The Journal of Experimental Biology 213, 1503-1512

doi:10.1242/jeb.038844

Use it or lose it? Sablefish, *Anoplopoma fimbria*, a species representing a fifth teleostean group where the βNHE associated with the red blood cell adrenergic stress response has been secondarily lost

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Accepted 19 January 2010

SUMMARY

Like most teleosts, sablefish (*Anoplopoma fimbria* Pallas 1814) blood exhibits a moderate Root effect (~35% maximal desaturation), where a reduction in blood pH dramatically reduces O_2 carrying capacity, a mechanism important for oxygenating the eye and filling the swim bladder (SB) in teleosts. Although sablefish lack a SB, we observed a well-defined choroid rete at the eye. The adrenergically mediated cell swelling typically associated with a functional red blood cell (RBC) β-adrenergic Na^+/H^+ exchanger (βNHE), which would normally protect RBC pH, and thus O_2 transport, during a generalized acidosis, was not observed in sablefish blood. Neither isoproterenol (a β-agonist) nor 8-bromo cAMP could elicit this response. Furthermore, RBC osmotic shrinkage, known to stimulate NHEs in general and βNHE in other teleosts such as trout and flounder, resulted in no significant regulatory volume increase (RVI), further supporting the absence of a functional RBC βNHE. The onset of the Root effect occurs at a much lower RBC pH (6.83–6.92) than in other teleosts, and thus RBC βNHE may not be required to protect O_2 transport during a generalized acidosis *in vivo*. Phylogenetically, sablefish may represent a fifth group of teleosts exhibiting a secondary reduction or loss of βNHE activity. However, sablefish have not lost the choroid rete at the eye (unlike in the other four groups), which may still function with the Root effect to oxygenate the retina, but the low pH onset of the Root effect may ensure haemoglobin (Hb)-O_2 binding is not compromised at the respiratory surface during a general acidosis in the absence of RBC βNHE. The sablefish may represent an anomaly within the framework of Root effect evolution, in that they possess a moderate Root effect and a choroid rete at the eye, but lack the RBC βNHE and the SB system.

Key words: βNHE, catecholamine, Root effect, regulatory volume increase, amiloride, hyperosmotic stress.

INTRODUCTION

The Root effect, a phenomenon limited almost exclusively to teleost fishes, is defined as the decrease in O_2 carrying capacity of the blood due to a decrease in pH (Brittain, 1987; Ingermann, 1982; Pelster and Weber, 1991; Root, 1931; Root and Irving, 1943). This mechanism at the level of the haemoglobin (Hb) evolved 400 million years ago (mya) (Berenbrink et al., 2005) and is used in many living ray-finned fishes to deliver O_2 to the retina, a recognized avascular yet metabolically active tissue (Wittenberg and Wittenberg, 1974), and the swim bladder (SB), a specialized gas-filled structure within the body cavity used to maintain buoyancy, against extremely high partial pressure gradients (>50 atm, ~5050 kPa) associated with the deep sea (Alexander, 1966). A recent evolutionary reconstruction suggests, however, that the Root effect evolved long before (~150 million years) the single evolutionary appearance of the counter-current capillary network of the eye, the choroid rete, which permits exploitation of the Root effect at the eye, and 270 million years before the multiple appearances of the counter-current capillary network of the SB (Berenbrink et al., 2005). Berenbrink and colleagues convey that the choroid rete is the most ancient anatomical structure known that is associated with the Root effect. They demonstrated that if a choroid rete is present, loss of the SB rete mirabile or the SB altogether is not associated with a change in the magnitude of the Root effect; therefore it is not surprising to observe species possessing a moderate Root effect that is utilized for O_2 delivery to the eye but not the SB.

Nevertheless, negative side effects can be associated with the Root effect. When a fish experiences a generalized acidosis, which can occur during exposure to environmental hypoxia or hypercarbia or following exhaustive exercise, the presence of a Root effect could result in incomplete blood-O_2 saturation at the gills, compromising O_2 uptake (Milligan and Wood, 1987; Salama and Nikinmaa, 1988; Tufts and Randall, 1989). Approximately 150 mya, in teleosts more derived than Osteoglossomorpha, subsequent to the evolution of the choroid rete but prior to the appearance of the SB rete mirabile, a β-adrenergically stimulated sodium (Na^+) proton (H^+) exchanger (βNHE) on the surface of the red blood cell (RBC) membrane evolved (Berenbrink et al., 2005). Following development of a blood acidosis, adrenaline and noradrenaline are released, activating the RBC βNHE which extrudes protons to protect RBC intracellular pH (pH_i), Hb–O_2 affinity, O_2 uptake at the gill, and therefore O_2 supply to the tissues (Borgese et al., 1987; Cossins and Kilbey, 1991; Nikinmaa et al., 1984; Primmett et al., 1986; Salama and Nikinmaa, 1988) (reviewed by Berenbrink and Bridges, 1994; Fievet and Motais, 1991; Jensen, 2004; Thomas and Perry, 1992). During this cascade of events, not only is pH_i protected, sometimes elevated, but also H^+ extrusion and Na^+ influx initiate a further chain of events requiring anion exchange to remove HCO_3^- from the RBC in exchange for Cl^-, Na^+/K^+-ATPase activation, as well as influx of

osmotically obliged water, resulting in a RBC regulatory volume increase (RVI) and associated increase in haematocrit (Hct) (Borgese et al., 1987; Cossins and Gibson, 1997; Cossins and Kilbey, 1991; Guizouarn et al., 1993; Nikinmaa et al., 1990). Species possessing a RBC βNHE generally possess a pronounced Root effect, and therefore extremely pH-sensitive Hbs (Berenbrink et al., 2005); however, the βNHE has been secondarily lost in four groups of derived teleosts (Berenbrink et al., 2005), a loss that is also associated with a substantial decrease in the magnitude of the Root effect.

Sablefish (*Anoplopoma fimbria* Pallas 1814) are a species that already represent a small industry in aquaculture, yet little is known regarding blood-O_2 transport characteristics. Information about the physiology of sablefish is limited to what has been investigated to assess post-release survival in the increasingly popular fisheries industry and the efficacy of protocols for rearing this new species in aquaculture (Davis, 2002; Davis, 2005; Davis and Parker, 2004; Lupes et al., 2006). Sablefish do not exhibit high release mortality rates prevalent in other deep-water teleosts, largely due to the absence of a SB (Davis, 2002; Davis, 2005; Davis and Parker, 2004; Furnell, 1987; Lupes et al., 2006). Sablefish are also thought to be relatively resistant to stress, are notably tolerant to hypoxia and air exposure (Davis and Parker, 2004; Moser et al., 1994), and are known to colonize the unforgiving oxygen minimum zone (OMZ) off the California coast (Moser et al., 1994), implying that their O_2 transport system may be resilient to stress and other environmental challenges. The present study was conducted to characterize the blood-O_2 transport system of sablefish, with the specific aim to (i) characterize the magnitude and pH onset of the Root effect to examine the potential effect of a generalized acidosis on O_2 transport, and (ii) determine whether the red blood cells of sablefish exhibit a functional βNHE, or an NHE that can be stimulated through changes in RBC volume (RVI response). Data collected for sablefish were compared with data on rainbow trout (*Oncorhynchus mykiss*), the species for which we currently have the most comprehensive understanding of O_2 delivery mechanisms, as well as copper rockfish (*Sebastes caurinus*), a species from the same taxonomic order and similar, albeit shallower, habitat to that of the sablefish.

