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

Jodie L. Rummer* and Colin J. Brauner
Department of Zoology, University of British Columbia, 6270 University Boulevard, Vancouver, BC V6T 1Z4, Canada
*Author for correspondence (jodie.rummer@gmail.com)

Accepted 14 April 2011

**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 (pHᵢ) and subsequent O₂ 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 O₂ 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 extravascular pH (pHₑ) and O₂ tension (Pₒ₂) 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 Pₒ₂ by >30 mmHg, depending on the starting Hb-O₂ saturation and degree of initial acidification. Interestingly, in the absence of adrenergic stimulation, addition of CA still elevated Pₒ₂ 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 O₂ 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 O₂ 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)–O₂ affinity with a decrease in 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–O₂ affinity but also greatly reduces the O₂ 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 O₂ 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 O₂ delivery.

Most teleost fish that exhibit a pronounced Bohr–Root effect adrenergically regulate RBC pH to maintain O₂ 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 adenylylate cyclase and 3',5'-cyclic adenosine monophosphate (cAMP), activate β-adrenergic Na⁺/H⁺ exchange (βNHE) (Mahé et al., 1985). The CA-catalyzed hydration of CO₂ inside the RBC produces H⁺ that are removed in exchange for Na⁺ via βNHE (Baroin et al., 1984; Cossins and Richardson, 1985), and HCO₃⁻ that is removed via anion exchange for Cl⁻ at a slower rate. This combination results in an increase in intracellular pH (pHᵢ) and an increase in Hb–O₂ 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 (pHₑ) (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 HCO₃⁻ at an uncatalyzed rate to form CO₂, 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 O₂ uptake at the respiratory surfaces during a
generalized acidosis in the presence of Bohr–Root shift Hb (Nikinmaa et al., 1984; Primett 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 (Motaïs et al., 1989; Nikinmaa et al., 1990). Although plasma-accessible CA is not present in the least 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 understudied (reviewed in Gilmour and Perry, 2009). We propose that if CA is available in the plasma in tissue capillaries, H⁺ removed from the RBC via βNHE could combine with plasma HCO₃⁻ to reform CO₂, which would back-diffuse into the RBC, decrease pHᵢ and ultimately create a larger arterial to venous pH gradient (ΔpHᵥᵛ) at the tissues than would otherwise occur (Fig. 1B). The large acidosis transferred to the RBC would elevate the partial pressure of O₂ (Pₒ₂) via the combined Bohr–Root effect, thus greatly facilitating tissue O₂ delivery. Furthermore, provided the rate of short-circuiting of βNHE RBC pH regulation in the tissue and subsequent pHᵢ 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 ΔpHᵢ) with every pass through the circulation, thus elevating tissue Pₒ₂ at a time when O₂ delivery is especially needed.

As a first step, this study was designed to demonstrate proof-of-principle for enhanced O₂ delivery when adrenergically stimulated RBC βNHE pH regulation is short-circuited. Rainbow trout blood was pre-equilibrated at pre-defined Hb–O₂ saturations and then, in a closed system, acidified, β-adrenergically stimulated and then exposed to CA. Changes in both pHᵢ and Pₒ₂ 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 O₂ delivery, ΔPₒ₂, 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 O₂ 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 201 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 24h prior to sampling, during which time cannulae were flushed twice with heparinized Cortland’s saline (101.0.1 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

