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RESEARCH ARTICLE

Plasma-accessible carbonic anhydrase at the tissue of a teleost fish may greatly enhance oxygen delivery: *in vitro* evidence in rainbow trout, *Oncorhynchus mykiss*

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SUMMARY

During a generalized acidosis in rainbow trout, catecholamines are released into the blood, activating red blood cell (RBC) Na^+/H^+ exchange (βNHE), thus protecting RBC intracellular pH (pH_i) and subsequent O_2 binding at the gill. Because of the presence of a Root effect (a reduction in oxygen carrying capacity of the blood with a reduction in pH), the latter could otherwise be impaired. However, plasma-accessible carbonic anhydrase (CA) at the tissues (and absence at the gills) may result in selective short-circuiting of RBC βNHE pH regulation. This would acidify the RBCs and greatly enhance O_2 delivery by exploitation of the combined Bohr–Root effect, a mechanism not previously proposed. As proof-of-principle, an *in vitro* closed system was developed to continuously monitor extracellular pH (pH_e) and O_2 tension (P_{O_2}) of rainbow trout blood. In this closed system, adding CA to acidified, adrenergically stimulated RBCs short-circuited βNHE pH regulation, resulting in an increase in P_{O_2} by >30 mmHg, depending on the starting Hb-O_2 saturation and degree of initial acidification. Interestingly, in the absence of adrenergic stimulation, addition of CA still elevated P_{O_2}, albeit to a lesser extent, a response that was absent during general NHE inhibition. If plasma-accessible CA-mediated short-circuiting is operational *in vivo*, the combined Bohr–Root effect system unique to teleost fishes could markedly enhance tissue O_2 delivery far in excess of that in vertebrates possessing a Bohr effect alone and may lead to insights about the early evolution of the Root effect.

Key words: combined Bohr–Root effect, haemoglobin, βNHE, oxygen delivery, carbonic anhydrase, catecholamine, isoproterenol, short-circuiting.

INTRODUCTION

A reduction in blood pH during blood capillary transit enhances O_2 delivery in vertebrates through the Bohr effect, a physiological mechanism that has been studied extensively for over a century and defined as the decrease in haemoglobin (Hb)–O_2 affinity with a reduction in blood pH (Bohr et al., 1904; Nikinmaa and Soivio, 1979; Nikinmaa, 1997). In addition to the Bohr effect, teleost fishes also possess a Root effect, where a reduction in pH not only decreases Hb–O_2 affinity but also greatly reduces the O_2 carrying capacity of blood (Root, 1931; Root and Irving, 1943; Scholander and Van Dam, 1954). Within a given teleost blood system, it may be impossible to separate the shift associated with the Root effect from the traditionally understood Bohr shift; therefore, in teleosts possessing Root effect Hbs, the shift is referred to as the combined Bohr–Root effect. The Root effect is used to great advantage for filling a swimbladder against large pressure gradients (>5066.5 kPa) associated with depth (Scholander and Van Dam, 1954) and for oxygenating the metabolically active yet avascular retinal tissue of the eye (Wittenberg and Wittenberg, 1962; Wittenberg and Wittenberg, 1974; Waser and Heisler, 2005). Harnessing this potential is thought to be dependent on localizing and recycling an acidosis *via* a unique vascular architecture, the rete mirabile at the swimbladder (Scholander, 1954) and the choroid rete at the eye (Wittenberg and Haedrich, 1974; Wittenberg and Wittenberg, 1974). With respect to general O_2 delivery, however, the role of the Root effect has received little attention probably because it is understood that the associated Haldane effect would actually minimize blood pH changes in the tissues (Lapennas, 1983). In this study, we propose a novel mechanism in fish blood that exploits the presence of plasma-accessible carbonic anhydrase (CA) in the tissues to increase H^+ influx to the red blood cells (RBCs) during blood capillary transit and exploit the combined Bohr–Root effect to greatly enhance general O_2 delivery.

Most teleost fish that exhibit a pronounced Bohr–Root effect adrenergically regulate RBC pH to maintain O_2 loading at the gills (Berenbrink et al., 2005). Catecholamines (e.g. adrenaline and noradrenaline) are released into the general circulation and bind to β-adrenergic receptors on the RBC membrane that, *via* adenylate cyclase and 3′,5′-cyclic adenosine monophosphate (cAMP), activate β-adrenergic Na^+/H^+ exchange (βNHE) (Mahé et al., 1985). The CA-catalyzed hydration of CO_2 inside the RBC produces H^+ that are removed in exchange for Na^+ *via* βNHE (Baroin et al., 1984; Cossins and Richardson, 1985), and HCO_3^- that is removed *via* anion exchange for Cl^- at a slower rate. This combination results in an increase in intracellular pH (pH_i) and an increase in Hb–O_2 affinity (Nikinmaa, 1983; Baroin et al., 1984; Cossins and Richardson, 1985; Borgese et al., 1986; Borgese et al., 1987). The H^+ removed from the RBCs acidify the plasma, resulting in a decrease in extracellular pH (pH_e) (Nikinmaa, 1983; Baroin et al., 1984; Cossins and Richardson, 1985; Borgese et al., 1986; Borgese et al., 1987). In the plasma, the H^+ will eventually combine with HCO_3^- at an uncatalyzed rate to form CO_2, resulting in a slow plasma alkalinization after the initial pH decrease (Fig. 1A) (Lessard et al., 1995; Geers and Gros, 2000). Adrenergic RBC βNHE is thought to have evolved to safeguard O_2 uptake at the respiratory surfaces during a

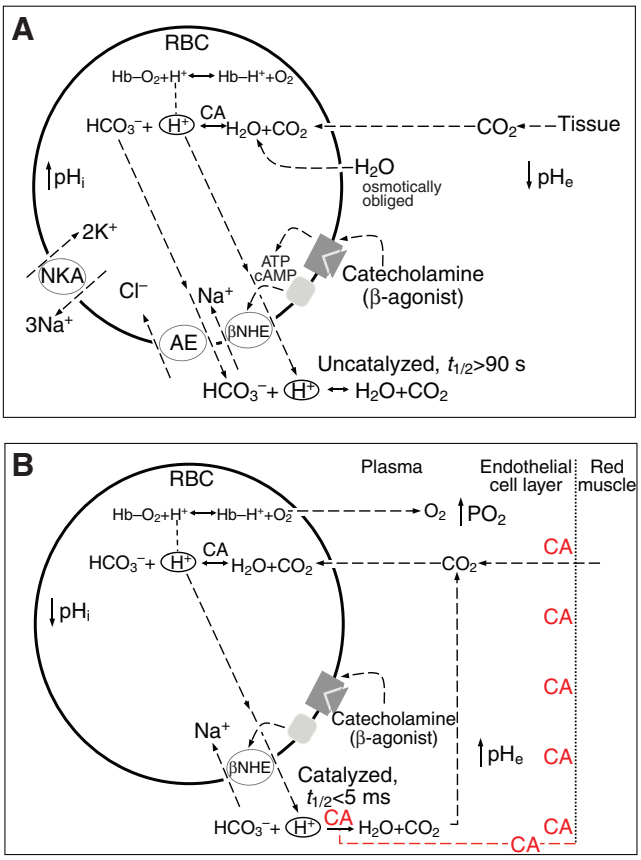

Fig. 1. (A) Schematic illustrating the cascade associated with red blood cell (RBC) adrenergic stimulation at the level of the tissue. Modified from Heming (Heming, 1984), Bidani and Crandall (Bidani and Crandall, 1988) and Cardenas et al. (Cardenas et al., 1998). (B) Simplified mechanism, such that the proposed short-circuiting of RBC βNHE pH regulation upon initial contact with plasma-accessible carbonic anhydrase (CA; in red) at the tissues and associated changes in extracellular pH (pH_e), intracellular pH (pH_i) and the partial pressure of oxygen (P_{O_2}) can be outlined. AE, anion exchange; ATP, adenosine triphosphate; cAMP, 3′,5′-cyclic adenosine monophosphate; Hb, haemoglobin; NKA, Na^+/K^+-ATPase; βNHE, β-adrenergically activated sodium proton exchanger.

generalized acidosis in the presence of Bohr–Root shift Hb (Nikinmaa et al., 1984; Primmett et al., 1986; Borgese et al., 1987; Perry and Kinkead, 1989; Malapert et al., 1997).