MATERIALS AND METHODS

Experimental animals and sampling

Juvenile sablefish (size range, 60–150 g wet mass) were purchased from Cluxewe Enterprises (Cedar, BC, Canada) and transported to the Department of Fisheries and Oceans/University of British Columbia (UBC), Centre for Aquaculture and Environmental Research (West Vancouver, BC, Canada). Upon arrival, fish were separated into three 1100 l fibreglass tanks and fed a commercial Atlantic salmon feed (Ewos Canada Ltd, Surrey, BC, Canada; 2 mm pellets) at 2% body mass per day. Fish were maintained on a natural photoperiod (daylight fluorescent lights) and were provided with running (11–15 l min^{-1}), filtered and oxygenated seawater. Temperature, dissolved oxygen and salinity of the sea water were measured daily at 12:00 h and these parameters ranged from 7.7 to 12°C, 7.5 to 10.4 mg l^{-1} and 28 to 31‰, respectively, over the duration that fish were held. Fish were maintained in the facility for approximately 2 years until this project commenced, at which point fish weighed between 900 and 1800 g (wet mass).

Rainbow trout, *O. mykiss* (size range, 300–600 g wet mass), were obtained from Spring Valley Trout Farm (Langley, BC, Canada). Fish were maintained in the Department of Zoology, UBC, in 4000 l flow-through tanks supplied with Vancouver dechlorinated municipal tap water (average 12°C) under a natural photoperiod. Fish were fed every other day to satiation using commercial trout pellets (Skretting, Orient 4-0; Vancouver, BC, Canada). All protocols comply with the guidelines approved by the Canadian Council on Animal Care, protocol no. A07-0080.

Copper rockfish, *S. caurinus* (size range, 159–905 g wet mass), were collected *via* hook and line from shallow water (<4 m depth) off the coast of Bamfield Marine Sciences Centre (BMSC, BC, Canada). Fish were transported back to the BMSC laboratory in buckets filled with aerated seawater and were maintained in aquaria equipped with flow-through seawater for at least 48 h until experiments commenced.

At the beginning of each set of experiments, fish were anaesthetized in a benzocaine solution (2×10^{-4} mol l^{-1} *p*-aminobenzoate), and blood was drawn from the caudal vein and collected in heparinized syringes. RBCs were prepared as described elsewhere (Caldwell et al., 2006). Briefly, RBCs were rinsed twice with and resuspended in ice-cold Cortland's saline (Wolf, 1963), Hct was standardized to 25%, and the samples were stored at 4°C overnight. In preliminary investigations using rinsed and resuspended rainbow trout RBCs stored overnight (see Caldwell et al., 2006), we determined we could generate oxygen equilibrium curves (OECs) that were not significantly different from those generated using blood freshly drawn from resting, cannulated fish. Furthermore, the rinse and resuspend procedure had no significant effect on the RBC βNHE response to isoproterenol in rainbow trout RBCs stored for up to 96 h (Caldwell et al., 2006). Following blood collection, five sablefish were killed with an overdose of benzocaine solution (*p*-aminobenzoate), so that the eyes could be dissected and observations made for the presence or absence of the choroid rete (for details, see Berenbrink et al., 2005).

Series 1: oxygen equilibrium curves and quantification of the Root effect

A 3 ml aliquot of washed and resuspended sablefish or copper rockfish RBCs that had been adjusted to 25% Hct was placed into one of four individual Eschweiler tonometers thermostatically regulated to 12°C (the temperature at which both species were acclimated at the time of sampling). Resuspended RBCs were equilibrated for 45 min with 0.5, 1, 2 or 4% CO_2 (0.5, 1, 2, 3 or 5% CO_2 for copper rockfish) balanced with air (21% O_2; N=4 per CO_2 tension). Samples were then exposed to stepwise decreases in O_2 tension (approximately 21, 15, 12.5, 8.5, 4.5 and 0%) by decreasing air while proportionally increasing N_2, as regulated by a gas-mixing pump (DIGAMIX 275 6KM 422 Wösthoff, Bochum, Germany) (trout and sablefish blood throughout the entire experiment) or Smart-Trak mass flow meters (Series 100; Sierra Instruments, Inc., Monterey, CA, USA) (copper rockfish samples only). Blood samples (0.8 ml) were drawn from each tonometer at each incubation interval (20 min at each O_2 tension) into pre-gassed Eppendorf™ tubes or a pre-gassed Hamilton™ syringe to measure O_2 content, Hct, [Hb], pH_i and extracellular pH (pH_e), each parameter being measured in duplicate for each blood sample. Total O_2 content was measured according to previous methods (Tucker, 1967). A separate 10 µl aliquot of blood was added to Drabkin's solution (Sigma-Aldrich cat. no. D5941; St Louis, MO, USA) and the absorbance measured spectrophotometrically at 540 nm; [Hb] (mmol l^{-1} per tetramer) was calculated applying a millimolar extinction coefficient of 11 mmol l^{-1} cm^{-1}. Hb saturation (expressed as a percentage) was calculated by converting the values obtained for O_2 content to reflect millimoles of O_2 per litre of blood, dividing by 4, and dividing by the tetrameric Hb concentration obtained spectrophotometrically.

Hct was determined in duplicate as the ratio of packed RBCs to total blood volume, from blood-filled microcapillary tubes centrifuged at 3000 r.p.m. for 5 min. Mean corpuscular Hb concentration (MCHC) was calculated as Hb/(Hct/100). Whole blood was analysed to determine pH_e, and the freeze–thaw method (see Zeidler and Kim, 1977) was used to prepare isolated RBCs to determine pH_i; both pH measurements were conducted for each sample in duplicate using a BMS 3 Mk2 Blood Microsystem (Radiometer, Copenhagen, Denmark).

