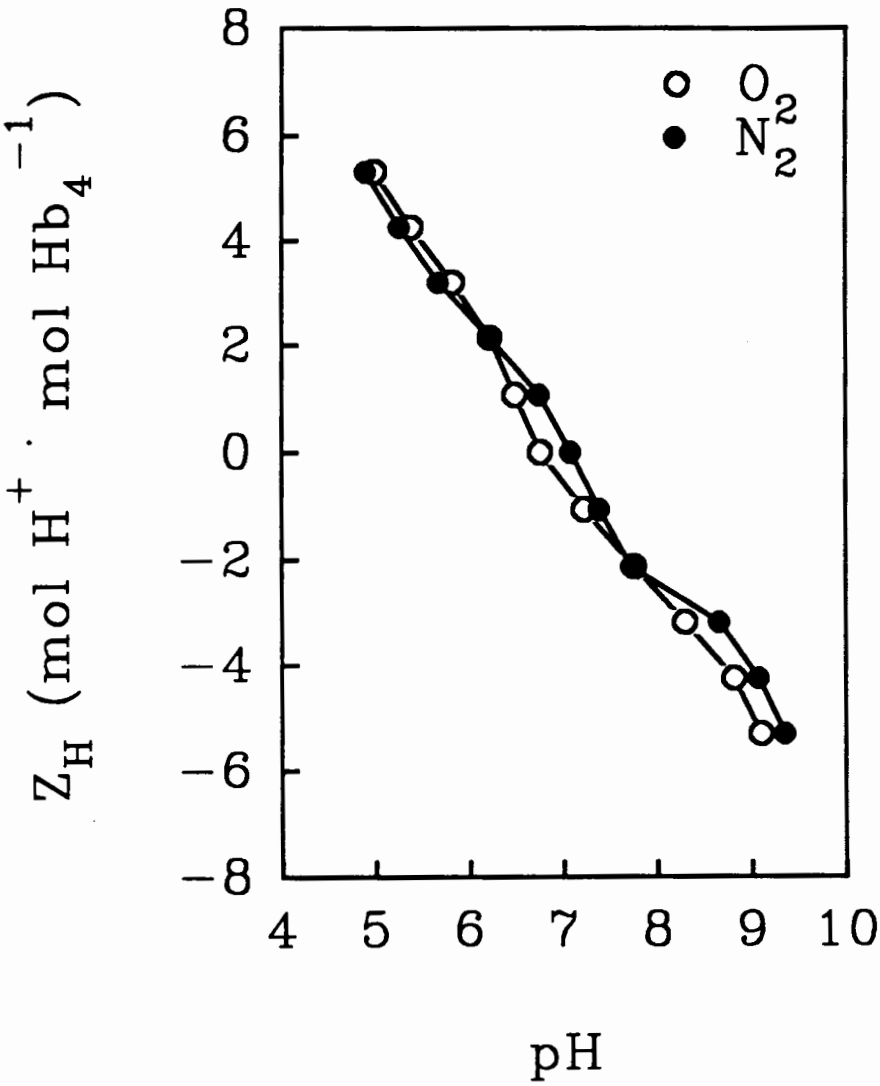
	A. gigas	*O. mykiss*
$\Delta Z_H max$	0.84	2.6
BUFFER CAPACITY	3.0/2.0	7.1/6.2
ROOT EFFECT	44.4 $\pm$ 0.95%	

$\Delta Z_H max$ refers to the maximum number of protons released per mole of Hb oxygenated at pH = 7.0 [calculated from figure 4.1 for *A. gigas* and Jensen, (1989) for *O. mykiss*]. See Fig. 4.1 legend for further details.

Buffer capacity ($-dZ_H/dpH$) was calculated from figure 4.1 for *A. gigas* and Jensen, 1989 for *O. mykiss* in deoxygenated/oxygenated haemolysates respectively, at a pH of 7.0.

Root effect refers to % reduction in oxygen saturation of stripped haemolysates in a pH 5.5 buffer relative to haemolysates in a pH 8 buffer.

FIGURE 4.1: H^+ titration curves, Z_H (net H^+ charge, mol H^+ mol Hb^{-1}) as a function of pH in oxygenated (open circles) and deoxygenated (closed circles) isolated stripped haemoglobins from *Arapaima gigas*, an obligate air breather. Temperature = 26 °C, Ionic strength = 0.1 M KCl, Tetrameric Hb concentrations = 0.3-0.5 mM. The vertical distance (ΔZ_H) between the titration curves for oxygenated and deoxygenated haemoglobin indicates the protons released from haemoglobin upon oxygenation at constant pH (Haldane effect) while the slope of the curve ($-dZ_H/dpH$) indicates the buffer capacity of the haemoglobin in the oxygenated or deoxygenated state. This is a representative trace of one of six titrations conducted.


8
6
4
2
0
−2
−4
−6
−8
4
5
6
7
8
9
10
O2
N2
Z_H (mol H^+ · mol Hb_4^{-1})
pH

The maximum number of protons released per mole of haemoglobin upon oxygenation (ΔZ_H) was 0.84 and occurred at pH 7.0.

DISCUSSION

78% of the O_2 uptake occurred across the air breathing organ and 86% of the CO_2 was excreted into the water, resulting in only 37% of O_2 and CO_2 exchange occurring across the same respiratory structure. This represents a slightly greater spatial uncoupling of O_2 and CO_2 movements than that determined by Randall *et al.* (1978). The Hb of *A.gigas* possess a large Root effect but a low buffer capacity and small Haldane effect. This is in contrast with rainbow trout which also possess a large Root effect and a low buffer capacity, but exhibit a large Haldane effect.

Haemoglobin characteristics with respect to O_2 uptake and transport:

The Root effect in the haemolysates of *A. gigas* was large, resulting in a 44% reduction in oxygen carrying capacity of the blood at a pH of 5.5. The magnitude of the Root effect is less than that in the stripped haemolysates of rainbow trout which exhibits a 60% reduction at pH 6.2 (Pelster and Weber, 1990). The magnitude of the Root effect was not investigated in the presence of organic phosphates, which have been demonstrated to increase both the magnitude of the Root effect, and the pH at which the maximum effect is observed (Pelster and Weber, 1990). The red cells in *A. gigas* possess inositol pentaphosphate (IP5; Isaacs *et. al.,* 1977; Val *et. al.,* 1992) which is a stronger modulator of haemoglobin-oxygen affinity than ATP and GTP which are the cofactors most commonly found in the red cells of fishes (Wood and Johansen, 1972; Lykkeboe

and Weber, 1978). Thus, the Root effect may be large within the physiological pH range in *A. gigas* and has been demonstrated to exist in whole blood at Pco_2 values measured *in vivo* (Randall *et al.* 1978).

The Hb in *A. gigas* possessed a small Haldane effect (Fig. 4.1) In the haemolysates of both *A. gigas* and *Oncorhynchus mykiss,* the maximum ΔZ_H occurred at pH 7.0 but was less than one third the magnitude in *A. gigas* (Table 4.3). The small Haldane effect in *A. gigas* relative to rainbow trout, minimizes pH changes associated with the release of Bohr protons during Hb oxygenation. Thus, despite the presence of a Root effect (Table 4.3) coupled with a gas exchange strategy where O_2 uptake from the air breathing organ occurs predominantly in the absence of CO_2 removal (Table 4.1), oxygenation of the haemoglobin in the air breathing organ will not be compromised.

