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**The origin and physiological significance of high plasma
buffering in brown bullhead (*Ameiurus nebulosus*)**

by

Cheryl Szebedinszky B. Sc. (Hon)

A thesis submitted to the School of Graduate Studies and Research

In partial fulfillment for the Degree

Master of Science

Carleton University

The Ottawa-Carleton Institute of Biology

Ottawa, Ontario

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**The origin and physiological significance of high plasma
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Submitted by
Cheryl Szebedinszky, B.Sc. (Hons.)

in partial fulfilment of the requirements
for the degree of Master of Science

Chair, Department of Biology

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Carleton University

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Master of Science (2001)**Biology**

Title: The origin and physiological significance of high plasma buffering in brown bullhead (*Ameiurus nebulosus*)

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Cheryl Szebedinszky

**The origin and physiological significance of high plasma
buffering in brown bullhead (*Ameiurus nebulosus*)**

Abstract

The separated plasma of brown bullhead (*Ameiurus nebulosus*) was found to exhibit an unusually high non-bicarbonate buffer capacity (β) in relation to that of other teleost fish. In brown bullhead, the non-bicarbonate buffer capacity of separated plasma (β_{plasma}), at $-5.72 \pm 0.34 \text{ mmol L}^{-1} \text{ pH unit}^{-1}$ (mean $\pm$ SEM, $N = 30$), constituted 37% of whole blood β and was 2.5 times higher than β_{plasma} in rainbow trout (*Oncorhynchus mykiss*) ($-2.33 \pm 0.42 \text{ mmol L}^{-1} \text{ pH unit}^{-1}$; $N = 7$). The strong buffering power of bullhead plasma was not the result of unusually high plasma protein levels. Size separation chromatography in conjunction with a spectrophotometric assay for buffering capacity were used to isolate a plasma fraction of high buffering power. SDS-polyacrylamide gel electrophoresis revealed that this fraction contained four proteins, but was dominated by a protein of approximately 68-70 kDa molecular mass. On the basis of the amino acid composition of this fraction, the dominant protein was identified as albumin. In comparison to other fish albumins, bullhead albumin appears to be histidine-rich (6.7%). Thus, the unusually high non-bicarbonate buffer capacity of bullhead plasma appears to stem from the presence of a histidine-rich albumin in the plasma.

Two hypotheses concerning the potential physiological role of the unusually high non-bicarbonate buffer capacity of brown bullhead plasma were tested. First, by analogy with the model of CO_2 excretion in elasmobranch fish, in which high plasma buffering provides protons for catalysed HCO_3^- dehydration in the plasma, high plasma buffering in bullhead was hypothesised to play a role in CO_2 excretion. The presence of a Haldane effect in bullhead, the low CO_2 capacitance of bullhead separated plasma relative to that of true plasma and the absence of plasma and gill membrane-bound CA activities were

inconsistent with this hypothesis. Second, the high buffer capacity of bullhead plasma was postulated to function as an alternative mechanism for the protection of red blood cell (RBC) intracellular pH (pHi) in the absence or attenuation of a RBC adrenergic response. The requirement for protecting RBC pHi arises from the presence of a Root effect haemoglobin in bullhead. In support of this hypothesis, bullhead RBCs incubated *in vitro* with isoproterenol (10^{-8} to 10^{-5} mol L⁻¹) or forskolin (10^{-4} mol L⁻¹) exhibited significant cyclic AMP accumulation, but failed to exhibit cell swelling or significant Na⁺ or Cl⁻ accumulation; plasma pH (pHe) was also unaffected. Similarly, no significant effect on RBC water content, Na⁺ or Cl⁻ concentration, or pHe was detected in bullhead blood incubated with 8-bromo cyclic AMP (10^{-4} to 10^{-2} mol L⁻¹) *in vitro*. These results suggest that while bullhead RBCs possess a β -adrenoreceptor linked to cyclic AMP formation, stimulation of this adrenergic receptor does not result in measurable activation of a Na⁺/H⁺ exchanger (β -NHE).

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List of Abbreviations

B	buffer capacity
B_{blood}	blood buffer capacity
B_{plasma}	plasma buffer capacity
B_{haemolysate}	haemolysate buffer capacity
β-NHE	adrenergically-activated Na⁺/H⁺ exchanger
CA	carbonic anhydrase
cAMP	cyclic adenosine monophosphate
CO₂	carbon dioxide
CCO₂	total carbon dioxide content
CM	carboxymethyl
DEAE	diethylaminoethyl
Hb	haemoglobin
Hct	haematocrit
NTP	nucleoside triphosphate
MW	molecular weight
PCO₂	partial pressure of carbon dioxide
pHe	extracellular pH
pHi	intracellular pH
RBC	red blood cell
SDS	sodium dodecyl sulfate
SEM	standard error of the mean

CHAPTER 1

INTRODUCTION

The mechanisms involved in respiratory gas transfer and acid-base balance in teleost fish are complex and closely associated with one another. The complexity reflects, at least in part, interspecific variation resulting from adaptations to different environments. The purpose of this thesis was to investigate the blood buffering characteristics of the brown bullhead (*Ameiurus nebulosus*) in relation to those of the rainbow trout (*Oncorhynchus mykiss*), a species often considered a “typical” teleost. The following chapter introduces the mechanisms associated with oxygen uptake and carbon dioxide excretion in fish, particularly in relation to interactions between haemoglobin binding affinity and capacity. The focus of this chapter is acid-base regulation, with an emphasis on intracellular and extracellular buffering properties and constituents.

Gas transfer

The respiratory system allows the uptake of oxygen (O_2) from the water to the tissues and the excretion of carbon dioxide (CO_2) in the opposite direction, during environmental and metabolic variation (see Gilmour et al., 1997). Oxygen transport in the blood depends in large part on the quantity and affinity for oxygen of the oxygen-binding protein haemoglobin. Thus, haemoglobin must possess the ability to load O_2 at the gills and unload O_2 in the tissue capillaries (see Brauner and Randall, 1998; Nikinmaa, 1992). By contrast, approximately 90-95% of total CO_2 is carried in the plasma as bicarbonate ions (HCO_3^-) (Perry et al., 1982); only 5% of CO_2 is transported in the blood as carbamino CO_2 (CO_2 bound to haemoglobin) owing to acetylation of the N-terminal chains of haemoglobin (Jensen, 1991). Therefore, the capacitance of whole blood for CO_2

primarily reflects the capacity for HCO_3^- carriage due to the inter-conversion of CO_2 and HCO_3^- within the red blood cell.

The process by which CO_2 excretion occurs in teleost fish begins as metabolic CO_2 diffuses from the tissues to the blood where it is hydrated to HCO_3^- and H^+ in the red blood cell (RBC) in a reaction catalyzed by carbonic anhydrase (CA):

(see Fig 1.1) (Perry, 1986; Klocke, 1987; 1988; Tufts and Perry, 1998). The resultant H^+ ions are buffered by haemoglobin while HCO_3^- is transferred across the RBC membrane in exchange for plasma Cl^- ions by the membrane-associated band 3 anion exchanger (AE1 isoform) (Cameron, 1978; Romano and Passow, 1984). The buffering power of haemoglobin, which binds H^+ , and the anion exchanger, which enables HCO_3^- removal, sustain the hydration reaction within the red blood cell by removing the reaction products (Fig. 1.1) (Perry, 1986; Tufts and Perry, 1998). The CO_2 capacitance of separated plasma is generally low in teleosts due to the limited buffering power of plasma proteins (see Tufts and Perry, 1998). Therefore, the importance of haemoglobin as a buffer in this context is clear from the low CO_2 capacitance of plasma relative to whole blood – buffering in plasma is provided by plasma proteins, which amount to ~3% (Wood et al., 1982; Tufts and Perry, 1998). As blood flows through the branchial vasculature, the direction of these reactions is reversed and intracellular Cl^- is exchanged for extracellular HCO_3^- , which is then dehydrated to molecular CO_2 (Fig 1.1). The production of CO_2

within the erythrocyte is driven by CO_2 diffusion from the blood to the water (Weber and Jensen, 1988).

Carbonic anhydrase is of great importance to CO_2 excretion (see Maren, 1967) because the uncatalyzed rate of HCO_3^- dehydration (~ 90 sec at 10°C) (Edsall, 1969) exceeds the blood transit time through the gills (0.5 – 2.5 sec) (Randall and Daxboeck, 1984; Henry and Heming, 1998; Henry and Swenson, 2000). The primary role of the red blood cell in HCO_3^- dehydration is indicated both by the existence of RBC $\text{Cl}^-/\text{HCO}_3^-$ exchange (Obaid et al., 1979; Wood et al., 1982; Heming and Randall, 1982), and by the observation that CO_2 excretion rates are correlated with haematocrit (Perry et al., 1982; Wood et al., 1982). As CA is present in the red blood cell in excess of requirements for CO_2 excretion (see Swenson and Maren, 1987), the rate-limiting process in CO_2 excretion is thought to be the supply of plasma HCO_3^- to RBC CA via the band 3 anion exchanger, as this step is relatively slow in comparison to the rate of CO_2 diffusion or catalyzed HCO_3^- dehydration (Desforges et al., 2001).

CO_2 excretion is also dependent upon proton availability/buffering (Brauner et al., 1996; Perry and Gilmour, 1993). Protons are supplied for HCO_3^- dehydration via the dissociation of non-bicarbonate buffers (Bidani and Heming, 1991) and as Bohr protons derived from haemoglobin during oxygenation (Haldane effect) (Perry et al., 1996b; Perry and Gilmour, 1993; Brauner et al., 1996). Protons produced from the hydration of CO_2 in the RBC are buffered by haemoglobin, and also contribute to O_2 off-loading via the Haldane and Bohr effects (reduction in Hb- O_2 affinity with decreasing pH) (see Brauner and Randall, 1998). Thus, O_2 transfer and the transport of O_2 within the blood

are closely related to the intracellular acid-base status of the RBC (Thomas and Perry, 1992).

As previously mentioned, the Root effect, found only in fish (Brittain, 1987; Riggs, 1988), refers to the reduction in haemoglobin O₂-carrying capacity during acidosis (Nikinmaa, 1990), and has been correlated with the presence of a swimbladder and/or a choroid rete (see Pelster and Randall, 1998). The inability of Root effect haemoglobins to bind O₂ at low pH (even at 110 atm pure O₂) is due to the stabilization of the low-affinity T-form of haemoglobin to such an extent that haemoglobin fails to undergo allosteric transition from the T-form to the high O₂ affinity R-form (Brittain, 1987). The presence of a Root effect is often correlated with the activity of circulating catecholamines on red blood cell function (Nikinmaa, 1983; Primm et al., 1986). Adrenergic stimulation of red blood cell β -adrenoreceptors acts to stimulate proton transport out of the red cell via a or the Na⁺/H⁺ exchanger (β -NHE) (Motaïs et al., 1987), thus preserving intracellular pH in the face of an extracellular blood acidosis (see below). Indeed, it has been suggested that the functional significance of the adrenergic pH response in fish erythrocytes (see below) may be to prevent any reduction in blood oxygen carrying capacity that would otherwise be caused by the Root effect during extracellular acidoses (Perry and Wood, 1989; Vermette and Perry, 1988; Tufts and Randall 1989).

Red blood cell β -adrenergic response

Although generally changes in extracellular pH (pHe) induce changes in intracellular pH (pHi) as a result of the passive Donnan-like distribution of H⁺ across the red cell

membrane (Heming et al., 1986; Albers, 1986), adrenergic activation of the red blood cell membrane β - NHE enables pH_i regulation essentially independently of pH_e (Baroin et al., 1984, Cossins and Richardson, 1985; Parker, 1988). Catecholamines are released from the chromaffin tissue of the head kidney in response to various physical and environmental disturbances in which blood O_2 transport is threatened (see reviews by Randall and Perry, 1992; Nikinmaa, 1992; Thomas and Perry, 1992) (see Fig. 1.2).

Catecholamines bind to β -adrenoreceptors (β_1 subtype; Nikinmaa and Tufts, 1989) on the RBC surface, activating adenylate cyclase, which in turn, increases the intracellular adenosine 3', 5' – cyclic adenosine monophosphate (cAMP) concentration. Cyclic AMP accumulation ultimately elicits activation of the RBC membrane Na^+/H^+ exchanger which acts to displace protons from electrochemical equilibrium during the initial minutes of stimulation (Motais et al., 1989). The efflux of protons from the RBC is accompanied by an equimolar influx of Na^+ ions (Motais and Garcia-Romeu, 1987; 1988; Nikinmaa, 1986; 1990; Nikinmaa and Huestis, 1984). Red blood cell pH_i increases (Nikinmaa, 1982; Heming et al., 1987; Cossins and Richardson, 1985; Borgese et al., 1987), shifting the $CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$ equilibrium within the RBC to the right. The resultant HCO_3^- accumulation within the RBC generates a driving force for a net efflux of HCO_3^- from the RBC in exchange for Cl^- ions by means of the band 3 anion exchanger (Borgese et al., 1986) (Fig. 2). Osmotically obliged water follows the entry of Na^+ and Cl^- ions into the erythrocyte, and therefore the cell volume increases. The increase in pH_i and decrease in pH_e are possible, despite functional anion exchange, because the extracellular dehydration reaction is uncatalyzed and hence H^+ efflux via the

Na^+/H^+ exchanger exceeds H^+ influx via the Jacobs-Stewart cycle (Motaïs et al., 1989; Nikinmaa, 1990).

Acid-base Regulation

One major strategy that has evolved to minimize changes in intracellular and extracellular pH is the binding/release of protons by buffers (molecules that resist changes in pH upon the depletion or addition of H^+) (Abe et al., 1985). Differing whole blood and separated plasma buffering characteristics are exhibited by various fish species and reflect their individual ability to face both intrinsic and environmental challenges. Fish inhabiting hypoxic environments often possess a high non-bicarbonate buffer capacity (β) and high blood O_2 affinities (Cech et al., 1994). Brown bullhead typically are a bottom-living species that will likely face environments that are deficient in O_2 and high in CO_2 . Bullhead are known to thrive in stagnant, hypoxic waters (Haws and Goodnight, 1962).