Series 2: RBC βNHE, activation and response characterization

To assess the RBC βNHE response, a 2 ml aliquot of washed and resuspended sablefish or trout RBCs that had been adjusted to 25% Hct was placed into one of four individual Eschweiler tonometers thermostatically regulated to 12°C. Resuspended RBCs were equilibrated for 45 min under acidic and hypoxic conditions generated by gas mixtures consisting of 0.5% CO_2 and 50% air, 2% CO_2 and 15% air, or 2% CO_2 and 0% air, balanced with N_2 to potentiate the βNHE response. Isoproterenol (ISO; Sigma-Aldrich cat. no. 286303), a β-adrenergic analogue of noradrenaline, was added to each tonometer to achieve a final concentration of 10^{-5} mol l^{-1} (Caldwell et al., 2006). In another experiment performed to bypass the β-adrenergic receptor to activate the βNHE directly, the membrane-permeable 8-bromo cyclic AMP (cAMP; Sigma-Aldrich cat. no. 372447) was added to each tonometer to achieve a final nominal concentration of 10^{-2} mol l^{-1} (Mahe et al., 1985; Perry et al., 1996; Thomas and Gilmour, 2006). Additional RBC samples were injected with propranolol (PROP; Sigma-Aldrich cat. no. P0884), a β-adrenergic antagonist, to achieve a final concentration of 2×10^{-5} mol l^{-1} (Fuchs and Albers, 1988; Motais et al., 1989). A final group was injected with Cortland's saline only to control for volume disturbance. Prior to injection of ISO, cAMP, PROP, the ISO+PROP combination or saline, samples were drawn from each tonometer and analysed to establish baseline [Hb], Hct, MCHC, pH_i and pH_e values. Following initiation of the treatment, samples were drawn at 5, 30, 60 and 90 min and analysed in the same manner as above. Sampling and treatments were performed in random order (N=6 for each treatment).

Series 3: characterization of the RBC RVI response

The magnitude of the RBC RVI was investigated in rinsed and resuspended sablefish RBCs upon incubation in tonometers exactly as described in series 2, except RBCs were incubated in gas mixtures consisting of 0.5% CO_2 and 50% air or 0.5% CO_2 and 0% air, balanced with N_2. Hyperosmotic shrinkage was induced by introducing a modified (10× concentrated, hypersaline) Cortland's saline solution (~3000 mosmol l^{-1}) into each tonometer to increase the final osmolarity by approximately 50%, a concentration cited to yield at least 25% cell shrinkage (Brauner et al., 2002; Koldkjær et al., 2002; Kristensen et al., 2007; Kristensen et al., 2008). Cell shrinkage was initiated in the absence and presence of EIPA [5-(*N*-ethyl-*N*-isopropyl)amiloride; Sigma-Aldrich cat. no. A3085), a general NHE inhibitor (Kristensen et al., 2007), which was used to block the NHE-related RVI response. Cortland's saline, hypersaline, EIPA (final concentration of 10^{-4} mol l^{-1}) and hypersaline+EIPA were injected as four separate treatments into individual tonometers (Kristensen et al., 2007). Baseline samples were drawn and analysed from each tonometer prior to treatment. Post-treatment samples were drawn and analysed after 5, 30, 60 and 90 min. All parameters mentioned in the previous two experiments were also measured for this RVI experiment (N=6).

Calculations and statistical analyses

Mean ± s.e.m. per cent Hb saturation (S) values were plotted as a function of incubation P_{O_2} (mmHg) for each CO_2 (0.5, 1, 2 and 4%) incubation treatment for sablefish blood, and OECs were fitted to data using the Dynamic Fit Wizard function in SigmaPlot for Windows 10.0.1.25 (Systat Software Inc. 2006). Half-saturation O_2 tensions (P_{50}) and the Hill cooperativity coefficients (n_H) were interpolated from $\log[S/(1–S)]$ *vs* $\log P_{O_2}$ plots (20–80% saturation), where S is the fractional Hb saturation with O_2 (Hb-O_2 saturation). Bohr coefficients (Φ) were calculated as $\Delta\log P_{50}/\Delta pH_e$ for pH values corresponding to CO_2 incubation tensions. Partial pressures are given in mmHg. For unit conversion, 1 mmHg=0.1333 kPa. Curves were fitted for each data set for sablefish and copper rockfish to determine the total magnitude and the pH onset of the Root effect. Statistical differences between Hb-O_2 saturation values for each CO_2 treatment were determined by ANOVA and *post-hoc* Holm–Sidak multiple comparisons procedures using SigmaStat for Windows 3.5.0.54 (Systat Software, Inc. 2006). Changes in Hct for NHE and RVI experiments were assessed for significance following an ANOVA and compared between all treatment groups, at all sampling intervals, and between sablefish and trout. All *post-hoc* comparisons in both NHE and RVI experiments (Dunnett's method) were made in comparison to the initial mean baseline Hct value. Additionally, for the RVI experiments, data were plotted as relative change in Hct (%) as a function of treatment and time. All statistical tests were performed with α=0.05.

RESULTS

Choroid rete observations

Sablefish were found to possess a pronounced, dense network of capillaries clustered around the optic nerve and adjacent to the retina of the eye (Fig. 1), which has not previously been documented.

Series 1: oxygen equilibrium curves and quantification of the Root effect

Incubating washed and resuspended sablefish RBCs at progressively higher CO_2 tensions (from 0.5 to 1, 2 and 4%) resulted in a statistically significant reduction in pH_e at each CO_2 tension (Table 1) and both a rightward (Bohr effect) and a downward (Root effect) shift in the OEC (Fig. 2). Mean Hb-O_2 saturation at each incubation P_{O_2} was significantly lower at each increasing CO_2 tension, with the exception of 0.5% and 1% CO_2 at 60 mmHg, and 2% and 4% CO_2 at air saturation (160 mmHg). At air-saturated oxygen tensions, the Root effect resulted in a reduction in Hb-O_2 saturation from 96% at 0.5% CO_2, to 84% at 1% CO_2, 68% at 2% CO_2 and 65% at 4% CO_2. The P_{50} values calculated for samples incubated at 0.5% and 1% CO_2 (29.3 and 30.7 mmHg) were not significantly different, but were significantly lower than the P_{50} calculated for 2% and 4% CO_2 incubation conditions (51.8 and 78.1 mmHg). The resulting Bohr coefficients were 0.06 (based upon the change in P_{50} between 0.5% and 1% CO_2 treatments), 1.32 (for 1% and 2% CO_2 treatments) and 0.91 (for 2% and 4% CO_2 treatments) (Table 1), the last two values being significantly different from the first. Low co-operativity in Hb-O_2 binding was exhibited, as demonstrated by Hill coefficients (n_H) of 1.40, 1.17, 0.97 and 1.09 for each OEC curve (0.5, 1, 2 and 4% CO_2, respectively), all of which were significantly different (P<0.001, except between 1% and 4%, and 2% and 4% CO_2 where P<0.05; Table 1). The relationship between pH_e and pH_i is depicted in Fig. 3, where changes in pH_i were significantly correlated with changes in pH_e (Fig. 3). Both [Hb] and Hct remained constant throughout all CO_2 treatments and did not significantly deviate over

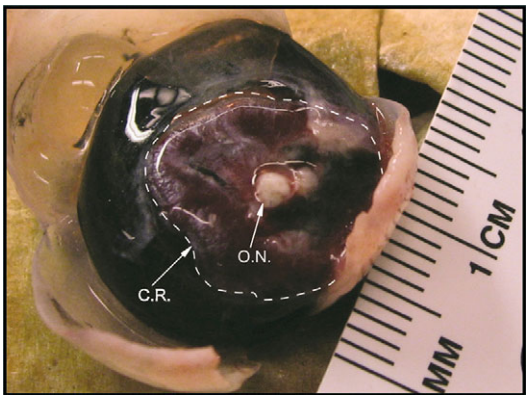

Fig. 1. Representative photograph depicting the choroid rete (C.R) and optic nerve (O.N) observed upon dissecting each sablefish eye. The choroid rete is outlined for clarity (white dashed line).

