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THE EFFECTS OF REDUCED GILL SURFACE AREA ON GAS TRANSFER IN THE RAINBOW TROUT

(*Oncorhynchus mykiss*)

By

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THE EFFECTS OF REDUCED GILL SURFACE AREA ON GAS TRANSFER IN THE RAINBOW TROUT

(*Oncorhynchus mykiss*)

Abstract

The total functional surface area of the gills is a key component in gas exchange and any reduction would predictably limit both oxygen uptake and carbon dioxide excretion. But one might also hypothesise that under these conditions the fish might compensate to maintain optimal tensions of O_2 and CO_2 in the blood. These are some of the questions examined in this thesis.

To study these questions, a reduction of 15, 30 and 40% of total anatomical gill surface area in rainbow trout was accomplished by the ligation of 1, 2 and 3 gill arches, respectively. With the use of an extracorporeal shunt, arterial blood gases were continuously monitored during normoxia and graded hypoxia. As well, a dorsal aortic cannula allowed for periodic blood sampling for such variables as total arterial oxygen content (CaO_2), total arterial carbon dioxide content ($CaCO_2$), haemoglobin concentration, haematocrit and circulating catecholamines. Oxygen uptake ($\dot{M}O_2$), carbon dioxide excretion rates ($\dot{M}CO_2$) and ventilation convection requirements for both gases were determined through measurements of inspired and expired water carbon dioxide content (CCO_2) and partial pressure of O_2 (PO_2) differences as well as in-flowing/out-flowing water CCO_2 and PO_2 differences. A final series of experiments examined the effects of carbonic anhydrase injections in ligated versus sham-ligated fish as well as comparing these results to ligated fish injected with physiological saline. Results reaffirm the hypothesis that, under normal conditions, the gill is perfusion limited for O_2 . Conversely, after a 40% reduction in surface area, oxygen uptake is diffusion limited as represented by the significantly lower arterial PO_2 levels at water PO_2 levels

below 120 Torr. In terms of CO_2 exchange, there is clear evidence for diffusion limitations as indicated by significantly elevated arterial PCO_2 levels under both normoxic and hypoxic conditions. As well, pHa values were significantly lowered. Ligation of the gills did not affect $\dot{M}O_2$, $\dot{M}CO_2$ or the respiratory exchange ratio (Re). However, ventilation volume ($\dot{V}w$) was significantly increased in fish from 1186.5 ± 188.4 ml/kg/min in control fish to 4463.3 ± 1303.2 ml/kg/min in experimental fish with 40% gill surface area reduction. Injection of carbonic anhydrase performed in fish with 2 gill arches ligated was sufficient to return elevated PCO_2 levels to control values after 80 minutes post-injection.

These results indicate that the apparent diffusion limitations for CO_2 transfer reflect the relatively slow rate of conversion of plasma HCO_3^- to CO_2 as blood flows through the gill. This may in fact refer to chemical equilibrium limitations rather than true diffusion limitations, *per se*.

Abstrait

La superficie fonctionelle totale est une constituante essentiel des échanges gazeux. Si cette variable est limité d'une forme artificielle, est-ce que ça limiterait automatiquement l'absorption d'oxygène et l'élimination du gaz carbonique ou les poissons, pouraient ils maintenir des tensions d'oxygène (O_2) et du gaz carbonique (CO_2) optimums dans le sang ? Ces questions seront examinés dans cette thèse.

Pour étudier ces questions, la superficie fonctionelle totale des branchies a été réduite de 15, 30 et 40% après avoir ligaturé1, 2 et 3 arcs branchiaux. En utilisant une circulation sanguine extracorporelle, l'analyze des gases sanguins a été effectué d'une façon continu, sous conditions normoxiques et hypoxiques. Une canule introduit dans l'aorte dorsale a été utilisé pour prendre des échantillons sanguins périodiquement durant l'expérience pour l'analyzes du contenu total d'O_2 dans le sang (CaO_2), le contenu total du CO_2 dans le sang ($CaCO_2$), la concentration d'hémoglobine, l'hématocrite et les catécholamines. L'absorption d'O_2 ($\dot{M}O_2$), l'élimination du CO_2 ($\dot{M}CO_2$) et la mesure de la convection ventilatoire requise furent déterminés en utilisant les différences du contenu total du CO_2 (CCO_2) et les différences des pressions partielles d'O_2 (PO_2) dans l'eau inspiré et expiré ainsi que les mêmes facteurs dans l'eau entrant et sortant de la boîte. La dernière expérience examinait les effets d'une injection d'anhydrase carbonique chez les poissons ligaturés versus les poissons non ligaturés ainsi que comparant ces résultats aux poissons ligaturés injectés avec du salin Cortland. Les resultats réaffirment la théorie que le transfert de l'O_2 à travers les branchies est limité par les constraintes de perfusion. Par contre, après une réduction de 40% de la superficie des branchies, le transfert de l'O_2 devient limité par les contraintes de diffusion. En terme du transfert du CO_2, il est

clairment limité par des contraintes de diffusion indiqué par des élévations de PCO_2 dans le sang sous conditions normoxiques et hypoxiques et des abaisements de pH dans le sang sous les mêmes conditions. Pas de différences apparentes ont été disernées dans $\dot{M}O_2$, $\dot{M}CO_2$ ou dans le rapport d'échange respiratoire (Re) entre les controles et les poissons ligatués. Parcontre, la ventilation (débit de l'eau ventilé, $\dot{V}w$) a augmenté significativement chez les poissons avec une réduction de 40% de leur surface de branchie, de 1186.5 ± 188.4 ml/kg/min chez les controles à 4463.3 ± 1303.2 ml/kg/min chez les poissons expérimentals. Une injection d'anhydrase carbonique chez les poissons avec deux arcs branchiaux ligaturés a été suffisante pour retourner les valuers élévés de PCO_2 aux valeurs des controles 80 minutes après l'injection en ayant des élévations de pHa correspondants.

Même si CO_2 diffuse facilement on croît que les limites diffusionelles reflètent la formation relativement lente du CO_2 à partir de HCO_3^- lorsque le sang pénètre les branchies. En effet, ceci peut être une limite d'équilibre chimique au lieu d'une limite de diffusion.

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Abbreviations

α, Greek letter alpha

Δ, Greek letter delta

Ca_{O_2}, arterial oxygen content

$CaCO_2$, arterial total carbon dioxide content

C_eCO_2, total carbon dioxide content in expired water

C_iCO_2, total carbon dioxide content in inspired water

C_lCO_2, total carbon dioxide content in in-flowing water

C_OCO_2, total carbon dioxide content in out-flowing water

Hb, haemoglobin

Hct, haematocrit

$\dot{M}O_2$, oxygen consumption

$\dot{M}CO_2$, carbon dioxide excretion

n, number of samples or individuals

PO_2, partial pressure of oxygen

PaO_2, partial pressure of oxygen in arterial blood

P_eO_2, partial pressure of oxygen in expired water

P_iO_2, partial pressure of oxygen in inspired water

P_lO_2, partial pressure of oxygen in in-flowing water

P_OO_2, partial pressure of oxygen in out-flowing water

PCO_2, partial pressure of carbon dioxide

$PaCO_2$, partial pressure of carbon dioxide in arterial blood

PwO_2, partial pressure of oxygen in water

$PwCO_2$, partial pressure of carbon dioxide in water

pHa, arterial pH

SEM, standard error of the mean

$\dot{V}b$, cardiac output

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

CHAPTER 1

GENERAL INTRODUCTION

Introduction

The fish gill is a complex, multi-functional organ primarily responsible for gas exchange as well as osmo-regulation, iono-regulation and acid-base balance. Unlike in air breathing vertebrates, the respiratory organs of fish are suspended in water and as such must compromise between 1) a thick, sturdy structure capable of sustaining constant water pressure and water flow past the gills; and 2) minimizing diffusion thickness of the blood-water barrier as well as maximizing total surface area for efficient gas exchange. These compromises have led to a net influx of water and net efflux of ions in freshwater fish. It is due to such compromises, necessary to accommodate all gill functions, that the gill has both a complex organization and an intricate control over the total functional gill surface area. Total gill surface area (or anatomical surface area), refers to the total surface area, perfused and non-perfused, potentially capable of respiratory exchange, and can be measured morphometrically at any point in time. Total functional gill surface area, refers to the total perfused and ventilated gill surface area that is actively involved in gas exchange at a specific point in time.

General structure of the teleost gill

The respiratory organs of teleosts are formed on either side of the pharynx, covered by a bony opercular flap. They are arranged as four gill arches on either side, each gill arch composed of a double row of filaments. Above and below these filaments are numerous, thin, closely spaced sheets called lamellae. These lamellae are the site of gas exchange and are made up of a double epithelial layer separated by pillar cells. The channels formed by these pillar cells allow movement of blood countercurrent to the flow

of water (see Hughes, 1984; Olson, 1991 for reviews on gill structure as related to physiological function). Countercurrent exchange is essential in an environment with about 1/30 of the oxygen content of air (Perry & McDonald, 1993). It allows constant renewal of the respiratory water, maintaining high water partial pressures of oxygen (PO_2's) and consequently a high driving force for gas diffusion.

Scanning electron micrographs of the epithelial layers reveal three main cell types: pavement cells, mitochondria-rich chloride cells and mucous cells. All three cell types are found throughout the lamellar surface but chloride cells are more abundant between lamellae. Their thick, round structure makes them ill suited for gas transfer and instead they are primarily involved in Ca^{2+} and Cl^- transport (see Perry, 1997 for review). The more abundant pavement cells are thinner and well suited for respiratory function, however they may also be involved in Na^+ uptake, possibly linked to a H^+- ATPase (Goss *et al.*, 1992). The third cell type, the mucous cell, provides a glycoprotein coating for epithelial cells and may in fact hamper gas exchange by increasing diffusion distance. It has been suggested by Perry & Laurent (1993) that this mucous layer traps Na^+ and Cl^- thereby aiding uptake of these ions. Mucus is in greater abundance in freshwater fish and may help in trapping essential ions, making them available for transport before they are lost to the external medium. Proliferation of both mucous cells (and hence increased mucus production) and chloride cells after cortisol injections and exposure to pollutants, irritants or soft water (Bindon *et al*, 1994; Greco *et al*, 1996; reviews by Laurent & Perry, 1991 and Perry, 1997) are adaptations necessary to maintain proper ion balance and acid-base balance but as we know from earlier discussion, there may be compromises with respiratory functions. In this case increased diffusion distance due to chloride and

mucous cell proliferation has been shown to impair carbon dioxide excretion with no apparent effect on oxygen uptake (Bindon *et al.*, 1994; Perry, 1998). Decreases in arterial partial pressures of oxygen (PaO_2) have been observed following lamellar chloride cell proliferation but O_2 content remained constant due to increases in Hb-O_2 affinity (Perry *et al.*, 1996a).

General principles of gas transfer

The key principles of gas transfer can be summarized by Fick's equation:

$\dot{M}O_2 = K*A*\Delta PO_2/D$ where K= Krogh's permeation coefficient (μmol/μm/cm^2/kPa); A = surface area available for gas transfer in the gills (cm^2/g); ΔPO2 = mean O_2 partial pressure gradient between blood and water (kPa) and D = mean blood-water diffusion distance (μm). Similarly, the diffusive movement of CO_2 across the gills is $\dot{M}\,CO_2 = K_{CO2}* A*\Delta PCO_2/D$.

Each one of these parameters can directly affect gas exchange and will be discussed individually, beginning with Krogh's permeation coefficient. This is a constant that so far has not been determined for the fish gill epithelia but considering the presence of three different cell types, this may not be a truly constant factor in the fish gill. It is important to note however, that CO_2 has a significantly greater permeation coefficient than O_2.

Total surface area of the gill as discussed previously is relatively easy to calculate, however an estimation of functional surface area at any given time is a more complicated process. This is due to the fact that at rest, only about 60% of gill lamellae are perfused, preferential perfusion is of the proximal lamellae and of these lamellae that are perfused;

the favored route for blood flow is through the marginal channels of the lamellae, rather than through the central channels. Functional respiratory surface area can be increased by lamellar recruitment and a preferred central perfusion of lamellae. Active control of surface area involves the release of catecholamines into the circulation following a severe stress (such as hypoxia). Alpha adrenergic receptors constrict efferent lamellar arteriols and the resultant increased lamellar pressure forces distal lamellae to open thereby increasing surface area. Beta adrenergic receptors provide the dominating effect by decreasing branchial vasculature resistance, dilating afferent lamellar arterioles and increasing surface area (Nilsson, 1983). Passive control involves increases in ventral aortic pressure to overcome critical opening pressures and increasing surface area.

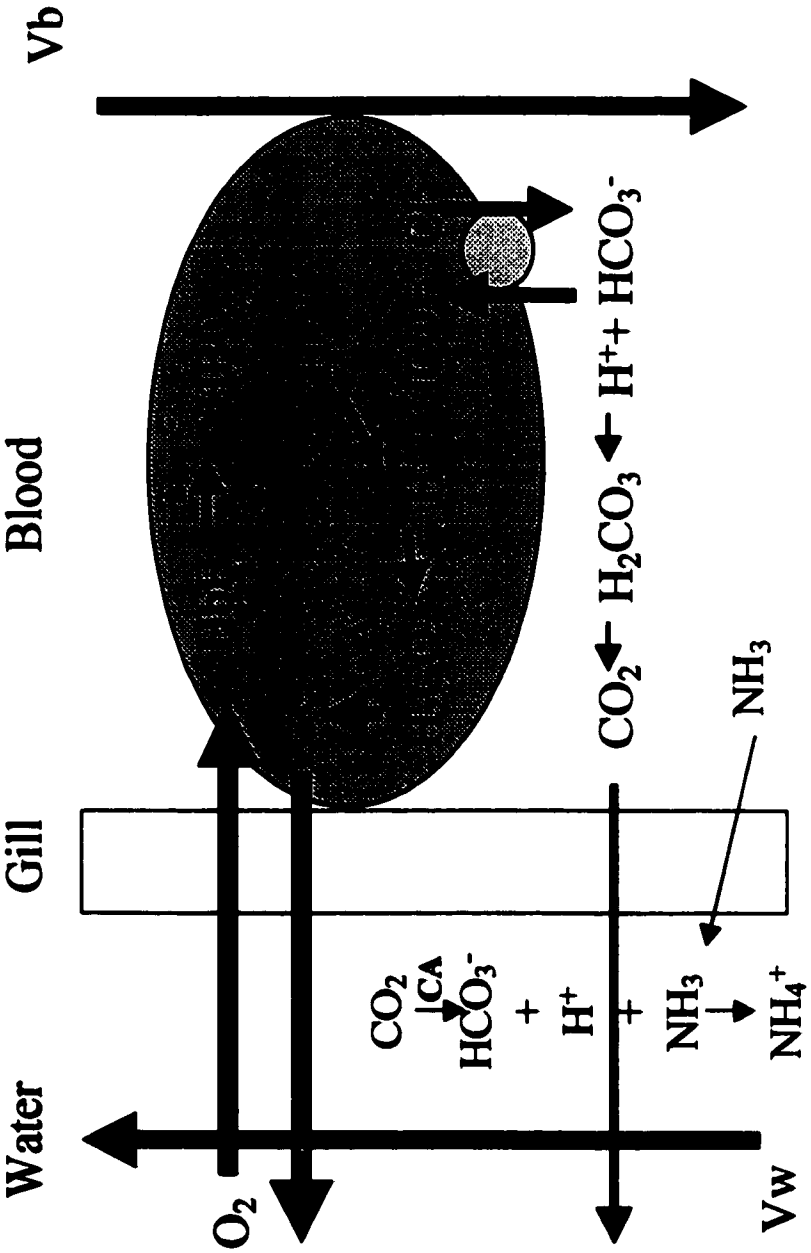
The thickness of the blood-water barrier is not only determined directly by the animal but may be influenced by the quality of the respiratory water (changes in ionic quality and amounts of pollutant) affecting the cellular composition of the epithelial layer. Other factors that could reduce the thickness of the blood-water barrier are increases in ventilatory water flow rates and ventral aortic pressure. This would be accomplished by increasing the width of the respiratory sheet thereby allowing even distribution of blood through individual lamellae.