Haemoglobin characteristics with respect to CO_2 excretion:

As pointed out by Jensen (1989), vertebrates generally possess haemoglobins with either a large buffering capacity and low Haldane effect (ie. pig and dogfish), or a low buffering capacity and large Haldane effect (ie. carp and trout) *A. gigas* differs from this general pattern because it possessed Hb with a small Haldane effect and a low buffer capacity (Fig. 4.1). In fact the Hb buffer capacity at fixed oxygenation status was lower than that reported in the stripped haemolysates in trout (Table 4.3). The rate of proton flux across the red cells (Forster and Steen, 1969) is very slow relative to the blood transit time through the gills. Thus, the possession of a Hb with a low Haldane effect

and low buffer capacity is seemingly maladaptive for CO_2 excretion. This is because haemoglobin cannot act as an efficient "store" for protons during HCO_3^- dehydration in the red cell when blood enters the gills. This may partly explain the high blood Pco_2 and total CO_2 levels measured in *A. gigas* in this study and by that of Randall *et al.* (1978).

When blood first enters the gills, any dissolved CO_2 in pre-branchial blood will rapidly diffuse from the blood to the water, creating conditions for HCO_3^- entry into the red cell and subsequent dehydration to CO_2. In *A. gigas* exposed to normocapnic water (Pco_2 approximately 1 mmHg), venous blood Pco_2 was 25 mmHg, much greater than that in trout. A pre-branchial blood Pco_2 of this magnitude may greatly reduce the dependence on HCO_3^- dehydration to total CO_2 excretion during blood transit through the gills due to the large Pco_2 gradient between blood and water. This may facilitate CO_2 excretion in a fish possessing haemoglobin with a low buffer capacity and Haldane effect. The Haldane effect is not completely absent in *A. gigas* and undoubtedly plays a role in the 36.6% of CO_2 excretion which occurs in the presence of O_2 uptake (Table 4.1). It is also possible that the Bohr protons released from haemoglobin during oxygen uptake in the air breathing organ can be utilized for CO_2 excretion in the gills; however, this is only possible if the blood transit time from the air breathing organ to the gills is rapid relative to the rate of proton flux across the red cell, which is not known.

In conclusion, the Hb characteristics of *A. gigas* were different from that in rainbow trout. Despite the presence of a Root effect, the Hb of *A. gigas* possessed a small Haldane effect and therefore oxygen uptake across the air breathing organ was not impaired in the absence of CO_2 removal. In contrast with the typical scenario in

vertebrates where Hb's possess either a small Haldane effect and high buffer capacity or vice versa (Jensen, 1989), the Hb of *A. gigas* possessed both a low Haldane effect and buffer capacity, seemingly maladaptive for CO_2 removal.

SUMMARY

1) Gas exchange in *A. gigas* is spatially uncoupled. 78% of the oxygen uptake occurs across the swim bladder, while 86% of the CO_2 is excreted into the water, 79% across the gills and 6% through the kidney.

2) The Hb of *A. gigas* possess a large Root effect but unlike rainbow trout, they possess a small Haldane effect and low Hb buffer capacity which may permit uncoupled movements of O_2 and CO_2.

GENERAL DISCUSSION

Haemoglobin is an intriguing molecule which is designed to optimize the transport and exchange of O_2 and CO_2, from the level of the tissues to the level of the gas exchange organ, in almost all vertebrates. A remarkable diversity in functional characteristics of Hb is found throughout the animal kingdom. Interestingly, nearly the entire spectrum of these characteristics is found in fish. In some fish, HbO_2 affinity is high while in others it is very low. In some fish, the Bohr and Haldane effects are large while in others they are virtually non-existent. Fish also represent the only vertebrates which have developed an O_2 multiplication system capable of generating O_2 tensions over 20 times that found in arterial blood (Fairbanks *et. al.*, 1969). This is achieved with a Hb which exhibits a large reduction in HbO_2 affinity as pH of the blood is lowered (Root effect), coupled with a structure (rete) capable of creating a large localized acidosis. The acidosis drives O_2 from the Hb to the respective structure, either the retina or swim bladder. In addition to the great diversity of Hb characteristics which exist between fish species, the degree of Hb heterogeneity found within individual animals is unsurpassed by any other vertebrate group. In a survey of teleost fishes from the Amazon, of 77 genera examined only 8% possessed only one Hb component, while the mean value was 4 Hb components per species (Fyhn *et. al.*, 1979). The Hb characteristics often differ greatly from one component to another and it has been hypothesized that the possession of multiple Hbs permits a "division of labour" between the individual Hb components (Weber, 1990). At the other extreme there are fish which do not possess Hbs whatsoever (Holeton, 1970; Acierno *et. al.*, 1995).

In addition to the great diversity in functional characteristics of Hb, the Hb in fish is modulated by different organic phosphates from those in other vertebrates. In contrast to most other vertebrates, the Hb in most fishes does not directly bind CO_2 (carbamate) and the gas exchanger lacks plasma accessible CA. All these characteristics together bring to light the great potential which exists for the study of gas exchange in fish.

Many teleost fishes possess haemoglobins which exhibit large Root and Haldane effects and a low buffer capacity (Jensen, 1989). This thesis has examined the hypothesis that the combination of these characteristics gives rise to an extensive interaction between the movements of O_2 and CO_2, without which gas exchange would be compromised. It has been shown that in trout the magnitude of the Root effect could impair oxygen transport in the blood. Due to the presence of a large Root effect, an accumulation of protons released during Hb oxygenation at the gills could acidify the red cell and reduce oxygen carrying capacity of the blood by up to 49%. This does not happen in trout because protons are removed by HCO_3^- dehydration, illustrating the importance of CO_2 removal at the gills to O_2 uptake in trout.

Arapaima gigas is an obligate air breathing teleost fish from the Amazon. It possesses two respiratory surfaces for gas exchange: gills and a highly vascularized swimbladder which acts as an air-breathing organ (ABO). The movements of O_2 and CO_2 are spatially uncoupled in normoxia: 78% of the O_2 consumed was from the air and 85% of the CO_2 excreted was into the water. Therefore, a large proportion of the oxygen uptake across the ABO occurred in the absence of CO_2 removal. The Hb in this species possessed a large Root effect and therefore, an acidosis induced by Hb oxygenation in

the absence of CO_2 removal, could impair O_2 uptake. The Haldane effect in this Hb, however, was small preventing an acidosis during Hb oxygenation. Interestingly, the Hb buffer capacity was also low relative to that in rainbow trout, seemingly maladaptive for CO_2 excretion. Thus, Hb characteristics appear to be modified to prevent impairment of O_2 uptake in the absence of CO_2 removal in *A. gigas*; however, the effect of these changes on CO_2 excretion is less clear (Chapter 4).