Active physiological buffers consist of non-bicarbonate (organic and inorganic phosphates and residues of polypeptide chains; histidine, cysteine and terminal NH_2 -groups) and bicarbonate buffers (the CO_2 -bicarbonate buffer system) (Heisler, 1986a). Intracellular non-bicarbonate buffering is usually present to a larger extent than in extracellular compartments, however, bicarbonate buffering plays a relatively small role in both intracellular and extracellular buffering in fish (Heisler, 1986a). The non-bicarbonate buffer capacities of separated plasma (β_{plasma}) and whole blood (β_{blood}) are determined by measuring the changes in pH induced by increasing levels of PCO_2 . The resultant changes in $[\text{HCO}_3^-]$ are equivalent to the number of H^+ ions buffered by non-

bicarbonate buffers (Heisler, 1986a). Therefore, the non-bicarbonate buffer value is determined separately from bicarbonate buffering. The most important extracellular non-bicarbonate buffers are inorganic phosphates, and residues of plasma proteins (Heisler, 1986b). The principle intracellular buffer in the blood is haemoglobin (Wilkes et al., 1981; Wood et al., 1982; Cameron and Kormanik, 1982; Perry et al., 1985; Jensen, 1989) and the buffering capacity of the haemoglobin molecule is the major determinant of the buffering power of both the RBC and of whole blood.

The number of histidine residues present in a protein is the major determinant of its buffering power (Jensen, 1989). The most important buffer groups are those that possess pK values close to physiological pH, such as the imidazole moieties and terminal α -amino groups (Jensen, 1989). Teleost haemoglobins contain two titratable α -amino groups in comparison to four reported for most other vertebrate haemoglobins (Jensen, 1989). Histidine-related compounds (histidine residues in peptides and proteins as well as histidine dipeptides) play a dominant role in buffering systems because they have pK values near 7 at physiological temperatures and the net charge of histidines may influence the conformation of a protein and, consequently, its activity (Jensen, 1991). It has been observed that histidine and its associated dipeptides [anserine (β -alanyl- π -methyl-L-histidine) and carnosine (β -alanyl-L-histidine)] are compounds that have strong buffering abilities within the physiological pH range (Abe et al., 1985; Ogata and Murai, 1994). The number of histidine residues varies amongst species. For example, the number of histidine residues per haemoglobin chain is 2 in lampreys (*Lampertra* and *Petromyzon*), 2-6 in teleost fish (Jensen, 1989), and 4 and 2 in the hagfish (*Myxine glutinosa*) and river lamprey, respectively (Kleinschmidt and Sgouros, 1987). Blood buffering capacity, in

turn, is positively correlated with haemoglobin histidine content (Jensen, 1989). In addition, differences in histidine-related compounds could account for variations in buffer capacity found between species.

Therefore, fish must co-ordinate both O₂ uptake and CO₂ excretion while maintaining internal acid-base regulation during variations in their environmental and/or metabolic state. Differences in blood buffering characteristics exist between various species of fish, enabling them to cope with various acid-base challenges. In order to better understand these differences in buffering properties the following two chapters explore the possible origin and physiological significance of the unusual buffering abilities of brown bullhead, a teleost, in comparison to a well known teleost species of fish, rainbow trout.

Figure 1.1. Schematic diagram of the movements of CO_2 and O_2 between the tissue and red blood cell in a teleost fish. CA, carbonic anhydrase; Hb, haemoglobin (Modified from Tufts and Perry, 1998).

Figure 1.2. Schematic diagram of the B-adrenergic response in a typical teleost red blood cell. 1) Catecholamines bind to β -adrenoreceptor, activating adenylate cyclase. 2) The concentration of cAMP increases and ultimately 3) the Na^+/H^+ exchanger is activated. 4) $\text{CO}_2/\text{HCO}_3^-$ equilibrium is shifted towards HCO_3^- production which is moved out of the RBC in exchange for Cl^- via the anion exchanger (Modified from Nikinmaa, 1992).

CHAPTER 2

The buffering power of separated plasma in brown bullhead

(*Ameiurus nebulosus*)

A modified version of this chapter has been submitted for publication:

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Introduction

The principal means of regulating acid-base balance include transmembrane and/or transepithelial ion transfer processes and buffering (Heisler, 1986c). Buffers are most effective when their pK values are close to physiological pH and, consequently, the main buffers active in physiological systems are the $\text{CO}_2\text{-HCO}_3^-$ buffer system, certain amino acids such as histidine and cysteine (as free amino acids, peptides or protein residues) and phosphates (Heisler, 1986b; Abe, 2000); the latter two are referred to collectively as the non-bicarbonate buffers. The importance of the $\text{CO}_2\text{-HCO}_3^-$ buffer system is reduced in water-breathing animals relative to air-breathers owing to their lower HCO_3^- levels and similarly, the role of non-bicarbonate buffers is relatively greater intracellularly than extracellularly as a result of the low HCO_3^- levels typical of intracellular fluids (Heisler, 1986b).

Blood buffering provides a first line of defence for the protection of tissue intracellular pH. In fish, the non-bicarbonate buffer (β) values for blood are quite variable (Perry et al., 1985; Cech et al., 1994; Tufts and Perry, 1998; Gilmour et al., 2001b), ranging from approximately $-3 \text{ mmol L}^{-1} \text{ pH unit}^{-1}$ for lamprey (Ferguson et al., 1992) to $-21 \text{ mmol L}^{-1} \text{ pH unit}^{-1}$ for blue marlin (following exhaustive exercise; Dobson et al., 1986). While few studies have systematically examined the significance of this wide range, high blood buffer values have been suggested to correlate with hypoxia tolerance (Cech et al., 1994) or reliance on burst-swimming activity (Perry et al., 1985; Dobson et al., 1986), *i.e.* situations where resistance to lactacidosis would be important. Red blood cell (RBC) haemoglobin is the principal blood buffer, as evidenced by the

significant dependence of β on haematocrit (or haemoglobin concentration) (Wilkes et al., 1981; Wood et al., 1982; Cameron and Kormanik, 1982; Turner et al., 1983; Perry et al., 1985; Dobson et al., 1986; Hyde et al., 1987). The buffering value of the plasma, which resides primarily in histidine residues of plasma proteins (Heisler, 1986a), tends to be low and accounts for only 20 – 40% of the whole blood value in most fish (Tufts and Perry, 1998; Gilmour et al., 2001b).

In several fish, however, plasma buffering appears to be considerably more important. Icefish, for example, lack haemoglobin yet exhibit a blood buffer value comparable with that of red-blooded teleosts (Acierno et al., 1997), owing primarily to high plasma protein and inorganic phosphate levels (Feller et al., 1994; Acierno et al., 1997). Unusually high plasma β (accounting for 50 – 70% of whole blood buffering), has also been documented for several chondrichthyan fish, despite unremarkable plasma protein concentrations (Lenfant and Johansen, 1966; Graham et al., 1990; Gilmour et al., 2001b). Similarly, the buffer value reported for plasma from channel catfish (*Ictalurus punctatus*), a teleost, at - 5.8 mmol L⁻¹ pH unit⁻¹ (Cameron and Kormanik, 1982), is approximately twice that of other teleosts. Whether high plasma buffering is unique to the channel catfish or is a common feature within the catfish order is uncertain. In addition, plasma protein levels in the channel catfish are not unusually high (Ellsaesser and Clem, 1987), raising the possibility that the high plasma β may instead reflect unusual buffering properties of the plasma proteins.

Thus, the present study was conceived with two objectives. First, the blood and plasma buffer values of the brown bullhead (*Ameiurus nebulosus*) were measured to determine whether high plasma buffering is unique to channel catfish or is present in

other catfish species. The relative contributions of plasma and red cell buffering to the whole blood buffer value were also examined. Second, the sources of plasma buffering in bullhead plasma were investigated in order to test the hypothesis that the high non-bicarbonate buffer capacity of bullhead plasma is the result of plasma proteins with unusual buffering properties. Rainbow trout, which exhibit typical teleost blood and plasma buffer values (Wood et al., 1982), was used as a reference species.

Materials and Methods

Experimental animals and sample preparation

Brown bullhead (*Ameiurus nebulosus*; 307 – 500 g. experimental $N = 68$) of either sex were obtained from the St. Lawrence River by a commercial fisherman (LaFrambois Fisheries, Lancaster, Ontario). Rainbow trout (*Oncorhynchus mykiss*; 120 – 297 g. experimental $N = 35$) of either sex were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario). All fish were maintained on a 12L:12D photoperiod in large fibreglass aquaria supplied with flowing, aerated and dechloraminated City of Ottawa tap water at 10 – 13°C. Trout were allowed at least 2 weeks to acclimate to the holding conditions before experiments were initiated, and were fed to satiation on alternate days on a diet of commercial trout pellets. Bullhead were allowed at least 1 week to acclimate to the holding conditions before experiments were initiated, and because they refused food, were sampled within six weeks of capture.

All fish were fitted with an indwelling catheter for blood withdrawal. Fish were anaesthetised by immersion in an aerated solution of benzocaine (ethyl-*p*-aminobenzoate; 0.1g L⁻¹) prior to being transferred to an operating table that permitted continuous irrigation of the gills with aerated anaesthetic solution. The caudal artery or caudal vein was cannulated using flexible polyethylene tubing (Clay-Adams PE 50). Briefly, a lateral incision was made at the level of the caudal peduncle to allow the epaxial and hypaxial musculature to be separated and the haemal arch to be exposed. A catheter was then inserted into the caudal artery or vein in the anterior direction, the incision was closed with silk sutures and the cannula was secured with ligatures to the skin. Fish were

revived and transferred to individual holding boxes provided with flowing, aerated water for a 24 h recovery period prior to experimentation. Cannulae were flushed with heparinised (100 IU mL^{-1} sodium heparin) Cortland saline (Wolf, 1963).

Following the recovery period, blood was withdrawn from the cannula, heparinised (100 IU mL^{-1}) and held on ice. Separated plasma was obtained by centrifugation. The haemoglobin concentration and haematocrit of whole blood were measured, while protein and haemoglobin concentrations were assessed for separated plasma (the latter as an index of haemolysis). The non-bicarbonate buffer capacities of whole blood (β_{blood}) and/or separated plasma (β_{plasma}) were then determined.

The buffering capacities of red cell stripped haemolysates from trout and bullhead were also assessed. Stripped haemolysate solutions were prepared according to the method of Jensen (1989). Red cells separated from freshly drawn blood by centrifugation were washed three times with cold 0.9% NaCl. The RBCs were then lysed by the addition of 3 volumes of distilled water and subsequent freezing in liquid N_2 , following which the cell debris was removed by centrifugation. The resultant haemolysates were dialyzed for a minimum of 18 h against distilled water (at 4°C) and passed repeatedly through a mixed-bed ion-exchange column (Amberlite, IRN 150, BDH) to remove ions and organic phosphates. Haemoglobin, methaemoglobin and NTP levels were measured before and after each stage of preparation of the stripped haemolysates.

Measurement of non-bicarbonate buffer capacity

The non-bicarbonate buffer capacity (β) of blood, separated plasma or haemolysate solution was determined by using a dual equilibrator (DEQ-1; Cameron Instruments) to

equilibrate 0.8 mL samples held at 12°C with one of five humidified gas mixtures of CO₂ in air (PCO₂ = 0 – 1.2 kPa) delivered by a gas mixing flowmeter (GF-3/MP; Cameron Instruments). Use of the dual equilibrator permitted simultaneous assessment of whole blood and separated plasma from the same fish, or of haemolysates from trout and bullhead. Following a 15 min equilibration period, the pH and total CO₂ concentration (CCO₂) of each sample were assessed. Whole blood samples were centrifuged in a sealed tube immediately after equilibration to yield true plasma for subsequent analysis. The pH measurements were carried out using E301 glass pH and reference electrodes (Cameron Instruments) housed in a thermostatted (12°C) cell and connected to a Radiometer PHM 71. A Capni-con Total CO₂ analyzer Model II (Cameron Instruments) was used to measure CCO₂ (20 µL, in duplicate). The non-bicarbonate buffer capacity (β) was approximated as the slope of the linear regression line of HCO₃⁻ concentration against pH.

Protein purification techniques

As an initial step towards identifying the origin of the high β of bullhead plasma, separated plasma samples were subjected to dialysis against distilled water (MWCO 6 – 8 kDa, 24 h, 4°C), boiling (5 min, followed by ultracentrifugation at 70 000 rpm for 30 min), or passage through ion exchange resins (diethylaminoethyl [DEAE] or carboxymethyl [CM] sephadex beads, 0.9% NaCl was used to elute the sample). In each case, separated plasma samples were divided into two equal aliquots, where one aliquot served as the control and the other was treated, and the β of control and treated plasma samples were assessed as described above.

Size separation chromatography was then used to separate plasma samples into various fractions on the basis of molecular weight. The gel filtration column consisted of cross-linked 4% beaded agarose (CL 4B-200) with a functional range of 60 – 200 kDa. Thyroglobulin (MW 669 kDa), apoferritin (MW 443 kDa), β -amylase (MW 200 kDa), alcohol dehydrogenase (MW 150 kDa), bovine serum albumin (MW 66 kDa), and carbonic anhydrase (MW 29 kDa) were used as marker proteins. Separated plasma samples (6 mL) were dehydrated by approximately 50% by means of a vacuum centrifuge prior to loading on the column and were eluted with saline (0.9% NaCl, 0.02% sodium azide; pH 7.8; 4°C). Elution was performed at a rate of 0.6 mL min⁻¹ and 6 mL fractions were collected.