Gas transfer will not occur without a partial pressure difference between the blood-water barrier; i.e. a driving force for diffusion. The driving force can be increased by increasing ventilation and maintaining high PwO_2 tensions flowing past the gas exchange surface. An increase in ventilation may also result in the recruitment of water channels toward distal ends of filaments from the normal water flow through basal and middle lamellar channels that occurs during "quiet" ventilation.

Figure 1 presents a general overview of gas transfer at the gills which combines all the above-mentioned principles of gas transfer. As indicated by arrows, oxygen diffuses across the gills into the plasma and diffuses into the red blood cell where it oxygenates hemoglobin (HbO_2). Favorable driving forces for O_2 diffusion are maintained by high water ventilatory water flow ($\dot{V}w$) and by the countercurrent flow of water versus blood and transit times for red blood cells are determined by cardiac output ($\dot{V}b$). As haemoglobin is oxygenated, protons necessary for the dehydration of HCO_3^- within the cell are released. At the gills, CO_2 is in the form of HCO_3^- therefore CO_2 excretion requires the additional step of converting HCO_3^- to CO_2 before it diffuses into the expired water. This dehydration is slow in the plasma because no carbonic anhydrase (CA) is available to catalyze this reaction. Plasma HCO_3^- must then enter the red blood cell in exchange for intracellular Cl^- and is rapidly dehydrated to CO_2 in the presence of carbonic anhydrase. CO_2 then diffuses into the plasma and into the ventilatory water. Driving force for CO_2 excretion is maintained by coupling this movement of CO_2 into the boundary layer with the movement of NH_3. CO_2 in the boundary layer is hydrated to $HCO_3^- + H^+$ catalyzed by carbonic anhydrase on the apical layer of pavement cells. H^+ from this reaction combines with NH_3 to produce NH_4^+ (Randall, 1990).

At the tissues, CO_2 is hydrated within the red blood cells in the presence of carbonic anhydrase and the resultant H^+ ions are buffered by Hb while HCO_3^- is transferred across the red blood cell membrane. Haemoglobin is the most important blood buffer therefore CO_2 capacitance is primarily a function of haematocrit. Carbon

Figure 1. General scheme for gas exchange at the gills. $\dot{V}b$ = cardiac output; $\dot{V}w$ = ventilatory water flow; CA= carbonic anhydrase. Arrows indicate countercurrent movement of water with respect to blood flow. Cooperativity of O_2 uptake with CO_2 excretion is clear as well as the relatively small contribution to CO_2 excretion from the plasma due to the uncatalysed dehydration of HCO_3^- versus the reaction in the red blood cell.


Vb
Blood
Gill
Water
O_2
CO_2 CA HCO_3^- + H^+ + NH_3 → NH_4^+
CO_2 ← H_2CO_3 ← H^+ + HCO_3^-
NH_3
Vw

dioxide excretion is not only complex due to chemical reactions at the gill and tissues but due to its involvement in acid-base balance.

Diffusion versus perfusion limitations

A concept that will be discussed repeatedly throughout this thesis is that of diffusion versus perfusion limited transfer. Diffusion limited gas transfer has low efficiency and occurs when capacitance for the gas in the blood is high. Efficiency in this context is determined by how closely and quickly gas partial pressures in lamellar blood come into equilibrium with the respiratory water. The term capacitance incorporates both the solubility of a particular gas in a liquid (plasma) and the additional carrying capacity of a respiratory pigment in the blood (haemoglobin). The gradient for exchange remains high across the gas exchange surface, however gas partial pressures change slowly and often do not reach equilibrium. Although improvements of diffusive properties will affect transfer, (decreasing diffusion distance, increasing surface area and increasing partial pressure gradients) changes in cardiac output will also hinder transfer. Perfusion limited gas transfer has a high efficiency and does not have as high a capacitance in the blood, therefore partial pressure in the blood increases quickly and soon reaches equilibrium. The amount of gas taken up depends only on the amount of blood in contact with the respiratory surface. However by definition, arterial blood gas tensions in perfusion-limited systems are insensitive to changes in cardiac output over the physiological range.

With these concepts in mind the following goals and hypotheses were set out for this thesis.

Goals

Record *in vivo* respiratory parameters in fish with varying degrees of gill surface area reduction and compare these results to those of sham ligated fish.

Observe the same parameters under hypoxic stress to see if fish compensate or are unable to cope with reductions in total surface area.

Test hypotheses: 1) that the gill is diffusion limited for CO_2 exchange and primarily, perfusion limited for O_2 exchange under normoxic conditions; 2) that fish with severe reductions in surface area will be unable to cope with environmental stress due to their inability to recruit further respiratory surface; 3) that CO_2 diffusion limitations will be overcome by injections of carbonic anhydrase by catalyzing the dehydration of HCO_3^- in the plasma.

CHAPTER 2

THE EFFECTS OF REDUCED GILL SURFACE AREA ON RESTING BLOOD GAS VARIABLES IN THE RAINBOW TROUT (*Oncorhynchus mykiss*)

Introduction

Previous work by Davis (1971) examined reductions of 40-57% of gill surface area by ligation of gill arches and its effects on oxygen uptake, ventilation and cardiac output. This qualitative study (due to low N numbers, insufficient to run statistical analyses) found that fish responded to decreases in surface area by increasing calculated cardiac output, ventilation volume and oxygen uptake rate, therefore increasing flow of blood and water past the respiratory exchange surface. Duthie and Hughes (1987) also studied reduction in gill surface area up to 30% by cauterizing gill arches. Their findings indicated no effects on oxygen consumption with reductions up to 30% under resting, normoxic conditions. Fish were exposed to swim trials and only at $\dot{V}O_2$ max was a difference observed from control values. While the two previous studies focused exclusively on the impacts of gill surface area reduction on O_2 transfer, the primary focus of this thesis was to assess the consequences on CO_2 transfer. Both theoretical and mathematical models for gas transfer predict that CO_2 transfer across the gills is limited by diffusion whereas the transfer of O_2 is thought to be predominantly limited by perfusion (Malte & Weber, 1985; Perry, 1986). Consequently changes in gill surface area, a diffusive property, should have a greater impact on CO_2 exchange. To this end, the combined effects on oxygen uptake and carbon dioxide excretion were evaluated using an extracorporeal blood loop, continuously monitoring arterial PO_2, PCO_2 and pH as well as other blood gas parameters analyzed through periodic blood sampling. As a result of diffusion limitations, it was predicted that $PaCO_2$ levels would be elevated in fish with ligated gill arches versus sham ligated fish and that no changes in PaO_2 would be detected between experimental and control groups. Elevation of CO_2 in the blood of

ligated fish would lead to increases in $[H^+]$ and consequently a decrease in pHa. It was also anticipated that fish with the greatest reduction in gill surface area would have the greatest difficulties when encountering an environmental stress such as hypoxia.

Materials and Methods

Experimental animals

Rainbow trout (*Oncorhynchus mykiss*) weighing 600.3 ± 40.4g, N=27, were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario). Fish were maintained in large fiberglass aquaria supplied with running, dechlorinated, city of Ottawa tap water at 14°C and on a 12h light: 12h dark photoperiod. Trout were fed to satiation on alternate days with commercial trout pellets until 24h prior to experimentation. All fish were allowed at least one week to acclimate to the holding conditions before any experiments were performed.

Surgical procedures

Fish were anaesthetized with 40 mg L^{-1} ethyl p-amino-benzoate (Sigma Chemical co.) (1g benzocaine dissolved in 10 ml 95% ethanol/ 25L water) and placed on a surgical table allowing continual flow of aerated anaesthetic solution over the gills. An indwelling cannula (Clay-Adams PE 50 polyethylene tubing) was implanted into the dorsal aorta (Soivio *et al*, 1975) for periodic blood sampling throughout the experiment. For continuous measurements of blood respiratory variables, a lateral incision (~2 cm in length) was made at the level of the caudal peduncle approximately 4 mm below the lateral line. The caudal vein and caudal artery were cannulated in orthograde and

retrograde directions, respectively (Clay-Adams PE 50 polyethylene tubing). The incision was sutured using a running stitch and both cannula were then secured to the body wall with silk ligatures.

Reduction of gill surface area

The gills were exposed by lifting the opercular flap. Gill arches were ligated by tying surgical silk (2-0) at their bases. Reductions of 15, 30 and 40% of total gill surface area were obtained by ligating a total of 1, 2 and 3 gill arches, respectively (see Davis, 1971 for estimates of total gill surface area). Ligation of the first gill arch was purposely avoided owing to the known presence of chemoreceptors on the first pair of arches (Daxboeck & Holeton, 1978; Burleson & Milsom, 1993).

Fish were revived by irrigating the gills with aerated water and later transferred into individual, opaque acrylic boxes supplied with running, aerated water. Fish were allowed to recover for 24h prior to experimentation.

Experimental protocol

Blood was monitored continuously for arterial O_2 tension (PaO_2), arterial CO_2 tension ($PaCO_2$) and arterial pH (pHa) using an extracorporeal blood shunt (Thomas, 1994). A peristaltic pump (flow = 0.6 ml min^{-1}) was used to withdraw blood from the dorsal aorta and pass it through a series of O_2, CO_2 and pH electrodes before returning it to the caudal vein. Immediately prior to experimentation, the extracorporeal shunt was rinsed for 15 – 20 min with a solution of ammonium heparin (540 units ml^{-1} in Cortland (Wolf, 1963) saline) to prevent blood from clotting in the tubing and electrode chambers.

Water PO_2 was monitored continuously by using a second peristaltic pump to pass water over an additional O_2 electrode. Analog signals were converted to digital data and collected and stored on a computer using a data acquisition system (Biopac) and accompanying software (AcqKnowledge 3.03).

Series 1- Exposure to a graded hypoxia after a reduction in gill surface area

After connecting the artificial blood shunt, it was allowed to run for ~20 min or until stable readings were obtained for all gas variables. Data were collected for a 20 min period prior to initiating hypoxia. After 10 min, an initial normoxic (control) blood sample was taken (0.8 ml) for measurement of arterial blood O_2 content (CaO_2), CO_2 content ($CaCO_2$), haematocrit (Hct), haemoglobin (Hb) concentration and catecholamine levels. At 20 min, hypoxia was initiated by substituting N_2 for air to a gas equilibration column that was delivering water to the fish. Blood samples of 0.8 ml were taken at approximately every 10 mm Hg (PaO_2) intervals until signs of struggling were observed. Water PO_2 was restored to normoxic levels and fish were allowed to recover.

Analytical techniques

In the extracorporeal shunt experiments, arterial blood pH, PCO_2 and PO_2 were monitored using Cameron Instruments Inc. (CO_2, O_2) and Metrohm (pH) electrodes housed in temperature controlled cuvettes and connected to a Radiometer PHM 73 meter. Water PO_2 was measured using an additional O_2 electrode connected to a dual channel O_2/CO_2 meter (Cameron Instruments). The O_2 electrodes were calibrated by pumping (using the peristaltic pump of the extracorporeal shunt) a zero solution (2% (w/v) sodium

sulfite) or air-saturated water continuously through the electrode sample compartments until stable readings were obtained. The CO_2 electrode was calibrated in a similar manner using mixtures of 0.5% and 1.0% CO_2 in air that were provided by a Cameron gas flowmeter. The pH electrode was calibrated using precision buffers. All electrodes were calibrated prior to each experiment.

Arterial blood samples (20μl) were analyzed in triplicate for oxygen content (CaO_2) using an Oxycon blood oxygen content analyzer (Cameron Instruments). Total CO_2 ($CaCO_2$) was analyzed in triplicate using true plasma (20μl) with a Capnicon carbon dioxide analyzer (Cameron Instruments). HCO_3^- concentrations were calculated by rearrangement of the Henderson-Hasselbalch equation

$pH = pK + \log [HCO_3^-]/\alpha CO_2 - PCO_2$ to give the following equation

$[HCO_3^-]$ = Total CO_2 – (αCO_2 * PCO_2). PCO_2 values were taken from acquisition files and constants from Boutilier *et al* (1984).

Haemoglobin concentration was determined in duplicate on 20μl blood samples using a commercial spectrophotometric haemoglobin assay kit (Sigma). Haematocrit was determined in duplicate by centrifuging microcapillary tubes at 5000 x g for 10 min.

Blood samples of 400 μl, collected for catecholamine measurements, were centrifuged immediately, the plasma was collected and placed in liquid N_2, then stored at -80°C for later analysis. Catecholamines were extracted using general methods by Woodward (1982) through HPLC analysis with electrochemical detection. An internal standard, 3,4-dihydroxybenzylamine was used in all samples analyzed. Detection limits for adrenaline and noradrenaline were 0.1 nmol l^{-1}.

Statistical analyses

All data are represented as means ± 1 SEM unless otherwise stated. Figures 2-1 – 2-3 represent mean continuous traces (compressed to 1 sample/minute) with the standard errors plotted at 10 Torr intervals. Data in these figures were analyzed using a two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison. Data from Figure 2-5 and Table 2-1 were analyzed using a one-way ANOVA followed by Dunnet's comparison with control value or Dunn's multiple comparison. P values < 0.05 were considered to be statistically significant. Calculations were performed using SigmaStat software package.

Results

Exposure of control (sham-ligated) and experimental (gill-ligated) fish to a graded external hypoxia resulted in an expected significant decrease in arterial PO_2 when compared to resting values (PwO_2 = 150 Torr; Figure 2-1). However below PwO_2=120 Torr, fish with three gill arches ligated exhibited a mean arterial PO_2 that was significantly lower than the corresponding control value; this trend continued until a PwO_2 of 70 Torr was reached. At PwO_2s of 70 and 80 Torr (moderate hypoxia), fish with one gill arch ligated had significantly lower PaO_2 values from corresponding control values.