The lack of plasma accessible CA in the gills in the teleost fishes examined to date (Perry *et. al.*,1982; Henry *et. al.*., 1988; Perry and Laurent, 1990) and the reduced dependence upon carbamate for CO_2 transport and excretion (Farmer, 1979; Heming, 1984) elevates the relative importance of HCO_3^-/Cl^- exchange during CO_2 excretion in teleost fish, relative to that in other vertebrates. In resting and exercising trout, about 60% of the total CO_2 excreted was dependent upon HCO_3^-/Cl^- exchange (Chapters 1 and 3). In resting humans, about 53% of the CO_2 excreted is due to changes in the plasma HCO_3^- pool (Klocke, 1987); however, this includes HCO_3^- dehydration in the plasma due to the presence of plasma accessible CA which may account for 7% of total CO_2 excreted (Crandall and Bidani, 1981). The limitation to CO_2 excretion in vertebrates is thought to lie at the level of HCO_3^-/Cl^- exchange (Perry *et. al.*, 1982; Weith *et. al.*,1982; Perry, 1986; Klocke, 1987) thus any conditions which influence the rate of HCO_3^-/Cl^- exchange will influence the rate of CO_2 excretion. In blood which exhibits a low Hb buffer capacity but a large Haldane effect, Hb oxygenation will increase the pH gradient, and therefore the HCO_3^- gradient across the red cell. Rapid oxygenation of trout blood in vitro, elevated HCO_3^- flux through the red cell by 30% (Chapter 2). The release of Bohr

protons and the associated increase in HCO_3^- flux through the red cell; however, only occurred between 60 and 100% of Hb So_2 (Chapter 2). In fish subjected to different levels of sustained exercise, the magnitude of the Haldane effect, calculated from *in vivo* arterial-venous differences in blood gas and acid-base status of the blood, was consistent with these data obtained *in vitro* (Chapter 3). That is, the majority of Bohr protons were released in the upper region of the Hb-O_2 equilibrium curve permitting an almost complete exploitation of the Haldane effect at the lowest swimming velocity. At 16% of Ucrit, there was a tight stoichiometric coupling between protons released during Hb oxygenation and CO_2 excretion. As swimming velocity increased and the range over which the Hb-O_2 equilibrium curve used for gas exchange increased, the degree of coupling decreased to less than 50%.

In general, control of the cardio-respiratory system in water breathing fishes is geared to ensure O_2 uptake from water (Randall and Cameron, 1973; Smith and Jones, 1982; Randall, 1990). Because the solubility and diffusivity of CO_2 is about 20-25 times that for O_2 in aqueous solutions (Dejours, 1988), the conditions at the gills are more than sufficient for CO_2 transfer. Consequently, Pco_2 and Cco_2 levels in water breathing fishes are very low in comparison with air breathing vertebrates. The relationship between P_aco_2 and pH is log-linear (Albers *et. al.*, 1983). Given the low absolute values of P_aco_2 in fish, it is clear that even small changes in blood P_aco_2 could have significant effects on acid-base balance (Iwama *et. al.*, 1987). The large Haldane effect and low buffer capacity in the blood of trout permits an indirect influence on CO_2 transport and excretion through conditions for O_2 uptake. The disproportionate Bohr proton release

observed in the blood of trout and tench (Jensen, 1986) permits a fine tuning of this indirect influence depending upon the region of the Hb-O_2 equilibrium curve used for gas transport. During sustained exercise there is a marked increase in the gill ventilation:blood perfusion ratio (Kiceniuk and Jones, 1977) to satisfy the metabolic demand for oxygen. At this time when the convective conditions for CO_2 removal is enhanced, the non-linear release of Bohr protons has been hypothesized to limit HCO_3^- dehydration at the gills and conserve tissue CO_2 stores (Chapter 3). The same also applies during exposure to hypoxia when ventilation volume is elevated to maintain O_2 uptake, with little change in cardiac output (Holeton and Randall, 1967; Smith and Jones, 1982; Randall, 1990). During exposure to hypoxia; however, arterial Cco_2 and Pco_2 levels generally decrease (Chapter 1; Boutilier *et. al.*, 1986; Lessard *et. al.*, 1995), due to the markedly increased convective conditions.

In addition to influencing CO_2 levels during hypoxia, the disproportionate Bohr proton release will benefit O_2 uptake at the gills. If So_2 is about 50%, the absence of Bohr proton release during oxygen binding to Hb will elevate red cell pH raising Hb-O_2 affinity. This oxygenation state dependent red cell alkalosis at the gills, may optimize conditions for oxygen uptake before circulating catecholamines are elevated causing an increase in red cell pH. The release of catecholamines during hypoxemia, has been demonstrated to benefit oxygen uptake at the gills (Primmett *et. al.*, 1986; Perry *et. al.*, 1989), but is metabolically costly. The maximal alkalosis due to the disproportionate Bohr proton release will be attained when arterial Hb-O_2 saturation is about 50% (Jensen, 1986). Interestingly, this is also the threshold at which catecholamines are

released in both eel and trout (Perry and Reid, 1992). Some fish, such as tench, may lack β-adrenergic sensitivity in red cells, in which case the disproportionate Bohr proton release may play a larger role in maintaining oxygen uptake during exposure to hypoxia (Jensen, 1986), than it does in other fishes.

At the level of the Hb, the basis for the large Haldane and Root effects and the low Hb buffer capacity in fish is related to the structure of the molecule. Fish Hb, as in most vertebrates, is a tetrameric protein, most commonly comprised of 2 α and 2 β sub-units (Weber and Jensen, 1988). Although, the amino acid sequence of these sub-units varies considerably among vertebrates, the majority of the amino acid replacements appear to be functionally neutral (Perutz and Brunori, 1982). However, amino acid substitutions at key positions, confer large functional differences for the transport of O_2 and CO_2 in the blood (Weber, 1995).

The imidazole group of the histidine residues comprises the majority of proton buffering capacity in Hb within the neutral pH range (Tanford, 1962; Jensen, 1989). The low buffer capacity at constant So_2 in the Hb of teleost fishes has been correlated with a reduced histidine content and a reduced number of titratable histidine residues, relative to that observed in mammalian Hbs (Jensen, 1989). In carp, there appear to be seven titratable histidine residues per Hb molecule, considerably lower than the 20 to 22 residues which exist in horse Hbs (Jensen, 1989). The removal of two histidine residues from mammalian Hb is thought to be responsible for the greater ATP and GTP sensitivity observed in teleost blood in comparison with the blood of mammals (Perutz and Brunori, 1982); however, this accounts for only a minor difference in histidine

content between teleost and mammalian Hb.

The Haldane effect arises from changes in the pK of specific amino acid groups due to oxygenation and deoxygenation induced conformational changes in Hb. Conversely, the Bohr effect results from changes in Hb conformation due to binding of protons to these specific amino groups as a function of pH. Thus, thermodynamically the Haldane and Bohr effects are indistinguishable (Wyman, 1973). The amino acid residues responsible for the Bohr/Haldane effect are located distant from the heme. In human Hb, in the absence of organic phosphates, the specific residues are thought to consist predominantly of β146 histidine (a histidine at the 146th residue from the N-terminus of the β subunit), α1 valine and β82 lysine, but several other groups are thought to be involved (Brittain, 1987; Riggs, 1988). In fish the α1 valine is acetylated (Farmer, 1979) and unable to contribute to the Bohr/Haldane effect but the β146 histidine is thought to play a major role. In fish Hb, however, a large portion of the Bohr/Haldane effect remains to be elucidated structurally (Jensen, 1989).