The gel filtration process was monitored in three ways; by photometry at 280 nm (A_{280}), by measurement of protein concentrations (in some cases), and by measurement of the relative buffering capacity of each fraction. The relative buffering capacity of each fraction was determined using a spectrophotometric assay adapted from that of Penketh et al. (1996). Owing to the large number and low volume of the fractions yielded by gel filtration, it was essential to develop a rapid assay for buffering capacity with which to screen the fractions. The standard method of measuring β (described above) was both too time intensive and required too high a sample volume to be useful in this context. The procedure employed was as follows: 200 μ L of phenol red was added to 2 mL of a fraction to achieve a final concentration of 80 mg L⁻¹. Absorbances were then determined at 560 nm before and after the addition of 100 μ L of 0.001 mol L⁻¹ HCl. The relative buffering capacity (ΔA_{560}) of the fraction was taken as the difference in absorbance before and after addition of the acid, where a small change in absorbance

indicated high buffering power.

The fraction(s) identified as being of interest were further investigated by discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and by amino acid analysis. SDS-PAGE was carried out with a vertical slab gel system (1.0 mm thick; Hoefer Scientific Instruments) at 4°C after the basic method of Laemmli (1970). Fractions were dialysed against distilled water (MWCO 6 – 8 kDa) for 24 h at 4°C (to eliminate NaCl) and were then dehydrated by freeze drying. The running buffer (pH 8.0) contained 25 mmol L⁻¹ Tris, 0.19 mol L⁻¹ glycine and 0.1% SDS. One volume of sample or molecular mass marker was mixed with two volumes of sample buffer (50% glycine, 5% mercaptoethanol, 2% SDS, 1% bromophenol blue and 0.125 mol L⁻¹ Tris, pH 6.8) and incubated for 5 min at 95°C. The stacking and separating gels contained 3% and 10% acrylamide, respectively. Gels were visualised using either silver stain (Chaudhuri and Green, 1987) or Coomassie blue (Anderson and Anderson, 1978). Molecular weight standards were purchased from Bio-Rad (broad range, 161-0317).

Amino acid analysis was carried out by the Alberta Peptide Institute (University of Alberta, Edmonton, Alberta) on fractions dialysed against distilled water (MWCO 6 – 8 kDa) for 24 h at 4°C (to eliminate NaCl) and then dehydrated by freeze drying. Samples were hydrolysed with 6 N HCl and then analysed using a Beckman 6300 amino acid analyser with Beckman Gold software.

Analytical techniques

Haematocrit was measured in duplicate using microcapillary tubes centrifuged at 6000 g for 6 min. Whole blood haemoglobin content ([Hb]) was determined by the

cyanmethaemoglobin method (Sigma). The [Hb] of separated plasma was determined via the same technique but the sensitivity of the assay was enhanced 4-fold as described by Wood et al. (1994). Plasma protein concentrations were measured using the Bio-Rad protein assay in combination with BioLynx 2.0 software. To assess the extent of organic phosphate removal, haemolysate ATP concentrations were measured (Sigma, 366-UV) before and after samples were processed through the ion-exchange column. To assess the extent of methaemoglobin formation induced by haemolysate preparation, haemolysate methaemoglobin content was determined just after lysis of the red blood cells and just prior to β measurements on the stripped haemolysate solution using the method of Benesch et al. (1973).

Statistical analysis

Data are presented as mean values $\pm$ 1 standard error of the mean (SEM). Differences between bullhead and trout were assessed using unpaired Student's *t*-tests. The statistical significance of differences between control and treatment values were determined by paired Student's *t*-tests or by repeated measures one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc multiple comparisons test, as appropriate. In all cases, 5% was taken as the fiducial limit of significance.

Results

Non-bicarbonate buffer capacity

The non-bicarbonate buffer capacities of both separated plasma (β_{plasma}) and blood (true plasma; β_{blood}) from brown bullhead were significantly higher than the corresponding values for rainbow trout (Fig. 2.1). The higher β_{plasma} of bullhead was not the result of significantly higher plasma protein levels in this species (Table 2.1, Fig. 2.1), nor was haemolysis a contributing factor since haemoglobin levels in all plasma samples were below the detection limit of the assay. Furthermore, bullhead β_{plasma} did not vary on a seasonal basis, with the reproductive status of the fish sampled, or with the degree of oxygenation of the plasma sample, suggesting that the high buffering power of bullhead plasma stems from a normal plasma component. The greater buffer capacity of bullhead blood over trout probably reflected the difference in β_{plasma} values, as haematocrits were similar, while haemoglobin levels were slightly but significantly higher in trout blood (Table 2.1).

To further investigate the contributions of plasma versus RBC buffers to blood buffering, the non-bicarbonate buffer capacities of stripped haemolysate solutions from both bullhead and rainbow trout were determined. Haemolysate solutions from bullhead ($N = 4$) and trout ($N = 4$) contained similar levels of haemoglobin (2.94 ± 0.39 and 2.96 ± 0.23 g 100 mL⁻¹, respectively), methaemoglobin (1.84 ± 0.30 and 1.93 ± 0.21 % of total haemoglobin, respectively) and NTPs (1.86 ± 0.56 and 2.12 ± 0.77 $\mu\text{mol g Hb}^{-1}$, respectively). The low NTP levels indicated that the stripping procedure was effective in removing organic phosphates, while methaemoglobin levels were similar to those

Table 2.1. Whole blood and separated plasma characteristics for brown bullhead (*Ameiurus nebulosus*) and rainbow trout (*Oncorhynchus mykiss*).

	Bullhead	Trout
Hct (%)	23.8 ± 2.1 (18)	26.5 ± 1.6 (13)
[Hb] (g 100 mL ⁻¹)	6.04 ± 0.47 (24)	7.81 ± 0.52 (18)*
[plasma protein] (mg mL ⁻¹)	41.5 ± 3.3 (16)	48.1 ± 0.1 (6)
$\beta_{\text{haemolysate}}$ (mmol g Hb ⁻¹ pH unit ⁻¹)	-0.12 ± 0.03 (4)	-0.09 ± 0.02 (4)

Values are means ± 1 SEM (*N*).

* indicates a statistically significant difference between bullhead and trout (unpaired Student's *t*-test, *P* < 0.05).

Hct haematocrit; *[Hb]* haemoglobin concentration; *[plasma protein]* concentration of plasma proteins; $\beta_{\text{haemolysate}}$ non-bicarbonate buffer capacity of stripped haemolysate solution.

Figure 2.1. The non-bicarbonate buffer capacities (β ; mmol L⁻¹ pH unit⁻¹) of separated plasma (unfilled bars; $N = 30$ bullhead, $N = 7$ trout) and blood (true plasma: filled bars; $N = 30$ bullhead, $N = 18$ trout) are presented for brown bullhead (*Ameiurus nebulosus*) and rainbow trout (*Oncorhynchus mykiss*). In the inset figure, separated plasma β values for bullhead ($N = 16$) and trout ($N = 6$) have been expressed per unit plasma protein to illustrate the observation that differences in plasma protein levels cannot account for the difference in plasma β between the species. Values are means ± 1 SEM; N numbers are indicated in the bars. * indicates a significant difference between bullhead and trout (unpaired Student's t -test, $P < 0.05$).

measured under control conditions *in vivo* (Brauner et al., 1993). Measurement of non-bicarbonate buffer capacities on these stripped haemolysate solutions yielded values that did not differ significantly between bullhead and rainbow trout (Table 2.1), further supporting plasma rather than RBC intracellular buffers as the origin of the greater buffering in bullhead blood over that of rainbow trout.

Protein purification

The initial steps taken to investigate the origins of the high β_{plasma} in bullhead involved confirming the involvement of one or more plasma proteins. Dialysis (MWCO 6 – 8 kDa) of bullhead plasma against distilled water did not significantly affect β_{plasma} (Table 2.2), indicating that the high buffering power of bullhead plasma was not due to low molecular weight plasma constituents such as inorganic phosphates, free amino acids, or dipeptides. By contrast, boiling bullhead plasma to denature all proteins effectively eliminated the buffering power (Table 2.2), confirming that the unusually high β_{plasma} is due to one or more plasma proteins. Proteins involved in buffering protons would be expected to carry a negative charge, and this was found to be the case for the protein(s) responsible for the high β_{plasma} of bullhead. A significant reduction in β_{plasma} was measured following diethylaminoethyl (DEAE) sephadex chromatography, which acts to bind negatively charged molecules, whereas passing plasma through carboxymethyl (CM) sephadex beads, which bind positively charged molecules, was without effect (Table 2.2).

Gel filtration in conjunction with a qualitative assay for buffering capacity was employed to determine the approximate size of the protein(s) responsible for the high

β_{plasma} of bullhead. Size separation chromatography yielded 80 fractions of 6 mL each, which were screened for protein concentration (absorbance at 280 nm: A_{280}) and relative buffer capacity (ΔA_{560}); typical chromatograms for bullhead and rainbow trout plasma are presented in Fig. 2.2. Two primary regions of high buffering (as indicated by low ΔA_{560} values) were detected for both species at elution volumes of approximately 340 mL and 425 mL, which corresponded to proteins with approximate molecular weights of 66 kDa and 25 kDa, respectively. Although these regions were common for both bullhead and rainbow trout, the buffering provided by the 66 kDa fraction (termed the high MW fraction) appeared to be consistently greater in bullhead (Fig. 2.2). Over 12 bullhead and 6 trout plasma samples subjected to gel filtration, mean ΔA_{560} values did not differ significantly for the fraction eluted at 425 mL, but were significantly (unpaired Student's *t*-test, $P < 0.05$) lower for the high MW fraction from bullhead ($\Delta A_{560} = 0.17 \pm 0.05$) than from trout ($\Delta A_{560} = 0.48 \pm 0.05$). These results indicated greater buffering in the bullhead high MW fraction at protein levels that were not statistically different ($A_{280} = 0.91 \pm 0.15$ and 0.76 ± 0.11 for bullhead and trout, respectively). To confirm this trend, high MW fractions from 6 bullhead and 6 trout plasma samples were analysed for buffer capacity and protein concentrations using the standard methods. Despite very similar protein concentrations for bullhead (0.74 ± 0.08 mg mL⁻¹) and trout (0.79 ± 0.09 mg mL⁻¹), the non-bicarbonate buffer capacity of the bullhead high MW fraction, at -0.94 ± 0.07 mmol L⁻¹ pH unit⁻¹, was significantly higher than that of trout, at -0.73 ± 0.07 mmol L⁻¹ pH unit⁻¹ (one-tailed Student's *t*-test, $P < 0.05$). Thus, further investigation was focused on the high MW fraction.

To determine the number and molecular weights of proteins in the high MW fraction,

SDS-gel electrophoresis was employed. SDS-PAGE of the high MW fraction isolated by gel filtration revealed four bands of approximate molecular weights 68, 64, 20 and 14 kDa (Fig. 2.3). It is important to note that silver staining produced identical bands as observed with staining with Coomassie blue. The largest (68 kDa) protein was by far the most abundant in the fraction. The bands were eliminated by DEAE-sephadex chromatography, but not by CM-cephadex or reactive blue agarose chromatography. The high MW fraction was also subjected to amino acid analysis and the resultant amino acid profile is presented in Table 2.3, together with those of fish albumins or albumin-like proteins for which compositions determined by amino acid analysis were available. The similarity of these amino acid compositions, together with the molecular weight of the predominant protein, suggests that the main protein present in the high MW fraction of bullhead plasma was albumin. Notably, the histidine content of the bullhead high MW fraction was approximately 6.7%, substantially higher than those of the other fish albumins (Table 2.3).

Table 2.2. The effects of various treatments (dialysis, boiling, charge separation chromatography) on β_{plasma} for separated plasma from brown bullhead (*Ameiurus nebulosus*).

	Control (mmol L ⁻¹ pH unit ⁻¹)	β_{plasma} Treatment (mmol L ⁻¹ pH unit ⁻¹)
Dialysis ^a	-0.15 ± 0.02	-0.15 ± 0.02
Boiling	-7.82 ± 0.38	-1.48 ± 0.22*
DEAE-sephadex chromatography	-9.19 ± 0.87	-3.32 ± 0.53*
CM-sephadex chromatography ^b	-9.19 ± 0.87	-7.30 ± 0.35

Values are means ± 1 SEM ($N = 6$).

* indicates a statistically significant difference between control and treatment values (paired Student's *t*-test or one-way repeated measures ANOVA, $P < 0.05$).

^a β_{plasma} values expressed as mmol g protein⁻¹ pH unit⁻¹ to take into account the dilution of the dialysed sample by distilled water.

^bThe same control group was used for both DEAE-sephadex and CM-sephadex chromatography.

β_{plasma} non-bicarbonate buffer capacity of plasma; DEAE diethylaminoethyl; CM carboxymethyl.

Table 2.3. Amino acid profile (in moles %) of the fraction believed to be the origin of the high non-bicarbonate buffer capacity of bullhead plasma, with those of fish albumins or albumin-like plasma proteins that have been determined by amino acid analysis.

Amino acid	Bullhead	Trout albumin ^a	Icefish IF1 ^b
Asx	10.4 ± 0.6	9.88 ± 0.34	9.10
Thr	4.50 ± 0.01	6.20 ± 0.47	6.02
Ser	4.98 ± 0.01	5.89 ± 0.53	9.69
Glx	12.1 ± 0.11	11.1 ± 0.9	9.25
Pro	5.47 ± 0.11	3.80 ± 0.63	3.96
Gly	6.75 ± 0.09	5.14 ± 0.55	8.22
Ala	7.63 ± 0.16	9.37 ± 0.32	10.3
Val	6.65 ± 0.12	7.32 ± 0.31	7.05
Cys	0.32 ± 0.07	2.37 ± 0.49	4.99
Met	0.56 ± 0.01	2.41 ± 0.19	1.32
Ile	4.87 ± 0.11	4.44 ± 0.53	4.41
Leu	8.00 ± 0.20	8.03 ± 0.39	7.78
Tyr	3.79 ± 0.07	2.66 ± 0.15	2.94
Phe	5.13 ± 0.07	4.45 ± 0.41	3.82

Trp	0.04 ± 0.04	ND	0.88
Lys	8.53 ± 0.12	8.68 ± 0.58	6.31
His	6.73 ± 0.16	4.63 ± 0.75	2.05
Arg	3.42 ± 0.08	3.38 ± 0.16	1.91

Values for bullhead are means ± 1 SEM ($N = 2$).