Conceptually, the presence of CA in the plasma would short-circuit pH regulation associated with adrenergic activation of RBC βNHE (Motais et al., 1989; Nikinmaa et al., 1990). Although plasma-accessible CA is not present in the teleost gill, membrane-bound plasma-accessible CA (e.g. CA IV-like isoforms) may exist in select locations such as bound to muscle endothelia (Effros and Weissman, 1979; Siffert and Gros, 1982; Decker et al., 1996; Henry et al., 1997; Geers and Gros, 2000). Indeed, fish are thought to possess plasma-accessible CA isoforms similar to mammalian CA IV, but their location and function remain undetermined (reviewed in Gilmour and Perry, 2009). We propose that if CA is available to the plasma in tissue capillaries, H^+ removed from the RBC *via* βNHE could combine with plasma HCO_3^- to reform CO_2, which would back-diffuse into the RBC, decrease pH_i and ultimately create a larger arterial to venous pH gradient (ΔpH_{a-v}) at the tissues than would otherwise occur (Fig. 1B). The large acidosis transferred to the RBC would elevate the partial pressure of O_2 (P_{O_2}) *via* the combined Bohr–Root effect, thus greatly facilitating tissue O_2 delivery. Furthermore, provided the rate of short-circuiting of βNHE RBC pH regulation in the tissue and subsequent pH_i recovery during transit to the gill was sufficiently rapid, a generalized acidosis could provide H^+ that could be repeatedly used at select tissues (to increase the ΔpH_{a-v}) with every pass through the circulation, thus elevating tissue P_{O_2} at a time when O_2 delivery is especially needed.

As a first step, this study was designed to demonstrate proof-of-principle for enhanced O_2 delivery when adrenergically stimulated RBC βNHE pH regulation is short-circuited. Rainbow trout blood was pre-equilibrated at pre-defined Hb–O_2 saturations and then, in a closed system, acidified, β-adrenergically stimulated and then exposed to CA. Changes in both pH_e and P_{O_2} were monitored continuously to assess both the magnitude and time course of the response. It was hypothesized that in this *in vitro* closed system, and in the presence of an acidosis, plasma-accessible CA short-circuits pH regulation associated with adrenergically stimulated RBC βNHE, thus creating a decrease in RBC pH that elevates the driving force for O_2 delivery, ΔP_{O_2}, because of the combined Bohr–Root effect. The overall aim was to determine whether βNHE short-circuiting could be operational *in vivo* and estimate the degree to which it might influence O_2 delivery.

MATERIALS AND METHODS

Animals and rearing conditions

Rainbow trout, *Oncorhynchus mykiss* Walbaum 1792 (300–600 g wet body mass), were obtained from Spring Valley Trout Farm (Langley, British Columbia, Canada) and maintained at the University of British Columbia Aquatic Facilities. Fish were held under a natural photoperiod at densities no greater than 10 kg m^{-3} (North et al., 2006) in 4000 l tanks supplied with flow-through 10°C Vancouver dechlorinated municipal tap water. Fish were fed every other day to satiation using commercial trout pellets (Skretting, Orient 4-0, Vancouver, BC, Canada). All experiments were completed during the spring months over two separate years. All procedures complied with the guidelines approved by the Canadian Council on Animal Care (UBC protocol no. A07-0080).

Sampling protocol

Fish were quickly anaesthetized in a 20 l bucket of clean, well-aerated water containing benzocaine solution (0.2 mmol l^{-1} final concentration p-aminobenzoate). Fish were then placed on a surgery table, and their gills were intubated and continuously irrigated with water containing a more dilute anaesthetic (0.02 mmol l^{-1} p-aminobenzoate). An indwelling cannula (PE50) was surgically implanted into the dorsal aorta according to Soivio et al. (Soivio et al., 1975). Following surgery, fish were placed in a Perspex box supplied with aerated 12°C clean water and gently force-ventilated until they regained equilibrium. Fish were left to recover for at least 24 h prior to sampling, during which time cannulae were flushed twice with heparinized Cortland's saline (10 i.u. ml^{-1} lithium heparin, Sigma-Aldrich catalog no. H0878, St Louis, MO, USA) (Wolf, 1963). Prior to experimentation, blood was removed from the cannula into a heparinized syringe, but at the first sign of struggling, no further blood was removed to ensure negligible plasma catecholamine levels. Blood was pooled from two to three fish and haematocrit (Hct) was measured in duplicate by centrifuging 60 µl of whole blood in heparinized micro-capillary tubes for 3 min at 17,000 ***g***. Prior to experimentation, the pooled blood sample was standardized to a Hct of 25% by removing either plasma or RBCs. Aliquots of approximately 2.5 ml were added to four Eschweiler tonometers. Tonometers containing blood were equilibrated for 1 h

Table 1. Concentrations of carbonic anhydrase (CA), catecholamines [noradrenaline (NA) and adrenaline (AD)] and adrenergic agonists [isoproterenol (ISO)] that have been used or measured in previous studies

[CA] (mmol l^{-1})	[CA] justification	[Catecholamine] or [β-agonist] (mmol l^{-1})	Type	[ISO] justification
5×10^{-5}	Mammalian white skeletal muscle (Henry et al., 1997)	1.2×10^{-7}	NA	Resting rainbow trout plasma (Tetens et al., 1988)
10^{-5}	Promotes rapid change in pH_e; tonometry experiments; rainbow trout (Motais et al., 1989)	5×10^{-7}	ISO	Used in rainbow trout blood *in vitro* (Motais et al., 1989; Nikinmaa et al., 1990)
1.5×10^{-4}	Rainbow trout red blood cells (J.L.R., unpublished data)	5.3×10^{-7}	NA	Resting rainbow trout plasma, overnight recovery from dorsal aorta cannulation surgery (J.L.R., unpublished data)
2×10^{-4}	Stopped flow experiments with spiny dogfish, *Squalus acanthias* (Perry et al., 1999)	2×10^{-5}	NA	Acute hypoxia, 60 min exposure in rainbow trout (Tetens et al., 1988)
6.7×10^{-3}	Final concentration, bovine CA II injected into rainbow trout (Wood and Munger, 1994)	8.5×10^{-5}	NA	After repeated burst swimming in rainbow trout (Butler et al., 1986)
5×10^{-3}	Mammalian red blood cell levels (Henry et al., 1997)	3×10^{-5} to 3.5×10^{-4}	NA AD	Elicits half-maximum β-adrengergic pH_i regulation in rainbow trout (reviewed in Nikinmaa, 1992)
0.01	Elicits a marked (>1 pH unit) pH_e recovery in β-adrenergically stimulated rainbow trout blood *in vitro* (Nikinmaa et al., 1990)	5×10^{-4}	ISO	*In vitro* studies on rainbow trout and eel (*Anguilla anguilla*) (Borgese et al., 1987; Romero et al., 1996)
		10^{-4}	NA, AD	*A. anguilla* blood *in vitro* (Hyde and Perry, 1990)
		10^{-3}	AD	Elicits half-maximum β-adrengergic pH_i regulation in rainbow trout (Nikinmaa, 1982)
		0.01	NA, AD	Following injection into *A. anguilla* circulatory system (Hyde and Perry, 1990)
			ISO	Following injection into rainbow trout circulatory system (Nikinmaa et al., 1990)
			ISO	Elicits maximum (saturated) response; rainbow trout blood *in vitro* (Caldwell et al., 2006)
		0.1	NA	Elicits maximum (saturated) response; rainbow trout blood *in vitro* (Tetens et al., 1988)

pH_e, extracellular, plasma pH; pH_i, intracellular, red blood cell pH.