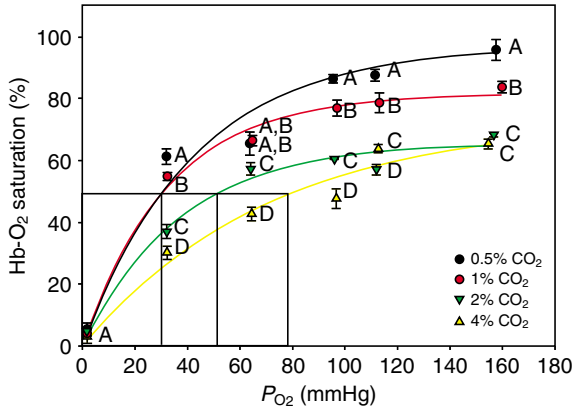

Fig. 2. Oxygen equilibrium curves (OECs) for sablefish red blood cells (RBCs), rinsed and resuspended (haematocrit, Hct 25%), and incubated at 0.5, 1, 2 and 4% CO_2 tensions with stepwise decreases in P_{O_2} (mmHg) from 21% (160 mmHg) to 0% (0 mmHg). Data are means ± s.e.m. Different letters indicate statistically significant differences between CO_2 treatments within a given O_2 incubation condition. Thin black lines extend from 50% Hb-O_2 saturation to indicate the half-saturation O_2 tension (P_{50}) for each CO_2 treatment.

the course of any treatment or between treatments (P<0.001; data not shown).

Washed and resuspended copper rockfish RBCs incubated under high CO_2 tensions (from 0.5% to 1, 2, 3 and 5%) showed a decrease in pH_e and pH_i as expected, which markedly right-shifted and down-shifted OECs (data not shown). The pH_e at atmospheric O_2 tension (~160 mmHg) for blood incubated at 0.5% CO_2 (7.91±0.07) was not significantly different from the pH_e upon incubation at 1% CO_2 (7.98±0.09) (P=0.49; data not shown). However, pH_e fell significantly to 7.62±0.06, 7.40±0.02 and 7.16±0.08 upon incubation at 2, 3 and 5% CO_2 (data not shown). pH_i fell dramatically from 7.29±0.11 at 0.5% CO_2 to 7.19±0.08, 7.00±0.06, 6.92±0.05 and 6.83±0.05, at 1, 2, 3 and 5% CO_2, respectively. Maximal Hb-O_2 saturation under these incubation conditions was 91.43±3.79%, 90.18±3.22%, 56.28±3.19%, 44.45±0.77% and 38.14±0.91%, all of which were plotted against pH_i to represent Root effect magnitude and pH onset (Fig. 4). Hct was 22.95±0.81% after the first incubation series (0.5% CO_2) and 25.27±1.42% after the final incubation series (5% CO_2), with a slight but significant difference detected between the two groups (P=0.025; data not shown).

Series 2: RBC βNHE, activation and response characterization

Compared with rainbow trout, sablefish RBCs did not significantly swell, as observed by the lack of change in Hct after blood samples were incubated under mildly hypoxic conditions (50% air, 0.5% CO_2, balance N_2) and stimulated with isoproterenol (ISO; P=0.422) (Fig. 5A). Trout RBCs swelled by at least 36% under mildly hypoxic conditions upon ISO exposure (P<0.001) (Fig. 5A). The trend of no RBC swelling persisted when sablefish blood samples were exposed to ISO following incubation under severely hypoxic and hypercapnic conditions (15% air, 2% CO_2, balance N_2; P=0.234) as well as under anoxic hypercapnic conditions (0% air, 2% CO_2, balance N_2; P=0.240) (Fig. 5A), both of which potentiate the response in other species. Exposing trout RBCs to a cAMP analogue resulted in significant swelling (up to 25%, P<0.001), but no swelling could be detected in sablefish RBCs under mild hypoxia (P=0.731) or severe hypoxia and hypercapnia (P=0.253) (Fig. 5B). However, under anoxic and hypercapnic incubation conditions, Hct was significantly elevated (by 14%), but only at the final sampling time (90 min; P<0.05) (Fig. 5B).

Table 1. Effects of CO_2 on whole blood oxygen parameters from sablefish, *Anoplopoma fimbria*

CO_2 (%)	0.5%	1.0%	2.0%	4.0%
P_{CO_2} (mmHg)	3.8	7.6	15.2	30.4
pH_e	7.69±0.03^a	7.33±0.02^b	7.16±0.022^c	6.96±0.01^d
P_{50} (mmHg)	29.3±0.7^a	30.7±0.4^a	51.8±1.1^b	78.1±1.3^c
n_H	1.40±0.03^a	1.17±0.02^b	0.97±0.020^c	1.09±0.02^d
Bohr coefficient	0.06±0.01^a	1.32±0.03^b	0.91±0.01^c	

Data are means ± s.e.m. Calculations: n_H=[Δlog(S/(1−S)]/(ΔlogP_{O_2}), Bohr coefficient (ΔlogP_{50}/ΔpH_e); where n_H is the Hill coefficient, S is the fractional Hb saturation with O_2, pH_e is extracellular pH and P_{50} is the half-saturation O_2 tension.
Different superscript letters indicate significant differences within a parameter.

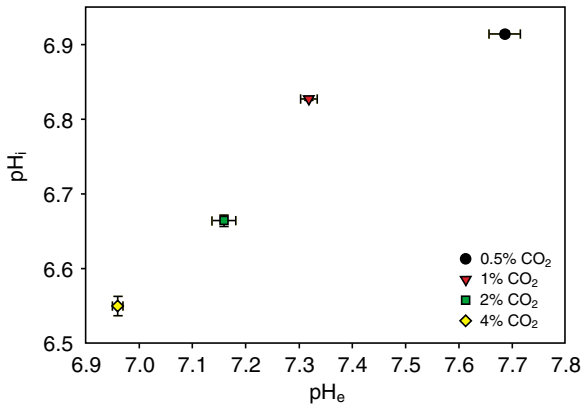

Fig. 3. The relationship between intracellular pH (pH_i) and extracellular pH (pH_e) in rinsed and resuspended sablefish RBCs (Hct 25%). Changes in pH were induced *via* incubation at 0.5, 1, 2 and 4% CO_2 tensions, balanced with air (21% O_2). pH_i=3.034+pH_e×0.5089, R^2=0.9179.