^aValues for trout are means ± 1 SEM ($N = 4$) of the values determined by Davidson et al., (1989), Ohkawa et al., (1987), and Maillou and Nimmo (1993a).

^bValues from Feller et al., (1994).

ND not determined

Figure 2.2. Representative chromatograms of separated plasma samples subjected to gel filtration for (A) bullhead and (B) trout are presented. The volumes at which marker proteins were found to elute are indicated by the vertical line symbols: T, thyroglobulin, 669 kDa; A, apoferritin, 443 kDa; β , β -amylase, 200 kDa; D, alcohol dehydrogenase, 150 kDa; B, albumin, 66 kDa; CA, carbonic anhydrase, 29 kDa. The gel filtration process was monitored by photometry at 280 nm to assess protein levels (A_{280} ; unfilled symbols) in each fraction. In addition, the relative buffer capacity (ΔA_{560} ; filled symbols) of each fraction was assessed using a spectrophotometric assay in which changes in the absorbance at 560 nm of samples to which phenol red had been added were measured following the addition of a known quantity of acid; a low ΔA_{560} value is indicative of high buffering. In both species, two regions (illustrated by the grey bars) exhibiting strong buffering were observed.

Figure 2.3. Analysis of the high MW fraction obtained by gel filtration of bullhead plasma, using SDS-polyacrylamide gel electrophoresis. Lane 2 contained 10 μ L of the high MW fraction, while lane 1 contained 10 μ L of standards (as marked).

Table 2.4. Non-bicarbonate buffer capacities for whole blood or true plasma from selected catfish species.

Species	β_{blood}^a (mmol L ⁻¹ pH unit ⁻¹)	Reference
<i>Ameiurus nebulosus</i>	-15.5 (24)	This study
<i>Ameiurus punctatus</i>	-14.3 (25)	Cameron and Kormanik. 1982
	-11.1	Albers et al.. 1981
<i>Ictalurus melas</i>	-14.8 (16)	Szebedinszky and Gilmour. unpublished
<i>Silurus glanis</i>	-11.8 (27)	Albers et al., 1981
<i>Hypostomus</i> sp.	-13.5	Wood et al., 1979

^aHaematocrits have been noted, where available, in parentheses.

β_{blood} non-bicarbonate buffer capacity of whole blood or true plasma.

Discussion

The non-bicarbonate buffer capacity of brown bullhead blood ($\beta_{\text{blood}} = -15.5 \text{ mmol L}^{-1} \text{ pH unit}^{-1}$) was among the highest of all fish species examined (Perry et al., 1985; Cech et al., 1994; Tufts and Perry, 1998; Gilmour et al., 2001b). Relatively high blood buffering appears to be common within the catfish order (Table 2.4) and in general among hypoxia-tolerant fish species such as tench, Northern squawfish or Sacramento blackfish (Cech et al., 1994). In bullhead, the unusually high β_{blood} reflects, in large part, a separated plasma buffer value that is two- to three-fold higher than typical β_{plasma} values for teleost blood (Gilmour et al., 2001b). This conclusion was supported by two lines of evidence. First, on the basis of the buffer values for stripped haemolysates and blood haemoglobin concentrations (Table 2.1), the contribution of haemoglobin to whole blood buffering was similar in trout ($-7.2 \text{ mmol L}^{-1} \text{ pH unit}^{-1}$) and bullhead ($-7.0 \text{ mmol L}^{-1} \text{ pH unit}^{-1}$). In addition, removal of the plasma component from blood buffering produced a value for the buffer capacity of bullhead blood that was comparable to those of other teleosts. For example, Packer and Sunkin (1979) reported a non-bicarbonate buffer capacity for brook trout blood of $-0.08 \text{ mmol g protein}^{-1} \text{ pH unit}^{-1}$ when both haemoglobin and plasma protein concentrations were taken into account. The equivalent calculation for the species used in the present study yielded values of -0.09 and $-0.17 \text{ mmol g protein}^{-1} \text{ pH unit}^{-1}$ for trout and bullhead, respectively. However, if the plasma contribution to buffering was removed, the resultant buffer capacities were essentially identical, at $-0.06 \text{ mmol g protein}^{-1} \text{ pH unit}^{-1}$. In the other catfish species for which plasma buffer values are available, channel catfish ($-5.8 \text{ mmol L}^{-1} \text{ pH unit}^{-1}$; Cameron and Kormanik, 1982)

and black bullhead ($-8.4 \text{ mmol L}^{-1} \text{ pH unit}^{-1}$; Szebedinszky and Gilmour, unpublished data), the unusually high β_{plasma} also appears to account for the higher than typical whole blood buffer values (Table 2.4). Thus, substantial buffering by separated plasma may be characteristic of the North American catfish (Ictaluridae) and/or of catfish species in general.

Since the effect of high plasma buffering in catfish is to raise the overall blood buffer value, the contribution of β_{plasma} to β_{blood} is approximately 37%, within the normal range for teleost fish (20 – 40%; Tufts and Perry, 1998; Gilmour et al., 2001b). In this respect, the catfish differ from the other red-blooded fish for which high plasma buffering has been reported, which belong to the class Chondrichthyes. High plasma buffer values have been measured for several species of elasmobranch but in the absence of elevated blood buffering, such that β_{plasma} accounts for 50 – 70% of β_{blood} (Lenfant and Johansen, 1966; Graham et al., 1990; Gilmour et al., 2001b). While the cause of the high plasma buffering in elasmobranch fish is unknown, the enhanced importance of β_{plasma} to β_{blood} may result, at least in part, from relatively low haemoglobin levels (Lenfant and Johansen, 1966; Graham et al., 1990). Interestingly, the buffering power of elasmobranch haemoglobin is greater than that of teleost haemoglobin (Albers and Pleschka, 1967; Jensen, 1989, 1991; Graham et al., 1990).

Having established that bullhead possess unusually high plasma buffering, the second objective of the present study focused on the origins of this buffering power. Both quantitative and qualitative explanations were investigated. Greater than typical levels of plasma proteins or other plasma constituents known to possess buffering properties, such as inorganic phosphates, free amino acids or peptides, were considered as quantitative

possibilities. The possibility that unusual qualities of the plasma proteins conferred high buffering power was also examined.

Icefish lack haemoglobin, yet exhibit blood buffer values comparable to those of teleosts in which haemoglobin is the principal buffer (Acierno et al., 1997). Protein and inorganic phosphate concentrations that are two- to six-fold higher in icefish plasma than in red-blooded species help explain the apparent discrepancy (Feller et al., 1994; Acierno et al., 1997). This strategy cannot, however, account for the unusually high buffer capacity of bullhead plasma. Plasma protein levels in bullhead and trout were similar (Table 2.1), serving to underline the difference in plasma buffering between these species (Fig. 2.1). Because the buffer capacity of bullhead plasma was not reduced by dialysis (Table 2.2), which would remove all low molecular weight compounds, inorganic phosphates do not appear to be the source of the high β_{plasma} . Moreover, the results for dialysis also eliminated the possibility that high plasma buffering in bullhead originated from free histidine or histidine dipeptides such as anserine, carnosine or balenine. Such histidine-related compounds have been implicated in explaining the high buffer capacity of fish muscle (Abe et al., 1985; Okuma and Abe, 1992; Ogata and Murai, 1994; Abe, 2000).

The possibility that unusual buffering properties of the plasma proteins contributed to the high buffer capacity of bullhead plasma was also investigated. Size separation chromatography, in combination with a spectrophotometric assay, was used to isolate plasma fractions of high buffering power. Chromatograms for both bullhead and trout revealed two regions of interest (Fig. 2.2). In both species, strong buffering was exhibited by a late-eluting fraction that was expected to contain protein(s) of around 25

kDa as well as low molecular weight plasma components. Owing to the similarity between the species in this fraction and the previous elimination of low molecular weight constituents as the source of the unusually high β_{plasma} in bullhead, the other fraction that exhibited strong buffering was considered to be more promising. This fraction (termed the high MW fraction for convenience) was expected to contain protein(s) of approximately 66 kDa, and despite very similar protein levels in both species, higher buffering was always present in bullhead samples.

Several lines of evidence suggested that the major component of the high MW fraction was albumin. Albumin, which has a molecular mass of 65 – 70 kDa, is typically the major anionic protein in vertebrate plasma, where its principal roles include functioning as a transport protein and maintaining the oncotic pressure of blood. While there is no single property by which albumins can be identified, they are often distinguished by their abundance, high electrophoretic mobility and ability to bind specific ligands, including palmitate (Davidson et al., 1989; Gray and Doolittle, 1992; Maillou and Nimmo, 1993a; Metcalf et al., 1998). Some fish species lack albumin (Maillou and Nimmo, 1993a; Ballantyne, 1997; De Smet et al., 1998; Metcalf et al., 1999). However, albumin(s) with a molecular weight of 65 – 70 kDa is a major constituent of the plasma protein complement in those species in which it is found (Yanagisawa and Hashimoto, 1984; Ohkawa et al., 1987; Davidson et al., 1988, 1989; Maillou and Nimmo, 1993a,b; Feller et al., 1994; Metcalf et al., 1998); *Petromyzon marinus* albumin has a unique molecular weight of ~175 kDa (Gray and Doolittle, 1992; Filosa et al., 1998; Danis et al., 2000). The elution volume and high protein content of the high MW fraction were in accordance with these properties (Fig. 2.2).

Moreover, SDS-PAGE revealed that the high MW fraction was dominated by a protein of approximately 68 kDa (Fig. 2.3) that could be eliminated by DEAE-sephadex chromatography of the fraction; DEAE-sephadex chromatography is frequently used to purify albumin from plasma (Davidson et al., 1989; Metcalf et al., 1998).

Finally, the amino acid profile of the high MW fraction was similar to those of other fish plasma proteins (Table 2.3) that have been subjected to amino acid analysis and identified as albumin on the basis of their composition and/or other properties, such as palmitate binding, antigenicity, or ability to bind specific dyes (Ohkawa et al., 1987; Davidson et al., 1989; Maillou and Nimmo, 1993a; Feller et al., 1994). The presence in the high MW fraction of up to three other proteins (Fig. 2.3) likely introduced some noise into the amino acid analysis. However, the minute levels of these proteins relative to that of the 68 kDa protein and the striking similarity between the composition of the high MW fraction and those of fish albumins suggested that such confounding factors were of minor significance.

Fish albumins appear to be distinguished from those of other vertebrates by their low cysteine content (Davidson et al., 1989; Maillou and Nimmo, 1993a) and this finding was particularly true of bullhead albumin, in which the cysteine content was lower than that of other fish albumins (Table 2.3). Bullhead albumin also differed in exhibiting a histidine content that appears to be the highest reported value for a fish albumin (Ohkawa et al., 1987; Davidson et al., 1989; Maillou and Nimmo, 1993a; Feller et al., 1994). Trout serum contains two proteins that may be classified as albumins and this factor, coupled with methodological differences, may account for the variability in the amino acid profiles obtained by amino acid analysis for trout albumin (Table 2.3). If the average

value for trout of 4.63% is considered, the histidine content of bullhead albumin was 1.5- to 3-fold higher than those of trout or icefish. Protein sequences for two albumins from Atlantic salmon (accession number P21848 and Q03156) as well as an eel (*Petromyzon marinus*) albumin (accession number Q91274) are available in the SWISS-PROT database. Estimation of the amino acid composition of these proteins (using tools available on the proteomics server of the Swiss Institute of Bioinformatics) yielded values of 3.7%, 3.7% and 5.1%, respectively, for histidine content; again, substantially lower than that of bullhead albumin. Histidine residues are considered to be one of the major buffers active in physiological systems (Heisler, 1986b; Abe, 2000) and it therefore seems likely that the unusually high buffering capacity of bullhead plasma is a reflection of a histidine-rich albumin in the plasma. Similarly, an enriched peptidic histidine content has been proposed to contribute to the non-bicarbonate buffer capacity of icefish plasma (Feller et al., 1994). The histidine content of icefish albumin was not unusual; rather, a second major plasma component identified as an immunoglobulin-like protein was found to be histidine-rich (Feller et al., 1994).

The physiological significance of the unusually high plasma buffering in bullhead and other catfish species is not clear. In elasmobranch fish, which possess gill membrane-bound carbonic anhydrase activity that is available to catalyse plasma CO_2 reactions (Gilmour et al., 1997, 2001a,b; Wilson et al., 2000), high plasma buffering may contribute to CO_2 excretion by providing protons for HCO_3^- dehydration in the plasma compartment (Gilmour et al., 2001a). However, it is unlikely that a similar mechanism operates in bullhead, given the prevailing view that teleost fish lack extracellular carbonic anhydrase activity at the gills (Gilmour et al., 1994, 2001a,b; Perry et al., 1997). In

muscle, high non-bicarbonate buffering is associated with an enhanced capacity for anaerobic exercise performance (Abe, 2000), and in a similar vein, the high blood buffer values of tuna and marlin were suggested to allow rapid recovery from burst-swimming activity (Perry et al., 1985; Dobson et al., 1986). High blood buffering has also been suggested to be indicative of hypoxia tolerance (Cech et al., 1994), yet whether the possession of high blood buffer values confers greater resistance to hypoxia and/or acidosis has not yet been tested. Strong plasma buffering could be beneficial by defending red cell intracellular pH from the effect of protons added to the vascular compartment, particularly in species having Root effect haemoglobins. Clearly, the physiological significance of the high plasma non-bicarbonate buffer capacity in bullhead and other catfish species warrants further investigation.