Arterial PCO_2 (Figure 2-2) was not significantly affected by the ligation of one gill arch although there was an obvious trend for elevated $PaCO_2$ during normoxia and mild hypoxia. The trend became significantly different with the ligation of 2 gill arches where values of 2.92 ± 0.33 Torr – 2.71 ± 0.29 Torr (taken at PwO_2s of 160 and 80 Torr, respectively) were statistically significant from the corresponding control values of 1.87

± 0.18 Torr – 1.81 ± 0.17 Torr. Removal of three gill arches was without effect on $PaCO_2$, although similar to the fish with one arch ligated, there was a trend for higher PCO_2 levels.

The significant increases in arterial PCO_2 (Figure 2-2) were mirrored by statistically significant decreases in pHa (Figure 2-3) in trout with two gill arches ligated, falling from 7.79 ± 0.02 (control value at PwO2 of 160 and 90 Torr) to 7.68 ± 0.05 – 7.69 ± 0.04, the corresponding values for trout with two gill arches ligated.

It is evident from the pH-HCO_3^- diagram (Figure 2-4) that under normoxic conditions, fish with one and two gill arches ligated experienced a pronounced respiratory acidosis, denoted by the leftward position of these points along the non-bicarbonate buffer line. Fish with three gill arches ligated appear to have a combined respiratory acidosis, partially compensated by a metabolic alkalosis, indicated by movement along the curved isopleths.

Control *in vivo* catecholamine levels (Figure 2-5), remained relatively low throughout the graded hypoxia, reaching maximum levels of 31.5 ± 13.6 mmol l^{-1} at PaO_2s ranging between 40-0 Torr. Trout with one gill arch ligated displayed a significant increase in catecholamines from resting and control values, reaching 359.0 ± 137.0 mmol l^{-1} at PaO_2s between 40 and 0 Torr whereas trout with two gill arches ligated showed no significant increase from control values or from resting values. Trout with three gill arches ligated had significantly elevated catecholamine levels from both control and resting values at PaO_2s ranging between 80-40 Torr, with total catecholamines of 191.7 ± 65.7 mmol l^{-1}.

Table 2-1 summarizes the respiratory variables for trout with reduced gill surface area as well as control fish under normoxic conditions. PaO_2, [haemoglobin], [noradrenaline], [adrenaline], haematocrit and PwO_2 were consistent between control and experimental fish. Although there appeared to be a trend for decreasing PaO_2 in the fish with reduced gill surface area, the high degree of variability in the data sets prevented statistical confirmation ($P > 0.23$). $PaCO_2$ was significantly elevated and pHa significantly lowered in fish with two gill arches ligated. There was a trend for decreasing arterial oxygen content but this was not statistically different.

Figure 2-1. The effects of graded external hypoxia on arterial PO_2 (Torr) in control trout (N=8; black circle); trout with 1 gill arch ligated (N=6; gray circle); trout with two gill arches ligated (N=7; open circle) and trout with 3 gill arches ligated (N=6; open triangle). All values are presented as means ± 1SEM. * denotes a statistically significant difference ($P<0.05$) from resting PaO_2 values. † indicates a statistically significant difference ($P<0.05$) from the control value.

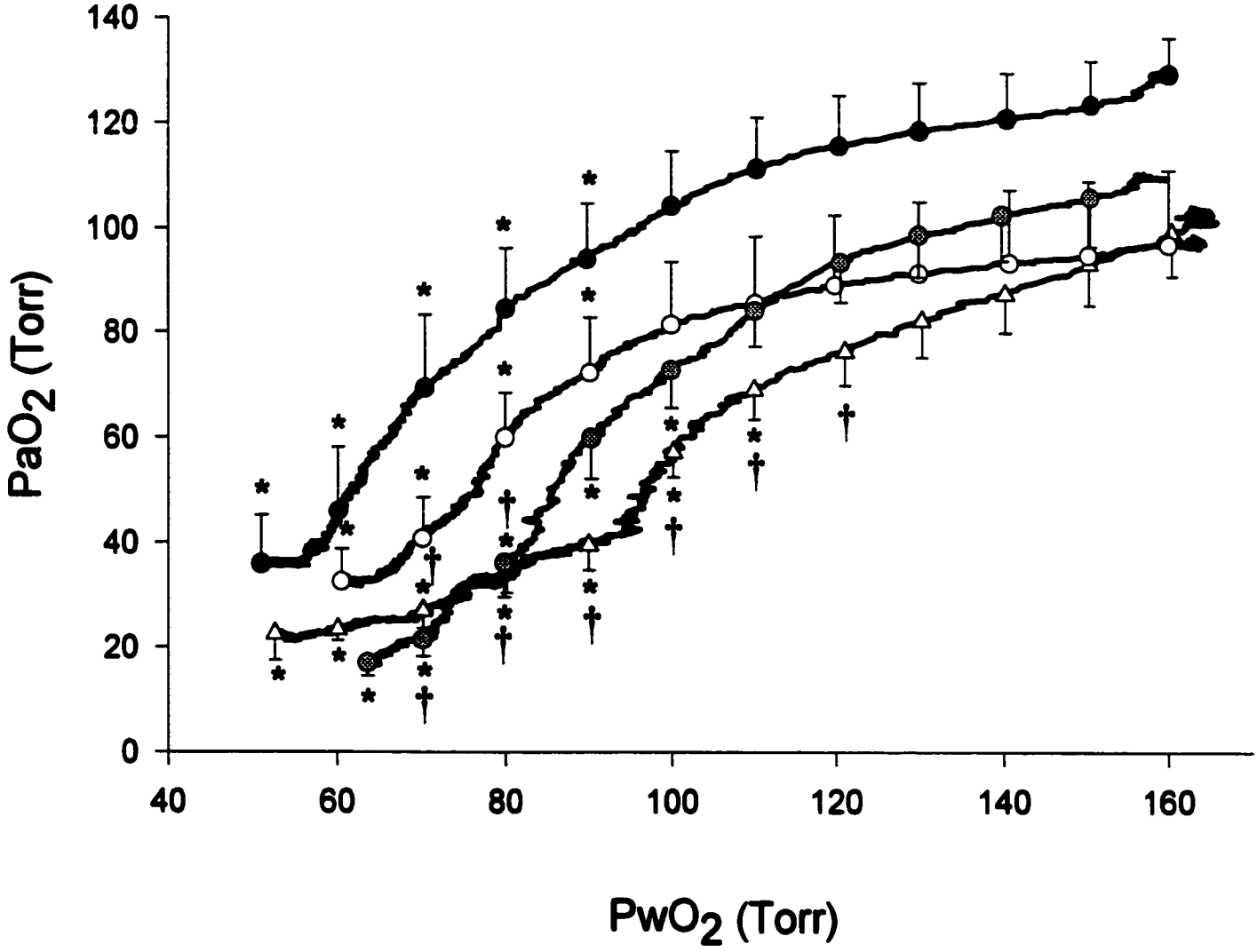

140
120
100
80
60
40
20
0
40
60
80
100
120
140
160
PaO2 (Torr)
PwO2 (Torr)

Figure 2-2. The effects of graded external hypoxia on arterial PCO_2 (Torr) in control trout (N=8; black circle); trout with 1 gill arch ligated (N=6; gray circle); trout with two gill arches ligated (N=7; open circle) and trout with 3 gill arches ligated (N=6; open triangle). All values are presented as means ± 1SEM. * denotes a statistically significant difference ($P<0.05$) from resting $PaCO_2$ values. † indicates a statistically significant difference ($P<0.05$) from the control value.

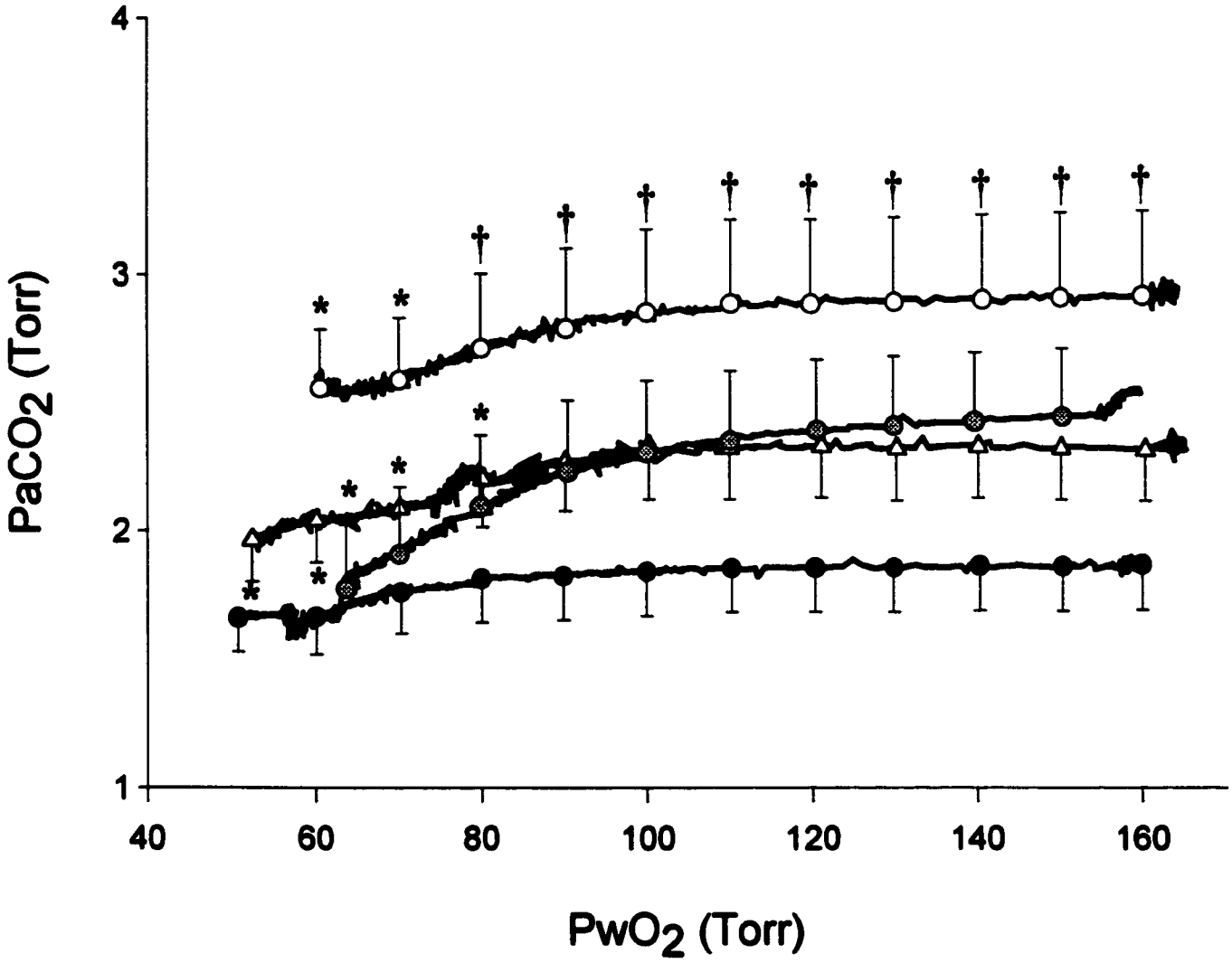

4
3
2
1
40
60
80
100
120
140
160
PaCO2 (Torr)
PwO2 (Torr)

Figure 2-3. The effects of a graded external hypoxia on arterial pH in control trout (N=8; black circle); trout with 1 gill arch ligated (N=6; gray circle); trout with two gill arches ligated (N=7; open circle) and trout with 3 gill arches ligated (N=6; open triangle). All values are presented as means ± 1SEM. † indicates a statistically significant difference ($P<0.05$) from the control value.

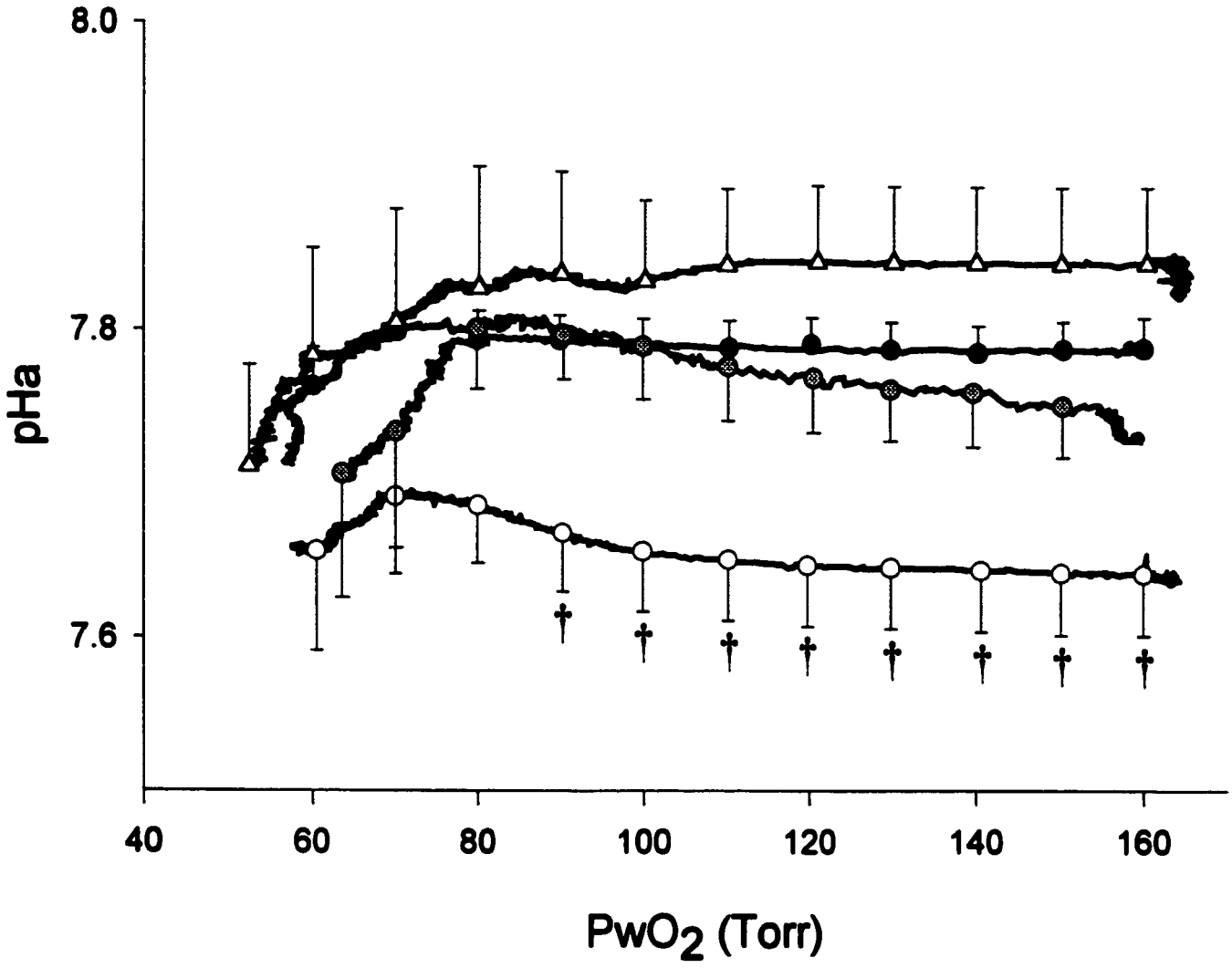

8.0
7.8
7.6
pHa
40
60
80
100
120
140
160
PwO2 (Torr)

Figure 2-4. A pH-HCO_3^- diagram depicting the whole blood acid-base status of trout subjected to sham ligation (N=8; black circle); 1 gill arch ligated (N=6; gray circle); 2 gill arches ligated (N=7; open circle) and 3 gill arches ligated (N=6; open triangle). Values are shown as means ± 1SEM. The dashed line represents the in-vitro non-bicarbonate buffer line (Wood et al., 1982). While the curved isopleths represent the bicarbonate buffering capacity of the blood. Movement of the experimental points along the buffer line represents a respiratory acidosis (↑ in $[HCO_3^-]$ and a ↓ in pHa).