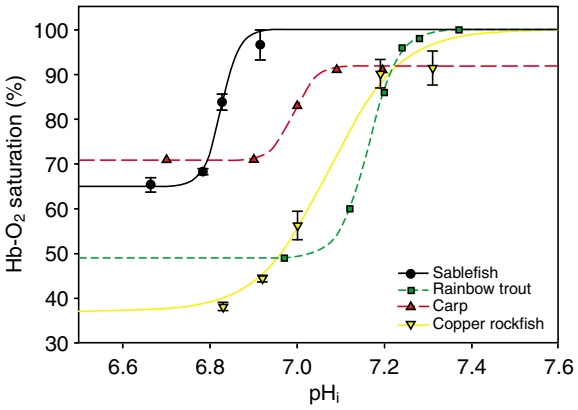

Fig. 4. The relationship between Hb-O_2 saturation and pH_i for sablefish whole blood (black solid line) compared with data from other studies on rainbow trout (*Oncorhynchus mykiss*, green dashed line) (Nikinmaa, 1990), carp (*Cyprinus carpio*, red dashed line) (Nikinmaa, 1990) and copper rockfish (yellow solid line, present study). Curves were fitted to data using the Dynamic Fit Wizard function in SigmaPlot (see Materials and methods).

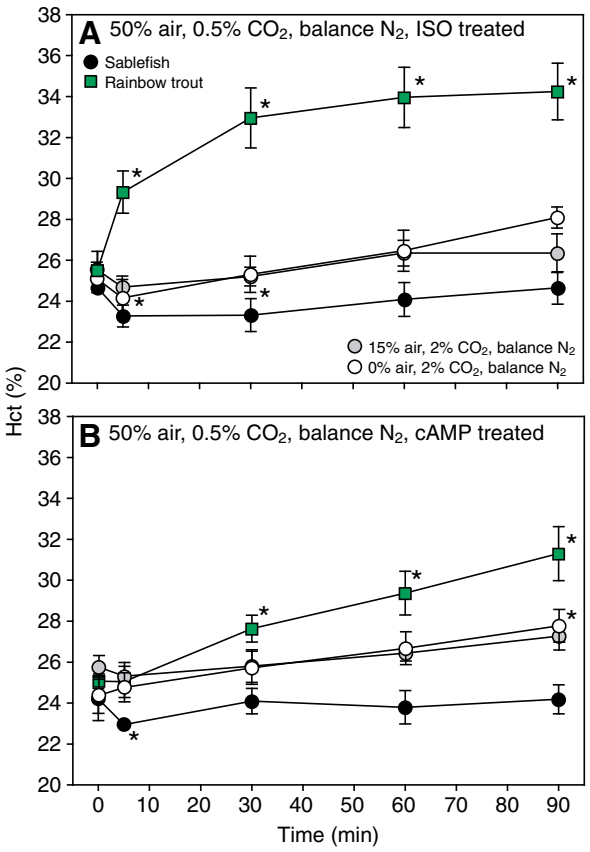

Fig. 5. Changes in Hct over time for rinsed and resuspended sablefish RBCs incubated under mildly hypoxic conditions (50% air, 0.5% CO_2), moderately hypoxic and hypercapnic conditions (15% air, 2% CO_2), and anoxic and hypercapnic conditions (0% air, 2% CO_2) and rainbow trout RBCs incubated under mildly hypoxic conditions, upon treatment with isoproterenol (ISO, A) or 8-bromo cyclic AMP (cAMP, B). Baseline Hct is displayed at time zero, and all sampling periods following time zero represent exposure to ISO or cAMP. Asterisks indicate statistically significant changes from the Hct at time zero.

Series 3: characterization of the RBC RVI response

Sablefish RBCs, incubated under mildly hypoxic conditions (50% air, 0.5% CO_2, balance N_2) and treated with a hypersaline solution shrank significantly (P<0.001), by at least 15% when compared with RBCs treated with Cortland's saline alone. This trend was observed as a decrease in absolute Hct values (Fig. 6A, top panel), but also as changes in Hct relative to control samples only exposed to Cortland's saline (Fig. 6A, bottom panel). Cell shrinkage persisted (by as much as 15%) when hypersaline-treated cells were also treated with the amiloride analogue EIPA (P<0.001) (Fig. 6A). The same trend persisted when RBCs were incubated under anoxic conditions (0% air, 0.5% CO_2, balance N_2) under hypersaline conditions (P<0.05) and in hypersaline conditions with EIPA (P<0.05) (Fig. 6B). However, the hypersaline+EIPA-treated RBCs had shrunk significantly more than RBCs under any other treatment at the 60 min sampling interval (P<0.05).

DISCUSSION

Sablefish blood exhibits a moderate Root effect, which is likely in place to facilitate O_2 delivery to the highly defined choroid rete we observed at the eye (Fig. 1), as sablefish lack the other anatomical feature most commonly associated with this mechanism, the SB (Davis, 2002; Furnell, 1987). Interestingly, however, the onset of the Root effect occurs at a much lower RBC pH than in other teleosts such as the copper rockfish, determined from this study, as well as rainbow trout and carp (Nikinmaa, 1990) (Fig. 4). Such low pH values have been measured in the rete mirabile at the SB in eel (Forster and Steen, 1969; Steen, 1970) and perhaps the choroid rete of the eye, but are not thought to occur in general circulation. This may be associated with the lack of a functional RBC βNHE that, in many teleosts, protects RBC pH, and thus O_2 transport, during a generalized acidosis. We were unable to pharmacologically stimulate the RBC βNHE in sablefish. Furthermore, osmotically shrinking RBCs, which is known to stimulate the βNHE in other teleosts such as rainbow trout, brown trout (*Salmo trutta*), carp, winter flounder (*Pseudopleuronectes americanus*) and European flounder (*Platichthys flesus*) (Brauner et al., 2002; Cala, 1977; Orlov and Skryabin, 1993; Orlov et al., 1994; Weaver et al., 1999), did not result in a significant RVI. Because the Root effect onset occurs at such a low RBC pH, the βNHE may not be necessary to protect O_2 transport during a generalized acidosis *in vivo*, because a pH fall of that magnitude *in vivo* would be unlikely. Sablefish may represent a fifth group of teleosts exhibiting a secondary reduction or loss of a functional βNHE.