at 12°C (LAUDA Brinkman™ Model S-1 recirculating chilling unit, Delran, NJ, USA) with a humidified gas mixture, varying in O_2 proportions regulated by a gas-mixing pump (DIGAMIX 275 6KM 422, Wösthoff, Bochum, Germany; P_{CO_2}=0.5%, balance N_2). The aim was to incubate blood at O_2 tensions above 20% but below 80% Hb–O_2 saturation to cover the range of Hb–O_2 saturation that likely occurs in venous blood *in vivo*. Nominal values of 30, 50, 65 and 75% Hb–O_2 saturation were targeted and the required incubation P_{O_2} was determined from the rainbow trout oxygen equilibrium curves (OECs) generated in a previous study at 12°C and 0.5% CO_2 (Rummer, 2010). Following incubation of the tonometers at respective gas proportions, a subsample of blood (600 µl) was removed so that haemoglobin concentration ([Hb]), Hct, pH_e and pH_i could be measured. The remaining blood was then loaded into the closed system for experimentation, as described below.

Closed-system preparation

Following blood tonometry, a 2 ml aliquot of blood was drawn into a pre-gassed Hamilton™ syringe and slowly ejected into a pre-gassed 2 ml glass vial until overflow, at which time the vial was sealed with a septum. A pre-calibrated fiber optic implantable O_2 sensor and a fiber optic implantable pH sensor (PreSens, Loligo Systems, Tjele, Denmark; tip diameters 50–140 µm), presoaked in heparinized Cortland's saline, were inserted through the septum to continuously monitor blood P_{O_2} and pH in the closed system. The vial, thermostatted at 12°C, was equipped with a small stir bar (7×2 mm) and positioned on a stir plate set at 400 revolutions min^{-1} to ensure adequate mixing throughout the experiment. Oxygen and pH signals were amplified using an Oxy-4 micro four-channel oxygen meter and signal amplifier (Loligo Systems, catalog no. OX11700) and a pH-1 micro single-channel meter (Loligo Systems, catalog no. PH10450), respectively. Data were collected throughout the duration of each experiment at a sampling rate of 1 s^{-1}, and integrated with the manufacturer's software packages for Windows. All data were saved as text files and analyzed using Acqknowledge® Data Acquisition Software (Version 3.7.3, BIOPAC Systems, Inc., Goleta, CA, USA). For representative traces, every other data point was imported into SigmaPlot for Windows 10.0.1.25 (Systat Software Inc., San Jose, CA, USA).

Series 1: β-adrenergic stimulation during an acidosis followed by CA exposure

In the closed system, rainbow trout RBCs were β-adrenergically stimulated with isoproterenol (ISO) during the HCl-induced acidosis and then subsequently exposed to CA. Blood P_{O_2} and pH were allowed to stabilize over the first 5 min in the closed system (time zero). When steady readings were observed for at least the last minute of this period, a 50 µl Hamilton™ syringe was used to inject 20 µl of 100, 150 or 200 mmol l^{-1} HCl prepared in Cortland's saline to achieve a final concentration of 1, 1.5 or 2 mmol l^{-1}, respectively. This resulted in a nominal 0.15, 0.30 or 0.50 pH unit reduction in blood pH, respectively (see Table 2 for actual pH values). Blood P_{O_2} and pH reached maximum change within 2–3 min following acidification, and after 5 min, 20 µl of the β-adrenergic agonist ISO (Sigma-Aldrich catalog no. I5627), prepared fresh in Cortland's saline, was added. The final concentration used (0.01 mmol l^{-1}) is known to elicit a maximum response in rainbow trout blood (Caldwell et al., 2006) (Table 1). After 5 min, CA (from bovine erythrocytes, E.C. 4.2.1.1, Sigma-Aldrich catalog no. C3934) prepared in Cortland's saline was injected into the system for a final concentration of 10^{-3} mmol l^{-1}. This concentration is similar to concentrations in mammalian RBCs and a concentration previously shown to short-circuit βNHE in rainbow

Table 2. The effect of subsequent additions of HCl, isoproterenol (ISO) and carbonic anhydrase (CA) on ΔP_{O_2} and pH_e, and associated half times ($t_{1/2}$) in rainbow trout blood *in vitro* in a closed system

Starting Hb–O_2 saturation (%)	Starting P_{O_2} (mmHg)	HCl-induced ΔP_{O_2} (mmHg)	$t_{1/2}$ (s)	pH_e disturbance from 7.93±0.02	ISO-induced ΔP_{O_2} (mmHg)	$t_{1/2}$ (s)	ISO-induced pH_e disturbance	CA-induced ΔP_{O_2} (mmHg)	$t_{1/2}$ (s)	CA-induced pH_e disturbance	Final pH_e	Final pH from 7.40±0.00	Final Hct from 25.02±0.12 (%)
33.7±0.4^{A}	29.5±0.5^{A}		44.0±4.6^{A}		–8.0±1.9^{A,*}	128.5±2.6^{A}		1.3±0.1^{A}	10.0±0.0^{A}		7.22±0.04^{A,C,*}	6.96±0.10*	42.66±0.74^{A,*}
54.1±1.3^{B}	55.4±1.9^{B}	76.9±18.6*	41.0±3.5^{A}	–0.15±0.04^{A,*}	–10.1±1.4^{A,B,*}	112.0±9.2^{A}	–0.02±0.03	5.5±1.1^{B,*}	26.0±0.0^{B}	0.04±0.02	7.49±0.08^{B,*}	n/a	37.75±1.04^{A,B,*}
63.4±0.7^{C}	69.3±1.1^{C}		39.7±3.3^{A}		–14.3±2.0^{B,*}	80.7±12.3^{B}		5.2±0.4^{B,*}	23.0±4.9^{B,C}		7.43±0.09^{A,B,*}	7.14±0.08*	35.13±0.99^{B,*}
67.8±0.8^{D}	77.3±1.6^{D}		33.7±1.8^{A}		–33.3±1.4^{C,*}	74.7±5.6^{B}		4.5±0.1^{B,*}	16.0±0.0^{A,C}		7.09±0.00^{C,*}	6.99±0.01*	32.75±2.20^{B,*}
46.7±0.0^{A}	45.3±0.0^{A}		32.7±12.1^{A}					0.3±0.0^{A}	21.0±1.4^{A}		7.53±0.00	7.05±0.02*	28.91±0.00*
59.0±1.6^{B}	62.5±2.4^{B}	48.8±5.2*	43.0±7.7^{A}	–0.15±0.03^{A,*}	n/a	n/a	n/a	3.6±1.2^{A,B}	22.0±4.3^{A}	0.02±0.00	7.57±0.10	7.10±0.01*	27.63±0.34*
65.2±0.7^{C}	72.5±1.2^{C}		26.9±7.1^{A}					5.7±1.1^{B,*}	14.7±4.9^{A}		7.61±0.14	7.03±0.03*	28.07±0.92*
73.7±1.3^{D}	88.5±2.8^{D}		29.5±3.5^{A}					5.9±2.2^{B,*}	30.0±0.0^{A}		7.67±0.08	7.05±0.05*	27.11±0.37*
32.4±1.2^{A}	28.0±1.3^{A}		45.0±0.1^{A}		–8.5±0.5^{A,*}	75.3±7.0^{A}		3.5±0.1^{A,*}	28.5±2.0^{A}		7.21±0.16	6.98±0.04^{A,*}	40.46±0.19^{A,*}
59.1±1.1^{B}	61.8±1.2^{B}	86.8±18.2*	46.7±1.9^{A}	–0.33±0.04^{B,*}	–31.2±4.8^{B,*}	82.7±7.8^{A}	–0.05±0.03	12.8±0.2^{B,*}	29.0±6.2^{A}	0.03±0.03	7.31±0.08	7.17±0.03^{B,*}	34.54±1.05^{B,*}
66.4±0.7^{C}	73.8±1.5^{B}		29.5±0.3^{B}		–29.2±2.7^{B,*}	99.0±30.0^{A}		14.1±0.3^{C,*}	7.0±0.0^{B}		7.39±0.05	n/a	38.03±1.78^{A,B,*}
78.0±3.8^{D}	99.6±10.5^{C}		34.3±1.1^{C}		–30.5±2.7^{B,*}	113.0±4.6^{A}		14.1±0.6^{C,D,*}	21.7±1.2^{C}		7.52±0.08	6.96±0.06^{A,*}	36.45±0.93^{A,B,*}
29.5±1.7^{A}	24.8±1.9^{A}		45.0±0.1^{A}		–4.6±1.1^{A}	144.0±26.4^{A}		7.0±1.4^{A,*}	23.7±3.2^{A,B,C}		7.44±0.06^{A,B}	7.20±0.06^{A,B,*}	36.51±1.70*
53.0±1.1^{B}	53.7±1.5^{B}		46.7±1.9^{A,B}		–7.1±1.6^{A}	84.7±4.0^{A}		13.0±0.3^{B,*}	35.0±3.3^{B}		7.44±0.03^{A,B}	7.28±0.06^{A}	37.40±1.36*
63.2±3.9^{C}	68.7±6.9^{C}	55.2±14.3*	29.5±0.3^{C}	–0.49±0.07^{C,*}	–27.7±4.0^{B,*}	121.0±22.0^{A}	–0.06±0.04	19.4±0.7^{C,*}	16.3±3.0^{C}	0.06±0.05	7.54±0.08^{A,*}	7.12±0.07^{B,*}	35.26±1.18*
75.1±1.5^{D}	91.4±3.2^{D}		34.3±1.1^{B,C}		–31.6±1.9^{B,*}	109.0±16.3^{A}		24.7±1.0^{D,*}	25.3±1.4^{A,B,C}		7.34±0.19^{B}	7.00±0.06^{B,*}	35.29±0.50*