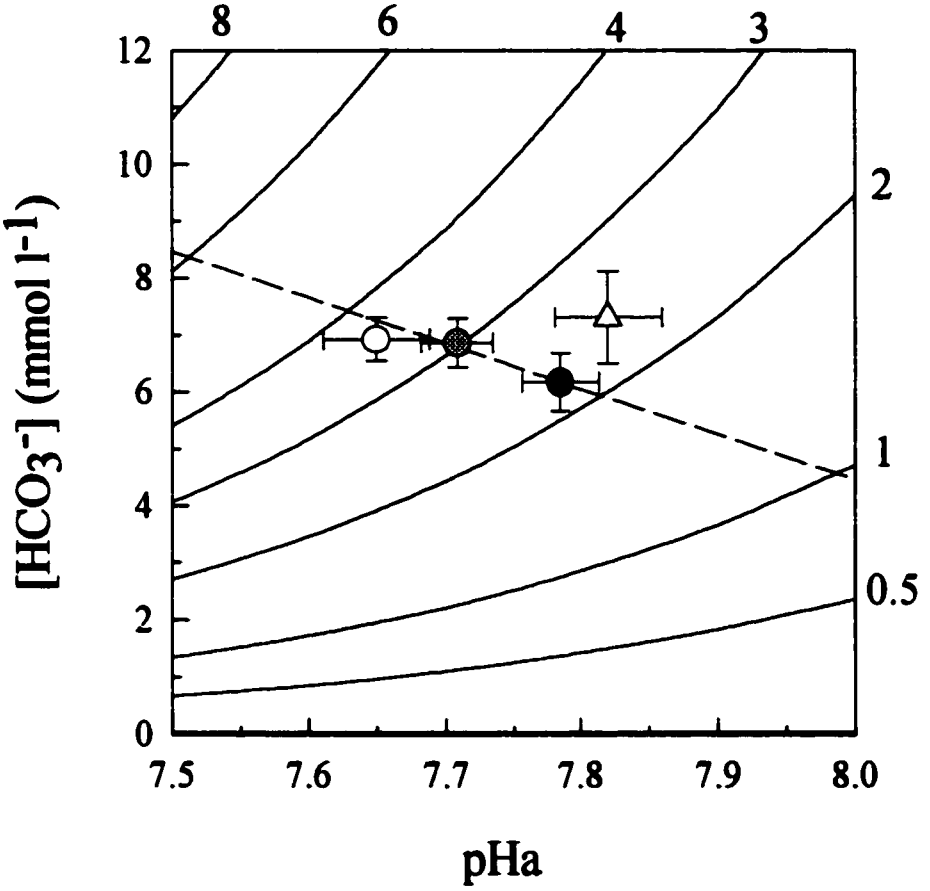

8
6
4
3
2
1
0.5
12
10
8
6
4
2
0
7.5
7.6
7.7
7.8
7.9
8.0
[HCO3-] (mmol l-1)
pHa

Figure 2-5. Total in vivo catecholamines (nmol l^{-1}) during a graded hypoxia in control trout (N=8; black bars); trout with 1 gill arch ligated (N=6, open bars); trout with 2 gill arches ligated (N=7; gray bars) and trout with 3 gill arches ligated (N=6; hatched bars). Values represent means ± 1SEM. * denotes a statistically significant difference ($P<0.05$) from resting PaO_2 values. † indicates a statistically significant difference ($P<0.05$) from the control value.

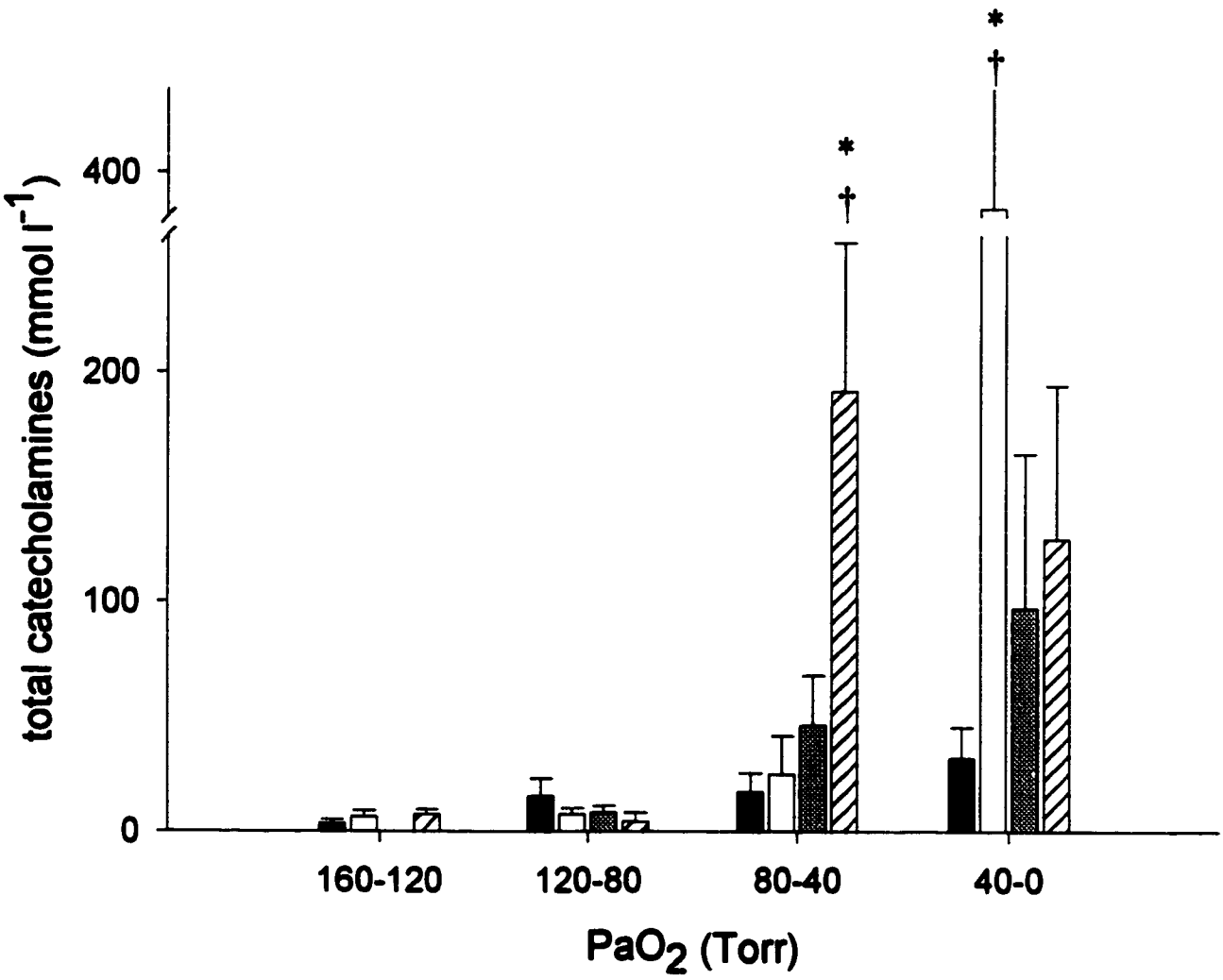

total catecholamines (mmol l-1)
400
200
100
0
*
†
*
†
160-120
120-80
80-40
40-0
PaO2 (Torr)

Table 2-1: *The effects of gill surface area reduction on selected arterial blood respiratory variables in rainbow trout (oncorhynchus mykiss), under normoxic conditions.*

Parameter	Control (n=7)	1 gill arch ligated (n=6)	2 gill arches ligated (n=7)	3 gill arches ligated (n=6)
PaO_2 (Torr)	122.9 ± 12.2	104.3 ± 10.3	103.6 ± 6.5	105.0 ± 8.4
$PaCO_2$ (Torr)	1.97 ± 0.13	2.59 ± 0.20	2.95 ± 0.33 *	2.39 ± 0.21
pHa	7.78 ± 0.03	7.71 ± 0.02	7.65 ± 0.04 *	7.82 ± 0.04
CaO_2 (mmol l^{-1})	3.25 ± 0.35	3.27 ± 0.48	2.79 ± 0.47	2.15 ± 0.54
Hb (g Hb 100 ml^{-1})	4.46 ± 0.24	3.04 ± 0.21	4.82 ± 0.37	4.17 ± 0.97
[HCO_3-] (mmol l^{-1})	6.17 ± 0.51	6.86 ± 0.43	6.70 ± 0.38	7.31 ± 0.80
[Noradrenaline] (nmol l^{-1})	0.9 ± 0.3	1.3 ± 0.5	2.7 ± 1.2	1.2 ± 0.5
[Adrenaline] (nmol l^{-1})	3.8 ± 1.3	5.3 ± 1.7	6.9 ± 3.0	5.6 ± 2.2
haematocrit (%)	20.9 ± 0.8	25.3 ± 2.0	22.6 ± 1.0	18.6 ± 2.5
PwO_2 (Torr)	165.6 ± 1.9	157.4 ± 7.7	171.9 ± 2.7	170.6 ± 6.3

All data represent means ± SEM

* denotes a statistically significant difference ($P<0.05$) from control values.

Discussion

Under normoxic conditions (water PO_2s above 120 Torr), oxygen transport was determined to be a perfusion-limited system in all treatments (ligated and sham ligated fish). Similar results were obtained with the use of a saline perfused trout head preparation by Part *et al.* (1984) and with the use of a blood perfused trout preparation by Daxboeck *et al.* (1982). Both studies concluded that the trout gill is strictly perfusion-limited owing to constant post-branchial PO_2 values following increases in flow rate. Fish subjected to a 15% reduction in total gill surface area (one gill arch ligated) in the present study, had significantly lowered PaO_2 values from the corresponding control values at water PO_2s between 80 and 70 Torr (moderate hypoxia). This may imply that fish with only a 15% reduction in total surface area are not actively compensating for this reduction and are simply following known responses to hypoxia. These responses, mediated by O_2 receptors result in increased stroke volume, increased dorsal aortic and ventral aortic blood pressure as well as an increase in ventilatory amplitude and frequency (Holeton & Randall, 1967a,b; Fritsche & Nilsson, 1989, 1993; Nonnotte et al., 1993). It is possible that these known strategies were insufficient to compensate for both an environmental hypoxia and a disruption in total respiratory surface area. The metabolic costs for a further increase in any of these parameters may not be favorable after only a 15% reduction in gill surface area if PaO_2s can be maintained comparable to control values at water PO_2s above 80 Torr. However, significant elevations in circulating catecholamines at the corresponding PaO_2s (below 40 Torr) were observed in fish with a 15% reduction in gill surface area and may signify recruitment of gill surface area (previously un-perfused lamellae) by means of adrenergic receptors, a common

response to hypoxia (Fritsche & Nilsson, 1994). The performance of fish with two gill arches ligated, or a 30% reduction in gill surface area, maintaining comparable arterial PO_2s to control values throughout the hypoxic bout may be due to changes in overall perfusion of the gill as discussed by Davis (1971). Although cardiovascular parameters were not examined in this thesis, these parameters may be involved in optimizing perfusion of the remaining respiratory surface and allow these fish to maintain optimal O_2 tensions in the blood; strategies such as increases in blood pressure or cardiac output would decrease residence times of red blood cells in the gills thereby maintaining a greater driving force for diffusion of O_2 into the blood. In a perfusion-limited system such as O_2 transfer, physiological changes in cardiovascular parameters (i.e. cardiac output; $\dot{V}b$) do not affect arterial gas tension, however they do maintain favourable conditions for gas exchange. These changes do however have a great impact on diffusion limited systems as will be discussed with respect to CO_2 transfer. Severe reductions following ligation of 3 gill arches, or a reduction of 40% of total gill surface area, indicate that O_2 transfer as a system can become solely dependent on diffusion following exposure to mild hypoxia. PaO_2 levels were statistically lowered from control levels following a decrease in water PO_2 past 120 Torr. Therefore from this point onwards only changes to diffusion distance, surface area, and partial pressure gradients would alleviate lowered oxygen tensions in the blood. To this end, fish experiencing severe reductions in gill surface area significantly increase circulating catecholamines (see Figure 2-5) at the corresponding arterial PO_2s (between 80 and 40 Torr) to increase functional gill surface area and decrease diffusion distance. In view of these opposing ideas, it can be theorized that oxygen transfer is predominantly perfusion limited. Under extreme conditions it may

extreme conditions it may display aspects of a diffusion limited system however it is not as sensitive to these limitations as CO_2 transfer.