CHAPTER 3

The physiological significance of high plasma buffering in brown bullhead (*Ameiurus nebulosus*)

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Introduction

The non-bicarbonate buffer capacity of separated plasma from brown bullhead (*Ameiurus nebulosus*), at $-5.7 \text{ mmol L}^{-1} \text{ pH unit}^{-1}$ (Chapter 2), is unusually high relative to values for other teleost fish which are typically -2 to $-3 \text{ mmol L}^{-1} \text{ pH unit}^{-1}$ (Tufts and Perry 1998; Gilmour et al., 2001a,b). Strong plasma buffering appears to be common among North American catfish species, and contributes to the generally high blood buffer values measured for these fish (Chapter 2). While high blood buffering has been suggested to correlate with hypoxia tolerance (Cech et al., 1994) or reliance on burst-swimming activity (Perry et al., 1985; Dobson et al., 1986), *i.e.* situations where resistance to lacticidosis would be important, the potential role of high plasma buffering remains largely unexplored. Thus, the objective of the present study was to test hypotheses concerning the physiological significance of the unusually high plasma buffering in bullhead.

High plasma relative to whole blood buffering has been documented in several elasmobranch fish (Tufts and Perry 1998; Gilmour et al., 2001a,b). Elasmobranchs possess both plasma carbonic anhydrase (CA) activity (Wood et al., 1994; Gilmour et al., 1997, 2001; Henry et al., 1997) and gill membrane-bound CA activity that is available to catalyse plasma CO_2 reactions (Gilmour et al., 1997, 2001; Wilson et al., 2000). In these species, high plasma buffering may contribute to CO_2 excretion by providing protons for HCO_3^- dehydration in the plasma compartment (Gilmour et al., 2001a). Elasmobranchs, in addition, lack endogenous plasma CA inhibitors (Henry et al., 1997; Gilmour et al., 2001b) and a Haldane effect (Butler and Metcalfe, 1988; Jensen, 1989; Wood et al., 1994), and exhibit separated and true plasma CO_2 capacitances that

are very similar (Lenfant and Johansen, 1966; Tufts and Perry, 1998). By contrast, the plasma of teleost fish lacks CA activity (e.g. Henry et al., 1993, 1997; Perry et al., 1996b; Gilmour et al., 1997; Acierno et al., 1997) and indeed often contains a CA inhibitor (Dimberg, 1994; Henry et al., 1997; Peters et al., 2000). Furthermore, membrane-associated CA activity is present only at very low levels in the gills of teleost fish (Henry et al., 1988, 1993, 1997), and the CA activity that is present is not accessible to plasma CO_2 reactions (Gilmour et al., 1994, 1997; Perry et al., 1997; see reviews by Gilmour 1998a,b). Given the high buffer capacity of bullhead plasma (Chapter 2) coupled with the absence of an endogenous plasma CA inhibitor (Henry et al., 1997), CO_2 excretion in bullhead was postulated to follow the elasmobranch pattern. To test this hypothesis, the CO_2 combining characteristics of bullhead plasma and blood were assessed, and plasma and gill CA activity was investigated. Pacific spiny dogfish, ratfish and longnose skate were used as comparison species due to their well documented distribution of CA.

Strong plasma buffering could alternatively be of benefit by defending red blood cell (RBC) intracellular pH (pHi) from the effect of protons added to the vascular compartment, particularly in species having Root effect haemoglobins. While the Root effect plays key roles in swim bladder function and/or in oxygen delivery to the retina, blood oxygen transport is negatively impacted by a reduction in RBC pHi in species having Root effect haemoglobins (reviewed by Pelster and Randall 1998). In many teleosts, RBC pHi is maintained even during severe extracellular acid-base disturbances by the mobilisation of catecholamines (see review by Randall and Perry 1992), which interact with RBC β -adrenoreceptors, triggering a cyclic AMP-mediated signal transduction pathway that ultimately activates the RBC membrane Na^+/H^+ exchanger (β -

NHE; Borgese et al., 1992) such that protons are extruded from the RBC in exchange for plasma Na^+ (for reviews see Thomas and Perry, 1992; Nikinmaa 1992; Motais et al., 1992; Nikinmaa and Salama, 1998). The functional significance of the teleost RBC adrenergic response is thought to be protection against the Root-shift induced reduction in blood oxygen carrying capacity that would otherwise occur (Tufts and Randall, 1989). Marked species differences exist, however, in the activity of the β -NHE (Nikinmaa and Salama, 1998). Since plasma buffering provides a first line of defence for the protection of RBC pH_i , the unusually high buffer capacity of bullhead plasma could reflect an alternative strategy for the protection of RBC pH_i owing to an attenuated RBC β -adrenergic response. This hypothesis was tested by first confirming that bullhead exhibit a Root effect, and then by assessing the adrenergic responsiveness of bullhead RBCs. Rainbow trout was used as a reference species because the adrenergic response of trout RBCs has been well characterised.

Materials and Methods

Experimental animals

Brown bullhead (*Ameiurus nebulosus*; 195 – 510 g, experimental $N = 85$) of either sex were obtained from the St. Lawrence River by a commercial fisherman. while rainbow trout (*Oncorhynchus mykiss*; 130 – 310 g, experimental $N = 64$) of either sex were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario). All fish were maintained on a 12L:12D photoperiod in large fibreglass aquaria supplied with flowing, aerated and dechloraminated city of Ottawa tap water at 10 – 13°C. Trout were fed to satiation on a diet of commercial trout pellets on alternate days and were allowed at least 2 weeks to acclimate to the holding conditions before experiments were initiated. Bullhead refused food and were therefore sampled within 6 weeks of capture. Longnose skate (*Raja rhina*; 800 – 3700 g; experimental $N = 7$) and spotted ratfish (*Hydrolagus coliei*; 900 – 1200 g; experimental $N = 6$) and Pacific spiny dogfish (*Squalus acanthias*; 400-2100 g; experimental $N=8$) were trawled off the coast of Vancouver Island, Canada, and held at Bamfield Marine Station (British Columbia, Canada) for up to 2 weeks in a 75 000 L circular tank provided with flowing sea water at 11°C. Fish were maintained under a 12L:12D photoperiod and were not fed. These cartilaginous fish were used as a positive control; i.e. all were expected to possess both plasma and branchial membrane-bound CA activities.

A catheter (Clay-Adams, PE50) was placed in the caudal artery of bullhead and trout (Axelsson and Fritsche 1994), or the coeliac artery of dogfish (Graham et al., 1990), for the withdrawal of blood samples. Cannulae were filled with heparinised (100 i.u. mL⁻¹

sodium heparin) saline; Cortland saline (Wolf 1963) was used for the teleosts while dogfish saline consisted of 500 mmol L⁻¹ NaCl. Fish were anaesthetised by immersion in an aerated solution of benzocaine (ethyl-*p*-aminobenzoate; 0.1 g L⁻¹) then transferred to an operating table that permitted continuous irrigation of the gills with the same anaesthetic solution. A lateral incision made at the level of the caudal peduncle was used to expose the haemal arch, and a cannula (Clay-Adams PE 50) was inserted into the caudal artery or vein in the anterior direction. Following a 24 h recovery period, blood samples were withdrawn from the arterial cannula. Ratfish and skate were not cannulated. Rather, blood samples were withdrawn from anaesthetised ratfish by caudal puncture, and from anaesthetised skate by cardiac puncture, using a wide bore (18 gauge) needle to minimise the chance of haemolysis. Following the collection of blood samples, the gills were perfused with heparinised saline to obtain erythrocyte-free tissue which was dissected out of the fish, frozen in liquid N₂ and stored at -80°C until analysis as described by Henry et al. (1997) and Gilmour et al. (2001b).

Most experiments required approximately 6 mL of blood, which was obtained either from a single fish or by pooling blood from 2 fish. A 6 mL blood sample was considered as a single *N* number for statistical purposes regardless of whether it was obtained from one or more fish. Blood sampling was terminated at the first signs of agitation. Following withdrawal, blood was heparinised (100 IU mL⁻¹) and held on ice until use; separated plasma was obtained by centrifugation and maintained on ice until use.

Approximately 3 g of gill tissue was added to 8 volumes of ice-cold buffer (in mmol L⁻¹, 225 mannitol, 75 sucrose, 10 Tris base, adjusted to pH 7.40 with 30% phosphoric acid) and homogenised by a minimum of 30 passes in a motor-driven Teflon/glass

homogeniser. The resultant crude homogenates were analysed for protein concentration and CA activity and then subjected to differential centrifugation according to the procedure of Henry et al. (1988, 1993, 1997): 750 g for 20 min to produce a pellet containing whole cells, nuclei and large cell fragments; 8,500 g for 20 min to yield a mitochondrial pellet (both on a Sorvall RC5C plus centrifuge at 4°C); and 100,000 g for 60 min at 4°C to obtain a microsomal pellet containing membrane-bound CA activity and cytoplasmic supernatant containing gill epithelial intracellular CA activity (Beckman Optima LE-80K ultracentrifuge). Pellets were re-suspended in 1 – 10 mL of buffer and each fraction was then assayed for CA activity and protein concentration. Protein concentrations were measured using the Bio-Rad standard (Bradford) protein assay with bovine serum albumin (Sigma) as a standard.

CO₂ combining curves

To quantify the Haldane effect in both species, CO₂ combining curves were constructed *in vitro* using oxygenated and deoxygenated blood samples. In addition, CO₂ combining curves were constructed *in vitro* for blood and separated plasma from bullhead to enable determination of CO₂ capacitances. The construction of a pair of CO₂ combining curves (oxygenated and deoxygenated blood, or blood and separated plasma) required approximately 6 mL of blood. The haemoglobin concentration ([Hb]) of each blood sample was determined prior to the construction of CO₂ combining curves. A dual equilibrators (DEQ-1; Cameron Instruments) was used to equilibrate 0.8 mL blood or separated plasma samples held at 12°C with one of five humidified gas mixtures of either CO₂ in air or CO₂ in N₂. Gas mixtures were delivered by a gas mixing flowmeter (GF-

3/MP; Cameron Instruments) and had nominal PCO_2 values of 0.5, 1, 2, 4 and 8 Torr. Blood and separated plasma samples for the determination of CO_2 capacitances could be equilibrated simultaneously, whereas for the quantification of the Haldane effect, it was necessary to alternate between oxygenated and deoxygenated equilibration conditions. Samples were equilibrated for 15 min and then analysed for total CO_2 content (CCO_2) and plasma pH (pHe). From these values, PCO_2 and HCO_3^- concentration ($[\text{HCO}_3^-]$) were calculated using the Henderson-Hasselbalch equation and the appropriate constants listed in Boutilier et al. (1984).

The magnitude of the Haldane effect in each species was quantified by fitting hyperbolic curves to the data obtained from individual fish using iterative curve-fitting software (Sigmaplot; SPSS Science Inc.). From these curves, the values of $[\text{HCO}_3^-]$ in oxygenated and deoxygenated blood were determined for a single PCO_2 of 4 Torr (Perry et al., 1996b). The difference in $[\text{HCO}_3^-]$ between oxygenated and deoxygenated blood at a PCO_2 of 4 Torr ($\Delta[\text{HCO}_3^-]$) was then normalised for differences in $[\text{Hb}]$ to yield final estimates of the Haldane effect ($\Delta[\text{HCO}_3^-]/[\text{Hb}]$).

Determination of carbonic anhydrase activity

The CA activity of gill homogenates and fractions, plasma, and RBC lysates was measured using the electrometric ΔpH method of Henry (1991). In brief, samples containing CA activity were added to 6 mL of reaction buffer (in mmol L^{-1} , 225 mannitol, 75 sucrose, 10 Tris base, adjusted to pH 7.40 with 30% phosphoric acid) held in a temperature-controlled (4°C) vessel, and the reaction was initiated by the addition of CO_2 -saturated water (200 μL). The initial velocity of the reaction was measured over a

change of approximately 0.15 pH units. To obtain the true catalysed rate, the uncatalysed rate (addition of CO₂-saturated water in the absence of any CA source) was subtracted from the observed rate. A GK2401C pH electrode and PHM 84 pH meter (both Radiometer) were used to measure pH.

Gill homogenate and fraction CA assays were carried out using 50 µL of sample. To characterise the CA activity associated with the microsomal fraction, CA activity was measured in the presence of various inhibitors. The sensitivity of microsomal CA to sodium dodecylsulphate (SDS) was determined by measuring CA activity in the presence of 0.005% SDS in the reaction chamber; in mammals, CA IV is less susceptible to inhibition by SDS than are cytoplasmic CA isozymes (Whitney and Briggie 1982; Waheed et al., 1992).

The effects of washing and incubation with the enzyme phosphatidylinositol-specific phospholipase C (PI-PLC) on microsomal CA activity were also assessed. Microsomal pellets were re-suspended in reaction buffer, agitated by mild sonication (5 W for 3 sec) and then re-pelleted by ultracentrifugation. The CA activity of the pellet was determined before and after washing. This procedure enabled the CA activity that was not integrally associated with the microsomal pellet to be assessed. After washing, microsomal pellets were re-suspended in reaction buffer, divided into two aliquots, and incubated (22°C for 90 min) with or without 1 unit of PI-PLC (Sigma). This enzyme cleaves phosphatidylinositolglycan-linked CA from its membrane anchorage and releases it to the supernatant (Zhu and Sly 1990; Böttcher et al., 1994; Gervais and Tufts 1998; Gilmour et al., 2001b). Following the 90 min incubation period, the suspensions were subjected to ultracentrifugation to re-pellet the microsomes and the CA activities of the resulting

supernatants and microsomal pellets were assessed.