Stereochemically, the Root effect was proposed to arise predominantly through the replacement of Cysteine with a Serine residue at position 93 on the β chain (β93) (Perutz and Brunori, 1982). This single substitution is not exclusively responsible for the Root effect because it is also found in *Xenopus*, which does not exhibit a Root effect (Bridges *et. al.*. 1985). It has been postulated that the Cysteine →Serine substitution in conjunction with an Aspartic acid 94β→Glutamic acid substitution may comprise the minimum amino acid replacements required for the Root effect (Brittain, 1987). Several other amino acid substitutions have also been implicated in the Root effect (Brunori and

Perutz, 1982).

The molecular structure of Hb intrinsically alters the magnitude of the Root, Bohr and Haldane effects; however heterotrophic ligands permit a rapid and metabolically inexpensive means of modulating the magnitude of these effects. In general, the most important heterotrophic ligands in fish are GTP and ATP (together referred to as NTP; Wood and Johansen, 1972; Weber and Lykkeboe, 1978). Organic phosphate concentrations within the red cell are modulated in response to pertubations such as hypoxia (Tetens and Lykkeboe, 1985; Boutilier *et. al.*. 1988; Weber, 1992) and anaemia (Val *et. al.*. 1994). An increase in NTP:Hb ratio elevates the magnitude of the Root effect (Weber and DeWilde, 1975; Vaccaro *et. al.*. 1977; Pelster and Weber, 1990) and the magnitude of the Bohr and Haldane coefficients (Jensen and Weber, 1985),

In conclusion, the combination between a large Root and Haldane effect and low Hb buffer capacity results in a tight coupling between the movement of O_2 and CO_2 in rainbow trout, *in vivo*. The magnitude of this interaction is greatest when venous Hb-O_2 saturation is above 50% such as during rest, and decreases when the lower reaches of the Hb-O_2 equilibrium curve are utilized. There are many levels at which the interaction between O_2 and CO_2 can be modulated. The non-linear release of Bohr protons observed in this study represents only one of these levels and permits a fine tuning of the degree of interaction between movements of O_2 and CO_2 depending upon the region of the Hb-O_2 equilibrium curve used for gas exchange *in vivo*.

APPENDIX

Calculations:

In calculating the partitioning of CO_2 excretion, the following equations 1-3 were used. The assumptions and limitations of these equations are described in the text. All CO_2 excreted was assumed to be due to the movement of molecular CO_2 from pre-branchial blood ($Pbco_2$, equation 1a), or HCO_3^- dehydrated to CO_2 during gill blood transit ($HCO_3^- \rightarrow CO_2$, equation 1b). See text of chapter 3 for further explanation.

1 a) Proportion of total CO_2 excreted which was due to movement of physically dissolved CO_2 in pre-branchial blood ($Pbco_2$). (ie. did not involve HCO_3^- dehydration during blood transit through the gills).

$$\frac{((P_vco_2-P_aco_2) \times ((1-Hct/100)+(Hct/100 \times 0.86)) \times \alpha CO_2)}{(C_vco_2-C_aco_2)} \times 100$$

where P_vco_2 and P_aco_2 are the pre-branchial and arterial partial pressure of CO_2 (Pco_2), Hct is haematocrit, 0.86 is to the solubility of CO_2 in red cells relative to that in plasma (Van Slyke et al., 1928), αCO_2 is the CO_2 solubility in plasma from Boutilier et al., (1984), and C_vco_2 and C_aco_2 are the total CO_2 content of pre-branchial and arterial blood, respectively.

1 b) Proportion of total CO_2 excreted which consisted of HCO_3^- dehydrated to CO_2 ($HCO_3^- \rightarrow CO_2$) during gill blood transit.

100% - equation 1a)

This proportion of CO_2 excreted (1 b) is comprised of HCO_3^- which entered the red cell via HCO_3^-/Cl^- exchange (equation 2) and that which resided within the red cell prior to blood entry into the gills (equation 3).

2) Proportion of total CO_2 excreted which is dependent upon HCO_3^-/Cl^- exchange (% HCO_3^-/Cl^-):

$$\frac{(HCO_{3\,vp}^- \times (1\text{-}Hct/100)) - (HCO_{3\,ap}^- \times (1\text{-}Hct/100))}{(C_vco_2\text{-}C_aco_2)} \times 100$$

where $HCO_{3\,vp}^-$ and $HCO_{3\,ap}^-$ are $[HCO_3^-]$ in venous and arterial plasma, respectively. Hct was assumed to be equal in arterial and venous blood.

3) The proportion of total CO_2 excreted which is dependent upon HCO_3^- existing in the red cell prior to gill blood transit was calculated as:

100% - (equation 1a + equation 2)

4) Calculation of modified respiratory exchange ratio (RE'):

This equation describes the amount of HCO_3^- dehydrated to CO_2 relative to the amount of oxygen which is bound to Hb during gas exchange across the gills.

$$\frac{(C_vco_2-C_aco_2)-((P_vco_2-P_aco_2) \times ((1-Hct/100)+(Hct/100 \times 0.86)) \times \alpha CO_2)}{(C_ao_2-C_vo_2)-((P_ao_2-P_vo_2) \times ((1-Hct/100)+(Hct/100 \times 0.86)) \times \alpha O_2)}$$

where symbols related to CO_2 are the same as those described in equation 1. P_vo_2 and P_ao_2 are the pre-branchial and arterial partial pressure of O_2 (Po_2). 0.86 refers to the solubility of CO_2 and O_2 in red cells relative to that in plasma (Van Slyke et al., 1928), αO_2 is the O_2 solubility in plasma from Boutilier et al., (1984), and C_vo_2 and C_ao_2 are the total O_2 content of pre-branchial and arterial blood respectively.

5) Calculation of the Haldane coefficient over the region of the Hb-O_2 equilibrium curve used during exercise:

The Haldane coefficient describes the moles of protons released per mole of oxygen bound to Hb. An *in vivo* Haldane coefficient was calculated from data from arterial and mixed-venous blood (chapter 3), using the following equations (A-E):

A) Total CO_2 excreted as HCO_3^- dehydrated to CO_2 during gill blood transit i.e. ($HCO_3^- \rightarrow CO_2$):

$$(C_vco_2-C_aco_2)-((P_vco_2-P_aco_2)x((1-Hct/100)+(Hct/100x0.86)) \times \alpha CO_2)$$

where abbreviations refer to the same parameters described above in equation 1.

B) Total protons titrated from plasma during gill transit:

$$(pH_a\text{-}pH_v) \times -1.271 \times ((Hb_a + Hb_v)/2) \times (1 - (Hct_a + Hct_v)/2)$$

where -1.271 is the slope of the true plasma buffer line ($HCO_3^- \cdot L^{-1} \cdot pH^{-1}$) from Wood et al. 1982. It should be noted that the buffer line was calculated at 13 °C, while the data described in chapter 3 were obtained from fish at 9 °C.