The ΔP_{O_2} here is defined as what would be the increase in the driving force for O_2 delivery to a tissue. Data are presented for Series 1, to which a representative trace corresponds in Fig. 2, and Series 2 (shaded region), to which a representative trace corresponds in Fig. 4. Data are categorized by starting Hb–O_2 saturation (first column) and the magnitude of the initial pH_e disturbance (fifth column) of –0.15 (Series 1 and 2), –0.33 (Series 1) or –0.49 (Series 1) pH units. When no significant differences were observed for a variable within a pH_e disturbance group, data were pooled for the four starting Hb–O_2 saturations and a single value is reported.

For values presented for each Hb–O_2 saturation within a pH_e disturbance, capital letters that differ indicate significant differences.

Asterisks indicate a significant difference from zero.

All data are presented as means ± s.e.m. n/a, not applicable.

trout blood *in vitro* (Table 1) (Nikinmaa et al., 1990; Henry et al., 1997). This experimental treatment will be referred to as HCl+ISO+CA (Fig. 2).

Series 2: acidosis followed by CA exposure

In the closed system, rainbow trout RBCs were exposed to an HCl-induced acidosis and subsequently exposed to CA, omitting β-adrenergic stimulation from the series. Only one acidification level (100 mmol l^{-1} HCl) was used, which decreased pH_e by 0.15 units. The CA concentration used and all other preparations and analyses were identical to those use in Series 1. This experimental treatment will be referred to as HCl+CA. Although it was assumed that βNHE activation would be absent in this series because ISO was omitted, a separate trial was conducted in the presence of a β-antagonist, propranolol (Sigma-Aldrich catalog no. P0884), using a final concentration of 2×10^{-5} mol l^{-1} (Fuchs and Albers, 1988; Motais et al., 1989) (data not shown). Propranolol competes with β-agonists at the level of the receptor, and so if catecholamines were present in the plasma, they would not bind at the RBC and activate the βNHE under this series of exposures.

Series 3: inhibiting RBC Na^+/H^+ exchange during an acidosis followed by CA exposure

In the closed-system, rainbow trout RBCs were exposed to an HCl-induced acidosis and then in the presence of a Na^+/H^+ exchange (NHE) inhibitor, subsequently exposed to CA. Ethylisopropylamiloride (EIPA) (Sigma-Aldrich catalog no. A3085) is a potent inhibitor of NHE, specifically NHE1 (Kristensen et al., 2007). In this series, it was used to validate the role of an NHE (adrenergic or non-adrenergically activated) in the short-circuiting model. The only incubation condition used was 0.5% CO_2, 65% air, balance N_2. At 5 min following HCl addition, EIPA was injected into the system (0.1 mmol l^{-1} final concentration). After 5 min, CA was injected into the system for a final concentration identical to that used in Series 1. All other preparations and analyses were identical to those used in Series 1. This experimental treatment will be referred to as HCl+EIPA+CA.

Controls and blood analysis

Sham injections of Cortland's saline were also introduced at every interval in a separate and final control experiment to account for potential injection effects (data not shown, as no effects were observed). At 30 min in all experiments, blood was removed for final analysis. Hct was determined in duplicate after centrifuging two filled heparinized Hct tubes at 17,000 ***g*** for 3 min. Hb was measured in duplicate using the cyanomethaemoglobin method and an extinction coefficient of 11 mmol l^{-1-1} cm^{-1} at 540 nm. The remaining aliquot of blood was centrifuged at 4000 ***g*** for 3 min, plasma was removed and discarded, and RBCs were immediately frozen in liquid nitrogen and stored at –80°C until later analysis. pH_i was measured in duplicate using a thermostatted BMS 3 Mk2 Blood Microsystem (Radiometer, Copenhagen, Denmark) in conjunction with a Radiometer PHM73 acid-base analyzer after samples were prepared using the freeze/thaw method of Zeidler and Kim (Zeidler and Kim, 1977).

Data analyses

Representative traces were chosen for both the HCl+ISO+CA and the HCl+CA experiments. Otherwise, data are presented

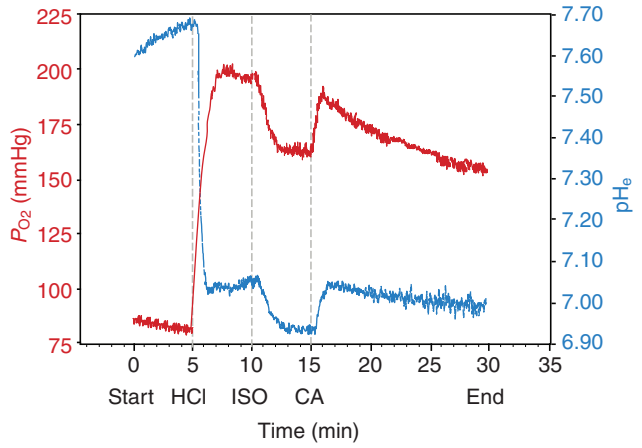

Fig. 2. Representative trace documenting changes (min) in *Oncorhynchus mykiss* blood P_{O_2} (red) and pH_e (blue) in the *in vitro* closed system over the 30 min duration of the experiment for Series 1 (HCl+ISO+CA). Dashed vertical lines represent the time at which the blood was exposed to the respective treatment indicated on the *x*-axis. CA, carbonic anhydrase; HCl, hydrochloric acid; ISO, isoproterenol. Means ± s.e.m. for all variables measured or calculated in Series 1 are reported in Table 2.

as means ± s.e.m. For every level of acidification at every starting Hb–O_2 saturation used and in each experiment, sample size was N=6 (Table 2). For all responses, time to half-maximal response ($t_{1/2}$) was calculated by using a double reciprocal plot, therefore likening the parameters to Michaelis–Menton enzyme kinetics and using a Lineweaver–Burke plot. Data were compared statistically within acidification treatments and with baseline values. When necessary, statistical differences were detected *via* one-way ANOVA. All data satisfied the assumptions of normality (Kolmogorov–Smirnov test) and homogeneity of variance (Bartlett's test). When a significant difference was identified, a *post hoc* Holm–Sidak multiple comparisons test was applied to compare means. All statistical analyses were performed using SigmaStat 3.5 (Systat Software) statistics software using a significance level of α<0.05.