Carbon dioxide excretion, as predicted, demonstrated clear diffusion limitations as illustrated by increases in arterial PCO_2 in fish with a 15% reduction in surface area and further statistically significant increases in $PaCO_2$ in fish with a 30% reduction in gill surface area from control values. Bindon *et al.* (1994) and Greco *et al.* (1996) predicted and observed diffusion limitations for carbon dioxide excretion due to proliferation of chloride cells. This proliferation lead to increased diffusion thickness and impairment of gas transfer. A similar study by Perry *et al.* (1996a) found lowered PaO_2 values in fish with soft water exposure (increased diffusion thickness from chloride cell proliferation), but the blood saturation and O_2 content remained stable. $PaCO_2$ levels were unchanged, but this study concluded that the resulting hyperventilation allowed for constant removal of CO_2. What may be occurring in the present study as mentioned above for O_2 transport after a 30% reduction, is increased perfusion of the gill by increasing blood pressure, cardiac output and thereby increasing the amount of blood perfusing the respiratory surface and decreasing residence time in the gills. Before CO_2 can be excreted into the respiratory water, bicarbonate (HCO_3^-) the preferred form of carbon dioxide in gill plasma must be dehydrated in the red blood cell in the presence of the enzyme carbonic anhydrase and with the use of a Bohr proton for the dehydration. Clearly this process is compromised by increasing the flow of blood past the gills and decreasing the length of time red blood cells are in contact with the respiratory surface. Several studies have investigated this limitation (Perry & Gilmour, 1993; Perry *et al.*, 1996b; Brauner *et al.*, 1996) concluding that the release of Bohr protons through oxygenation of haemoglobin -

the Haldane effect, is one of the key limiting factors of carbon dioxide excretion. To further complicate the scenario fish with a 40% reduction in gill surface area were able to maintain $PaCO_2$ levels similar to fish with a 15% reduction in gill surface area, therefore, lower than fish with 30 % reduction in surface area and not significantly elevated from control values. Hyperventilation, as described by Perry *et al.* (1996a) may be a compensatory factor used by these fish to deal with diffusion limitations of O_2 transfer as well as CO_2 excretion and will be assessed in Chapter 3.

It is clear from the acid-base status of the control and experimental groups that fish with 3 gill arches ligated (40% reduction) are compensating in a different manner than fish with a 15 or 30% reduction. From the pH/HCO_3^- diagram under normoxic conditions, fish with a 15 and 30% reduction in gill surface area demonstrate a respiratory acidosis 24 hours post-ligation however fish with a 40% reduction in gill surface area exhibit a combined respiratory acidosis and metabolic alkalosis. This is comparable to acid-base status in fish exposed to external hypercapnia (Cameron, 1978; Clairborne & Heisler, 1984; Thomas; 1983; Heisler, 1993) where increases in external PCO_2 were mirrored by increases in arterial PCO_2 and decreases in arterial pH. These changes in acid base status are compensated for by increasing plasma $[HCO_3^-]$ to minimize the decrease in arterial pH over a period of hours to days.

It is clear that an intricate network of inputs regulate gas exchange under normal resting conditions as well as under constrained, stressful conditions. These components will be further discussed in the following chapter as well as possible mechanisms to alleviate diffusional constrains put on carbon dioxide excretion.

CHAPTER 3

CONSEQUENCES OF ARTIFICIAL REDUCTION OF GILL SURFACE AREA IN RAINBOW TROUT: CAN LIMITATIONS TO GAS TRANSFER BE REVERSED ?

Introduction

Findings in Chapter 2 demonstrated that CO_2 transfer across the gill was more sensitive to reductions in surface area than was O_2 transfer. Thus, CO_2 excretion exhibits greater diffusion limitations than does O_2 uptake, despite the more rapid rate of CO_2 diffusion across biological membranes. In spite of a presumed, significantly greater branchial permeation coefficient (Krogh's permeation coefficient; K) for CO_2 than O_2, it appears the ability of lamellar blood to achieve CO_2 equilibrium with the external water may be constrained by several factors that are unrelated to CO_2 diffusion *per se*. These factors include i) the high capacitance of blood for CO_2 (Swenson, 1990), ii) the reliance of CO_2 excretion on the oxygenation of haemoglobin [the Haldane effect (Perry and Gilmour, 1993; Brauner and Randall, 1998; Brauner *et al.*, 1996], iii) the low driving force (blood-to-water PCO_2 difference) for trans-branchial diffusion of CO_2, and iv) the requirement to convert plasma HCO_3^- to CO_2 during blood transit through the gill circulation. The last point is extremely significant because the majority of CO_2 excreted across the gill must first be derived from the dehydration of plasma HCO_3^- within a very brief period (estimated transit time of blood within the gill = 1 – 3 sec). The presence of carbonic anhydrase (CA) within the red blood cell ensures that the dehydration reaction proceeds at a non-limiting catalysed rate (see reviews by Perry and Laurent, 1990; Henry and Heming, 1998). However, the availability of plasma HCO_3^- to CA is limited by its relatively slow entry *via* electroneutral Cl^-/HCO_3^- exchange (Cameron, 1978; Romano and Passow, 1984) into the red blood cell. Indeed, the entry of HCO_3^- into the rbc via Cl^-/HCO_3^- exchange is thought to be the rate-limiting step in CO_2 excretion in teleost fish (Perry, 1986; Tufts and Perry, 1998). Thus, the relatively slow rate of Cl^-/HCO_3^-

exchange, coupled with the requirement of the Haldane effect to furnish Bohr protons, effectively constrains the conversion of HCO_3^- to CO_2 as blood flows through the gill. These constraints lower the effective period for CO_2 diffusion and are believed to be the cause for apparent diffusion limitations for CO_2 transfer across the gill (Swenson, 1990).

With these factors in mind, the prevailing question for this chapter was: can the apparent diffusion limitations on CO_2 excretion be reduced or obliterated by accelerating the rate of plasma HCO_3^- dehydration with the administration of intravascular bovine carbonic anhydrase injections? At the same time, questions concerning the overall status of these fish in terms of oxygen uptake rates, carbon dioxide excretion rates, ventilation flow rates and convection requirements were assessed. Intuitively, one would expect ventilation rates to increase as soon as surface area was compromised. Similarly, convection requirements would be expected to increase. However, convection requirements in fish are already maintained significantly higher than air-breathing vertebrates due to the low oxygen content in water, therefore it may not be beneficial to further increase metabolic costs to maintain constant tensions of O_2 and CO_2. These parameters were calculated from inspired/expired water CCO_2 differences and PO_2 differences as well as in-flowing/out-flowing water CCO_2 and PO_2 differences. Carbonic anhydrase injections were performed on ligated and sham ligated fish and a third group, ligated fish with saline injection was used as a second control group. Arterial PCO_2 levels were expected to decrease in ligated fish after carbonic anhydrase injection by facilitating the dehydration of HCO_3^- to CO_2 in the plasma.

Materials and Methods

Experimental animals

Rainbow trout (*Oncorhynchus mykiss*) weighing 529.5 ± 33.8g, N = 21, were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario). An additional group of smaller trout weighing 235.1 ± 3.9g, N = 34, were used for experiments that measured ventilation. Fish were maintained in large fiberglass aquaria supplied with running, dechlorinated, city of Ottawa tap water at 14°C and on a 12h light: 12h dark photoperiod. Trout were fed to satiation on alternate days with commercial trout pellets until 24h prior to experimentation. All fish were allowed at least one week to acclimate to the holding conditions before any experiments were performed.

Surgical procedures

Fish were anaesthetized with 40 mg L^{-1} ethyl p-amino-benzoate (Sigma Chemical co.) (1g benzocaine dissolved in 10 ml 95% ethanol/ 25L water) and placed on a surgical table allowing continual flow of aerated anaesthetic solution over the gills. For continual measurements of blood respiratory variables, a lateral incision (~2 cm in length) was made at the level of the caudal peduncle below the lateral line and both the caudal vein and artery were cannulated in orthograde and retrograde directions respectively (Clay-Adams PE 50 polyethylene tubing). The incision was sutured using a running stitch and both cannula were then secured to the body wall with silk ligatures.

Ventilation and convective requirements

To calculate ventilation volume and to estimate ventilatory convection requirements, an additional series of experiments was performed. For these experiments, measurement of inspired and expired water PO_2 differences and total CO_2 (CCO_2) differences as well as in-flowing and out-flowing water PO_2 and CCO_2 differences were made. Inspired water was sampled using a heat-flared PE 160 cannula, that was inserted and secured in the buccal cavity. To sample expired water, a small hole was drilled on either opercular flap using an 18 gauge needle, into which hear-flared PE 160 tubing was inserted; the cannula were secured with silk thread. In-flowing/out-flowing water was sampled though PE 160 tubing inserted into the inflow and outflow of the plexiglass box containing the experimental fish.

Artificial reduction of gill surface area

The gills were exposed by lifting the opercular flap. Gill arches were ligated by tying surgical silk (2-0) at their bases. Reductions of 15 or 30% of total gill surface area were obtained by ligating 1 or 2 of the second pair of gill arches, respectively (see Davis, 1971 for estimates of total gill surface area). Ligation of the first gill arch was purposely avoided owing to the known presence of chemoreceptors on the first pair of arches (Daxboeck & Holeton, 1978; Burleson & Milsom, 1993).

Fish were revived by irrigating the gills with aerated water and later transferred into individual, opaque acrylic boxes supplied with running, aerated water. Fish were allowed to recover for 24 h prior to experimentation.

Experimental protocol

Blood was monitored continuously for arterial O_2 tension (PaO_2), arterial CO_2 tension ($PaCO_2$) and arterial pH (pHa) using an extracorporeal blood shunt (Thomas, 1994). A peristaltic pump (flow = 0.6 ml min^{-1}) was used to withdraw blood from the dorsal aorta and pass it through a series of O_2, CO_2 and pH electrodes before returning it to the caudal vein. Immediately prior to experimentation, the extracorporeal shunt was rinsed for 15 – 20 min with a solution of ammonium heparin (540 units ml^{-1} in Cortland (Wolf, 1963) saline) to prevent blood from clotting in the tubing and electrode chambers. Water PO_2 was monitored continuously by using a second peristaltic pump to pass water over an additional O_2 electrode. Analog signals were converted to digital data and collected and stored on computer using a data acquisition system (Biopac) and accompanying software (AcqKnowledge 3.03).

Series 1 – Estimates of ventilation volume

Two peristaltic pumps were used to provide flowing water to two O_2 electrodes. One electrode received either inspired water from the buccal cannula or expired water from the opercular cannula; the second electrode received either inflowing or outflowing water. Under normoxic conditions, inspired and inflowing water were monitored for ~10 minutes or until stable readings were achieved. At this point a mean PO_2 value was determined (derived from 5 min of stable recording) and a water sample (1 ml) was taken to measure CCO_2. Sampling was then switched to expired and outflowing water by means of a series of 3-way valves and PO_2's were monitored until a new equilibrium was achieved; additional water samples were taken for measurement of CCO_2. Preliminary

experiments were performed under hypoxic conditions but owing to hyperventilation, it was not feasible to accurately measure inspired – expired PO_2 differences.

Oxygen consumption ($\dot{M}O_2$), carbon dioxide excretion ($\dot{M}CO_2$), ventilation volume ($\dot{V}w$), ventilatory convection requirements and the respiratory exchange ratio (Re) were calculated using the following formulas.

$\dot{M}O_2$ = water flow rate * $(P_IO_2 - P_OO_2)\ \alpha O_2$

$\dot{M}CO_2$ = water flow rate * $(C_OCO_2 - C_ICO_2)$

Ventilatory water flow ($\dot{V}w$) was calculated using two different formulas:

i) $\dot{V}w = \dot{M}O_2 / (P_iO_2 - P_eO_2)\ \alpha O_2$, and

ii) $\dot{V}w = \dot{M}CO_2 / C_eCO_2 - C_iCO_2$

An average was taken from the two calculated values

O_2 ventilatory convection requirement = $\dot{V}w / \dot{M}O_2$

CO_2 ventilatory convection requirement = $\dot{V}w / \dot{M}CO_2$

$Re = \dot{M}CO_2 / \dot{M}O_2$

Where the subscripts I, i, e, and O represent in-flowing, inspired, expired and out-flowing water, respectively. CCO_2 represents total CO_2 content and αO_2 is O_2 solubility coefficient in fresh water (from Boutilier *et al.*, 1984).

Series 2- Injection of CA into fish with reduced gill surface area

Experiments were performed on fish that were subjected to a sham ligation or a 30% reduction in gill surface area. After connecting the extracorporeal blood shunt, it

was allowed to run for ~20 min or until stable readings were obtained for all blood gas variables. After 10 min of data recording, fish were injected (1 ml kg^{-1} via the caudal vein cannula) with either Cortland saline (ligated fish) or bovine CA [(5 mg kg^{-1}) ligated and sham ligated fish]. Data were recorded for 120 min post injection.

Analytical analysis

Water samples taken from inspired, expired, in-flowing and out-flowing water were analyzed in triplicate for total CO_2 using a Capnicon carbon dioxide analyzer. Larger volumes of 40µl were necessary for the analysis of water samples. Data were then combined with water PO_2 differences to calculate water ventilatory convection requirements using the formulas described in series 2.

Statistical analysis

All data were presented as means ± 1 SEM unless otherwise noted. Absolute changes in PaO_2, $PaCO_2$ and pHa seen in Figures 3-2, 3-4 and 3-6, respectively were calculated by assigning a value of 0 to the injection point (10 min value) and subtracting this value from all pre and post-injection values. Data in Table 3-1 were analyzed using a one-way analysis of variance followed by Dunnett's comparison to control values. All remaining data were analyzed using a two-way ANOVA followed by Tukey's multiple comparison. P values of < 0.05 were considered to be statistically significant. Calculations were performed using SigmaStat software package.

Results

Oxygen consumption ($\dot{M}O_2$), carbon dioxide excretion ($\dot{M}CO_2$) and the respiratory exchange ratio (Re) remained constant between control and all experimental groups (Table 3-1). However, there was a dramatic elevation in ventilatory water flow ($\dot{V}w$) in trout with three gill arches ligated (4463.3 ± 1303.2 ml/kg/min) compared with the control value (1186.5 ± 188.4 ml/kg/min) as well as an increase in the ventilatory convection requirement for oxygen, from 41.0 ± 9.2 L/mmol in control fish to 106.8 ± 23.2 L/mmol in fish with three gill arches ligated. The ventilatory convection requirement for carbon dioxide appeared elevated in trout with three arches ligated (100.0 ± 26.0 L/mmol) from the control value (42.2 ± 7.6 L/mmol) but no statistical difference was found.

There were no effects on PaO_2 after carbonic anhydrase injection in either sham ligated or ligated fish (Figure 3-1) and no effects of saline injection on ligated fish. When overall changes in PaO_2 before and after injection were calculated there was no effect of either carbonic anhydrase injection, saline injection or effect of ligation over time or between treatments (Figure 3-2).

Injection of carbonic anhydrase (CA) into fish with two ligated gill arches did significantly decrease arterial PCO_2 (Figure 3-3) from 3.33 ± 0.08 Torr (pre-injection point) to 2.49 ± 0.35 Torr (final 130 minute point). Sham ligated with CA injection and ligated fish with saline injection remained constant following injection and for the duration of the experiment. When overall changes were examined (Figure 3-4), not only was $PaCO_2$ dramatically lowered in ligated fish after CA injection but values 60 minutes

injection and ligated fish with saline injection. There were no changes over time or due to injection in either sham, CA injected or ligated, saline injected group.