<table>
<thead>
<tr>
<th>[CA] (mmol l⁻¹)</th>
<th>[CA] justification</th>
<th>[Catecholamine] or [β-agonist] (mmol l⁻¹)</th>
<th>Type</th>
<th>[ISO] justification</th>
</tr>
</thead>
<tbody>
<tr>
<td>5×10⁻⁵</td>
<td>Mammalian white skeletal muscle (Henry et al., 1997)</td>
<td>1.2×10⁻⁷</td>
<td>NA</td>
<td>Resting rainbow trout plasma (Tetens et al., 1988)</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>Promotes rapid change in pH₄; tonometry experiments; rainbow trout (Motais et al., 1989)</td>
<td>5×10⁻⁷</td>
<td>ISO</td>
<td>Used in rainbow trout blood in vitro (Motais et al., 1989; Nikinmaa et al., 1990)</td>
</tr>
<tr>
<td>1.5×10⁻⁴</td>
<td>Rainbow trout red blood cells (J.L.R., unpublished data)</td>
<td>5.3×10⁻⁷</td>
<td>NA</td>
<td>Resting rainbow trout plasma, overnight recovery from dorsal aorta cannulation surgery (J.L.R., unpublished data)</td>
</tr>
<tr>
<td>2×10⁻⁴</td>
<td>Stopped flow experiments with spiny dogfish, Squallus acanthias (Perry et al., 1999)</td>
<td>2×10⁻⁴</td>
<td>NA</td>
<td>Acute hypoxia, 60 min exposure in rainbow trout (Tetens et al., 1988)</td>
</tr>
<tr>
<td>6.7×10⁻³</td>
<td>Final concentration, bovine CA II injected into rainbow trout (Wood and Munger, 1994)</td>
<td>8.5×10⁻⁵</td>
<td>NA</td>
<td>After repeated burst swimming in rainbow trout (Butler et al., 1986)</td>
</tr>
<tr>
<td>5×10⁻³</td>
<td>Mammalian red blood cell levels (Henry et al., 1997)</td>
<td>3×10⁻⁴ to 3.5×10⁻⁴</td>
<td>AD</td>
<td>Licit half-maximum β-adrenergic pHi regulation in rainbow trout (reviewed in Nikinmaa, 1992)</td>
</tr>
<tr>
<td>0.01</td>
<td>Elicits a marked (&gt;1 pH unit) pHi recovery in β-adrenergically stimulated rainbow trout blood in vitro (Nikitmaa et al., 1990)</td>
<td>5×10⁻⁴</td>
<td>ISO</td>
<td>In vitro studies on rainbow trout and eel (Anguilla anguilla) (Borgese et al., 1987; Romero et al., 1996)</td>
</tr>
</tbody>
</table>

pHₑ, extracellular, plasma pH; pHᵢ, 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 O₂ and CO₂ proportions regulated by a gas-mixing pump (DIGAMIX 275 6KM 422, Wösthoff, Bochum, Germany; P₀₂=0.5% oxygen, balance N₂). The aim was to incubate blood at O₂ tensions above 20% but below 80% Hb–O₂ saturation to cover the range of Hb–O₂ saturation that likely occurs in venous blood in vivo. Nominal values of 30, 50, 65 and 75% Hb–O₂ saturation were targeted and the required incubation P₀₂ was determined from the rainbow trout blood oxygen equilibrium curves (OECs) generated in a previous study at 12°C and 0.5% CO₂ (Rummer, 2010). Following incubation of the tonometers at respective gas proportions, a subsample of blood (600µl) was removed so that haemoglobin concentration ([Hb]), Hct, pHₑ and pHᵢ 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 O₂ sensor and a fiber optic implantable pH sensor (PreSens, Loligo Systems, Tjele, Denmark; tip diameters 50–140µm), presoaked in heparinized Cortland’s saline, were inserted through the septum to continuously monitor blood P₀₂ 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-I 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 P₀₂ 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 P₀₂ 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. 15627), 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 10⁻⁴ mmol l⁻¹. This concentration is similar to concentrations in mammalian RBCs and a concentration previously shown to short-circuit βNHE in rainbow trout.
### Table 2. The effect of subsequent additions of HCl, isoproterenol (ISO) and carbolic anhydride (CA) on $\Delta p_{0_2}$ and pH, and associated half times ($t_{1/2}$) in rainbow trout blood in vitro in a closed system

