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REVIEW

A unique mode of tissue oxygenation and the adaptive radiation of teleost fishes

D. J. Randall[1,*], J. L. Rummer[2], J. M. Wilson[3], S. Wang[4] and C. J. Brauner[1]

ABSTRACT

Teleost fishes constitute 95% of extant aquatic vertebrates, and we suggest that this is related in part to their unique mode of tissue oxygenation. We propose the following sequence of events in the evolution of their oxygen delivery system. First, loss of plasma-accessible carbonic anhydrase (CA) in the gill and venous circulations slowed the Jacobs–Stewart cycle and the transfer of acid between the plasma and the red blood cells (RBCs). This ameliorated the effects of a generalised acidosis (associated with an increased capacity for burst swimming) on haemoglobin (Hb)–O_2 binding. Because RBC pH was uncoupled from plasma pH, the importance of Hb as a buffer was reduced. The decrease in buffering was mediated by a reduction in the number of histidine residues on the Hb molecule and resulted in enhanced coupling of O_2 and CO_2 transfer through the RBCs. In the absence of plasma CA, nearly all plasma bicarbonate ultimately dehydrated to CO_2 occurred via the RBCs, and chloride/bicarbonate exchange was the rate-limiting step in CO_2 excretion. This pattern of CO_2 excretion across the gills resulted in disequilibrium states for CO_2 hydration/dehydration reactions and thus elevated arterial and venous plasma bicarbonate levels. Plasma-accessible CA embedded in arterial endothelia was retained, which eliminated the localized bicarbonate disequilibrium forming CO_2 that then moved into the RBCs. Consequently, RBC pH decreased which, in conjunction with pH-sensitive Bohr/Root Hbs, elevated arterial oxygen tensions and thus enhanced tissue oxygenation. Counter-current arrangement of capillaries (retia) at the eye and later the swim bladder evolved along with the gas gland at the swim bladder. Both arrangements enhanced and magnified CO_2 and acid production and, therefore, oxygen secretion to those specialised tissues. The evolution of β-adrenergically stimulated RBC Na^+/H^+ exchange protected gill O_2 uptake during stress and further augmented plasma disequilibrium states for CO_2 hydration/dehydration. Finally, RBC organophosphates (e.g. NTP) could be reduced during hypoxia to further increase Hb–O_2 affinity without compromising tissue O_2 delivery because high-affinity Hbs could still adequately deliver O_2 to the tissues via Bohr/Root shifts. We suggest that the evolution of this unique mode of tissue O_2 transfer evolved in the Triassic/Jurassic Period, when O_2 levels were low, ultimately giving rise to the most extensive adaptive radiation of extant vertebrates, the teleost fishes.

KEY WORDS: Carbon dioxide, Oxygen, Teleosts

[1]Department of Zoology, University of British Columbia, 6270 University Boulevard, Vancouver, BC V6T 1Z4, Canada. [2]Australian Research Council Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, QLD, 4811 Australia. [3]Ecofisiologia Centro de Investigação Interdisciplinar Marinha e Ambientral (CIIMAR), Rua dos Bragas 289, 4050-123 Porto, Portugal. [4]Fisheries College, Jimei University, 43 Yindou Road, Xiamen 361021, China.

*Author for correspondence (randall@zoology.ubc.ca)

Introduction

Teleost fishes comprise 95% of all extant fishes, with approximately 26,000 named species (Helfman et al., 1997). Teleosts first appeared in the early Triassic period 200–250 million years ago (MYA) and have since radiated into almost all aquatic environments (Nelson, 1994; Near et al., 2012). The ray-finned fishes, to which the teleost fishes belong, diverged from the lobe-finned fishes (e.g. lungfishes) around the Devonian, 400 MYA. The lobe-finned fishes were very successful until the Permian crisis 252 MYA, and gave rise to nearly all terrestrial vertebrates (Clack, 2007). In contrast, only a limited number of ray-finned fishes evolved into semi-terrestrial air-breathing species, while the vast majority remained completely aquatic (Randall et al., 1981; Graham, 1997). The Permian crisis, also known as the Permian mass extinction, was caused by extensive volcanic activity and global fires, and resulted in the loss of 96% of marine fish species. Following the Permian, the ray-finned fishes invaded many habitats from which the lobe-finned fishes had been extirpated. Explanations of the teleost success involve modifications in swimming and feeding, with genome duplication (3R) enhancing the rate of evolution of these processes (Ilves and Randall, 2007).