Injection of CA into fish with 2 gill arches ligated resulted in a significant increase in arterial pH 70-85 minutes after injection (Figure 3-5) followed by a gradual decrease in pHa towards control values. Sham ligated with CA injection and ligated fish with saline injection remained constant following injection and for the duration of the experiment. After calculations of overall changes in pHa (Figure 3-6), fish with two arches ligated after CA injection showed significantly elevated pHa levels from pre-injection values between 70 and 85 minutes post-injection as well as significantly elevated pHa levels from saline injected fish between 60 and 100 minutes post injection. There were no changes over time or due to injection in either sham, CA injected or ligated, saline injected group.

Table 3-1: *Selected ventilatory convection requirements for rainbow trout with 1, 2 and 3 gill arches ligated and sham ligated(control).*

	$\dot{M}O_2$ mmol/kg/h	$\dot{V}w$ ml/kg/min	$\dot{V}w/\dot{M}O_2$ L/mmol	$\dot{M}CO_2$ mmol/kg/h	$\dot{V}w/\dot{M}CO_2$ L/mmol	Re
Control (n=8)	2.3 ± 0.3	1186.5 ± 188.4	41.0 ± 9.2	2.0 ± 0.4	42.2 ± 7.6	0.9 ± 0.2
1 gill arch ligated (n=8)	2.55 ± 0.56	2793.3 ± 882.9	74.8 ± 25.7	2.9 ± 0.9	85.4 ± 31.2	1.1 ± 0.4
2 gill arches ligated (n=10)	1.2 ± 0.2	1032.3 ± 212.8	60.9 ± 10.7	2.2± 0.4	37.6 ± 9.8	1.7 ± 0.6
3 gill arches ligated (n=8)	2.6 ± 0.7	4463.3 ± 1303.2*	106.8 ± 23.2*	3.0 ± 0.4	100.0 ± 26.0	1.4 ± 0.3

$\dot{M}O_2$, oxygen consumption; $\dot{V}w$, ventilatory water flow; $\dot{V}w/\dot{M}O_2$, ventilatory convection requirement for O_2; $\dot{M}CO_2$, carbon dioxide excretion; $\dot{V}w/\dot{M}CO_2$, ventilatory convection requirement for CO_2; $Re = \dot{M}CO_2 / \dot{M}O_2$, respiratory exchange ratio. Values are means ± SEM.

* indicates a statistically significant difference from the control value

Figure 3-1. Arterial PO_2 (Torr) over time (minutes) in trout sham ligated after a bolus carbonic anhydrase injection (5mg/kg) N=7, black circles; trout with 2 gill arches ligated after a bolus CA injection, N=8, open circles; and trout with 2 gill arches ligated after a bolus saline injection, N=6, gray circles. Point of injection is indicated with the vertical dotted line. Values represent means ± 1SEM.

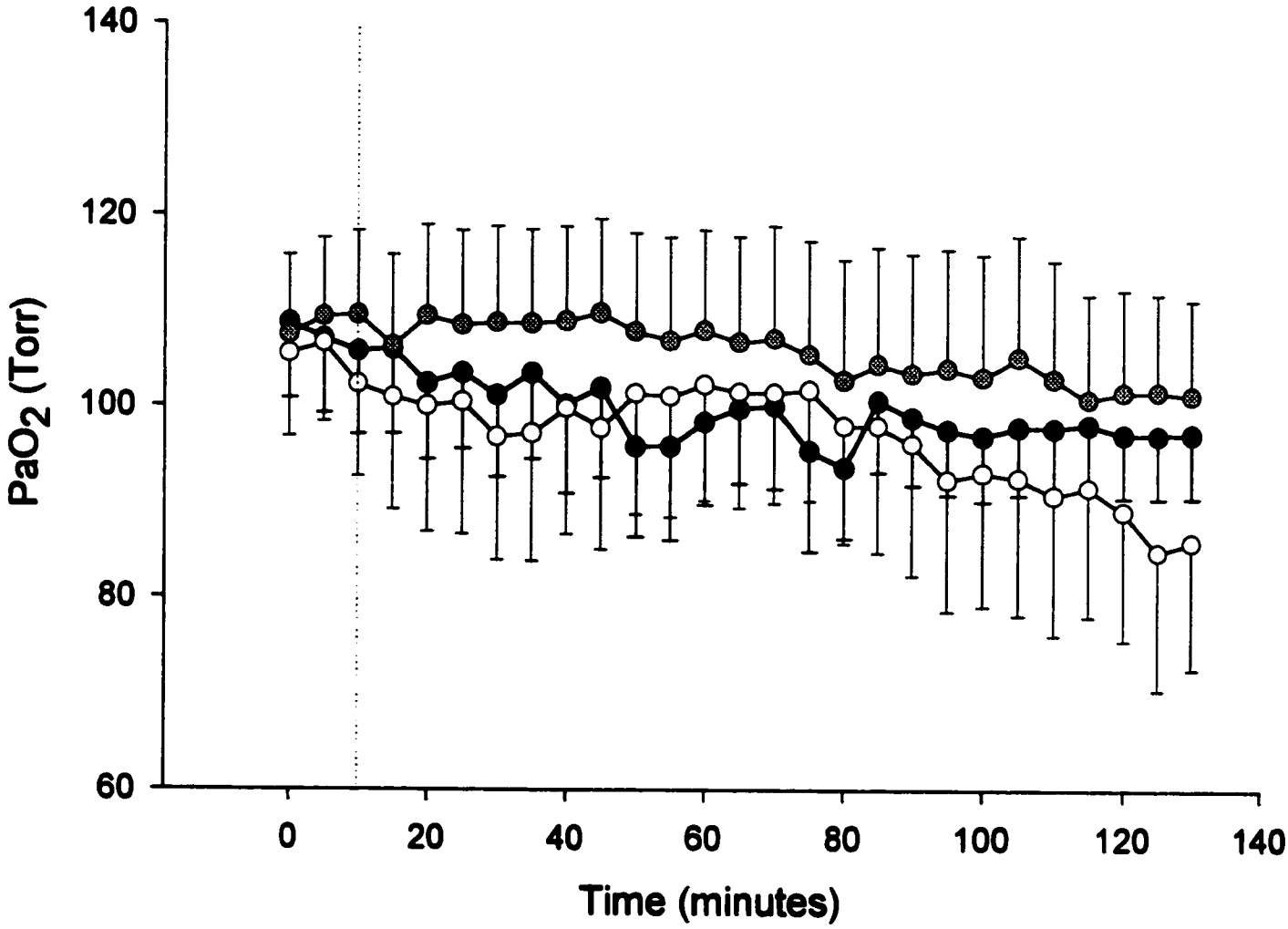

140
120
100
80
60
PaO2 (Torr)
0
20
40
60
80
100
120
140
Time (minutes)

Figure 3-2. Changes in arterial PO_2 (Torr) over time (minutes) in trout sham ligated after a bolus carbonic anhydrase injection (5mg/kg) N=7, black circles ; trout with 2 gill arches ligated, after a bolus injection of CA N=8, open circles; and trout with 2 gill arches ligated after a bolus saline injection N=6, closed circles. Injection point is indicated by the dotted vertical line and was assigned a value of zero to calculate overall changes before and after injection. Data points represent means ± 1SEM.

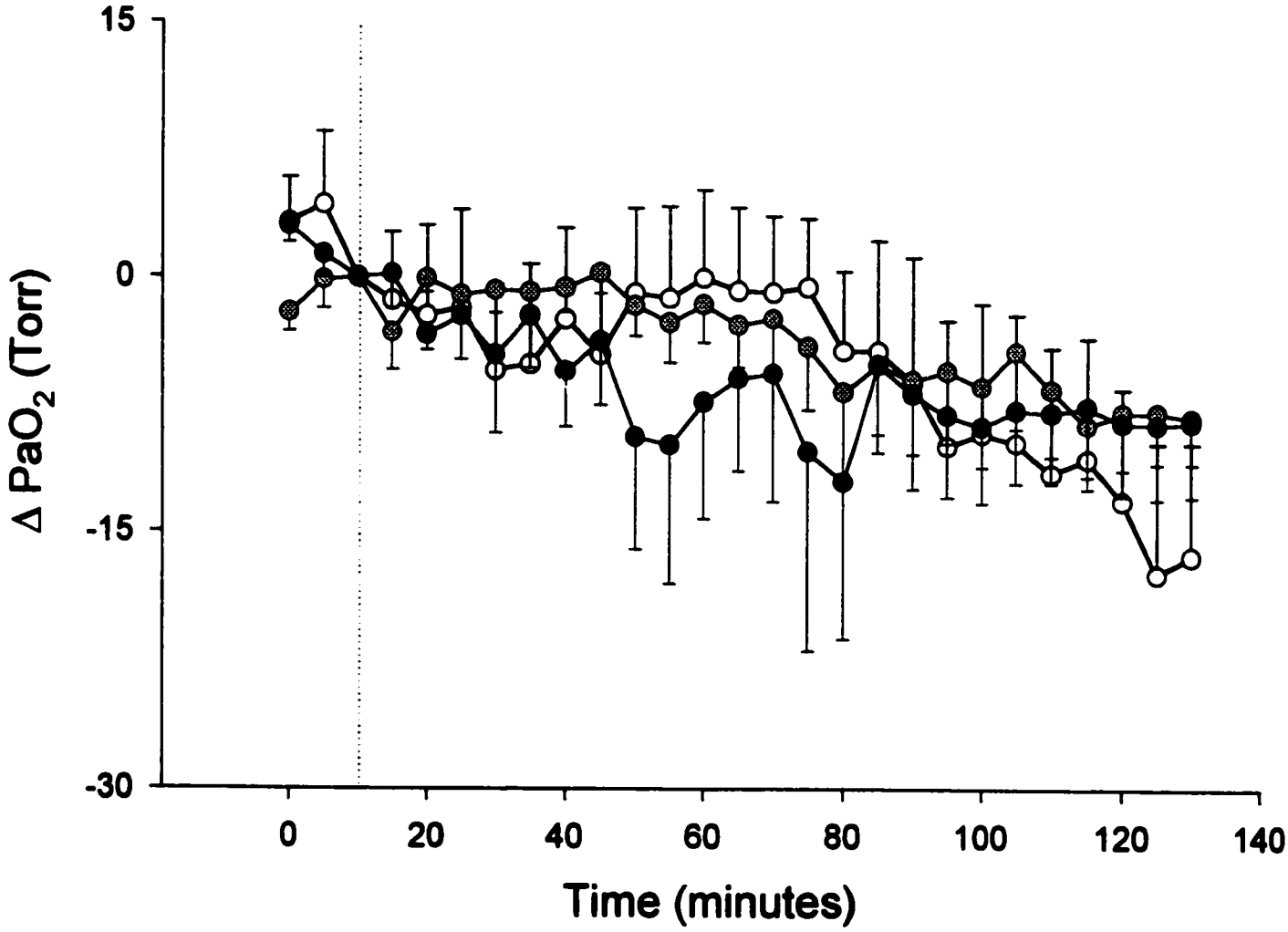

15
0
-15
-30
Δ PaO2 (Torr)
0
20
40
60
80
100
120
140
Time (minutes)

Figure 3-3. Arterial PCO_2 (Torr) over time (minutes) in trout sham ligated after a bolus carbonic anhydrase injection (5mg/kg) N=7, black circles; trout with 2 gill arches ligated after a bolus CA injection, N=8, open circles; and trout with 2 gill arches ligated after a bolus saline injection, N=6, gray circles. Point of injection is indicated with the vertical dotted line. * denotes a statistically significant difference ($P<0.05$) from pre-injection values. Values represent means ± 1SEM.

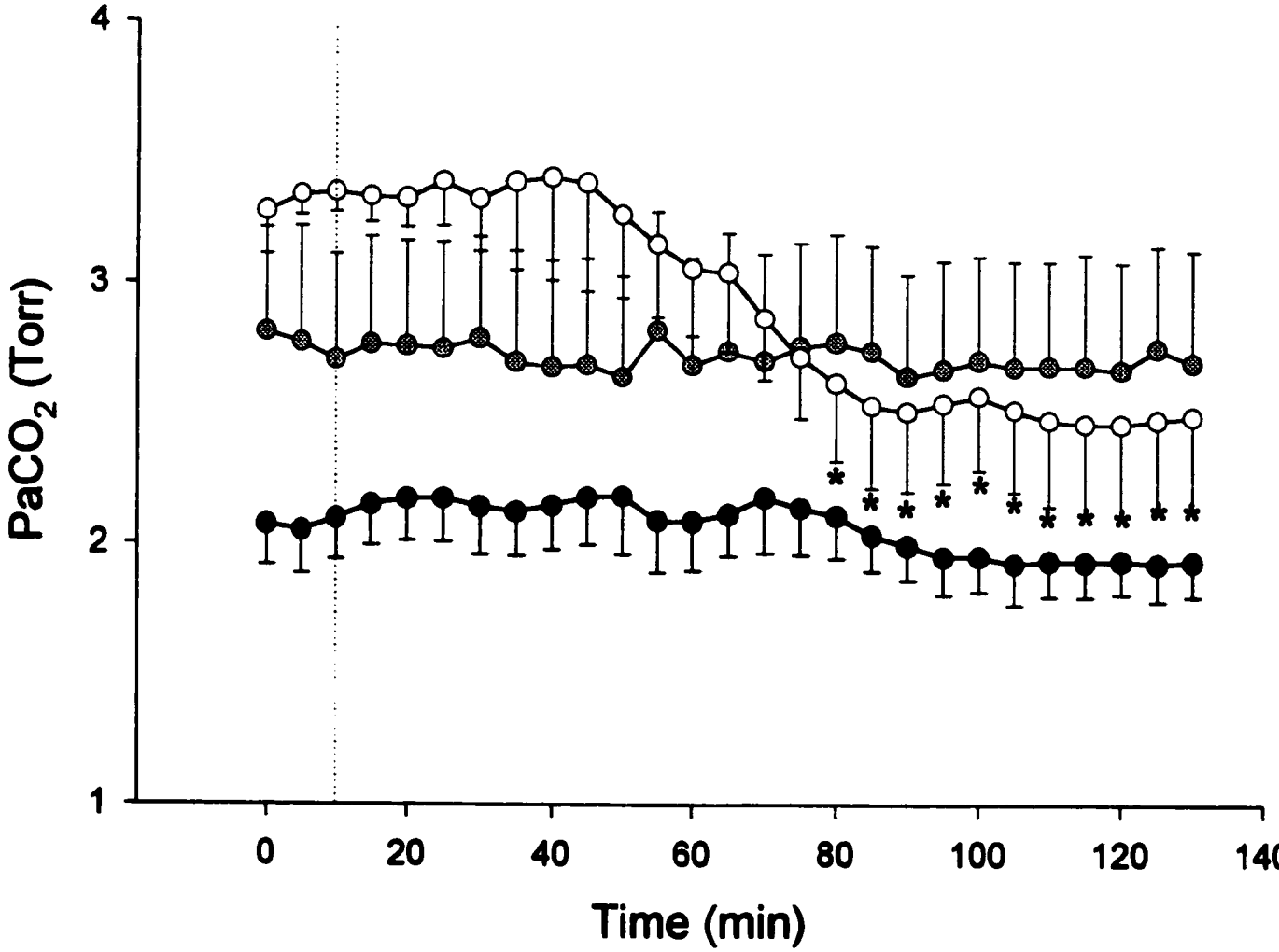

4
3
2
1
PaCO2 (Torr)
0
20
40
60
80
100
120
140
Time (min)
*
*
*
*
*
*
*
*
*
*

Figure 3-4. Changes in arterial PCO_2 (Torr) over time (minutes) in trout sham ligated after a bolus carbonic anhydrase injection (5mg/kg) N=7, black circles; trout with 2 gill arches ligated after a bolus CA injection, N=8, open circles; and trout with 2 gill arches ligated after a bolus saline injection, N=6, gray circles. Injection point is indicated by the dotted vertical line and was assigned a value of zero to calculate overall changes before and after injection. * denotes a statistically significant difference ($P<0.05$) from pre-injection values. † indicates a statistically significant difference ($P<0.05$) from the control value (saline injection). Data points represent means ± 1SEM.