<table>
<thead>
<tr>
<th>Starting Hb–O2 saturation (%)</th>
<th>Starting $P_{0_2}$ (mmHg)</th>
<th>HCl-induced $\Delta p_{0_2}$ (mmHg)</th>
<th>$t_{1/2}$ pH disturbance from 7.30 to 7.35</th>
<th>ISO-induced $\Delta p_{0_2}$ (mmHg)</th>
<th>$t_{1/2}$ pH disturbance</th>
<th>CA-induced $\Delta p_{0_2}$ (mmHg)</th>
<th>$t_{1/2}$ pH disturbance</th>
<th>CA-induced pH disturbance</th>
<th>Final pH</th>
<th>Final pH from 6.90 to 7.00</th>
<th>Final Hct from 25.0±0.12 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>33.7±0.5*</td>
<td>29.5±0.5*</td>
<td>44.0±4.3*</td>
<td>7.8±0.3</td>
<td>128.5±2.6*</td>
<td>1.3±0.1*</td>
<td>10.0±0.0*</td>
<td>7.2±0.0*</td>
<td>9.5±0.0*</td>
<td>42.6±0.74*</td>
<td>37.5±1.4*</td>
<td>35.3±0.99*</td>
</tr>
<tr>
<td>54.1±1.3*</td>
<td>55.4±1.9*</td>
<td>76.9±18.6*</td>
<td>–0.15±0.04*</td>
<td>112.0±9.2*</td>
<td>5.5±1.1*</td>
<td>26.0±0.0*</td>
<td>7.4±0.8*</td>
<td>37.5±1.4*</td>
<td>n/a</td>
<td>37.5±1.4*</td>
<td>35.3±0.99*</td>
</tr>
<tr>
<td>63.4±0.7*</td>
<td>69.3±1.1*</td>
<td>39.7±3.3*</td>
<td>–14.3±2.9*</td>
<td>23.0±4.9*</td>
<td>5.2±0.4*</td>
<td>23.0±4.9*</td>
<td>7.4±0.8*</td>
<td>37.5±1.4*</td>
<td>42.6±0.74*</td>
<td>37.5±1.4*</td>
<td>35.3±0.99*</td>
</tr>
<tr>
<td>67.8±0.8*</td>
<td>77.3±1.6*</td>
<td>33.7±1.8*</td>
<td>–33.3±14.4*</td>
<td>74.7±5.6*</td>
<td>4.5±0.3*</td>
<td>16.0±1.0*</td>
<td>7.4±0.8*</td>
<td>37.5±1.4*</td>
<td>42.6±0.74*</td>
<td>37.5±1.4*</td>
<td>35.3±0.99*</td>
</tr>
</tbody>
</table>

* $t_{1/2}$ is defined as the increase in the driving force for O2 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–O2 saturation (first column) and the magnitude of the initial pH disturbance (fifth column) of –0.15 (Series 1 and 2), –0.33 (Series 1) or –0.49 (Series 1) pH units. When no significant difference was observed for a variable within a pH disturbance group, data were pooled for the four starting Hb–O2 saturations and a single value is reported. For values presented for each Hb–O2 saturation within a pH 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.

### Controls and blood analyses

Animals were fed corn alongside the algae for acclimation. Control samples were chosen for both the HCl-ISO+CA experiments and the HCl+ISO+CA experiments. Otherwise, data are presented as means ± s.e.m. Hb was measured in duplicate using a thermostatted BMS 3 Mk2 cyanomethaemoglobin method and an extinction coefficient of 1 mmol l–1 cm–1. Blood was collected from a radish as a function of the NHE system for a final concentration identical to that used in Series 1. The only incubation condition was that the NHE system was exposed to CA. Blood was pooled for the four starting Hb–O2 saturations and a single value is reported for values presented for each Hb–O2 saturation within a pH disturbance, capital letters that differ indicate significant differences.

### Data analyses

### In vivo experiments

The effect of subsequent additions of HCl, isoproterenol (ISO) and carbolic anhydride (CA) on $\Delta p_{0_2}$ and pH, and associated half times ($t_{1/2}$) in rainbow trout blood in vitro in a closed system.
as means ± s.e.m. For every level of acidification at every starting Hb–O2 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–Menten 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_{b} 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–O2 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_{i} 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 PO_{2} (ΔPO_{2}) of between 55 and 87 mmHg, depending on the starting Hb–O2 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–O2 saturations and acidification levels. Within a given acidification group, ΔPO_{2} did not differ significantly among the four different starting Hb–O2 saturations, and therefore values were pooled. There were no significant differences in ΔPO_{2} among the three acidification groups (P=0.271) (Table 2). However, ΔPO_{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 PO_{2} in all acidification groups and at all Hb–O2 saturations except for in the lowest two starting Hb–O2 saturations in the group where pH_{i} was decreased by 0.49 units (P=0.05) (Table 2). Qualitatively, pH_{i} 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–O2 saturations and acidification levels, P<0.001; Table 2, Fig. 2).