RESULTS

Series 1: β-adrenergic stimulation during an acidosis followed by CA exposure

The mean starting Hct, pH_e and pH_i immediately following tonometry was 25.0±0.1%, 7.93±0.02 and 7.40±0.00, respectively. Within each acidification group, experiments began with four statistically distinct Hb–O_2 saturations (P<0.001), nominally 34, 54, 63 and 68% for the lowest level of acidification, 32, 59, 66 and 78% for the middle level of acidification, and 30, 53, 63 and 75% for the highest level of acidification (Table 2). The addition of HCl significantly reduced blood pH_e by 0.15, 0.33 and 0.49 units, all of which differed significantly from one another (Table 2). Upon HCl addition, there was a rapid and significant increase in P_{O_2} (ΔP_{O_2}) of between 55 and 87 mmHg, depending on the starting Hb–O_2 saturation and the degree of acidification (P<0.001) (Table 2). The $t_{1/2}$ for this response was 40.9±2.1 s, pooled for all Hb–O_2 saturations and acidification levels. Within a given acidification group, ΔP_{O_2} did not differ significantly among the four different starting Hb–O_2 saturations, and therefore values were pooled. There were no significant differences in ΔP_{O_2} among the three acidification groups (P=0.271) (Table 2). However, ΔP_{O_2} values were all significantly different from 0 (P<0.001). For reference, data for this experimental series are presented in tabular format (Table 2), and a representative trace from a single trial is depicted in Fig. 2.

Adrenergic stimulation significantly decreased P_{O_2} in all acidification groups and at all Hb–O_2 saturations except for in the lowest two starting Hb–O_2 saturations in the group where pH_e was decreased by 0.49 units (P>0.05) (Table 2). Qualitatively, pH_e decreased, but the change was not significant. Compared with the HCl-mediated response, the ISO-mediated response was twice as slow ($t_{1/2}$=102.1±8.1 s, pooled for all Hb–O_2 saturations and acidification levels, P<0.001; Table 2, Fig. 2).

Subsequent CA addition significantly increased P_{O_2} in every acidification group and at every starting Hb–O_2 saturation (P<0.001), except within the group where pH_e was decreased by 0.15 units, in the subgroup where starting Hb–O_2 saturation was 33.7% (P=0.104; Table 2). Qualitative increases in pH_e were evident on most traces (Fig. 2); however, changes in pH_e were not significant within or between groups. Overall, the CA-mediated response was two to five times faster than the HCl- and ISO-mediated responses, respectively ($t_{1/2}$=21.8±2.2 s, pooled for all Hb–O_2 saturations and acidification levels, P=0.003 and P<0.001 compared with HCl and ISO, respectively; Table 2, Fig. 2).

pH_i was measured only at the start and end of each experiment and was always significantly higher at the beginning of the experiment (P<0.001). The exception was at one Hb–O_2 saturation level in the group where pH_e was decreased by 0.49 units (P=0.127; Table 2). Differences in final pH_i between Hb–O_2 saturation levels within each acidification group were only observed in the groups where pH_e was decreased by 0.33 and 0.49 units (Table 2). Final Hct, measured as an additional proxy for RBC β-adrenergic stimulation, significantly increased relative to the initial value in all acidification groups at all starting Hb–O_2 saturations (P<0.001), resulting in up to a 70% increase in RBC volume (Table 2). A significant correlation existed between the starting Hb–O_2 saturation and the decrease in P_{O_2} following the addition of ISO to previously acidified blood (R^2=0.706, P<0.001; Fig. 3A). The correlation was also evident with the increase in P_{O_2} following CA addition (R^2=0.289, P<0.05; Fig. 3B). When the decrease in P_{O_2} due to ISO was pronounced, the increase in P_{O_2} due to CA was pronounced (R^2=0.355, P<0.05; Fig. 3C). This relationship was evident within and among each acidification group (Table 2). Consistent with these responses, in previously acidified blood a significant relationship could be detected between the degree of RBC swelling and the ISO-induced decrease in P_{O_2} (R^2=0.394, P<0.03; Fig. 3D).

Series 2: acidosis followed by CA exposure

When adrenergic receptors were inhibited (using the β-antagonist propranolol; data not shown) or stimulation was omitted from the sequence, starting Hb–O_2 saturations were nominally 47, 59, 65 and 74% (Table 2). Immediately following tonometry, Hct, pH_e and pH_i were not significantly different from values measured in Series 1; consequently, Series 1 and 2 starting values were pooled. Following HCl addition, pH_e was significantly reduced by 0.15 units, consistent with the lowest level of acidification in Series 1 (Fig. 4). However, as seen in the representative trace (Fig. 4), the HCl-mediated decrease was followed by a slight rise, with pH_e reaching a new apparent equilibrium prior to the CA exposure. This overshoot was reflected in the P_{O_2} trace as well. Upon acidification, P_{O_2} increased significantly (P<0.001) by a mean of 49 mmHg (Table 2). The time to half-maximal acidosis was 33.0±4.1 s (pooled for all starting Hb–O_2 saturations), but this was not significantly different than the $t_{1/2}$ for the same level of acidification in Series 1 experiments (Student's t-test, t=1.372, d.f.=6, P=0.219) (Table 2, Fig. 4). CA addition increased P_{O_2} by a