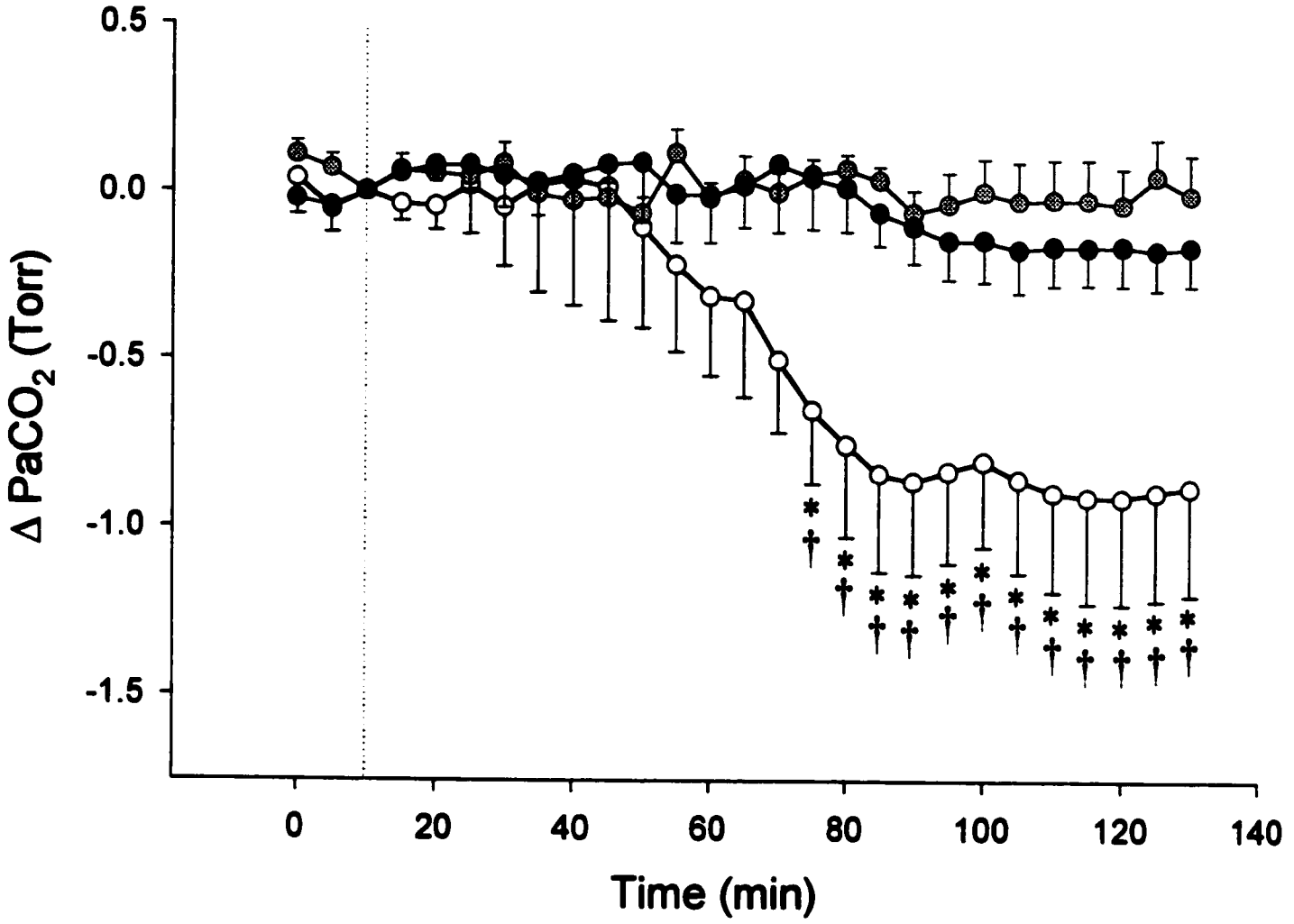

0.5
0.0
-0.5
-1.0
-1.5
Δ PaCO2 (Torr)
0
20
40
60
80
100
120
140
Time (min)

Figure 3-5. Arterial pH over time (minutes) in trout sham ligated after a bolus carbonic anhydrase injection (5mg/kg) N=7, black circles; trout with 2 gill arches ligated after a bolus CA injection, N=8, open circles; and trout with 2 gill arches ligated after a bolus saline injection, N=6, gray circles. Point of injection is indicated with the vertical dotted line. * denotes a statistically significant difference ($P<0.05$) from pre-injection values. Values represent means ± 1SEM.

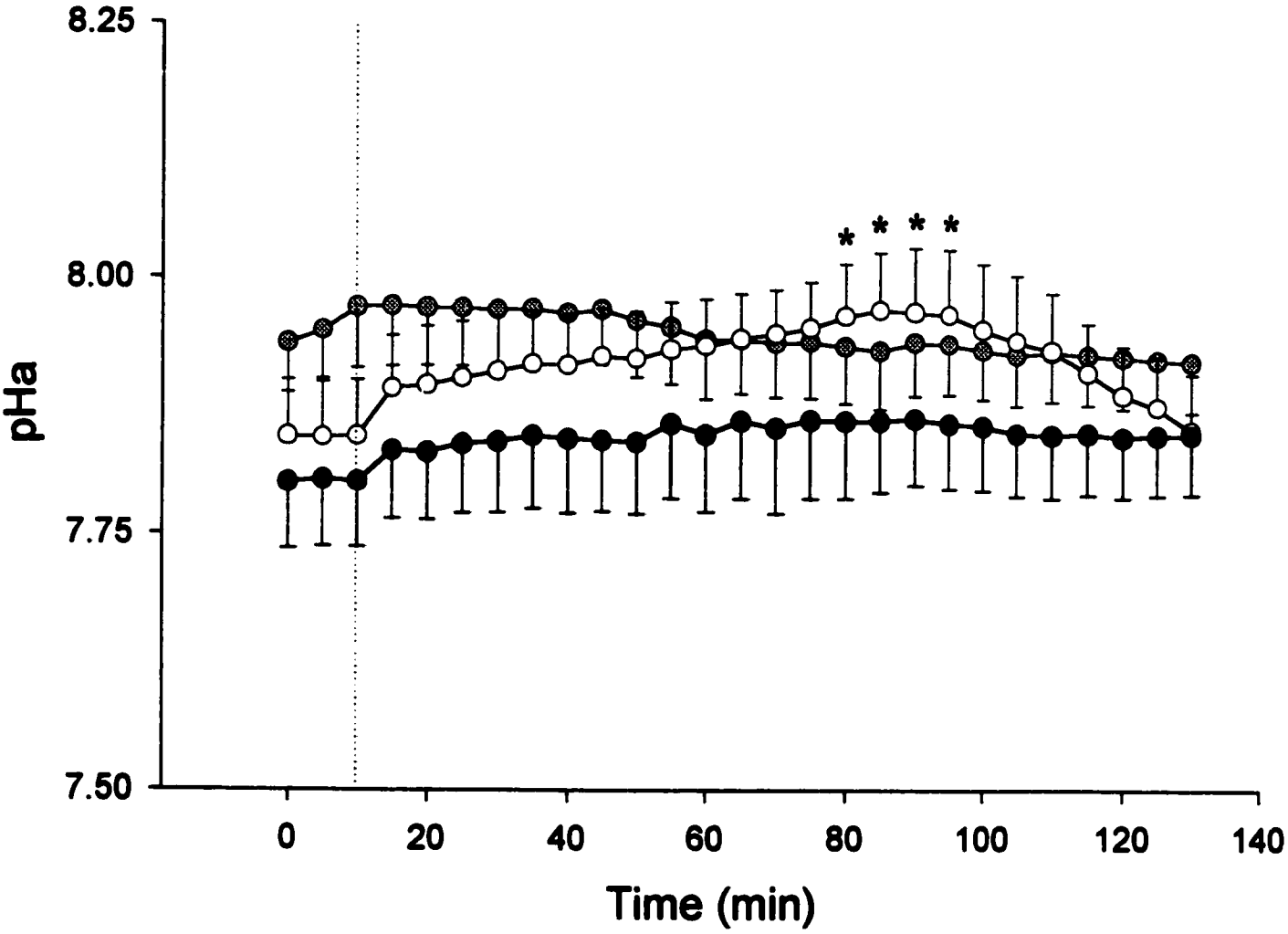
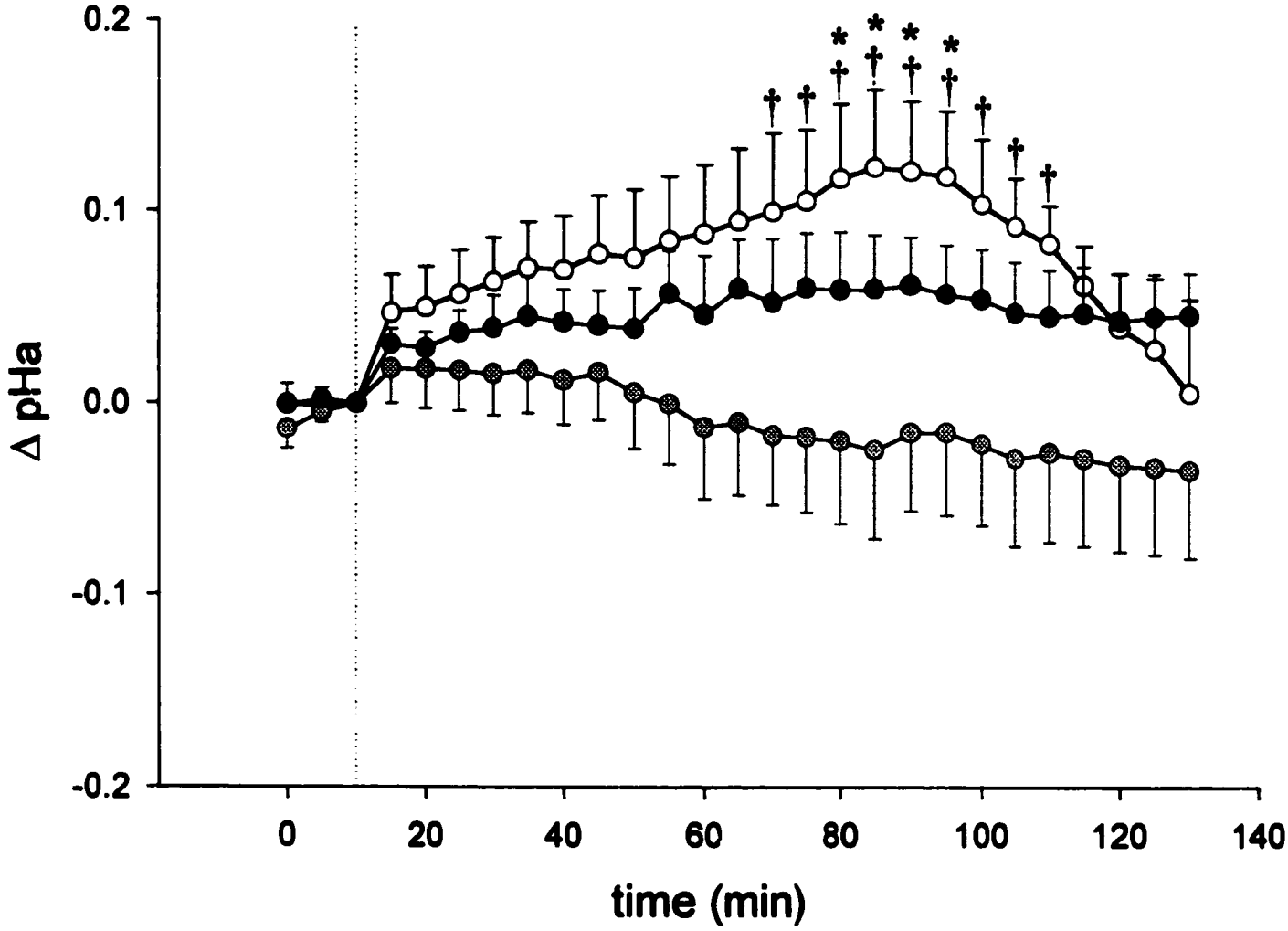

8.25
8.00
7.75
7.50
pHa
* * * *
0
20
40
60
80
100
120
140
Time (min)

Figure 3-6. Changes in arterial pH over time (minutes) in trout with 2 gill arches ligated, after a bolus injection of carbonic anhydrase(5mg/kg) N=8, open circles; or a bolus saline injection N=6, closed circles. Injection point is indicated by the dotted vertical line and was assigned a value of zero to calculate overall changes before and after injection. * denotes a statistically significant difference ($P<0.05$) from pre-injection values. † indicates a statistically significant difference ($P<0.05$) from the control value (saline injection). Data points represent means ± 1SEM.


0.2
0.1
0.0
-0.1
-0.2
Δ pHa
0
20
40
60
80
100
120
140
time (min)

Discussion

Although it appears that carbon dioxide excretion is more susceptible to diffusional constraints, under severe conditions oxygen uptake is also compromised. In Chapter 2, reductions of 40% of total gill surface area (ligation of three gill arches) imposed diffusion limitations on oxygen transfer and it was predicted that increased levels of catecholamines in the circulation were one possible mechanism for compensation. In theory, elevated catecholamines in the blood would allow for active recruitment of previously un-perfused lamellae and increase functional surface area (Randall & Perry, 1992; Wendelaar Bonga, 1997). A second compensatory response was a hyperventilation of 4463.3 ± 1303.2 ml/kg/min in fish with three gill arches ligated versus ventilatory flow of 1186.5 ± 188.4 ml/kg/min in sham ligated fish. A similar response was observed by Perry *et al.* (1996) after soft water exposure and subsequent increase in respiratory diffusion distance; hyperventilation allowed arterial PCO_2 levels to remain constant and prevented further decreases in arterial PO_2 values. Likewise in Chapter 2, arterial PCO_2 levels in fish with three gill arches ligated (40% reduction in gill surface area) were not significantly elevated from control levels as was the case for fish with two gill arches ligated (30% reduction in surface area). This implies that hyperventilation in fish with a 40% reduction in surface area was sufficient to aid in carbon dioxide excretion and, therefore, maintain $PaCO_2$ levels closer to control values.