C) Total protons titrated from red cells during gill transit:

$$(pH_{ia}\text{-}pH_{iv}) \times -16.5 \times (Hct_a + Hct_v)/2$$

where -16.5 refers to the slope of the erythrocyte buffer line (mmol $HCO_3^- \cdot L^{-1} \cdot pH^{-1}$) from Heming, 1984. It was assumed that the mean cell Hb concentration (MCHC) in this study was the same as that which was not reported by Heming (1984). MCHC did not change significantly during exercise (chapter 3).

D) Oxygen taken up across the gills which bound to Hb:

$$(C_aO_2\text{-}C_vO_2)\text{-}(((P_aO_2\text{-}P_vO_2) \times \alpha O_2) \times ((1\text{-}Hct/100) + (Hct/100 \times 0.86)))$$

E) Haldane coefficient:

$$\frac{A-(B+C)}{D}$$

The calculation of the Haldane coefficient depends upon a number of assumptions. Firstly, there is no H^+ excretion or HCO_3^- uptake across the gills independent of CO_2. That is protons and HCO_3^- can only traverse the gills as molecular CO_2.

A-(B+C) is an indirect measure of proton release from Hb during oxygenation and is inferred from the difference in HCO_3^- dehydrated to CO_2 which will consume a proton, and protons titrated (based upon the pH changes across the gills) from plasma and the red cell which can supply protons. The difference presumably represents protons released upon oxygenation. When this is divided by the moles of O_2 bound to Hb (D) it provides an approximation of the Haldane coefficient based upon the region of the Hb-O_2 equilibrium curve used for gas exchange during exercise *in vivo*.

6) Proportion of CO_2 excretion potentially linked to O_2 uptake through the release of Bohr protons during Hb oxygenation (%MCO_2:MO_2):

$$\frac{\text{Haldane coefficient(equation 5E) x } O_2 \text{ which bound to Hb (equation 5 D)}}{(C_vco_2-C_aco_2)}$$

REFERENCES

Acierno R., MacDonald J. A., Agnisola C. and Tota B. (1995) Blood volume in the hemoglobinless antarctic teleost *Chionodraco hamatus* Lonnberg. *J. Exp. Zool.* 272, 407-409.

Albers C., Goetz K.H. and Hughes G. M. (1983) Effect of acclimation temperature on intraerythrocytic acid-base balance and nucleoside triphosphates in the carp, *Cyprinus carpio*. *Respir. Physiol.* 34, 145-159.

Baroin A., Garcia-Romeu F., Lamarre T. and Motais R. (1984) Hormone-induced co-transport with specific pharmacological properties in erythrocytes of rainbow trout, *Salmo gairdneri*. *J. Physiol.* 350, 137-157.

Bartlett G. R., Schwantes A. R. and Val A. L. (1987) Studies on the influence of nitrite on methemoglobin formation in amazonian fishes. *Comp. Biochem. Physiol.* 86C(No. 2), 449-456.

Baumann R., Bartels H. and Bauer C. (1987) Chapter 9, Blood oxygen transport. In *Handbook of Physiology, Section 3, The Respiratory System.* (Fishman A. P., Farhi L. E., Tenney S. M. and Geiger S. R., eds.), Vol. IV, Gas Exchange, pp. 147-172. American Physiological Society, Bethesda.

Bell W. H. and Terhune L. D. B. (1970) Water tunnel design for fisheries research. *Fish. Res. Board Can., Tech. Rep.* 195, 1-69.

Bhargava V., Lai N. C., Graham J. B., Hempleman S. C., Shabetai R. (1992) Digital image analysis of shark gills: modeling of oxygen transfer in the domain of time. *Am. J. Physiol.*263, R741-R746.

Bohr C., Hasselbalch K. and Krogh A. (1904) Ueber einen in biologischer beziehung wichtigen einfluss, den die Kohlensauerspannung des blutes auf dessen sauerstoffbindung uebt. *Skand. Arch. Physiol.* 16, 402-412.

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

Boutilier R. G., Iwama G. K., Heming T. A. and Randall D. J. (1985) The apparent pK of carbonic acid in rainbow trout blood plasma between 5 and 15 C. *Respir. Physiol.* 61, 237-254.

Boutilier R. G., Iwama G. K. and Randall D. J. (1986) The promotion of catecholamine release in rainbow trout, *Salmo gairdneri*, by acute acidosis: interactions between red cell pH and haemoglobin oxygen-carrying capacity. *J. exp. Biol.* 123, 145-157.

Boutilier R. G., Dobson G., Hoeger U. and Randall D. J. (1988) Acute exposure to graded levels of hypoxia in rainbow trout *Salmo gairdneri*:metabolic and respiratory adaptations. *Respir. Physiol.* 71, 69-82.

Brauner C. J., Val A. L. and Randall D. J. (1993) The effect of graded methaemoglobin levels on the swimming performance of chinook salmon (*Oncorhynchus tshawytscha*). *J. exp. Biol.* 185, 121-135.

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

Brett J. R. and Glass N. R. (1973) Metabolic rates and critical swimming speeds of sockeye salmon (*Oncorhynchus nerka*) in relation to size and temperature. *J. Fish. Res. Board Can.* 30, 379-387.

Bridges C. R., Pelster B. and Scheid P. (1985) Oxygen binding in blood of *Xenopus laevis* (amphibia) and evidence against Root effect. *Respir. Physiol.* 61, 125-136.

Brittain T. (1987) The Root effect. *Comp. Biochem. Physiol.* 86B(3), 473-481.

Brunori M., Giardina B., Antonini E., Benedetti P. A. and Bianchini G. (1974) Distribution of the haemoglobin components of trout blood among the erythrocytes: Observations by single-cell spectroscopy. *J. Mol. Biol.* 86, 165-169.

Cameron J. N. (1978) Chloride shift in fish blood. *J. Exp. Zool.* 206, 289-295.

Cameron J. N. and Davis J. C. (1970) Gas exchange in rainbow trout (*Salmo gairdneri*) with varying blood oxygen capacity. *J. Fish. Res. Board Can.* 27, 1069-1085.

Cameron J. N. and Polhemus J. A. (1974) Theory of CO_2 exchange in trout gills. *J. exp. Biol.* 60, 183-194.

Cech J. J. Jr., Laurs R. M. and Graham J. B. (1984) Temperature-induced changes in blood gas equilibria in the albacore, *Thunnus alalunga*, a warm-bodied tuna. *J. exp. Biol.* 109, 21-34.

Christiansen J., Douglas C. G. and Haldane J. S. (1914) The absorption and dissociation of carbon dioxide by human blood. *J. Physiol. Lond.* 48, 244-277.

Comroe J. H. J. (1974) *Physiology of Respiration*. Year Book Medical Publishers, Inc., Chicago. pp. 316.

Cossins A. R. and Richardson P. A. (1985) Adrenaline-induced Na^+/H^+ exchange in trout erythrocytes and its effects upon oxygen-carrying capacity. *J. exp. Biol.* 118, 229-246.

Crandall E. D. and Bidani, A. (1981) Effects of red blood cell HCO_3^-/Cl^- exchange kinetics on lung CO_2 transfer: theory. *J. Appl. Physiol.* 50(2),265-271.