It should be noted that aquatic oxygen levels remained low for approximately 100 million years after the Permian crisis and only increased to levels approaching present-day conditions during the Cretaceous (Fig. 1). Teleosts radiated after the Permian (Bellwood and Hoey, 2004) during a prolonged period of low oxygen levels, and we propose that hypoxia tolerance was key to the teleost success after the Permian crisis; subsequent radiation depended on modifications in feeding and swimming. We propose that hypoxia tolerance is related to a unique mode of tissue oxygen delivery in teleost fishes as described below.

Hypoxia tolerance in teleost fishes

Many extant teleost fishes can survive environmental hypoxia. Their scope for activity is reduced, but they are able to maintain resting oxygen consumption rates ($\dot{M}_{O_2}$) as dissolved oxygen levels in the water decrease to a critical level (P_{crit}). Below P_{crit}, $\dot{M}_{O_2}$ falls with further reductions in water oxygen levels. Many hypoxia-tolerant teleost fishes do not exhibit this decrease until the partial pressure of oxygen (P_{O_2}) in water is below 30 mmHg, much lower than in other vertebrates (Nilsson and Randall, 2010). Numerous factors enable teleost fishes to survive in low-oxygen environments. First, counter-current flow of blood and water at the gills allows for up to 80% of oxygen extraction from water (reviewed by Randall and Daxboeck, 1984). During hypoxia, water flow over the gills and gill oxygen diffusing capacity both increase, while heart rate decreases (Holeton and Randall, 1967). Gill oxygen diffusing capacity increases during hypoxia via several mechanisms; for example, the heart beats in phase with the oscillations in ventilation, such that blood flow through the gills slows down during times of high water flow (Randall and Smith, 1967). Also during hypoxia, cardiac stroke volume approaches that of the vascular volume of the gills so that,

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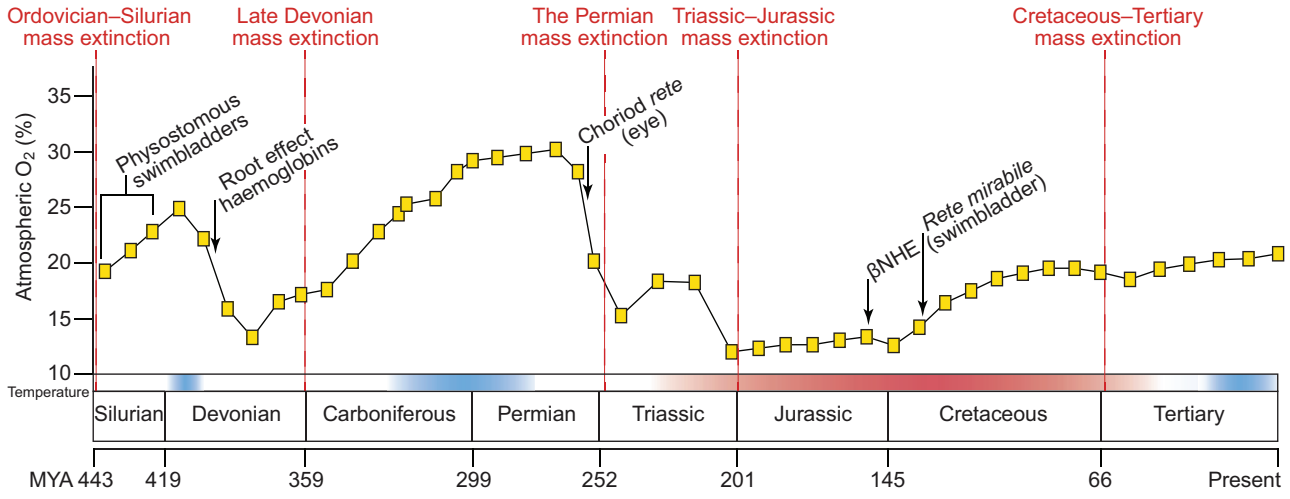

Fig. 1. Changes in oxygen levels (% atmospheric) over time (millions of years ago; MYA). Red and blue indicate global warm and cold periods [derived from information in previous studies (Berenbrink et al., 2005; Clack, 2007; Ward, 2006)].