Root effect magnitude and pH onset

Interestingly, the magnitude of the Root effect in sablefish resembles neither that of a species from the same taxonomic order like the copper rockfish, *S. caurinus* (order Scorpaeniformes), nor that of rainbow trout, the teleost for which the most general information on Hb–O_2 relationships has been collected, but rather that of the notably hypoxia tolerant common carp, *C. carpio* (Nikinmaa, 1990; Salama and Nikinmaa, 1988) (Fig. 4). Indeed, sablefish have been observed exploiting the OMZ off the coast of California, an area known to only contain species with clear hypoxia tolerance strategies (Levin, 2003). Although sablefish possess a moderate, perhaps pronounced Root effect and a relatively high P_{50} (low-affinity Hb), hypoxia-tolerant species (e.g. carp, catfishes, loaches and some air-

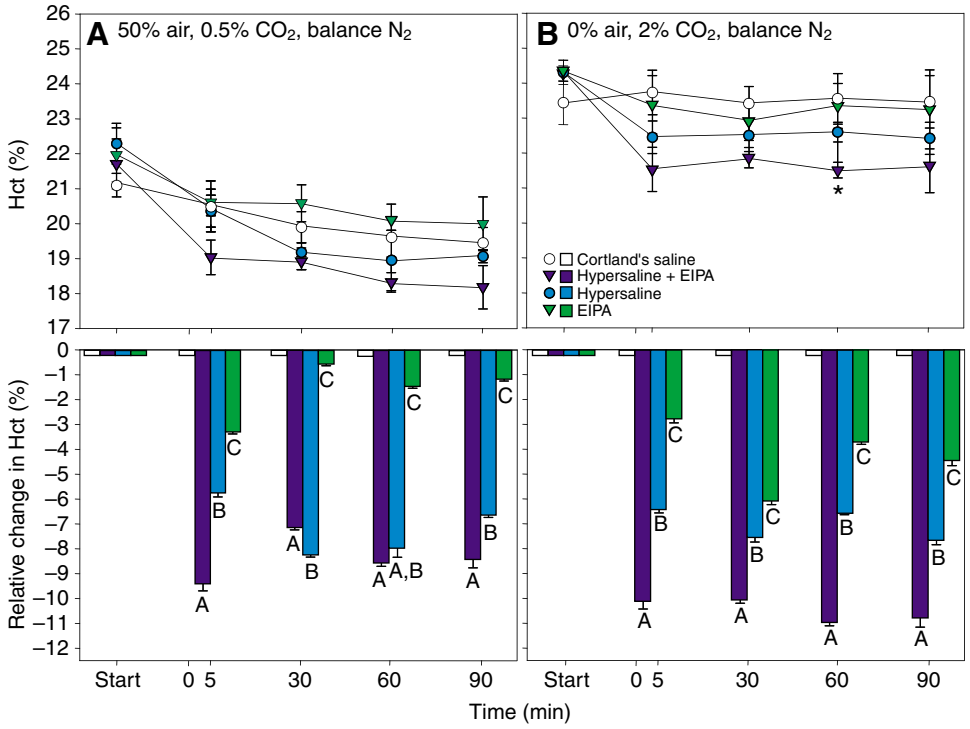

Fig. 6. Top panels represent absolute changes and bottom panels represent relative (compared with control, 0% change) changes in Hct with respect to time for sablefish RBCs rinsed and resuspended, and incubated under two conditions. Panel A (top and bottom) depicts changes in blood incubated under mildly hypoxic conditions (50% air, 0.5% CO_2), and panel B (top and bottom) depicts changes in blood incubated under anoxic and hypercapnic conditions (0% air, 2% CO_2). Treatments included a hypersaline solution with the amiloride analogue EIPA, hypersaline solution alone, EIPA alone or, as a control, Cortland's saline. Baseline Hct is displayed at time zero, and all sampling periods following time zero represent exposure to one of the four treatments. Asterisk indicates statistically significant changes from the Hct at time zero in the top panels; different letters represent statistically significant differences between treatments within each sampling interval in the bottom panels.

breathing fishes) are often relatively inactive and generally possess a reduced Root effect and high-affinity Hb, favouring O_2 loading in hypoxic environments (Berenbrink et al., 2005; Pelster and Weber, 1990; Wells et al., 1986). The sablefish's low-affinity Hb may be advantageous to tissue oxygenation in this highly predatory, powerful swimmer, but perhaps at a cost to O_2 uptake if sablefish are also exploiting hypoxic waters (Conway, 1967; Ryer, 2004; Sullivan and Somero, 1983). Sablefish in the wild reside at great depth and pressure, but animals used in this study had been reared at atmospheric pressure. It is well known that extreme pressure, like that associated with deepwater habitats, can affect protein stability, membrane integrity and ion transport, which could most definitely affect Hb conformation and affinity for O_2. This low Hb affinity, low pH onset paradox may be resolved by incorporating the pressures associated with habitat depth into our protocols, but such experiments were beyond the scope of this study. Sablefish can be reared very successfully in well-oxygenated water at atmospheric pressure, and the Hb function is seemingly unaffected. However, our findings suggest that many more research questions remain, revolving around how environmental conditions realistic to sablefish's natural habitat may be influencing their physiology.

The pH onset for the Root effect in sablefish, or the pH_i at which Hb-O_2 saturation falls below 100% (~6.9), is below that which would ever occur during a general acidosis in other teleosts (Nikinmaa, 1990) (Fig. 4). For example, in trout, plasma pH can fall to 7.7 and even, during the most extreme circumstances, down to 7.3, which would translate to an intracellular acidosis ranging from 7.3 to as low as 7.1 (Milligan and Wood, 1986), still higher than that required to elicit the Root effect in sablefish RBCs in this study. If the *in vivo* relationship between pH_e and pH_i in sablefish is similar to that of rainbow trout and other teleosts, it is unlikely that a generalized blood acidosis would ever elicit the Root effect in the general circulation. However, in this study, pH_i at 0.5% CO_2 in rinsed, resuspended sablefish RBCs was low (6.9) relative to values commonly observed in trout (7.4) (Heming et al., 1987). No other such data exist for this species; however, if pH_i is unusually low in sablefish, perhaps O_2 transport could be compromised during a severe acidosis in the absence of βNHE. Clearly, further *in vivo* studies are required to address this point. Regan and Brauner (Regan and Brauner, 2010) found that although the most phylogenetically basal fish species in the transition zone of Root effect evolution (specifically bowfin *Amia calva*, mooneye *Hiodon tergisus* and pirarucu *Arapaima gigas*) possess a moderate to pronounced Root effect, they also have very low pH onset values for the Root effect (ranging from 6.8 to 7.0) as observed in sablefish. All of these species also lack a functional RBC βNHE, but unlike the sablefish, these basal species appeared before the evolution of RBC βNHE.