Subsequent CA addition significantly increased PO_{2} in every acidification group and at every starting Hb–O2 saturation (P<0.001), except within the group where pH_{i} was decreased by 0.15 units, in the subgroup where starting Hb–O2 saturation was 33.7% (P=0.104, Table 2). Qualitative increases in pH_{i} were evident on most traces (Fig. 2); however, changes in pH_{i} 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–O2 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 end of the experiment (P<0.001). The exception was at one Hb–O2 saturation level in the group where pH_{i} was decreased by 0.49 units (P=0.127; Table 2). Differences in final pH_{i} between Hb–O2 saturation levels within each acidification group were only observed in the groups where pH_{i} 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–O2 saturations (P<0.001), resulting in up to a 70% increase in RBC volume (Table 2). A significant correlation existed between the starting Hb–O2 saturation and the degree of acidification (R^{2}=0.706, P<0.001; Fig. 3A). The correlation was also evident with the increase in PO_{2}, following the addition of ISO to previously acidified blood (R^{2}=0.289, P<0.05; Fig. 3B). When the decrease in PO_{2} due to ISO was pronounced, the increase in PO_{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 PO_{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–O2 saturations were nominally 47, 59, 65 and 74% (Table 2). Immediately following tonometry, Hct, pH_{i} and pH_{b} were not significantly different from values measured in Series 1; consequently, Series 1 and 2 starting values were pooled. Following HCl addition, pH_{i} 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_{i} reaching a new apparent equilibrium prior to the CA exposure. This overshoot was reflected in the PO_{2} trace as well. Upon acidification, PO_{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–O2 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 PO_{2} by a

**Fig. 2.** Representative trace documenting changes (min) in *Oncorhynchus mykiss* blood PO_{2} (red) and pH_{i} (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 every level of acidification at every starting Hb–O2 saturation and acidification levels. Within a given acidification group, experiments began with four statistically distinct Hb–O2 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_{i} 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 PO_{2} (ΔPO_{2}) of between 55 and 87 mmHg, depending on the starting Hb–O2 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–O2 saturations and acidification levels. Within a given acidification group, ΔPO_{2} did not differ significantly among the four different starting Hb–O2 saturations, and therefore values were pooled. There were no significant differences in ΔPO_{2} among the three acidification groups (P=0.271) (Table 2). However, ΔPO_{2} values were all significantly different from 0 (P<0.001). For reference, data for this experimental
Significantly lesser degree (16% versus P values (experimental and recording period, Hct was unchanged from starting affect blood either blood 0.22±0.03 units (Fig. 2324 significantly increased blood 92.8±2.3% respectively, and Hb–O2 saturation was 75.8±1.9%. Acidification The had decreased to 7.10 or lower, and although blood from this earlier studies (Motais et al., 1989; Nikinmaa et al., 1990), as is our significant lower than initial values (P<0.001), as was pH i 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 O2, pH i and pH e were 25.2±0.1%, 92.8±2.3 mmHg, 7.83±0.03 and 7.20±0.03, respectively, and Hb–O2 saturation was 75.8±1.9%. Acidification significantly increased blood P O2 by 73 mmHg, which reached a maximum value of 165.4±17.9, and pH i significantly decreased by 0.22±0.03 units (Fig. 5). Addition of EIPA did not significantly affect either blood P O2 or pH e. Likewise, CA addition did not significantly affect blood P O2 or pH i (Fig. 5). At the end of the 30 min experimental and recording period, Hct was unchanged from starting values (P>0.05). Blood P O2 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 i stabilized over the last 15 min of the recording period, but was still significantly lower than initial values (P<0.001), as was pH e (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–O2 affinity is reduced, resulting in a positive ΔP O2 in this closed system. The increase in P O2 upon CA exposure was in excess of 30 mmHg in some treatments, and occurred twice as rapidly as the increase in P O2 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 O2 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 O2 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 (Berensbrink 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–O2 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–O2 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 i) corresponded to in vivo changes in pH i 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...
high as $8.5 \times 10^{-5}$ mmol$^{-1}$ (Butler et al., 1986; Milligan and Wood, 1987). Furthermore, ISO is a more potent $\beta$-adrenergic agonist, and we used concentrations known to generate a maximal $\beta$NHE response at the RBCs (Teten 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 $\beta$NHE 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 ($K_{cat}$) $\sim 1.1 \times 10^{-6}$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 ($5 \times 10^{-3}$ mmol$^{-1}$) (Henry et al., 1997), but consistent with levels of bovine erythrocyte CA previously used to short-circuit $\beta$-adrenergically stimulated RBC pH regulation in rainbow trout in vivo (Motais et al., 1989; Nikinmaa et al., 1990) (Table 1).