Currie S. and Tufts B. L. (1993) An analysis of carbon dioxide transport in arterial and venous blood of the rainbow trout, *Oncorhynchus mykiss*, following exhaustive exercise. *Fish Physiol. Biochem.* 12(3), 183-192.

Dejours P. (1988) *Respiration in water and air: Adaptations, regulation, evolution* pp. 180, Elsevier, Amsterdam.

Eddy F. B. (1974) *In vitro* blood carbon dioxide of the rainbow trout (*Salmo gairdneri*). *Comp. Biochem. Physiol.* 47(A), 129-140.

Fairbanks M. B., Hoffert J. R. and Fromm P. O. (1969) The dependence of the oxygen-concentrating mechanism of the teleost eye (*Salmo gairdneri*) on the enzyme carbonic anhydrase. *J. Gen. Physiol.* 54, 203-211.

Farmer M. (1979) The transition from water to air breathing: effects of CO_2 on hemoglobin function. *Comp. Biochem. Physiol.* 62A, 109-114.

Farmer M., Fyhn H.J., Fyhn U.E.H. and Nobel R.W. (1979) The occurrence of Root effect haemoglobins in Amazonian fishes. *Comp. Biochem. Physiol.* 62A, 115-124.

Farrell A.P. (1978) Cardiovascular events associated with air breathing in two teleosts, *Hoplerythrinus unitaeniatus* and *Arapaima gigas*. *Can. J. Zool.* 56(4), 953-958.

Farrell A.P. and Randall D.J. (1978) Air-breathing mechanics in two Amazonian teleosts, *Arapaima gigas* and *Hoplerythrinus unitaeniatus*. *Can. J. Zool.* 56(4), 939-945.

Farrell A. P., Daxboeck C. and Randall D. J. (1979) The effect of input pressure and flow on the pattern and resistance to flow in the isolated perfused gill of a teleost fish. *J. Comp. Physiol.* 133, 233-240.

Feuerlein R. J. and Weber R. E. (1994) Rapid and simultaneous measurement of anodic and cathodic haemoglobins and ATP and GTP concentrations in minute quantities of fish blood. *J. exp. Biol.* 189, 273-277.

Forster R.E. and Steen J.B. (1969) The rate of the "Root shift" in eel red cells and eel haemoglobin solutions. *J. Physiol.* 204, 259-282.

Fyhn E. H., Fyhn H. J., Davis B. J., Powers D. A., Fink W. L. and Garlick R. L. (1979) Hemoglobin heterogeneity in Amazonian fishes. *Comp. Biochem. Physiol.* 62(A), 39-66.

Gallaugher P., Axelsson M. and Farrell A. P. (1992) Swimming performance and haematological variables in splenectomized rainbow trout, *Oncorhynchus mykiss*. *J. exp. Biol.* 171, 301-314.

Gilmour K. M. and Perry S. F. (1994) The effects of hypoxia, hypercapnia on the acid-base disequilibrium in the arterial blood of rainbow trout. *J. exp. Biol.* 192, 269-284.

Gilmour K. M., Randall D. J. and Perry S. F. (1994) Acid-base disequilibrium in the arterial blood of rainbow trout. *Respir. Physiol.* 96, 259-272.

Heming T. A. (1984) The role of fish erythrocytes in transport and excretion of carbon dioxide. Ph.D. Thesis, University of British Columbia, Vancouver, B.C. Canada. 177 p.

Heming T. A., Randall D. J., Boutilier R. G., Iwama G. K. and Primmett D. (1986) Ionic equilibria in red blood cells of rainbow trout (*Salmo gairdneri*): Cl^-, HCO_3^- and H^+. *Respir. Physiol.* 65,-223-234.

Henry R. P., Smatresk N. J. and Cameron J. N. (1988) The distribution of branchial carbonic anhydrase and the effects of gill and erythrocyte carbonic anhydrase inhibition in the channel catfish *Ictalurus punctatus*. *J. exp. Biol.* 134, 201-218.

Hlastala M. P., Woodson, R.D. and Wranne, B. (1977) Influence of temperature on hemoglobin-ligand interaction in whole blood. *J. Appl. Physiol.* 43, 545-550.

Holeton G. F. (1970) Oxygen uptake and circulation by a hemoglobinless antarctic fish (Chaenocephalus aceratus LONNBERG) compared with three red-blooded antarctic fish. *Comp. Biochem. Physiol.* 34, 457-471.

Holeton G. F. and Randall D. J. (1967) The effect of hypoxia upon the partial pressure of gases in the blood and water afferent and efferent to the gills of rainbow trout. *J. exp. Biol.* 46, 317-327.

Honig C. R., Feldstein M. L. and Frierson J. L. (1977) Capillary lengths, anastamoses, and estimated capillary transit times in skeletal muscle. *Am. J. Physiol.* 233(1), H122-H129.

Isaacks R. E., Kim H. D., Bartlett G. R. and Harkness D. R. (1977) Inositol pentaphosphate in erythrocytes of a fresh water fish, pirarucu (*Arapaima gigas*). *Life Sciences* 20, 987-990.

Iwama G. K., Boutilier R. G., Heming T. A., Randall D. J. and Mazeaud M. (1987) The effects of altering gill water flow on gas transfer in rainbow trout. *Can. J. Zool.* 65, 2466-2470.

Jensen F. B. (1986) Pronounced influence of Hb-O_2 saturation on red cell pH in tench blood *in vivo* and *in vitro*. *J. Exp. Zool.* 238, 119-124.

Jensen F. B. (1989) Hydrogen ion equilibria in fish haemoglobins. *J. exp. Biol.* 143, 225-234.

Jensen F. B. (1991) Multiple strategies in oxygen and carbon dioxide transport by haemoglobin. In *Physiological Strategies for Gas Exchange and Metabolism*. (Woakes A. J., Greishaber M. K. and Bridges C. R., eds.), Vol., pp. 55-78. Cambridge University Press, Cambridge.

Jensen F. B. and Weber R. E. (1985) Proton and oxygen equilibria, their anion sensitivities and interrelationships in tench hemoglobin. *Molecular Physiology* 7, 41-50.

Jensen F. B. and Brahm J. (1995) Kinetics of chloride transport across fish red blood cell membranes. *J. exp. Biol*. 198, 2237-2244.

Johansen K. (1970) Air breathing in fishes. In *Fish Physiology*. (Hoar W. S. and Randall D. J., eds.), Vol. IV, pp. 361-411. Academic Press, New York.

Jones D. R. and Randall D. J. (1978) The respiratory and circulatory systems during exercise. In *Fish Physiology*. (Hoar W. S. and Randall D. J., eds.), Vol. 7, pp. 425-501. Academic Press, New York.

Jones D. R., Brill R. W. and Mense D. C. (1986) The influence of blood gas properties on gas tensions and pH of ventral and dorsal aortic blood in free-swimming tuna, *Euthynnus affinis*. *J. exp. Biol.* 120, 201-213.

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

Klocke R. A. (1973) Mechanism and kinetics of the Haldane effect in human erythrocytes. *J. Appl. Physiol.* 35(5), 673-681.