RBC pH regulation

Derived teleosts have evolved adrenergically sensitive RBC βNHEs that, when activated during an acidosis, serve to elevate RBC pH and secure O_2 uptake at the gills, despite the presence of a Root effect, perhaps at a time when the stressed organism needs O_2 the most. The βNHE(1), unique to teleosts, remains one of the most well-characterized non-mammalian β-adrenergic-activated mechanisms (Motais et al., 1992; Nikinmaa, 1992) and is thought to have evolved as a paralogue to the adrenergic-insensitive, cAMP-insensitive NHE1 found in other vertebrates (Nickerson et al., 2003). Our findings suggest no adrenergic control over NHE in sablefish RBCs. The concentrations of the adrenergic agonist used in this study have been found to elicit the maximum (saturated) response in rainbow trout blood *in vitro* (Tetens et al., 1988), but it is also well known that the density and affinity of trout RBC β-adrenergic receptors can be dramatically affected by chronic stress, for example social status or confinement (Thomas and Gilmour, 2006), as well as season and temperature (Koldkjær et al., 2004; Lecklin et al., 2000). However, we addressed this, in part, by also attempting to activate the βNHE downstream from the adrenergic receptor on the RBC membrane with cAMP (Mahe

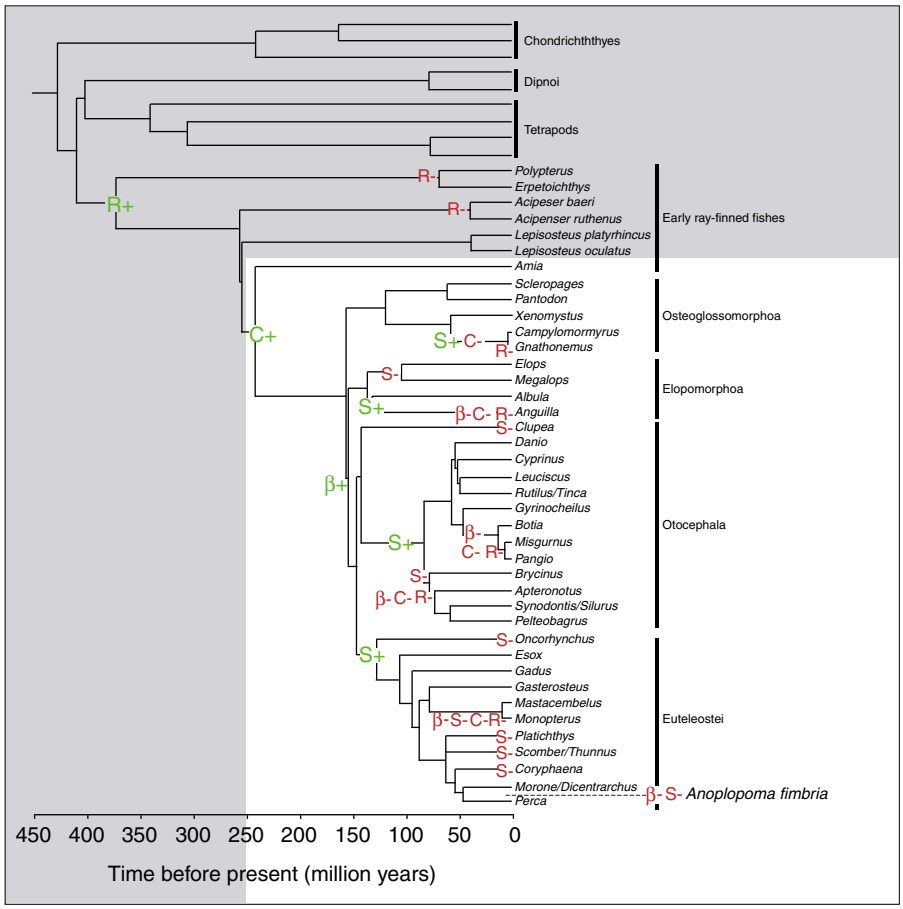

Fig. 7. Phylogenetic tree (modified from Berenbrink et al., 2005) depicting the relationships between species with respect to the first appearances and various losses of characteristics associated with the Root effect. Clades are grouped as per Berenbrink et al. (Berenbrink et al., 2005). Branch lengths are minimal divergence times as estimated from the fossil record. Terminal branches bearing two names denote studies where data from both species were used (Berenbrink et al., 2005). Bold letters demarcate the first appearance(s) (+) and each secondary loss (–) or reduction in a trait, such as the Root effect (R), where a loss would be a reduction in magnitude to <40% (in stripped haemolysates with saturating levels of organic phosphates), choroid rete (C), adrenergically activated βNHE on the RBC (β), and the rete mirabile at the swim bladder (S). The white inset represents all species that evolved subsequent to the first and only known appearance of the most ancient anatomical structure associated with the Root effect, the choroid rete, approximately 250 mya. Sablefish are placed as close to the Perciformes (*Perca*) lineage as possible, to stay in line with the original phylogenetic relationships (Nelson, 1994) accessed for this evolutionary reconstruction (Berenbrink et al., 2005). Although results from this study on washed and resuspended RBCs suggest the reduction in Hb-O_2 saturation is slightly <40%, which would categorize sablefish with a reduced Root effect (ancestral state) according to Berenbrink et al. (Berenbrink et al., 2005), a preliminary study where the magnitude of the Root effect was assessed in sablefish haemolysates with saturating organophosphate levels according to Berenbrink et al. (Berenbrink et al., 2005), and thus a procedure consistent with that used for other species presented in this figure, resulted in a Root effect magnitude >40%.

et al., 1985), as the βNHE is the only known form of the NHE to be activated by a cAMP/protein kinase A (PKA) pathway (Borgese et al., 1992). Although we did not find any evidence for a functional RBC βNHE in this study, it would be interesting to look at mRNA expression of RBC β3 adrenoreceptors and βNHEs to gain insight into the level at which function is lost. For example, the eel, which represents another teleost group where the function of the RBC βNHE has been secondarily lost, does possess RBC β-adrenoreceptors (Perry and Reid, 1992). Despite the lack of functional RBC βNHE, sablefish may be unlikely to experience reduced O_2 uptake at the gills even during the most severe acidosis, because the onset pH for the Root effect is so low. It may be that the only location in sablefish where the Root effect may be elicited *in vivo* is where an acidosis could be localized and perpetuated, like the dense counter-current capillary network of the choroid rete.