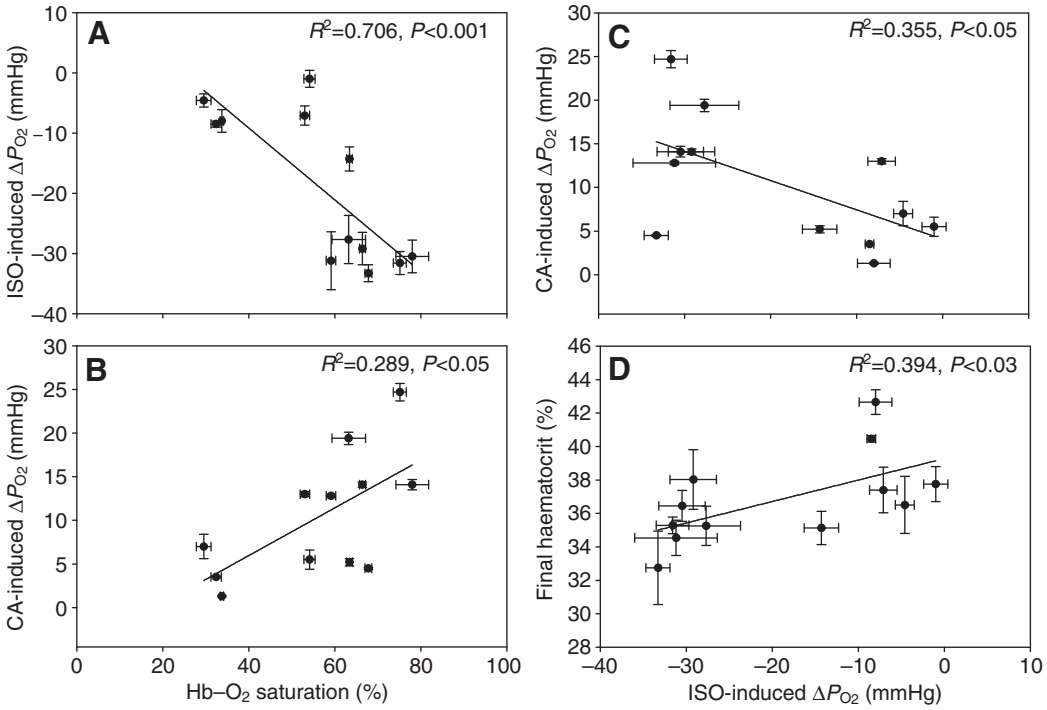

Fig. 3. For all acidification groups combined from Series 1 experiments (HCl+ISO+CA), a quantitative representation of the significant correlations between (A) starting Hb–O_2 saturation and the decrease in P_{O_2} following the addition of ISO to previously acidified *O. mykiss* blood, (B) the starting Hb–O_2 saturation and the increase in P_{O_2} following CA addition, (C) the decrease in P_{O_2} due to ISO and the increase in P_{O_2} due to CA, and (D) the decrease in P_{O_2} due to ISO and the degree of RBC swelling as represented by the final haematocrit (Hct). Note: starting Hct was 25.02±0.12%. These relationships were also evident within each acidification group; see Table 2 for data separated by acidification group.

mean of 0.3 to 5.9 mmHg, depending on the starting Hb–O_2 saturation (Table 2, Fig. 4). The time to half-maximal CA-mediated response was 21.9±3.6 s (pooled for all starting Hb–O_2 saturations); this was not significantly different than the pooled $t_{1/2}$ for the same level of acidification in Series 1 experiments (Student's *t*-test, t=–0.577, d.f.=6, P=0.585; Table 2). At the end of the 30 min monitoring period, pH_i had decreased to 7.10 or lower, and although blood from this experiment was not adrenergically stimulated with ISO, Hct had increased significantly over starting values (P<0.001), but to a significantly lesser degree (16% *versus* 70% increase) relative to Series 1 (P<0.001; Table 2). For reference, a representative trace is presented in Fig. 4 and mean values are listed in Table 2.

Series 3: inhibiting RBC NHE during an acidosis followed by CA exposure

For experiments conducted with EIPA, starting Hct, P_{O_2}, pH_e and pH_i were 25.2±0.1%, 92.8±2.3 mmHg, 7.83±0.03 and 7.20±0.03, respectively, and Hb–O_2 saturation was 75.8±1.9%. Acidification significantly increased blood P_{O_2} by 73 mmHg, which reached a maximum value of 165.4±17.9, and pH_e significantly decreased by 0.22±0.03 units (Fig. 5). Addition of EIPA did not significantly affect either blood P_{O_2} or pH_e. Likewise, CA addition did not significantly affect blood P_{O_2} or pH_e (Fig. 5). At the end of the 30 min experimental and recording period, Hct was unchanged from starting values (P>0.05). Blood P_{O_2} continued to fall over the duration of the experiment, reaching 133.8±8.1 mmHg at 30 min, but remained significantly elevated over initial values (P<0.01). Blood pH_e stabilized over the last 15 min of the recording period, but was still significantly lower than initial values (P<0.001), as was pH_i (7.09±0.03, P=0.016).

DISCUSSION

The *in vitro* results from this study are consistent with those from earlier studies (Motais et al., 1989; Nikinmaa et al., 1990), as is our overall hypothesis that during an acidosis, adrenergic RBC pH regulation *via* βNHE can be short-circuited by plasma-accessible CA. Because of this short-circuiting, Hb–O_2 affinity is reduced, resulting in a positive ΔP_{O_2} in this closed system. The increase in P_{O_2} upon CA exposure was in excess of 30 mmHg in some treatments, and occurred twice as rapidly as the increase in P_{O_2} upon acidification without CA. This response also occurred to a lesser degree in the absence of adrenergic stimulation (Fig. 4), but was abolished in the presence of EIPA, which directly inhibits all forms of NHE (Fig. 5). Thus, the addition of plasma-accessible CA to acidified blood, in the presence or absence of adrenergic stimulation, appears to result in a positive ΔP_{O_2} through short-circuiting of some NHE isoform(s). If this mechanism is operational *in vivo*, short-circuiting the pH regulation associated with RBC NHE in conjunction with a highly pH-sensitive combined Bohr–Root effect could markedly enhance tissue O_2 delivery over that which would occur in vertebrates possessing a Bohr effect alone (Rummer, 2010). This may result in further insight into the evolution of Root-effect Hbs, which evolved prior to RBC βNHE and specialized retia at the eye and swimbladder (Berenbrink et al., 2005).

Justification of the chosen parameters

The specific *in vitro* treatments were chosen to mimic *in vivo* conditions where possible (i.e. initial Hb–O_2 saturations and acidification levels). Concentrations of ISO were used based on information from past studies (Table 1), and excess levels of CA ensured maximal effects in demonstrating proof-of-principle that this mechanism is functional. Starting Hb–O_2 saturations (between 30 and 78%) encompassed the region of the OEC most commonly used during activity in rainbow trout venous blood *in vivo*. The levels of initial acidification (0.15, 0.3 and 0.5 unit decreases in pH_e) corresponded to *in vivo* changes in pH_a documented in rainbow trout following exposure to hypoxia or strenuous exercise (Kiceniuk and Jones, 1977; Milligan and Wood, 1987; Nikinmaa and Vihersaari, 1993; Brauner et al., 2000). An acid–base disturbance of this magnitude *in vivo* also rapidly elevates plasma catecholamine levels (both adrenaline and noradrenaline) from resting levels that are usually less than 2×10^{-7} mmol l^{-1} (Tetens et al., 1988) to levels as

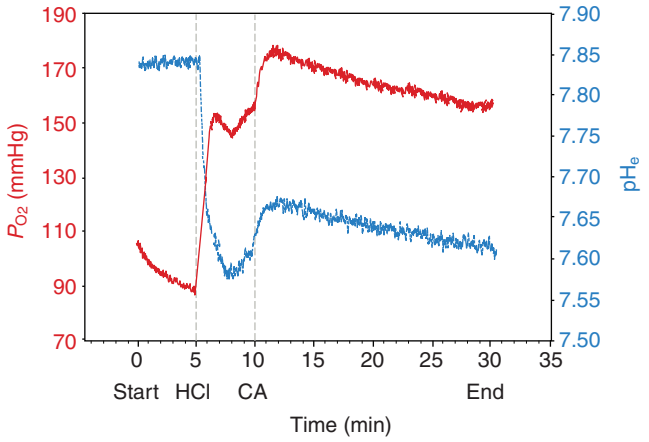

Fig. 4. Representative trace for Series 2 experiments (HCl+CA). Other details are as in Fig. 2.

high as 8.5×10^{-5} mmol l^{-1} (Butler et al., 1986; Milligan and Wood, 1987). Furthermore, ISO is a more potent β-adrenergic agonist, and we used concentrations known to generate a maximal βNHE response at the RBCs (Tetens et al., 1988) (Table 1).