Plasma CA activities were evaluated using 50 – 200 μ L of sample. For comparison, the CA activities of dogfish and trout plasma were also assessed as these species are known to exhibit and lack plasma CA activity, respectively (Gilmour et al., 1997; Henry et al., 1997). Plasma haemoglobin concentrations were measured on those species exhibiting plasma CA activity as a control for haemolysis; the standard cyanmethaemoglobin method (Sigma) was used but the sensitivity of the assay was increased four-fold (Wood et al., 1994). To test for the presence of an endogenous plasma CA inhibitor, 10 μ L of dilute RBC lysate of each species was assayed in the presence of 100 μ L of its own plasma. Unfortunately, no skate RBC lysate was available and therefore ratfish RBC lysate was used to test for the presence of an endogenous CA inhibitor in skate plasma.

Root effect

The Root effect was determined in both species by measuring the O_2 content of blood samples equilibrated to different CO_2 tensions at a constant O_2 tension of about 156 Torr. Blood samples (0.8 mL) held at 12°C were equilibrated (DEQ-1; Cameron Instruments) with one of six humidified gas mixtures of CO_2 in air (0 – 4% CO_2) delivered by a gas mixing flowmeter (GF-3/MP; Cameron Instruments). Following a 15 min equilibration period, blood samples were analysed for O_2 content and pHe; O_2 content values were corrected for dissolved O_2 using constants from Boutilier et al. (1984). Because nucleoside triphosphates (NTP) are a major cofactor modifying haemoglobin-oxygen (Hb- O_2) affinity, NTP levels were assessed for each pool of blood used in the

determination of the Root effect immediately prior to equilibration of the first blood sample and immediately following equilibration of the last blood sample. Bullhead blood sample NTP levels were found not to change over the course of the experiment (mean NTP levels were 32.2 ± 9.2 and $33.1 \pm 9.4 \mu\text{mol g}^{-1} \text{Hb}$, $N = 7$, respectively, for the initial and final equilibration times). Correspondingly, there was no significant difference in mean NTP levels for trout (mean NTP levels were 10.2 ± 1.5 and $8.6 \pm 1.6 \mu\text{mol g}^{-1} \text{Hb}$, $N = 7$, respectively, for the initial and final equilibration times). For ease of experimentation, blood rather than RBC intracellular pH was measured in Root effect experiments. In most cases, pH_e is a good indicator of pH_i (Perry and McDonald, 1993; Pelster and Randall, 1998); to ensure that this was the case under the conditions used in the present study, a separate set of blood samples for trout ($N = 5$) and bullhead ($N = 5$) were equilibrated to the same conditions used in the determination of the Root effect, and pH_e and pH_i were measured.

Red blood cell adrenergic responsiveness

The adrenergic response of bullhead and trout RBCs was assessed by measuring cyclic AMP accumulation, cell swelling, Na^+ and Cl^- accumulation and pH_e at 12°C in hypoxic ($\text{PCO}_2 = 2.3$ Torr, $\text{PO}_2 = 15.2$ Torr, $\text{pH}_\text{e} = 7.97$ for bullhead and 7.83 for trout, 30 min pre-incubation) blood samples (0.5 mL) after exposure to saline (control) and to one of a) the β -adrenergic agonist isoproterenol (6 levels, final nominal concentrations ranging from 10^{-8} to $10^{-5} \text{ mol L}^{-1}$), b) the adenylate cyclase activator forskolin ($10^{-4} \text{ mol L}^{-1}$), or c) the permeable cyclic AMP analogue 8-bromoadenosine 3',5'-cyclic monophosphate (8-bromo cyclic AMP; 3 levels, 10^{-4} to $10^{-2} \text{ mol L}^{-1}$ final nominal

concentrations). Forskolin was dissolved in dimethyl sulphoxide (DMSO; 0.1%) and therefore 0.1% DMSO was included in the saline control for this experiment. The haematocrit of blood samples pooled from 1 – 2 fish was adjusted to 20 – 25% and blood samples were then stored on ice in glass vials for a minimum of 4 h prior to experimentation to allow for catecholamine decay (Perry et al., 1996a).

Cyclic AMP levels were measured after 15 min incubation with isoproterenol [this incubation time was chosen because cyclic AMP accumulation was found to be maximal 5 min after the addition of agonist in previous work (Salama and Nikinmaa, 1990; Salama, 1993)], or 30 min incubation with forskolin. Blood samples were divided into two 250 μL aliquots. One aliquot was deproteinised with 15 μL of 50% perchloric acid, then centrifuged (10, 000 g for 2.5 min). The resultant supernatant was removed, neutralised with 45 μL of 2 mol L^{-1} KHCO_3 and stored at -80°C until analysis for cyclic AMP concentration ([cyclic AMP]). Red cell mass was determined on the remaining 250 μL aliquot by drying the pellet to a constant mass.

RBC adrenergic swelling was assessed by measuring cell water content. After 30 min incubation [chosen on the basis of previous work indicating that changes in cell water levels are detectable at this point (Salama and Nikinmaa, 1990; Salama, 1993)], blood samples (0.5 mL) were centrifuged and the supernatant and upper layer of the red cells were discarded. The RBC pellet was weighed, dried (at 60°C) to a constant mass overnight and re-weighed. The water content of the cells (RBC [water], %) was calculated as the percentage water of the total mass and was not corrected for any water contained within trapped plasma. To determine Na^+ and Cl^- accumulation, the dried RBC pellet was dissolved with shaking in 5 mL dH_2O at room temperature for 18 h.

deproteinised with 150 μL of 70% perchloric acid, and centrifuged (6,000 g for 2 min). The resultant supernatant was removed and stored at -20°C until analysed for Na^{+} and Cl^{-} concentrations ($[\text{Na}^{+}]$, $[\text{Cl}^{-}]$). Finally, pHe was measured (as an index of H^{+} extrusion) after 30 min incubation of a separate set of blood samples (0.5 mL).

Analytical techniques

All pH measurements were carried out using E301 glass pH and reference electrodes (Cameron Instruments) housed in a thermostatted (12°C) cell and connected to a Radiometer PHM71 meter. The freeze-thaw method of Zeidler and Kim (1977) was used for determination of pHi from RBC pellets. Plasma CCO_2 measurements were carried out in duplicate on 20 μL samples using a Capni-Con 5 total CO_2 analyser (Cameron Instruments), while blood O_2 content measurements were carried out in triplicate on 30 μL samples with an OxyconTM (Cameron Instruments). Red cell $[\text{Na}^{+}]$ was determined by flame emission spectrophotometry (Varian, model SpectraAA 250 Plus), and $[\text{Cl}^{-}]$ with the mercuric thiocyanate spectrophotometric assay of Zall et al. (1956). Commercial kits were utilised for the measurement of RBC [cyclic AMP] (Amersham Pharmacia Biotech catalogue no. RPN 225) and blood [NTP] (Sigma, 366-UV). The cyanmethaemoglobin method (Sigma) was used to determine [Hb] in duplicate. Haematocrit was measured in duplicate by means of microcapillary tubes centrifuged at 6000 g for 5 min. Plasma protein concentrations were measured using the Bio-Rad protein assay in combination with BioLynx 2.0 software.

Statistical analyses

Data are presented as mean values $\pm$ 1 standard error of the mean (SEM). The statistical significance of treatment effects was assessed by one-way repeated measures analysis of variance (ANOVA) followed by the Bonferroni test for post hoc multiple comparisons against the control value, or by paired Student's *t*-tests, as appropriate. Where assumptions of normality or equal variance were violated, equivalent non-parametric analyses were employed. In all cases, 5% was taken as the fiducial limit of significance and statistical analyses were carried out using commercial software (SigmaStat v2.03; SPSS Scientific Inc.).

Results

CO₂ and O₂ binding properties of bullhead blood

The construction of *in vitro* CO₂ combining curves for bullhead blood under oxygenated and deoxygenated conditions revealed the presence of a Haldane effect (Fig. 3.1A). The magnitude of the Haldane effect was quantified by comparing the plasma [HCO₃⁻] in the oxygenated and deoxygenated blood at a PCO₂ of 4 Torr ($\Delta[\text{HCO}_3^-] = 0.65 \pm 0.23 \text{ mmol L}^{-1}$; $N = 6$) and then normalising for differences in [Hb] ($5.51 \pm 0.82 \text{ g } 100 \text{ mL}^{-1} \text{ blood}$; $N = 6$), yielding a value of $0.77 \pm 0.29 \text{ mmol mmol}^{-1} \text{ Hb}$. This value was significantly different from zero (one-sample Student's *t*-test, $P < 0.05$). For purposes of comparison, CO₂ combining curves were also constructed for rainbow trout blood (Fig. 3.1B) and a Haldane effect of $1.26 \pm 0.06 (6) \text{ mmol mmol}^{-1} \text{ Hb}$ was calculated; again, this value was significantly different from zero (one-sample Student's *t*-test, $P < 0.05$).

Unlike the situation in some elasmobranch fish, where the CO₂ capacitances of separated and true plasma are similar (Lenfant and Johansen, 1966; Tufts and Perry, 1998), construction of *in vitro* CO₂ combining curves for separated and true plasma from bullhead indicated that the CO₂ capacitance of true plasma was more than three times that of separated plasma over most of the PCO₂ range examined (Fig. 3.2). Interestingly, however, separated and true plasma CO₂ capacitances were similar at very low PCO₂ values ($< 1 \text{ Torr}$).

Bullhead blood exhibited a Root effect; the O₂ content of blood equilibrated *in vitro* with gas mixtures of increasing CO₂ tension decreased as pHe decreased (Fig. 3.3A).

Figure 3.1. *In vitro* CO₂ combining curves for brown bullhead (*Ameiurus nebulosus*) (A) and rainbow trout (*Oncorhynchus mykiss*) (B) whole blood under oxygenated (filled symbols) and deoxygenated (unfilled symbols) conditions. Data are means $\pm$ 1 SEM (N = 6) for both species.

Figure 3.2. In vitro CO₂ combining curves for brown bullhead true (unfilled symbols) and separated (filled symbols) plasma. Data are means $\pm$ 1 SEM ($N = 6$).

Figure 3.3. The pH-dependence of blood O₂ content in brown bullhead (A) and rainbow trout (B) were determined by incubating blood samples *in vitro* with gas mixtures of increasing CO₂ tension. Blood O₂ content was expressed as the percentage decrease in haemoglobin-bound O₂ from the maximum value measured. Values for $N = 6$ for both species are included. Iterative curve-fitting software was used to fit a sigmoidal function to the data; a sigmoidal function was selected because it yielded a better fit to the data than either a linear or hyperbolic function. The equation of the line for brown bullhead was $\Delta \text{Hb-O}_2 = 56.6 / (1 + e^{(\text{pH}_e - 7.27)0.35})$, $P < 0.0001$, $R^2 = 0.56$ and for rainbow trout the equation was $\Delta \text{Hb-O}_2 = 61.8 / (1 + e^{(\text{pH}_e - 7.57)0.10})$, $P < 0.0001$, $R^2 = 0.62$. Inset figures correspond to the linear relationship between pH_i and pH_e for each species.

Blood O₂ content was significantly lower than the control value for the two highest CO₂ tensions at which samples were equilibrated, with reductions amounting to $19.0 \pm 5.7\%$ (6) and $27.4 \pm 4.1\%$ (6) for pHe decreases of 0.71 ± 0.14 units (6) and 0.81 ± 0.09 units (6), respectively (Fig. 3.3A). Again, for comparison, trout blood exhibited a significant decrease in O₂ content with a decrease in pHe (Fig. 3.3B). A significant linear relationship between pH_i and pHe was obtained for both bullhead ($\text{pH}_i = 4.69 + 0.29 \cdot \text{pHe}$, $P = 0.0015$, $R^2 = 0.40$) (Fig. 3.3A), and trout ($\text{pH}_i = 4.48 + 0.36 \cdot \text{pHe}$, $P = 0.0001$, $R^2 = 0.78$) (Fig. 3.3B).

Distribution of carbonic anyhydrase

The CA activity of dilute RBC lysates from bullhead, ratfish and skate was not affected by the presence of 100 μL of plasma (Fig. 3.5), indicating that naturally-occurring CA inhibitors are not present in the plasma of these species. By contrast, the inclusion of 100 μL of trout plasma in the CA assay abolished approximately 80% of the CA activity of the dilute trout RBC lysate (Fig. 3.5), a finding in agreement with those of previous studies (Dimberg, 1994; Henry et al., 1997). CA activity was detected in the plasma of ratfish and skate, but not bullhead (Fig. 3.4) in the absence of measurable plasma haemoglobin. For purposes of comparison, rainbow trout and dogfish plasma were also assayed for CA activity. As found previously (Gilmour et al., 1997; Henry et al., 1997), trout plasma lacked CA activity while dogfish plasma exhibited low but measurable activity.

CA activity was found in the gill tissue of all species examined (Table 3.1). Subcellular fractionation of gill homogenates revealed that the majority (60 – 75%) of

CA activity in the gill was found in the cytoplasmic fraction, with the microsomal fraction containing only 3 – 5% of total activity (Table 3.1). Microsomal pellet CA activity was significantly reduced by washing in bullhead (76%) and ratfish (47%) but not skate (Fig. 3.6). Microsomal CA activity was not significantly inhibited by 0.005% SDS in any species [inclusion of 0.005% SDS inhibited microsomal CA activity by $25.4 \pm 11.1\%$ (5), $21.1 \pm 10.8\%$ (3) and $20.9 \pm 2.3\%$ (3) in bullhead, ratfish and skate preparations, respectively] (Fig. 3.7). In ratfish and skate, incubation of suspensions of microsomal pellets with 1 unit of PI-PLC resulted in a significantly higher supernatant CA activity than that observed following incubation with the saline vehicle alone (Fig. 3.7). Furthermore, the elevated supernatant CA activity was accompanied by a corresponding reduction in pellet CA activity. In bullhead, supernatant CA activity was significantly higher for suspensions incubated with PI-PLC over those incubated with saline, but this difference was not accompanied by a corresponding significant difference in the pellet CA activity (Fig. 3.7).