Klocke R. A. (1987) Carbon dioxide transport. In *Handbook of Physiology: The Respiratory System.* (Fishman A. P., Farhi L. E., Tenney S. M. and Geiger S. R., eds.), Vol. IV, pp. 173-197. American Physiological Society, Bethesda, Maryland.

Kobayashi H., Pelster B. and Scheid P. (1990). CO_2 back-diffusion in the rete aids O_2 secretion in the swimbladder of the eel. *Resp. Physiol.* 78:59-71.

Kutty M. N. (1968) Respiratory quotients in goldfish and rainbow trout. *J. Fish. Res. Board Can.* 25(8), 1689-1728.

Lessard J., Val A. L., Aota A. and Randall D. J. (1994) Why is there no carbonic anhydrase activity available to fish plasma? *J. exp. Biol.* 198, 31-38.

Nikinmaa M. (1990) *Vertebrate red cells: Adaptations of function to respiratory requirements* Zoophysiology 28. Pp. 262. Springer Verlag, Berlin.

Nilsson S. and Grove D. J. (1974) Adrenergic and cholinergic innervation of the spleen of the cod: *Gadus morhua. Eur. J. Pharmac.* 28, 135-143.

Pelster B. and Weber R. E. (1990) Influence of organic phosphates on the Root effect of multiple fish haemoglobins. *J. exp. Biol.* 149, 425-437.

Pelster B. and Scheid P. (1992) Countercurrent concentration and gas secretion in the fish swim bladder. *Physiol. Zool.* 65(1), 1-16.

Pelster B., Scheid P. and Reeves R. B. (1992) Kinetics of the Root effect and of O_2 exchange in whole blood of the eel. *Respir. Physiol.* 90, 341-349.

Perry S. F. (1986) Carbon dioxide excretion in fishes. *Can. J. Zool.* 64, 565-572.

Perry S. F. and Laurent P. (1990) The role of carbonic anhydrase in carbon dioxide excretion, acid-base balance and ionic regulation in aquatic gill breathers. In *Animal Nutrition and Transport Processes 2. Transport, Respiration and Excretion: Comparative and Environmental Aspects.* (Truchot J. P. and Lahlou B., eds.), Vol. 6, pp. 39-57. Karger, Basel, Switzerland.

Perry S. F. and Reid S. D. (1992) Relationship between blood O_2 content and catecholamine levels during hypoxia in rainbow trout and American eel. *Am. J. Physiol.* 263, R240-R249.

Perry S. F. and Gilmour K. (1993) An evaluation of factors limiting carbon dioxide excretion by trout red blood cells *in vitro. J. exp. Biol.* 180, 39-54.

Perry S.F., Davie P.S., Daxboeck C. and Randall D.J. (1982) A comparison of CO_2 excretion in a spontaneously ventilating blood-perfused trout preparation and saline-perfused gill preparations: contribution of the branchial epithelium and red blood cell. *J. exp. Biol.* 101, 47-60.

Perry S. F., Kinkead R., Gallaugher P. and Randall D. J. (1989) Evidence that hypoxemia promotes catecholamine release during hypercapnic acidosis in rainbow trout *(Salmo gairdneri). Respir. Physiol.* 77, 351-364.

Perry S. F., Wood C. M., Walsch P. J. and Thomas S. (1995) Fish red blood cell carbon dioxide excretion *in vitro*. A comparative study. *Comp. Biochem. Physiol.* (in press).

Perutz M. F. and Brunori M. (1982) Stereochemistry of cooperative effects in fish and amphibian haemoglobins. *Nature* 299, 421-426.

Piiper J. (1990) Modeling of gas exchange in lungs, gills and skin. In *Advances in Comparative and Environmental Physiology*. (Boutilier R. G., ed.), Vol. 6, pp. 15-44. Springer-Verlag, Berlin.

Primmett D. R. N., Randall D. J., Mazeaud M. and Boutilier R. G. (1986) The role of catecholamines in erythrocyte pH regulation and oxygen transport in rainbow trout (*Salmo gairdneri*) during exercise. *J. exp. Biol.* 122, 139-148.

Puckett K. J. and Dill L. M. (1984) Cost of sustained and burst swimming to juvenile coho salmon (*Oncorhynchus kisutch*). *Can. J. Fish. Aquat. Sci.* 41, 1546-1551.

Randall D. (1982) Blood flow through gills. In *Gills. Society for Experimental Biology Seminar Series*. (Houlihan, Rankin and Shuttleworth, eds.), Vol. 16, pp. 173-191. Cambridge University Press, U.S.A.

Randall D. (1990) Control and co-ordination of gas exchange in water breathers. In *Advances in Comparative and Environmental Physiology*. (Boutilier R. G., ed.), Vol. 6, pp. 253-278. Springer-Verlag, Berlin.

Randall D. J. and Cameron J. N. (1973) Respiratory control of arterial pH as temperature changes in rainbow trout *Salmo gairdneri. Am. J. Physiol.* 225(4), 997-1002.

Randall D.J. and Daxboeck C. (1984) Oxygen and carbon dioxide transfer across fish gills. In: *Fish Physiology* (W.S. Hoar and D.J. Randall eds.) Vol. 10A pp. 263-307. Academic Press, New York.

Randall D.J., Farrell A.P., Haswell M.S. (1978) Carbon dioxide excretion in the pirarucu (*Arapaima gigas*), an obligate air-breathing fish. *Can. J. Zool.* 56, 977-982.

Riggs A. (1979) Studies of the hemoglobins of Amazonian fishes: an overview. *Comp. Biochem. Physiol.* 62A, 257-272.

Riggs A. F. (1988) The Bohr effect. *Ann. Rev. Physiol.* 50, 181-204.

Ristori M. T. and Laurent P. (1985) Plasma catecholamines and glucose during moderate exercise in the trout: comparison with bursts of violent activity. *Exp. Biol.* 44, 247-253.

Romano L. and Passow H. (1984) Characterization of anion transport system in trout red blood cell. *Am. J. Physiol.* 246, C330-C338.

Root R. W. (1931) The respiratory function of the blood of marine fishes. *Biol. Bull. mar. biol. Lab. Woods Hole* 61, 427-456.

Saffran W. A. and Gibson Q. H. (1981) Asynchronous ligand binding and proton release in a Root effect hemoglobin. *J. Biol. Chem.* 256, 4551-4556.

Scholander P. F. and Van Dam L. (1954) Secretion of gases against high pressures in the swimbladder of deep sea fishes. I. Oxygen dissociation in blood. *Biol. Bull. mar. biol. Lab. Woods Hole.* 107, 247-259.

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

Smith F. M. and Bell G. R. (1967) Anaesthetic and surgical techniques for Pacific salmon. *J. Fish. Res. Board Can.* 24, 1579-1588.

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

Steffensen J. F., Tufts B. L. and Randall D. J. (1987) Effect of burst swimming and adrenaline infusion on CO_2 excretion in rainbow trout, *Salmo gairdneri*. *J. exp. Biol.* 131, 427-434.

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

Stevens E.D. and Holeton G.F. (1978) The partitioning of oxygen uptake from air and from water by the large obligate air-breathing teleost pirarucu (*Arapaima gigas*). *Can. J. Zool.* 56(4), 974-976.