Two factors were considered when choosing CA concentrations higher than what might be expected in muscle: the importance of accounting for high H^+ appearance in the plasma following RBC βNHE activation, and overwhelming any endogenous CA inhibitors potentially present in the plasma (Dimberg, 1994). The isoform used was from bovine erythrocytes, likely mammalian CA II, which is not expected to be affected by plasma inhibitors, which are thought to be not only species specific but also particular to the RBC isoform (Henry et al., 1997; Peters et al., 2000). The final CA concentrations used in this study exceeded, by 20 times, those found in rabbit white muscle [likely CA IV, enzyme catalytic activity (K_{cat}) ~1.1×10^{-6} s^{-1}, similar to CA II (Hilvo et al., 2008)] (Table 1), a membrane-bound isoform similar to what may be available to rainbow trout muscle *in vivo* (Effros and Weissman, 1979; Wang et al., 1998). However, the concentrations used were slightly lower than those determined for mammalian RBCs (5×10^{-3} mmol l^{-1}) (Henry et al., 1997), but

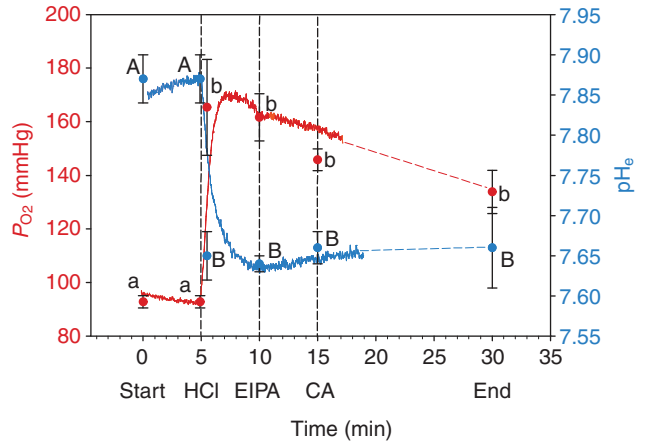

Fig. 5. Representative trace for Series 3 experiments (HCl+EIPA+CA). Means ± s.e.m. are plotted on this trace, as only one starting Hb-O_2 saturation and one level of acidification were used for this experiment. Letters that differ within a variable, indicate a significant difference. Other details are as in Fig. 2.

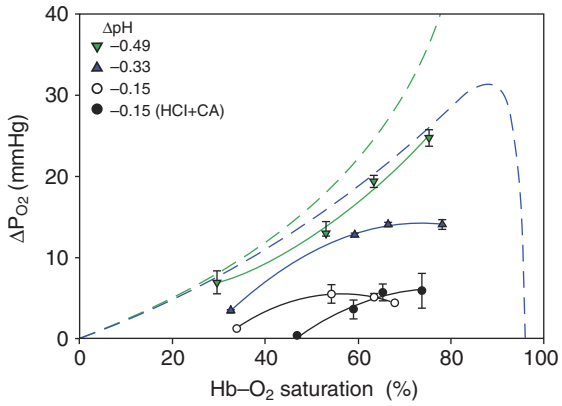

Fig. 6. ΔP_{O_2} (mmHg) following addition of CA to the *in vitro* closed system, representing the potential benefit to O_2 delivery that could result from short-circuiting of βNHE pH regulation at different starting Hb–O_2 saturations. Symbols are means ± s.e.m. from Series 1 and 2 (reported in Table 2) fitted with a second-order polynomial regression. Groups are separated by the change in pH (ΔpH) to which the blood was exposed. Green triangles and solid line (Series 1, R^2=0.9902) represent an initial pH_e decrease of 0.49 units prior to ISO and CA addition; blue triangles and solid line (Series 1, R^2=0.9994) represent an initial pH_e decrease of 0.33 units; and white (Series 1, R^2=0.9999) and black (Series 2, R^2=0.9782) circles and associated black lines represent an initial pH_e decrease of 0.15 units. All dashed lines represent the potential ΔP_{O_2} for the combined Bohr–Root effect system in rainbow trout blood for a decrease in pH_e of 0.5 (green) and 0.3 (blue) units as described in Rummer (Rummer, 2010).

consistent with levels of bovine erythrocyte CA previously used to short-circuit β-adrenergically stimulated RBC pH regulation in rainbow trout *in vitro* (Motais et al., 1989; Nikinmaa et al., 1990) (Table 1).

The ΔP_{O_2} associated with RBC βNHE short-circuiting

The ΔP_{O_2} quantified using this closed system served as proof-of-principle for short-circuiting of βNHE pH regulation in this study. Insight was also gained relative to the time course over which short-circuiting and subsequent pH_i recovery occurs. The optode response time for O_2 is much faster than for pH (pH optodes ≥30 s; P_{O_2} optodes <1 s). Thus, the ΔP_{O_2} was a very sensitive, indirect measurement of changes in RBC pH_i, which could not be measured directly and continuously. Therefore, regardless of the level of pH_e detection, which was limited by optode response time, even the subtlest changes in pH_i could be identified *via* changes in P_{O_2}.

The magnitude of the CA-mediated ΔP_{O_2} following *in vitro* acidification, where pH was decreased by 0.33 or 0.49 units, was very similar to the ΔP_{O_2} values calculated by direct interpolation between OECs generated at pH values that differed by a similar degree (Rummer, 2010) (Fig. 6). It should also be considered that values calculated for ΔP_{O_2} here may have been underestimated because of RBC metabolism, which may have changed under the various treatments, particularly adrenergic stimulation. Together, these data indicate that nearly the entire acid load initially added to the closed system may have been available for short-circuiting of βNHE pH regulation in this *in vitro* setup. If this system were operational *in vivo* (see below for a detailed discussion) the magnitude of ΔP_{O_2} could be reduced because the tissues are not a closed system and will continuously consume O_2. However, there would be additional acidification from the CO_2 produced from the tissues that could even further increase the ΔP_{O_2} reported here.

The Bohr effect, which is understood to be important in enhancing tissue O_2 delivery, elicits a ΔP_{O_2} in humans of only 2–3 mmHg with a ΔpH_{a-v} of –0.15 (Hutter et al., 1999; Jung et al., 1999; Behnke et al., 2001; Suttner et al., 2002). However, the ΔP_{O_2} associated with βNHE short-circuiting in the *in vitro* setup employed in this study with a similar pH difference of –0.15 can be up to 25 mmHg (Table 2). A change of this magnitude could have huge implications toward tissue oxygenation, and accounts of elevated blood P_{O_2} following an acidosis in past studies also support the potential for this system to be operational *in vivo*. For example, Nikinmaa et al. measured a 46% increase in blood P_{O_2} in arterial blood (dorsal aorta) of striped bass (*Morone saxitalis*) following 5 min chasing, an increase that exceeded environmental O_2 tensions and corresponded with a decrease in pH_e from 7.555 to 7.244 as well as substantial lactate production (Nikinmaa et al., 1984). If the dorsal aorta endothelium possesses plasma-accessible CA, arterial blood P_{O_2} could become elevated by the mechanism proposed herein and enhance O_2 delivery when blood entered the tissue capillaries. Short-circuiting of RBC βNHE pH regulation could also explain the high red muscle P_{O_2} values in trout prior to, during and following sustained and exhaustive exercise in comparison with much lower values seen in mammals with similar starting arterial P_{O_2} values (McKenzie et al., 2004). It may also explain the observation that red muscle P_{O_2} values were considerably higher than mixed venous blood P_{O_2} values (McKenzie et al., 2004). Whether this system can operate *in vivo* is discussed in more detail below.