Significant changes in oxygen uptake ($\dot{M}O_2$) and carbon dioxide excretion ($\dot{M}CO_2$) were not observed in any of the experimental (gill-ligated) groups and may indicate achievement of a new steady state 24 hours after ligation of gill arches. Metabolic and

ventilatory convection requirements are already 4-8 fold higher in fish than in other vertebrates (Milsom, 1989), therefore permanently increasing metabolic costs may not be favorable for these fish. Increased convection requirements for oxygen uptake were observed in fish with a 40% reduction in gill surface area but no corresponding increases in ventilatory convection requirements for CO_2 excretion were observed. Although this can be costly in terms of overall metabolism, it may represent a temporary state for the fish (ie. these levels may be reduced after fish are allowed more time to adjust to the diffusional constraints). Another reason for the discrepancy may be explained by the experimental protocol used. As fish began to hyperventilate, differences between inspired and expired PO_2 and total CO_2 became very small, therefore increasing variability in the calculations of convection requirements.

Theoretically, after injection of bovine carbonic anhydrase, limitations to CO_2 excretion imposed by the relatively slow conversion of bicarbonate (HCO_3^-) to CO_2 in the plasma of the gill should be abolished. The large quantities of HCO_3^- in the plasma would be quickly dehydrated to CO_2 in the presence of carbonic anhydrase both inside the red blood cell and the plasma. The only remaining component necessary for this reaction are H^+ ions, and are used diligently as indicated by the significant increase in arterial pH. In the red blood cell these protons (Bohr protons) are made available through the Haldane effect, or the release of protons from haemoglobin after oxygenation. Several studies have concluded this to be the limiting factor in carbon dioxide excretion *in situ* (Perry & Gilmour, 1993; Perry *et al.*, 1996; Brauner *et al.*, 1996). Injections of bovine carbonic anhydrase do in fact significantly lower arterial PCO_2 levels in fish with two gill arches ligated. These results are in agreement with studies by Wood & Munger

(1994) where a less severe respiratory acidosis was observed during exercise in the presence of extracellular carbonic anhydrase (CA) and Lessard *et al.* (1995) where CA injections significantly lowered both arterial PCO_2 and plasma total CO_2 content. Significantly elevated pHa values after CA injection were also observed in these studies. Although a significant decrease in arterial PCO_2 was observed after carbonic anhydrase injections in ligated fish, the final values two hours post-injection still exhibit a trend toward elevated $PaCO_2$ values from the sham ligated controls due to high variability. For this reason, absolute changes before and after injection were examined and revealed a dramatic decrease of 0.87 ± 0.32 Torr (Figure 3-4), essentially identical to the increase in $PaCO_2$ (1.04 ± 0.33 Torr) that was caused by the gill ligation (Table 2-1).

Thus, we believe that the apparent diffusion limitations for CO_2 transfer across the fish gill are a reflection of chemical equilibrium limitations. As explained previously, transfer of CO_2 across the gill appears to be limited by the relatively slow conversion of plasma HCO_3^- to CO_2 and the limiting access of plasma HCO_3^- to red blood cell CA through the red blood cell Cl^-/HCO_3^- exchanger. If this is indeed the case, dogfish (*Squalus acanthias* or *Svyliorhinus stellaris*) that are known to possess extracellular CA (Wood, 1994; Gilmour *et al.*, 1997) would conceivably be insensitive to reductions in gill surface area (i.e., $PaCO_2$ would remain at control levels regardless of gill surface reductions). Further studies would have to be performed to further clarify these mechanisms.

CHAPTER 4

GENERAL DISCUSSION

Although previous experiments have examined the impact of reduced gill surface area on gill O_2 transfer (Davis, 1971; Duthie and Hughes, 1987), this is the first study to assess the consequences for CO_2 transfer. The results clearly demonstrate that despite its high permeation coefficient, the excretion of CO_2 across the gill behaves in a diffusion-limited manner. Moreover, because the effects of surface area reduction on $PaCO_2$ were largely eliminated by injection of CA, it is obvious that the limitations on CO_2 transfer originate from chemical equilibrium limitations in which the accessibility of plasma HCO_3^- to red blood cell CA constrains the conversion of HCO_3^- to CO_2 as blood flows through the gill. As demonstrated previously (Davis, 1971), a 30% reduction in gill surface area did not significantly lower PaO_2, although there did appear to be a trend for lower PaO_2 values in the present study. Thus, O_2 transfer across the gill appears to be less influenced by diffusional constraints than does CO_2 transfer. The results of the present study, therefore, reaffirm current models of gas transfer in fish while providing, for the first time, experimental evidence that can explain the greater sensitivity of branchial CO_2 transfer to reductions in diffusion conductance.

Methods

This thesis aspired not only to asses the effects of gill surface area reduction but to perform this artificial manipulation as effectively as possible. Gill functional surface area was reduced *in vivo* by ligation of 1, 2 or 3 gill arches (Davis, 1971). Although cauterizing of gill arches as described by Duthie and Hughes (1987) ensured disruption of blood flow to the structure, it also increased mortality rates and possible infections. Using values for surface area of brown trout (*Salmo trutta*) gill arches (see Davis, 1971),

it was reasoned that ligation of one or two of the second pair of gill arches would yield reductions in functional surface area of 15 and 30%, respectively and ligation of two of the second pair of gill arches and one of the third pair of gill arches would yield an approximate reduction of 40% in functional surface area. Irrespective of the method used to reduce total surface area, it is likely that the blood flowing to the remaining gill arches was redistributed to initiate perfusion of previously un-perfused lamellae (lamellar recruitment) following ligation as discussed by Davis (1971). Thus, it is possible that the extent of the change in functional gill surface area after ligation was overestimated in the present study.

Because the experiments were performed *in vivo*, any effects on arterial blood gases after ligation will reflect the net effect of reduced surface area and any other secondary physiological adjustments that are activated to cope with the loss of area including possible increases in ventilation volume ($\dot{V}w$) and cardiac output ($\dot{V}b$). The ligation of two gill arches and associated reduction in functional surface area of 30% did not affect $\dot{V}w$ in the present study and thus any potential impact of ventilatory adjustments on arterial blood gas tensions need not be considered further. The absence of any ventilation changes differs from the finding of Davis (1971) who reported a significant increase in $\dot{V}w$ after a 38% reduction in gill surface area. Although $\dot{V}b$ was not monitored in this study, Davis (1971) demonstrated that a 38% reduction in functional surface area in rainbow trout was associated with a significant increase in $\dot{V}b$. An increase in $\dot{V}b$, coupled with a reduced total cross-sectional area of lamellar blood channels, may impose additional limitations on gas equilibration as blood flow velocity is

increased and hence residence time in the lamella is reduced. Possible changes in $\dot{V}b$, however, do not invalidate the conclusions of the present study for two reasons. First, by definition, arterial blood gas tensions in perfusion-limited systems are insensitive to changes in $\dot{V}b$ over the physiological range; i.e. an increase in $\dot{V}b$ would not elicit an increase in $PaCO_2$ in a perfusion-limited system. Indeed, experimental manipulation of cardiac output/blood flow has been used in previous studies (see below) to discern between perfusion- and diffusion-limited systems. Thus, any change in $PaCO_2$ after gill ligation, irrespective of any potential change in $\dot{V}b$, is evidence for apparent diffusion limitations. Second, the fact that PaO_2 was more-or-less insensitive to gill surface area reduction, whereas under identical conditions of $\dot{V}b$, $PaCO_2$ was increased, indicates that CO_2 transfer was more sensitive to diffusional constraints than was O_2 transfer.

Apparent diffusion limitations for CO_2 transfer

Based on theory and mathematical modelling (Cameron and Polhemus, 1974; Malte and Weber, 1985), it has been argued that CO_2 transfer across the fish gill would behave as a diffusion-limited system. In other words, a decrease in diffusion conductance (imposed either by a reduction in surface area or a thickening of the blood-to-water diffusion barrier) or a lowering of the residence time of blood in the lamellae would be expected to cause $PaCO_2$ to rise. This is precisely what occurred in the present study after functional surface area was reduced by 30%. A similar increase in $PaCO_2$ was reported for rainbow trout experiencing a thickening of the blood-to-water diffusion barrier caused by cortisol-induced proliferation of lamellar chloride cells (Bindon *et al.*, 1994; Perry, 1998). In contrast, a similar increase in diffusion distance induced by

lamellar chloride cell proliferation after prolonged exposure of trout to ion-poor water (Greco *et al.*, 1995; Perry *et al.*, 1996a) was not associated with any increase in $PaCO_2$. A likely explanation for the discrepant results obtained from the studies in which diffusion distance was experimentally increased is that the fish in ion-poor water were experiencing marked hyperventilation [e.g., $\dot{V}w$ was approximately doubled in the study of Perry *et al.* (1996a)] whereas ventilation was unaffected in the cortisol-treated fish. An increase in $\dot{V}w$ would be expected to lower $PaCO_2$ and thus counteract the diffusional limitations imposed upon CO_2 transfer by the increased diffusion distance. Thus, in the absence of hyperventilation, a decrease in diffusion conductance caused either by a 30% reduction in surface area (this study) or a thickening of the blood-to-water diffusion barrier (Bindon *et al.*, 1994), is associated with an increase in $PaCO_2$. These results provide evidence for apparent diffusion limitations for CO_2 transfer across the trout gill. Diffusion-limited CO_2 transfer can be explained by two possible mechanisms. First, there is the possibility that the movement of CO_2 across the gill epithelium truly is limited by the prevailing diffusion conductance (a 'true' diffusion limitation). We are unaware of any empirical studies that have directly tested this possibility. However, owing to its high Krogh's permeation coefficient [KCO_2; approximately 17 – 25 X greater than KO_2 in water and various tissues (see Table 2 in Swenson, 1990)] it seems unlikely that CO_2 diffusion, itself, would be a limiting factor. Second, there is the more likely possibility that CO_2 transfer across the fish gill is limited by the relatively slow rate at which plasma HCO_3^- is converted to CO_2 (an apparent diffusion limitation). This slow conversion, in turn, reflects the relatively slow rate at which plasma HCO_3^- gains access to red blood cell CA *via* the red blood cell Cl^-/HCO_3^- exchanger. The fact that addition of

bovine CA to the plasma of trout largely relieved the apparent diffusion limitations associated with gill surface area reduction, provides strong evidence in support of this idea. Similar effects of CA injection on $PaCO_2$ or blood acid-base status were observed in hypoxic trout (Lessard *et al.*, 1995) or trout recovering from exhaustive exercise (Wood and Munger, 1994). The reduction in $PaCO_2$ (0.87 ± 0.32 mm Hg) after CA injection into ligated fish was essentially identical to the increase in $PaCO_2$ (1.04 ± 0.33 mm Hg) that was caused by the gill ligation (see Table 2-1; Figure 3-4). Thus, although it has been suggested that the low buffering capacity of the plasma could limit the ability of extracellular CA to catalyse the dehydration of HCO_3^- by restricting availability of H^+ (Gilmour, 1998), the complete abolition of chemical equilibrium limitations after CA injection indicates that H^+ availability did not limit plasma CA activity under the present experimental conditions.

Limitations on O_2 transfer

The relative importance of diffusion limitations to O_2 transfer in fish are generally considered to be less than for CO_2 transfer (Gilmour, 1997). Indeed, the transfer of O_2 across the gill is thought to be predominantly limited by perfusion (Malte and Weber, 1985). However, an examination of the available literature reveals considerable inconsistencies. For example, Part *et al.* (1984) using a saline perfused trout head preparation and Daxboeck *et al.* (1982) using a blood-perfused trout preparation reported that post-branchial PO_2 was unaffected (i.e. not decreased) by increases in flow rate. Thus, both studies concluded that the trout gill is strictly perfusion-limited for O_2 uptake. On the other hand, Perry *et al.* (1985) reported a significant reduction in post-branchial

PO_2 when flow rate was increased in a saline-perfused trout head preparation and thus concluded that the trout gill is diffusion-limited for O_2 uptake. A possible explanation for the contradictory results is the different levels of adrenaline present in the various perfusion fluids that were used. High levels of adrenaline were present in the saline/blood used in the studies that failed to demonstrate any diffusion limitations (Daxboeck *et al.*, 1982; Part *et al.*, 1984) whereas no adrenaline was used in the study that identified diffusion limitations (Perry *et al.*, 1985). Adrenaline is known to enhance branchial O_2 transfer (Pettersson, 1983) by increasing functional surface area (Booth, 1979). This may explain why diffusion limitations for O_2 transfer were not revealed in the studies employing high levels of adrenaline. As pointed out by Malte and Weber (1985), the prevalence of a particular limitation (i.e. perfusion, ventilation or diffusion) on gas transfer does not exclude the contribution of another. Thus, although O_2 transfer across the fish gill is primarily perfusion-limited, diffusion limitations are also present (Perry *et al.*, 1985; Malte and Weber, 1985). Indeed, prior studies have reported a significant lowering of PaO_2 in fish experiencing an increase in the blood-to-water diffusion distance (Thomas *et al.*, 1988; Perry *et al.*, 1996). In the present study, PaO_2 was not statistically decreased after a 15 - 30% reduction of gill surface area under conditions of normoxia. However, there was an obvious trend for decreased PaO_2 by approximately 20 mm Hg. According to theory, diffusion limitations for branchial O_2 transfer are expected to increase as water PO_2 is lowered thereby lowering the mean water-to-blood diffusion gradient. In the present study, gill surface area reduction caused a significant reduction in PaO_2 only under conditions of hypoxia. Similarly, Bindon *et al.* (1994) demonstrated that a thickening of the diffusion barrier imposed by cortisol-

induced chloride cell proliferation in rainbow trout caused a reduction in P_aO_2 only at P_wO_2 values below 90 mm Hg.

Conclusions

Apparent diffusion limitations for branchial CO_2 excretion in rainbow trout were revealed after experimental reduction in gill surface area. Because these diffusion limitations were relieved by injections of carbonic anhydrase into the plasma, they likely originate from the relatively slow rate at which plasma HCO_3^- normally gains access to red blood cell carbonic anhydrase (chemical equilibrium limitation). P_aO_2 was also decreased by surface area reduction but these changes were only statistically significant under conditions of hypoxia. These results are consistent with the prevailing view that O_2 transfer, though largely perfusion-limited, also exhibits diffusion limitations.

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