Swenson E. R. (1990) Kinetics of oxygen and carbon dioxide exchange. In *Advances in Comparative and Environmental Physiology*. (Boutilier R. G., ed.), Vol. 6, pp. 163-209. Springer-Verlag, Berlin Heidelberg.

Swenson E. R. and Maren T. H. (1978) A quantitative analysis of CO_2 transport at rest and during maximal exercise. *Respir. Physiol.* 35, 129-159.

Tanford C. (1962) The interpretation of hydrogen ion titration curves of protein. *Adv. Protein Chem.* 17, 69-165.

Tetens V. and Lykkeboe G. (1985) Acute exposure of rainbow trout to mild and deep hypoxia: O_2 affinity and O_2 capacitance of arterial blood. *Respir. Physiol.* 61, 221-235.

Thomas S. and Le Ruz H. (1982) A continuous study of rapid changes in blood acid-base status of trout during variations of water Pco_2. *J. Comp. Physiol.* 148, 123-130.

Thomas S., Poupin J., Lykkeboe G. and Johansen K. (1987) Effects of graded exercise on blood gas tensions and acid-base characteristics of rainbow trout. *Respir. Physiol.* 68, 85-97.

Tucker V. A. (1967) A method for oxygen content and dissociation curves on microliter blood samples. *J. Appl. Physiol.* 23(3), 407-410.

Vaccaro A. M. T., Raschetti R., Salvioli R., Riciardi G. and Winterhalter K. H. (1977) Modulation of the Root effect in goldfish by ATP and GTP. *Biochim. Biophys. Acta* 496, 367-373.

Val A. L., Affonso E. G., Souza R. H. S., Almeida-Val V. M. F. and Moura M. A. F. (1992) Inositol pentaphosphate in the erythrocytes of an Amazonian fish, the pirarucu (*Arapaima gigas*). *Can. J. Zool.* 70, 852-855.

Val A. L., Mazur C. F., De Salvo-Souza R. H. and Iwama G. K. (1994) Effects of experimental anaemia on intra-erythrocytic phosphate levels in rainbow trout, *Oncorhynchus mykiss*. *J. Fish Biol.* 45, 269-277.

Van den Thillart G., Randall D. and Hoa-Ren L. (1983) CO_2 and H^+ excretion by swimming coho salmon, *Oncorhynchus kisutch*. *J. exp. Biol.* 107, 169-180.

Van Slyke D. D., Sendroy J. Jr., Hastings A. B. and Neill J. M. (1928) Studies of gas and electrolyte equilibria in blood. X. The solubility of carbon dioxide at 38 C in water, salt solution, serum and blood cells. *J. Biol. Chem.* 78, 765-799.

Weber R. E. (1990) Functional significance and structural basis of multiple hemoglobins with special reference to ectothermic vertebrates. In *Comparative Physiology; Animal Nutrition and Transport Processes; 2. Transport, Respiration and Excretion: Comparative and Environmental Aspects; II. Blood oxygen transport: Adjustment to Physiological and Environmental Conditions*. (Truchot J. P. and Lahlou B., eds.), Vol. 6, pp. 58-75. Karger, Basel, Switzerland.

Weber R. E. (1992) Molecular strategies in the adaptation of vertebrate hemoglobin function. In *Physiological Adaptation in Vertebrates: Respiration, Circulation, and Metabolism; Lung biology in Health and Disease*. (Wood S. C., ed.), Vol. 56, pp. 257-277. Marcel Dekker, Inc., New York.

Weber R. E. (1995) Hemoglobin adaptations to hypoxia and altitude-the phylogenetic perspective. In: *Hypoxia and the Brain*. (Sutton, Jr., Houston, CS and Coates, G, eds.) pp 31-44. Queen City Printers Inc., Burlington, USA.

Weber R. E. and DeWilde J. A. M. (1975) Oxygenation properties of haemoglobins from the flatfish plaice (*Pleuronectes platessa*) and flounder (*Platichthys flesus*). *J. Comp. Physiol.* 101, 99-110.

Weber R. E. and Lykkeboe, G. (1978) Respiratory adaptations in carp blood. Influences of hypoxia, red cell organic phosphates, divalent cations and CO_2 on hemoglobin-oxygen affinity. *J. Comp. Physiol.* 128, 127-137.

Weber R. E. and Jensen F. B. (1988) Functional adaptations in hemoglobins from ectothermic vertebrates. *Ann. Rev. Physiol.* 50, 161-179.

Weber R. E., Jensen F. B. and Cox R. P. (1987) Analysis of teleost hemoglobin by Adair and Monod-Wyman-Changeux models. Effects of nucleotide triphosphates and pH on oxygenation of tench hemoglobin. *J. Comp. Physiol.* 157, 145-152.

Wieth J. O., Andersen O. S., Brahm J., Bjerrum P. J. and Borders Jr C. L. (1982) Chloride-bicarbonate exchange in red blood cells: physiology of transport and chemical modification of binding sites. *Phil. Trans. R. Soc. Lond.* B 299, 383-399.

Wolf K. (1963) Physiological salines for fresh-water teleosts. *Prog. Fish. Cult.* 25, 135-140.

Wood C.M. and Randall D.J. (1973) The influence of swimming activity on water balance in the rainbow trout (*Salmo gairdneri*). *J. Comp. Physiol.* 82, 257-276.

Wood C. M. and Jackson E. B. (1980) Blood acid-base regulation during environmental hyperoxia in the rainbow trout (*Salmo gairdneri*). *Respir. Physiol.* 42, 351-372.

Wood C. M. and Perry S. F. (1985) Respiratory, circulatory, and metabolic adjustments to exercise in fish. In *Circulation, Respiration and Metabolism.* (Gilles R., ed.), p. 22. Springer-Verlag, Berlin Heidelberg.

Wood C. M. and Perry II S. F. (1991) A new *in vitro* assay for carbon dioxide excretion by trout red blood cells: effects of catecholamines. *J. exp. Biol.* 157, 349-366.

Wood C. M., McDonald D. G. and McMahon B. R. (1982) The influence of experimental anaemia on blood acid-base regulation *in vivo* and *in vitro* in the starry flounder (*Platichthys stellatus*) and the rainbow trout (*Salmo gairdneri*). *J. exp. Biol.* 96, 221-237.

Wood, S.C. and Johansen, K. (1972) Adaptation to hypoxia by increased HbO_2 affinity and decreased red cell ATP concentration. *Nature* 237: 278-279.

Woodward J. J. (1982) Plasma catecholamines in resting rainbow trout, *Salmo gairdneri* Richardson, by high pressure liquid chromatography. *J. Fish Biol.* 21, 429-432.

Wyman J. (1973) Linked functions and reciprocal effects in haemoglobin: a second look. *Adv. Protein Chem.* 19, 223-286.

Yamamoto K. I., Itazawa Y. and Kobayashi H. (1980) Supply of erythrocytes into the circulating blood from the spleen of exercised fish. *Comp. Biochem. Physiol.* 65(A), 5-11.

Zeidler R. and Kim D. H. (1977) Preferential hemolysis of postnatal calf red cells induced by internal alkalinization. *J. Gen. Physiol.* 70, 385-401.