Red blood cell adrenergic responses

The accumulation of cyclic AMP, water, Na^+ and Cl^- by RBCs as well as changes in plasma pH in response to incubation *in vitro* with appropriate agonists were used in the present study as indices of the β -adrenergic responsiveness of bullhead RBCs. Trout RBCs were used as a standard in these experiments because the trout RBC adrenergic response is well defined. Bullhead RBCs exhibited significant cyclic AMP accumulation at isoproterenol concentrations at and above $10^{-7} \text{ mol L}^{-1}$ (Fig. 3.8). Relative to rainbow trout, however, both the extent of cyclic AMP accumulation by bullhead RBCs (876% vs.

Table 3.1. Total carbonic anhydrase (CA) activity (in $\mu\text{mol CO}_2 \text{ min}^{-1}$) associated with crude gill homogenates, as well as microsomal and cytoplasmic subcellular fractions.

Sample	Bullhead (<i>N</i> = 7)	Ratfish (<i>N</i> = 4)	Skate (<i>N</i> = 4)
Crude homogenate	10676 $\pm$ 1962	17486 $\pm$ 2100	7812 $\pm$ 1698
Cytoplasmic fraction	5153 $\pm$ 839 (59.6 $\pm$ 5.8%)	8827 $\pm$ 1223 (74.8 $\pm$ 4.5%)	5124 $\pm$ 761 (66.5 $\pm$ 4.4%)
Microsomal fraction	378 $\pm$ 80 (5.0 $\pm$ 1.4%)	386 $\pm$ 75 (3.3 $\pm$ 0.4%)	409 $\pm$ 160 (5.1 $\pm$ 1.7%)

Values are means $\pm$ 1 SEM. CA activities have been multiplied by fraction volumes (mL) to account for the dilution of each fraction and are therefore reported as total CA activities in $\mu\text{mol CO}_2 \text{ min}^{-1}$. Values in parentheses are CA activities reported as percentages of the total CA activity in the homogenate.

Figure 3.4. Plasma carbonic anhydrase (CA) activities (in $\mu\text{mol CO}_2 \text{ mL}^{-1} \text{ min}^{-1}$) measured for 50 μL plasma samples from rainbow trout, bullhead, ratfish, skate and dogfish using the electrometric ΔpH method. Values are means $\pm$ 1 SEM, and an asterisk (*) indicates that the mean CA activity is significantly greater than zero (one-sample Student's *t*-test, $P < 0.05$).

Figure 3.5. Percent inhibition of 10 μL dilute RBC lysate carbonic anhydrase (CA) activity for rainbow trout, bullhead, ratfish and skate by 100 μL of plasma from the corresponding species. Values are means $\pm$ 1 SEM ($N = 5$ for all species except rainbow trout, where $N = 4$). An asterisk (*) indicates a significant reduction in the RBC lysate CA activity upon inclusion of 100 μL of plasma in the assay (paired Student's *t*-test, $P < 0.05$). For skate, dilute ratfish RBC lysate was used as skate RBC lysate was not available.

Figure 3.6. The effect of washing by mild sonication on carbonic anhydrase (CA) activity associated with the microsomal fraction of bullhead, ratfish and skate gills. Values are means $\pm$ 1 SEM ($N = 3$). CA activities were multiplied by fraction volumes (mL) to account for the dilution of each fraction and are therefore expressed as total CA activities in $\mu\text{mol CO}_2 \text{ min}^{-1}$. An asterisk (*) indicates a significant difference from the initial value (paired Student's *t*-test, $P < 0.05$).

Figure 3.7. Effect of incubating a suspension of the microsomal fraction from bullhead, ratfish or skate gills with phosphatidylinositol-specific phospholipase C (PI-PLC) or a saline vehicle alone (control). The carbonic anhydrase (CA) activities of (A) the supernatant derived from centrifugation of the suspension following the incubation period, and (B) the resultant microsomal pellet are presented. Values are means $\pm$ 1 SEM ($N = 3$). CA activities have been reported on a per milligram of protein basis to allow comparisons among species. An asterisk (*) indicates supernatant CA activity that was significantly higher than the control value, or pellet CA activity that was significantly lower than the control value, with PI-PLC treatment (unpaired Student's *t*-test, $P < 0.05$). In (B), control pellet CA activities that share the same letter are not significantly different from one another (one-way ANOVA followed by Bonferroni's post hoc multiple comparisons test, $P < 0.05$)

3564% for trout) and the maximal RBC cyclic AMP level achieved (4-fold lower than trout; Fig. 3.8), were small. Despite the significant increases in bullhead RBC cyclic AMP concentration at the higher isoproterenol concentrations, no significant change in pHe was detected, nor did bullhead RBCs exhibit swelling or ion accumulation (Fig. 3.9). By contrast, trout blood exhibited the expected responses (Fig. 3.9) of a fall in pHe (of 0.21 pH units at 10^{-5} mol L⁻¹ isoproterenol), coupled with substantial red cell swelling (maximum increase of 4.4%) and ion accumulation (maximal increases of 52 μ mol g⁻¹ dry mass and 35 μ mol g⁻¹ dry mass in RBC [Na⁺] and [Cl⁻], respectively). Thus, bullhead RBCs accumulated cyclic AMP, but failed to demonstrate measurable activation of a β -NHE.

To further investigate the apparent absence of β -NHE activity from bullhead RBCs, blood was incubated with the permeable cyclic AMP analogue 8-bromo cyclic AMP, or with the adenylate cyclase activator forskolin. In rainbow trout blood, as previously demonstrated (Mahé et al., 1985; Salama and Nikinmaa, 1990), the effect of these agonists in increasing RBC intracellular cyclic AMP levels produced changes in pHe and RBC water and ion contents similar to the maximal effects induced by isoproterenol (Tables 3.2 and 3.3). For example, pHe decreased by a maximum of 0.2 units with all three agonists, while trout RBC swelling amounted to 4% with isoproterenol, 6% with 8-bromo cyclic AMP and 5% with forskolin. However, no reduction in pHe, cell swelling or RBC ion accumulation accompanied the elevation of RBC cyclic AMP levels resulting from incubation of bullhead blood with 8-bromo cyclic AMP or forskolin (Tables 3.2 and 3.3).

Figure 3.8. The effects of isoproterenol concentration on red blood cell (RBC) cyclic AMP concentration ([cyclic AMP]) for brown bullhead (filled symbols; $N = 6$) and rainbow trout (unfilled symbols, $N = 6$) blood incubated with isoproterenol *in vitro*. Data are means $\pm$ 1 SEM. * indicates a significant difference (one-way repeated measures ANOVA, $P < 0.05$) between the value for a given isoproterenol concentration and its associated control (blood incubated with saline alone, assumed to have [catecholamines] = 1 nmol L⁻¹).

Figure 3.9. The effects of isoproterenol concentration on (A) plasma pH (pHe), and on red blood cell (RBC) (B) water content, (C) Na^+ concentration and (D) Cl^- concentration for brown bullhead (filled symbols, $N = 6$) and rainbow trout (unfilled symbols, $N = 6$) blood incubated with isoproterenol *in vitro*. Values are means $\pm$ 1 SEM. * indicates a significant difference (one-way repeated measures ANOVA, $P < 0.05$) between the value for a given isoproterenol concentration and its associated control (blood incubated with saline alone, assumed to have [catecholamines] = 1 nmol L^{-1}).

● bullhead
○ trout

Table 3.2. The effects of 8-bromo cyclic AMP concentration on plasma pH (pHe) as well as red blood cell (RBC) water content and ion concentrations for blood from bullhead or rainbow trout incubated with 8-bromo cyclic AMP *in vitro*.

		Control	[8-bromo cyclic AMP] (mol L ⁻¹)		
			10 ⁻⁴	10 ⁻³	10 ⁻²
pHe	Bullhead	7.97 ± 0.02	7.97 ± 0.02	7.98 ± 0.02	7.98 ± 0.02
	Trout	7.90 ± 0.01	7.78 ± 0.02*	7.72 ± 0.02*	7.68 ± 0.02*
RBC [water] (%)	Bullhead	61.5 ± 1.3	61.7 ± 0.9	61.8 ± 0.9	59.5 ± 0.8
	Trout	62.5 ± 0.6	65.1 ± 0.8*	68.1 ± 0.4*	67.9 ± 0.4*
RBC [Na ⁺] (μmol g ⁻¹ dry mass)	Bullhead	37.4 ± 7.1	45.2 ± 7.3	32.2 ± 3.3	50.9 ± 3.9
	Trout	52.6 ± 11.8	72.4 ± 6.2	110.0 ± 8.7*	119.0 ± 10.1*
RBC [Cl ⁻] (μmol g ⁻¹ dry mass)	Bullhead	39.4 ± 15.7	42.2 ± 16.1	35.6 ± 9.8	37.6 ± 4.4
	Trout	33.8 ± 2.5	45.9 ± 5.8	57.5 ± 4.6	64.8 ± 6.9*

Values are means ± 1 SEM (N = 6).

* indicates a statistically significant difference between a treatment value and its associated control (one-way repeated measures ANOVA. $P < 0.05$).

Table 3.3. The effects on plasma pH (pHe) and red blood cell (RBC) cyclic AMP, water and ion concentrations of incubation *in vitro* of blood samples from bullhead or rainbow trout with 10^{-4} mol L⁻¹ forskolin.

		Control	Forskolin
RBC [cyclic AMP] ($\mu\text{mol kg}^{-1}$ dry mass)	Bullhead	0.52 ± 0.24	$2.09 \pm 0.48^*$
	Trout	2.22 ± 1.27	$19.8 \pm 7.2^*$
pHe	Bullhead	7.98 ± 0.01	7.98 ± 0.01
	Trout	7.84 ± 0.02	$7.66 \pm 0.03^*$
RBC [water] (%)	Bullhead	60.9 ± 0.3	60.6 ± 0.3
	Trout	65.4 ± 0.5	$70.7 \pm 0.5^*$
RBC [Cl ⁻] ($\mu\text{mol g}^{-1}$ dry mass)	Bullhead	63.4 ± 13.6	72.3 ± 4.7
	Trout	68.2 ± 4.4	$103.2 \pm 6.6^*$

Values are means $\pm$ 1 SEM ($N = 6$, except cyclic AMP data for bullhead, where $N = 5$)

* indicates that the value for forskolin is significantly greater ([cyclic AMP], RBC [water] and [Cl⁻]) or significantly lower (pHe) than its associated control (one-tailed paired Student's *t*-test, $P < 0.05$).

Discussion

Oxygen and carbon dioxide transport in vertebrates are intimately linked with RBC pH_i, RBC membrane transport proteins, and the properties of haemoglobin (see reviews by Brauner and Randall, 1996, 1998; Nikinmaa, 1997). In teleost fish, the buffering power of haemoglobin, and hence blood, is relatively low owing to few histidine residues per haemoglobin chain and the acetylation of N-terminal amino acids (Jensen, 1989, 1991). Oxygenation-linked changes in haemoglobin-proton binding are therefore of enhanced importance, and most teleost fish display a substantial Haldane effect (Jensen 1989) that contributes to CO₂ excretion by providing protons for HCO₃⁻ dehydration at the gills (Perry and Gilmour, 1993; Brauner et al., 1996; Perry et al., 1996b) and by removing protons produced during CO₂ hydration in the tissues. A markedly different strategy is employed by elasmobranch fish. The buffer capacity of elasmobranch haemoglobins is substantial relative to that of teleosts, while the Haldane effect is small or absent (Butler and Metcalfe, 1988; Jensen, 1989; Wood et al., 1994). Consequently, RBC CO₂ reactions are supported by the buffering properties of haemoglobin rather than by oxylabile proton binding and release (Jensen, 1991; Nikinmaa, 1997). In addition, plasma buffering may account for up to 70% of the whole blood buffer value (Lenfant and Johansen, 1966; Graham et al., 1990) and this high non-bicarbonate buffer capacity contributes to CO₂ excretion in two ways. First, the CO₂ capacitance of separated plasma is similar to that of true plasma, unlike in teleosts where separated plasma exhibits a much lower CO₂ capacitance than true plasma (Lenfant and Johansen, 1966; Tufts and Perry, 1998). Second, plasma buffering may provide protons for HCO₃⁻ dehydration

(Gilmour et al., 2001a) by the extracellular CA that in elasmobranchs is found both in the plasma and bound to gill epithelial cell membranes with an extracellular orientation (Wood et al., 1994; Gilmour et al., 1997, 2001a,b; Henry et al., 1997; Wilson et al., 2000); elasmobranch fish lack an endogenous plasma CA inhibitor (Henry et al., 1997; Gilmour et al., 2001b). Analysis of plasma and gill CA activities in bullhead yielded results that were also in agreement with this conclusion (Figs 3.4, 3.6, 3.8).

Like elasmobranch fish (Henry et al., 1997), brown bullhead exhibit an unusually high plasma buffer capacity (Chapter, 2), and do not possess an endogenous plasma CA inhibitor (Fig. 3.5). In all other respects, however, bullhead follow the standard teleost pattern. Despite plasma buffering in bullhead that is two- to three-fold higher than typical teleost values, the CO₂ combining power of separated plasma was generally low relative to that of true plasma (Fig. 3.2). The low buffer capacity of bullhead haemoglobin, and hence the RBC coupled with the presence of a Haldane effect (Fig. 3.1A) suggests reliance on oxyliable proton binding to haemoglobin to support CO₂ excretion.