Potential for short-circuiting of RBC βNHE to be operational *in vivo*

In order for short-circuiting of RBC βNHE pH regulation to operate *in vivo*, there are many requirements that must be met. Minimally, CA must be plasma accessible. The rate at which βNHE is short-circuited in acidified blood must also be sufficiently fast to significantly decrease RBC pH_i in the time required for blood transit from the gills to the tissues. Furthermore, the rate of βNHE to recover the pH_i and secure O_2 uptake at the gills must be faster than that required for transit from the tissues to the gills. A resting fish has a cardiac output of 26.6 ml min^{-1} kg^{-1} (Thorarensen et al., 1996; Brauner et al., 2000). Thus, for a 1 kg fish with 5% blood volume, blood transit time through the entire circulatory system is roughly 2 min. The $t_{1/2}$ for O_2 release from Hb is very rapid, <10 ms (Roughton, 1964). However, rates calculated from this experiment seem slow in comparison. This may be in part because the intracellular acidification in the first step of this *in vitro* model, as indirectly indicated by the ΔP_{O_2} following acid addition, may be rate limited. The H^+ from the initial HCl extracellular acidification must enter the RBC as CO_2 at the uncatalyzed rate ($t_{1/2}$>90 s) of formation in the plasma because H^+ do not typically cross plasma membranes over such short durations (Pelster and Niederstatter, 1997). There were immediate effects of the acidification, but in some instances an overshoot and recovery was observed, almost as if a new equilibrium was being established (Fig. 4). Furthermore, although immediate, the $t_{1/2}$ (28–44 s) was orders of magnitude slower than values published in the literature. Pelster and colleagues reported a that a 'Root-off' $t_{1/2}$ could be as fast as 44.8 ms (Pelster et al., 1992), almost 1000 times faster than that observed in this study. However, Pelster's group used dual wavelength spectrophotometry to investigate the Root-on and Root-off rates in unicellular blood layers of the eel between porous Gore-Tex™ membranes (Pelster et al., 1992). This minimized the effects of unstirred diffusion boundary layers around the RBCs that had resulted in notably slow rates in other studies, which likely also apply to the present system. Additionally, Pelster's studies also suggest that CA is available to the plasma in the vicinity of the acid-producing gas gland (Pelster and Scheid, 1992; Pelster, 1995; Pelster and Niederstatter, 1997), which would greatly facilitate a fast Root-off effect. This *in vitro* system was set to record every second, but the response time for the pH optodes is not matched with the faster (yet still not as fast as 1 ms) responding P_{O_2} optodes. It should be noted, however, that the $t_{1/2}$ for the increase in P_{O_2} was faster for the CA-mediated response, which may be a closer match to that observed in the eel, where CA was available (Pelster et al., 1992).

The role of general RBC NHE

In Series 2, it was determined that a CA-mediated increase in P_{O_2} could occur in the absence of RBC adrenergic stimulation (Fig. 4). Yet some isoform of NHE was key, as evidenced in Series 3 experiments, where blocking NHE eliminated all CA-mediated responses (Fig. 5). It is known that rainbow trout possess a highly sensitive βNHE (Nikinmaa, 1983; Nikinmaa and Huestis, 1984; Borgese et al., 1987; Nikinmaa and Tufts, 1989; Nikinmaa et al., 1990). However, it may be that other stimuli are activating the βNHE (Romero et al., 1996; Weaver et al., 1999) or that additional NHE1 isoforms are present on the RBCs. These transporters could be functioning as 'housekeeping' H^+ exchangers and be activated independent of adrenergic stimulation (Claiborne et al., 1999). Indeed, nearly all eukaryotes possess an isoform of NHE to regulate cell pH and volume (Yun et al., 1995; Wakabayashi et al., 1997; Claiborne et al., 1999; Deigweiher et al., 2008), and it has been suggested that at least one derived teleost species of the five groups that have secondarily lost the βNHE maintains a general RBC NHE for those purposes (Rummer et al., 2010). These data support this hypothesis, and raise questions regarding what may be activating NHE isoforms in the absence of catecholamines on the RBCs. Changes in RBC volume may activate NHE (Brauner et al., 2002; Koldkjær et al., 2002; Kristensen et al., 2007; Kristensen et al., 2008), and preliminary data from another study suggest that increases in RBC HCO_3^- may be activating NHE *via* a soluble adenylate cyclase (J.L.R., unpublished data). If catecholamines are not crucial to this mechanism for increasing ΔP_{O_2}, this mechanism could be more broadly applied. The conditions under which catecholamines are released and βNHE is activated may be limited to extremely stressful scenarios *in vivo*, such as when arterial P_{O_2} falls below 20 mmHg or 45–60% Hb–O_2 saturation or when water P_{O_2} falls below 60 mmHg (Perry and Thomas, 1991; Perry and Gilmour, 1996). If the system also functions *via* short-circuiting of pH regulation due to a general NHE and with pH disturbances of as small as –0.15, for example, O_2 delivery could be enhanced in select locations where CA is plasma accessible under much less stressful conditions that may occur more frequently.

If some form of NHE (or βNHE) can be activated quickly and the full response prolonged over several minutes, it may mean that pH_i has ample time to recover from an acidosis that is perpetuated at the muscle tissue by the time it returns to the gill to bind O_2, which could take 1 min. The CA-mediated response observed in this study, with $t_{1/2}$ ranging from 10 to 35 s (depending on the starting Hb–O_2 saturation and the level of initial acidification), occurred almost twice as fast as the HCl-induced P_{O_2} increases, where $t_{1/2}$ ranged from 29 to 46 s, and almost five times faster than the responses associated with βNHE activation (Table 2). If the P_{O_2} increase *in vivo* is as fast as a previous study suggests (45 ms) (Pelster et al., 1992), then it may be expected that the CA-mediated βNHE short-circuiting that elevates P_{O_2} also happens far more rapidly than measured here. Transit time of the RBCs through the capillaries can

take 1–3 s (Honig et al., 1977; Tetens and Lykkeboe, 1981; Randall, 1982; Bhargava et al., 1992), which is ample time for CA to short-circuit the effects of the H^+ extrusion mechanism on the RBCs. After this phenomenon permits enhanced O_2 delivery at the tissues, blood will leave the site of plasma-accessible CA and βNHE pH regulation will no longer be short-circuited. Therefore, blood returns to the respiratory surface with pH_i and Hb–O_2 binding once again protected by βNHE. Even if only a fraction of the response observed *in vitro* could be realized *in vivo*, this would significantly affect O_2 transport in comparison to systems possessing a Bohr effect alone, an idea that has been recently supported *in vivo* (Rummer, 2010).

Conclusions

The main conclusion of this study is that *in vitro*, RBC βNHE or NHE pH regulation can be short-circuited during an acidosis in the presence of plasma-accessible CA, effectively decreasing Hb–O_2 saturation and elevating the P_{O_2} of the blood. If operational *in vivo*, this finding has implications for the Bohr–Root effect to enhance O_2 delivery *via* this novel mechanism of increasing $pH_{a–v}$ during blood transit through the tissues. A pH_e decrease as small as 0.15 units can be recycled *via* the CA-mediated NHE short-circuiting mechanism in the presence as well as absence of catecholamines. This suggests that the mechanism may not be restricted to circumstances where a severe acidosis is created and/or where catecholamines are released and remain in circulation for an extended period of time.

Perhaps early teleosts utilized the combined Bohr–Root effect for general O_2 delivery before the appearance of the choroid rete and rete mirabile. If this was the case, then the driving force for O_2 delivery could be elevated over what is possible in vertebrates possessing a Bohr effect alone (Rummer, 2010). Root-effect Hbs may be considered an exaptation for O_2 delivery to the eye and swimbladder, as proposed by Berenbrink et al. (Berenbrink et al., 2005). Thus, an incipient function of Root Hbs – general O_2 delivery – may have been co-opted to give rise to the complex physiological system of the eye and swimbladder 150–270 million years later (Berenbrink et al., 2005). Thus, on a broader scale, the results of this study may help to elucidate one of the most successful adaptive radiations of an animal group in evolutionary history, that of the teleost fishes, and the selection pressures that may have been involved with the evolution of enhanced O_2 delivery.

LIST OF ABBREVIATIONS

CA	carbonic anyhydrase
cAMP	3′, 5′-cyclic adenosine monophosphate
EIPA	ethylisopropylamiloride
Hb	haemoglobin
Hct	haematocrit
ISO	isoproterenol
K_{cat}	enzyme catalytic activity (s^{-1})
NHE	Na^+/H^+ exchange
OEC	oxygen equilibrium curve
pH_a	arterial blood pH
pH_e	extracellular, plasma pH
pH_i	intracellular, red blood cell pH
P_{O_2}	partial pressure of O_2
RBC	red blood cell
$t_{1/2}$	the half-time of a reaction
βNHE	β-adrenergically activated Na^+/H^+ exchange
$\Delta pH_{a–v}$	arterial–venous pH difference

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