Cell volume regulation

The process of regulating and restoring RBC volume has been well characterized in most vertebrates, including mammals, amphibians, teleosts and agnathans (reviewed in Cossins and Gibson, 1997). Specifically, NHEs are ubiquitous in organisms across all phyla from all kingdoms (reviewed in Brett et al., 2005), and the NHE1 isoform is important for regulation of pH_i and cell volume in nearly all eukaryotic cells (Wakabayashi, 1997; Yun et al., 1995). It appears that an adrenergic-insensitive sablefish RBC NHE may be activated in both mild hypoxia and anoxia incubation conditions, because RBCs treated with the amiloride analogue EIPA (which is specific to NHE) exhibit shrinkage prior to exposure to hypersaline conditions (Fig. 6). Furthermore, RBCs subjected to hypersaline conditions exhibit a greater degree of shrinkage in the presence of EIPA, indicating that, again, a transporter is being activated to regulate cell volume, although not very effectively as recovery in

the hypersaline-treated RBCs was not evident over the 90 min time course of the experiment (Fig. 6). Although it would have been interesting to prolong the exposure to see whether volume recovery was possible, we observed problems with haemolysis. This observation suggests that prolonged RBC shrinkage may have compromised membrane integrity resulting in atypically fragile cells, a phenomenon that has also been observed in lungfish (Koldkjær et al., 2002). Regardless, it does appear that an NHE isoform may be operating on the sablefish RBC membrane to maintain pH and cell volume homeostasis, likely a 'housekeeping' NHE1 (Claiborne et al., 1999; Deigweiher et al., 2008); however, further studies are required to address this.

Role of the Root effect

The phylogenetic reconstruction executed by Berenbrink and colleagues (Berenbrink et al., 2005) revealed that the ancestral state of Root effect evolution corresponded with a Root effect magnitude of <40% reduction in maximal Hb-O_2 saturation upon acidification. For their reconstruction, however, Hb solutions (haemolysates) were prepared in citrate buffers to stabilize native organophosphate modulators and ensure maximal expression of the Root effect (Berenbrink et al., 2005). Washed and resuspended sablefish RBCs for our study exhibited a maximal desaturation of ~35%; however, in a preliminary (but *post-hoc*) investigation on sablefish haemolysates prepared and analysed according to Berenbrink et al. (Berenbrink et al., 2005), the magnitude of the Root effect exceeded 40%. If included in the Berenbrink phylogeny (Fig. 7), sablefish would likely be categorized as possessing a pronounced rather than reduced Root effect, but until a thorough study addressing Hb modulators can supplement the current study, we refer to the Root effect magnitude in sablefish as 'moderate'.

To date, the primary roles for the Root effect in teleosts are thought to be (1) oxygenating the retina and (2) filling the SB, but the possession of Root shift Hbs does not necessarily correlate with the presence of retia. The SB rete mirabile evolved independently at least four times in derived teleosts approximately 130 mya, and the structure has since been secondarily lost several times (Berenbrink et al., 2005) (Fig. 7). In contrast, the choroid rete at the eye evolved only once, approximately 250 mya in the common ancestor of *Amia* and teleosts, but has also been lost at least five times over the course of teleost evolution (Berenbrink et al., 2005) (Fig. 7). Basal actinopterygian species like the bowfin, mooneye and pirarucu (Regan and Brauner, 2010) and some of the Hb-possessing Antarctic Nototheniod species (Verde et al., 2008) possess moderate Root effects and only a choroid rete at the eye. A decrease in the Root effect magnitude (<40%) has only been noted in species where the choroid rete has also been lost, like the catfishes, loaches and some eels [*Gnathonemus*+ *Campylomormyrus*, *Anguilla* and *Misgurnus* clades (see Berenbrink et al., 2005)]. As long as the choroid rete is present, loss of the rete mirabile at the SB should have no impact on the magnitude of the Root effect (Berenbrink et al., 2005). Therefore it is not surprising that the deep-dwelling sablefish, which possesses a choroid rete but lacks a SB, exhibits a moderate Root effect. In fact, relative to body and eye size, the sablefish choroid rete is larger than what is observed in rainbow trout (Herbert and Wells, 2002) or other rockfish species (J.L.R., personal observation), which is likely correlated to the role of the choroid rete in retinal oxygenation (M. Berenbrink, personal communication) (Fig. 1). Fish species residing in water shallower than the aphotic bathypelagic zone but as deep as the mesopelagic zone (150–1000 m) experience progressively dimmer light conditions, and the highly developed vision on which they may come to rely could be coordinated with enhanced retinal oxygenation. Deepwater predatory species like sablefish (as deep as 2740 m) (McFarlane and Beamish, 1983a; McFarlane and Beamish, 1983b) may therefore be at an advantage if they are able to hone visual acuity.

Ever since the first appearance of the RBC βNHE in teleosts, there have been at least four, this instance being the fifth, secondary losses of RBC βNHE activity (Fig. 7). The four groups previously identified (Berenbrink et al., 2005), which include the eels, catfishes and loaches, however, have also exhibited a decrease in the magnitude of the Root effect (<40% desaturation) and lost the choroid rete at the eye (Berenbrink et al., 2005). Sablefish maintain the choroid at the eye and possess a moderate Root effect, which in the presence of saturating levels of organophosphates may be greater than 40%. This system is in place without the adrenergically stimulated protective mechanism at the RBC, the βNHE. We suspect that the absence of the βNHE is associated with the decrease in the Root effect onset pH, suggesting that the only place where pH would reach such low levels as to induce the Root effect would be in an isolated system like the choroid rete at the eye, rather than in the general circulation. However, the sablefish O_2 transport system may be very plastic, potentially dependent on life history stage, environmental conditions, and controlled by Hb modulators. While an intriguing species to study from a purely scientific, perhaps even an evolutionary perspective, many questions remain to be answered before we can fully understand the interesting respiratory and stress physiology of the sablefish.

LIST OF ABBREVIATIONS

βNHE	β-adrenergically stimulated sodium (Na^+) proton (H^+) exchanger
Φ	Bohr coefficient
cAMP	8-bromo cyclic adenosine monophosphate
EIPA	5-(*N*-ethyl-*N*-isopropyl)amiloride
Hb	haemoglobin
Hct	haematocrit
ISO	isoproterenol
MCHC	mean cellular haemoglobin concentration
mya	million years ago
n_H	Hill cooperativity coefficient
OEC	oxygen equilibrium curve
OMZ	oxygen minimum zone
P_{50}	P_{O_2} at 50% saturation
pH_e	extracellular pH
pH_i	intracellular pH
PKA	protein kinase A
P_{O_2}	oxygen tension
PROP	propranolol
RBC	red blood cell
RVI	regulatory volume increase
S	fractional saturation
SB	swim bladder

ACKNOWLEDGEMENTS

This study was supported by a Discovery grant from the Natural Sciences and Engineering Research Council (NSERC) of Canada to C.J.B. Funding for J.L.R. was through the University of British Columbia Graduate Fellowship program and for M.R.-M. through an NSERC undergraduate research scholarship. The authors wish to thank D. W. Baker, S. K. Hanna and F. Zahedieh for technical assistance, the staff and scientific support at Bamfield Marine Science Centre, including Drs B. Cameron and G. Goss, as well as Drs T. D. Clark and D. J. Randall for interesting discussions and editorial comments, and finally, two anonymous reviewers whose contributions have resulted in dramatic improvements to this study and manuscript.

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