The $\Delta P_{O2}$ associated with RBC $\beta$NHE short-circuiting

The $\Delta P_{O2}$ quantified using this closed system served as proof-of-principle for short-circuiting of $\beta$NHE pH regulation in this study. Insight was also gained relative to the time course over which short-circuiting and subsequent pH recovery occurs. The optode response time for $O_2$ is much faster than for pH (pH optodes $\geq 30$ s; $P_{O2}$ optodes $<1$ s). Thus, the $\Delta P_{O2}$ was a very sensitive, indirect measurement of changes in RBC pH, which could not be measured directly and continuously. Therefore, regardless of the level of pH detection, which was limited by optode response time, even the slightest changes in pH could be identified via changes in $P_{O2}$.

The magnitude of the CA-mediated $\Delta P_{O2}$ following in vivo acidification, where pH was decreased by 0.33 or 0.49 units, was very similar to the $\Delta P_{O2}$ 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 $\Delta P_{O2}$ 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 $\beta$NHE pH regulation in this in vivo setup. If this system were operational in vivo (see below for a detailed discussion) the magnitude of $\Delta P_{O2}$ 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 $\Delta P_{O2}$ reported here.
The Bohr effect, which is understood to be important in enhancing tissue \( O_2 \) delivery, elicits a \( \Delta P_{O_2} \) in humans of only 2–3 mmHg with a \( \Delta pH \), of \(-0.15\) (Hutter et al., 1999; Jung et al., 1999; Behnke et al., 2001; Suttner et al., 2002). However, the \( \Delta P_{O_2} \) associated with \( bNHE \) short-circuiting in the \textit{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 \textit{in vivo}. For example, Nikinmaa et al. measured a 46% increase in blood \( P_{O_2} \) in arterial blood (dorsal aorta) of striped bass (\textit{Morone saxatilis}) following 5 min chasing, an increase that exceeded environmental \( O_2 \) tensions and corresponded with a decrease in \( pH \), 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 \( RBC bNHE \) \( pH \) 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 \textit{in vivo} is discussed in more detail below.

\section*{Potential for short-circuiting of RBC \( bNHE \) to be operational \textit{in vivo}}

In order for short-circuiting of RBC \( bNHE \) \( pH \) regulation to operate \textit{in vivo}, there are many requirements that must be met. Minimally, \( CA \) must be plasma accessible. The rate at which \( bNHE \) is short-circuit- ed in acidified blood must also be sufficiently fast to significantly decrease RBC \( pH \) in the time required for blood transit from the gills to the tissues. Furthermore, the rate of \( bNHE \) to recover the \( pH \) 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\(^{-1}\) (Thorarsen 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 \textit{in vitro} model, as indirectly indicated by the \( \Delta P_{O_2} \) 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 (\textit{in vitro} model, as 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 Gilmore, 1996). If the system also functions \textit{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 \( bNHE \)) can be activated quickly and the full response prolonged over several minutes, it may mean that \( pH \) 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 \( bNHE \) activation (Table 2). If the \( P_{O_2} \) increase \textit{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 \( bNHE \) short-circuiting that elevates \( P_{O_2} \) also happens far more rapidly than measured here. Transit time of the RBCs through the capillaries can 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 \textit{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).

\section*{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 \( bNHE \) (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 \( bNHE \) (Romero et al., 1996; Weaver et al., 1999) or that additional NHE 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; Deigweiler et al., 2008), and it has been suggested that at least one derived teleost species of the five groups that have secondarily lost the \( bNHE \) 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; Koldkjaer 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 \( NHE \) \textit{via} soluble adenylate cyclase (J.L.R., unpublished data). If catecholamines are not crucial to this mechanism for increasing \( \Delta P_{O_2} \), this mechanism could be more broadly applied. The conditions under which catecholamines are released and \( bNHE \) is activated may be limited to extremely stressful scenarios \textit{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 Gilmore, 1996). If the system also functions \textit{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.

THE JOURNAL OF EXPERIMENTAL BIOLOGY
In vitro βNHE short-circuit in rainbow trout

References


The acknowledgements section of the provided material is not required to be included in the natural text representation.
hemodilution on distribution of blood flow and tissue oxygenation in dog skeletal muscle. J. Appl. Physiol. 86, 860-866.