Despite the absence of plasma CA inhibitors (Fig. 3.5), bullhead plasma failed to exhibit CA activity. Moreover, analysis of bullhead branchial CA in the present study yielded results that were, in general, consistent with the standard teleost pattern of little or no branchial membrane bound CA activity. While PI-PLC treatment of microsomal suspensions had a significant impact on supernatant CA activity, this difference was not accompanied by a corresponding reduction in microsomal pellet CA activity (Fig. 3.8). The data suggest that CA was released by PI-PLC treatment, but that the activity released was not a significant proportion of the total activity associated with the microsomal

pellet. In addition, bullhead microsomal CA activity was not significantly inhibited by 0.005% SDS (Fig. 3.7). In mammals, CA IV is more stabilised by intramolecular disulphide bonds and hence more resistant to denaturation by SDS than is cytoplasmic CA, and therefore susceptibility to inhibition by SDS is used to distinguish between these isozymes (Whitney and Briggie 1982; Waheed et al., 1992, 1996). However, SDS appears to be a less effective tool for differentiating membrane-bound from cytoplasmic CA in at least some fish and invertebrates (Böttcher et al., 1994; Gervais and Tufts 1998; but see also Maffia et al., 1996). Overall, the results of the present study suggest that the gills of bullhead, like those of other teleost fish, contain physiologically insignificant levels of membrane-bound CA activity. As bullhead lack plasma CA activity (Fig. 3.4) as well as significant levels of branchial membrane-bound CA (Figs. 3.6, 3.7), the hypothesis that the unusually high non-bicarbonate buffer capacity of bullhead plasma functions in CO₂ excretion was not supported by the experimental data.

In teleost fish, the effect of oxygenation status on proton binding by haemoglobin is reflected in the effect that proton binding has on the affinity (Bohr effect) and capacity (Root effect) of haemoglobin to bind oxygen (Jensen 1991; Brauner and Randall 1998; Jensen et al., 1998). Blood O₂ transport can be protected from the consequences of the Root effect, however, by adrenergic activation of the RBC membrane β -NHE which permits regulation of RBC pHi effectively independently of plasma pH (Nikinmaa 1992). The interaction of catecholamines with β -adrenoreceptors on the RBC surface triggers, *via* the β -adrenergic signal transduction pathway, cyclic AMP formation that, in turn, elicits activation of the Na⁺/H⁺ exchanger (Guizouarn et al., 1993). Extrusion of protons in exchange for extracellular Na⁺ results in alkalisation of the RBC intracellular

environment coupled with an extracellular acidosis and RBC swelling, as osmotically-obliged water follows Na^+ entry (reviewed by Thomas and Perry 1992; Nikinmaa 1992; Motais et al., 1992; Perry and McDonald, 1993; Nikinmaa and Salama 1998); these effects were clear in rainbow trout blood exposed to isoproterenol *in vitro* (Figs. 3.9. Tables 3.2, 3.3).

Despite the presence of a Root effect in bullhead (Fig. 3.3A; Haws and Goodnight 1962) and significant cyclic AMP accumulation with exposure to isoproterenol (Fig. 3.9), no evidence for adrenergic activation of a RBC membrane Na^+/H^+ exchanger was obtained in the present study. Bullhead blood failed to show any cell swelling, RBC ion accumulation or an extracellular acidosis with adrenergic stimulation *in vitro* (Fig. 3.9), or following experimental elevation of RBC cyclic AMP levels via incubation with 8-bromo cyclic AMP or forskolin (Tables 3.2, 3.3). Although the change in pHe is generally a reliable indicator of the intensity of adrenergically-induced Na^+/H^+ exchange (Thomas et al., 1991), the unusually high buffer capacity of bullhead plasma would minimise the extent of the pHe disequilibrium resulting from β -NHE activity and could make the detection of a pHe decrease difficult (Perry and Reid, 1992). Thus, multiple indices of β -NHE activity (cell swelling, change in pHe, ion accumulation) were used in the present study; regardless of the index used, bullhead RBCs failed to demonstrate measurable β -NHE activity. Because oxygen and/or pH dependencies of the RBC β -adrenergic response have been documented in several teleosts, blood was equilibrated to hypoxic conditions in the present study. The magnitude of the RBC adrenergic response in carp, for example, was reported to be negligible under normoxic conditions, but to increase with decreasing O_2 tension (Salama and Nikinmaa, 1988, 1990). Exposure to

hypoxic conditions results in increases in both the number of β -adrenoreceptors (Marttila and Nikinmaa 1988; Reid et al., 1993) and in the activity of the β -NHE (Motaïs et al., 1987). However, even in species such as carp where the normoxic RBC β -adrenergic response was minimal, incubation with forskolin or 8-bromo cyclic AMP elicited significant cell swelling (Salama and Nikinmaa, 1990), which contrasts with the results for bullhead RBCs. While the accumulation of cyclic AMP by adrenergically-stimulated bullhead red cells implies the presence of a RBC β -adrenergic receptor, which has been confirmed by J. Nickerson (personal communication), the results of the present study indicate that stimulation of this receptor does not activate a β -NHE and hence, that bullhead RBCs are incapable of adrenergic pHi regulation.

Pronounced species differences exist in the activity of the β -NHE, with salmonid fish typically exhibiting greater Na^+/H^+ exchange activity, particularly under normoxic conditions and/or at physiological pHe, than cyprinid or percid fish (Milligan and Wood, 1987; Jensen, 1987; Tufts and Randall, 1989; Salama and Nikinmaa, 1989; Cossins and Kilbey, 1991; Lowe et al., 1998). Like bullhead, eel (*Anguilla rostrata* and *A. anguilla*) RBCs appear incapable of adrenergic pHi regulation (Hyde and Perry, 1990; Perry and Reid, 1992; Romero et al., 1996) despite the presence of RBC membrane β -adrenoreceptors (Bennett and Rankin, 1985; Perry and Reid, 1992) and significant cyclic AMP accumulation following adrenergic stimulation (Perry and Reid, 1992). Although eel RBCs were found to accumulate greater quantities of cyclic AMP than trout (Perry and Reid, 1992) while bullhead RBC cyclic AMP accumulation was small relative to trout (Fig. 3.9; Table 3.3), incubation with 8-bromo cyclic AMP failed to stimulate Na^+/H^+ exchange in both eel (Romero et al., 1996) and bullhead (Table 3.2). Eel RBC

membranes contain an amiloride-sensitive Na^+/H^+ antiporter that is activated by cell shrinkage and functions in regulatory volume increases (Romero et al., 1996). The presence of a similar volume-activated NHE in bullhead red cells must still be assessed. In addition, the physiological role of the β -adrenergic signal transduction system that is clearly present in the RBCs of bullhead and eel remains to be determined.

Since eel RBCs lack the capacity for adrenergic pHi regulation, RBC pHi *in vivo* during acid-base disturbances induced by air-exposure or hypercapnic exposure conforms to the *in vitro* relationship between pHe and RBC pHi (Hyde et al., 1987; Hyde and Perry 1990). Decreases in RBC pHi, in turn, elicit reductions in arterial O_2 content that can be predicted from measurement of the Root effect *in vitro* (Hyde et al., 1987; Hyde and Perry 1990). A similar situation would be envisaged to exist in bullhead. However, the impact on pHe, and hence RBC pHi, of proton addition to the vascular compartment in bullhead should be mitigated by the unusually high plasma non-bicarbonate buffer capacity. Hyde et al. (1987) determined plasma buffering in eel to be $-2.73 \text{ mmol L}^{-1} \text{ pH unit}^{-1}$, a value typical of most teleosts (Tufts and Perry, 1998; Gilmour et al., 2001b), whereas plasma buffering in bullhead, at $-5.7 \text{ mmol L}^{-1} \text{ pH unit}^{-1}$, is two- to three-fold higher than standard teleost values, likely owing to the presence in the plasma of a histidine-rich albumin (Chapter 2). Thus, in the absence of adrenergic RBC pHi regulation, the unusually high non-bicarbonate buffer capacity of separated plasma in bullhead is likely to be of physiological significance in defending RBC pHi against the effect of protons added to the vascular compartment, and hence in protecting blood O_2 transport.

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UMI

Teleost fish generally possess haemoglobins that have lower buffering capacities (Perry et al., 1985; Wood et al., 1982), than those of elasmobranch fish or other vertebrates (Lenfant and Johansen, 1966; Graham et al., 1990; Wood et al., 1990; Brunori, 1966; Breepoel et al., 1980; Jensen and Weber, 1985). However, relatively high blood buffering appears to be common among hypoxia-tolerant fish (Cech et al., 1994) and within the catfish order (Cameron and Kormanik, 1982; Albers et al., 1981; Wood et al., 1979). In accordance with this trend, brown bullhead (*Ameiurus nebulosus*), a teleost, was found to possess an unusually high separated plasma non-bicarbonate buffer capacity (Figure 2.1), which tends to elevate whole blood buffer capacity (Table 2.4). This large extracellular buffering power is likely due to a histidine-rich albumin that is present in the plasma (Table 2.3; Figs 2.2, 2.3).

Haemoglobin supplies the protons required for HCO_3^- dehydration and hence CO_2 excretion via its high buffer capacity and/or through the release of Bohr protons during the oxygenation of haemoglobin (Haldane effect) (Brauner and Randall, 1998). Bullhead haemoglobin displayed a Haldane effect (Fig. 3.1), like most teleosts (Jensen, 1986, 1989; Nikinmaa and Mattsoff, 1992; Ferguson et al., 1992; Cech et al., 1984; Jones et al., 1986). Conversely, the Haldane effect in most elasmobranchs is small or absent (Jensen, 1989), presumably because the high haemoglobin buffer capacity in elasmobranchs functions to provide sufficient protons to support CO_2 reactions. Plasma (Wood et al., 1994; Gilmour et al., 1997; Henry et al., 1997) and gill membrane-bound carbonic anhydrase (CA) activities are available for catalysing extracellular CO_2 reactions (Henry et al., 1997; Gilmour et al., 1997) in elasmobranchs. Teleosts, like elasmobranchs,

possess intracellular CA in the RBC and branchial tissue, but lack extracellular CA activity (Henry et al., 1988; 1993; Perry and Laurent, 1990; Perry et al., 1997). Additionally, teleosts typically possess an endogenous plasma CA inhibitor (Dimberg, 1994). Although bullhead, like most other teleosts, were observed to lack both plasma (Fig. 3.4) and branchial membrane-bound CA activities (Figs. 3.6, 3.7), this species was also found to lack a plasma CA inhibitor (Fig. 3.5). It is clear that the physiological significance of the high plasma buffer capacity in bullhead is not related to CO₂ excretion, because bullhead lack extracellular CA, are dependent upon oxyliable proton binding for CO₂ excretion, and exhibit a plasma CO₂ capacitance that is significantly lower than that of whole blood.

Root effect haemoglobins are present in most teleosts (Torracca et al., 1977; Noble et al., 1970; Brill and Bushnell, 1991). Likewise, brown bullhead haemoglobin displayed a pronounced decrease in haemoglobin-O₂ carrying capacity at decreased pH (Fig. 3.3A); the response was comparable to that observed for trout (Fig. 3.3B). The β -adrenergic response is thought to offset the decrease in haemoglobin-O₂ binding capacity that would otherwise occur during the Root effect. In agreement with this hypothesis, certain elasmobranch species that lack Root effect haemoglobins also lack a RBC β -adrenergic pH response (Tufts and Randall, 1989).

In many species of teleost fish, activation of the β -NHE (Nikinmaa, 1983; Primm et al., 1986) plays an important role in the control of Hb-O₂ affinity (Nikinmaa, 1997). Generally, teleost RBCs exhibit a β -adrenergic response to some extent (Salama and Nikinmaa, 1988; 1989; Nikinmaa and Huestis, 1984; Cossins and Kilbey, 1991), with the exception of the eels (*Anguilla anguilla* and *A. rostrata*) (Hyde and Perry, 1990; Perry

and Reid, 1992; Romero et al., 1996). Despite intracellular accumulation of cyclic AMP, eels do not show β -NHE activation and it is likely that these fish lack the β -NHE completely, having instead a volume-activated NHE (Romero et al., 1996). Similar to eels, bullhead did not exhibit red blood cell swelling, ion accumulation or extracellular pH differences with the addition of isoproterenol (Fig. 3.9), 8-bromo cyclic AMP (Table 3.2) or forskolin (Table 3.3), despite the increase in intracellular cyclic AMP concentration seen with isoproterenol (Fig. 3.8) and forskolin (Table 3.3). Therefore, the high plasma buffering power in bullhead may function to protect RBC intracellular pH, since bullhead appear to lack β -NHE activity despite possessing RBC β -adrenoreceptors.

FUTURE RESEARCH

Although this study provided an explanation for the origin of and a potential physiological role for the unusually high separated plasma buffer capacity in brown bullhead, the results obtained have in turn generated new questions. For example, can the high plasma buffering that appears to be common among catfish and/or hypoxia tolerant fish also be attributed to the presence of a histidine-rich albumin in other species or is it solely a characteristic of brown bullhead separated plasma? Furthermore, while the results of this study have postulated a role for high plasma buffering in protecting RBC pH_i, this hypothesis has yet to be tested. Does the high extracellular buffering power of bullhead protect blood O₂ transport against the effects of acidosis as a result of exhaustive exercise, hypoxia or hypercapnia? These questions could be investigated by analyzing changes in intracellular pH and haemoglobin-oxygen binding in fish with both a high extracellular buffer capacity (i.e. bullhead) and those that possess lower buffering

power (other species and/or bullhead with lower plasma buffering). Additionally, it would be of interest to further investigate the absence of plasma carbonic anhydrase inhibitors in bullhead. Perhaps plasma CA inhibitors are not necessary because the red cells are unusually resistant to lysis.

Studies further examining the RBC β -adrenergic response signal transduction pathway for species in which the response is attenuated (i.e. eels and bullhead) would aid in understanding the physiological significance of the presence of the first portion of the pathway (β -adrenoreceptor, cyclic AMP accumulation) in the absence of subsequent activation of the β -NHE. In light of the reported variability in both the magnitude and external conditions at which the β -adrenergic response is observed in fish, studies focusing on the association between high extracellular buffering and the absence of measurable β -NHE activation may yield a better understanding of the protective role that high plasma buffering may play. Moreover, experiments involving variations in physical and environmental stressors may provide insight into the type of situations for which bullhead secrete catecholamines as well as the additional function, if any, that RBC β -adrenoreceptors possess.

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