with each heartbeat, the gills are completely filled with new blood (Randall and Daxboeck, 1984). Blood pressure increases, and blood enters all gill lamellae, thus increasing the residence time of the blood within the gills (Booth, 1978). In addition, changes in blood flow through each lamella may enhance gill diffusing capacity (for details, see Randall and Daxboeck, 1984). Another means for increasing diffusing capacity during hypoxia (but not anoxia) has been observed in carp, where an interlamellar epithelial cell mass between the gill lamellae is removed through apoptosis (Sollid et al., 2003), which increases gill surface area and decreases epithelium diffusion distance (Matey et al., 2008). Fish increase blood Hb concentrations during hypoxia but, unlike mammals, many teleost fishes also increase Hb–oxygen (Hb–O_2) affinity (Val, 2000) by decreasing levels of organic phosphates (e.g. ATP and GTP) within the nucleated red blood cells (RBCs). Changes in ATP and GTP levels are linked to oxidative phosphorylation (Wells, 2009), formation of magnesium complexes (Houston, 1985), and the action of catecholamines, corticosteroids and changes in pH (Val, 2000). While the mechanism is not fully understood, what is clear is that, in many teleosts, RBC ATP and GTP levels decrease in response to hypoxia, and Hb–O_2 affinity increases. In contrast, a few air-breathing teleost fishes have high levels of 2,3 diphosphoglycerate in their RBCs, which increase during hypoxia, thus decreasing Hb–O_2 affinity (Val, 2000), a pattern similar to that observed in mammals. The increase in Hb–O_2 affinity observed in teleosts during hypoxia will increase gill oxygen diffusing capacity and enhance oxygen uptake across the gills.

Oxygen uptake at the tissues

Uptake into the blood is only the first step in the oxygen delivery system, and an increase in Hb–O_2 affinity could be detrimental for oxygen release to the tissues. What happens at the tissue level? Unfortunately, there are only limited data concerning this question. McKenzie et al. (McKenzie et al., 2004) reported red muscle oxygen levels (P_{RMO_2}) in rainbow trout (Fig. 2) that were much higher than mixed venous blood (P_{vO_2}) oxygen levels (Stevens and Randall, 1967). One explanation is that blood flow relative to metabolic rate is higher in red muscle compared with other tissues and $P_{RMO_2}>P_{vO_2}$ could simply be a function of high muscle blood flow rates. However, there is no evidence for such high blood flows to red muscle in resting animals, being only 9% of cardiac output in resting rainbow trout (Bushnell and Brill, 1992; Randall and Daxboeck, 1982).

Arterial oxygen content (C_{aO_2}) and $\dot{M}_{O_2}$ in rainbow trout were not significantly affected by hypoxia (McKenzie et al., 2004). Thus O_2 delivery to the tissues appears unchanged, despite a fourfold reduction in the apparent blood-to-muscle oxygen gradient [arterial

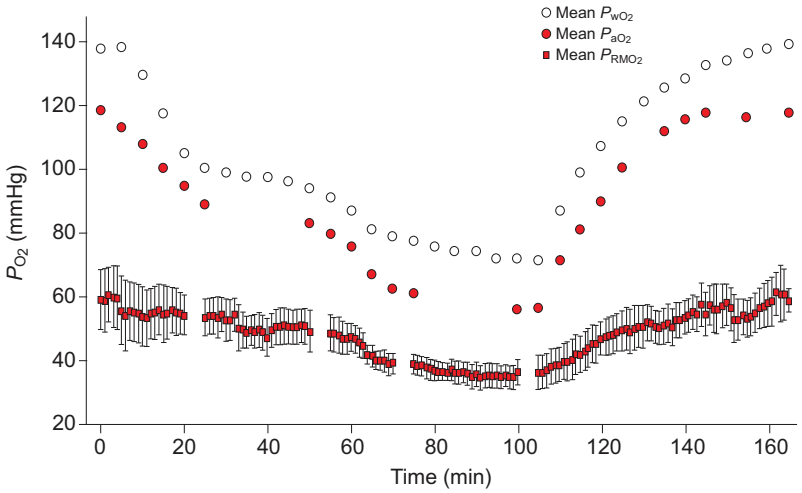

Fig. 2. Changes in red muscle P_{O_2} (P_{RMO_2}) and arterial P_{O_2} (P_{aO_2}) in rainbow trout during exposure to changes in water P_{O_2} (P_{wO_2}) during aquatic hypoxia (McKenzie et al., 2004). Data are means ± s.e.m., *N*=6 in all cases.

P_{O_2} (P_{aO_2}) minus P_{RMO_2}; Fig. 2] (McKenzie et al., 2004). Nevertheless, muscle oxygen levels are high, and oxygen transfer to muscle may be maintained by a mechanism somewhat analogous to that seen in the swim bladder and eye of many teleost fishes. High oxygen levels in the eye and swim bladder are clearly associated with the Root effect, a reduction in Hb–O_2 carrying capacity with a decrease in pH (Pelster and Randall, 1998; Waser and Heisler, 2005). Acidification necessary to drive oxygen from the Hb comes from CO_2 entering the RBCs. Elevated CO_2 levels are produced at the swim bladder gas gland largely via the pentose phosphate shunt; CO_2 enters the RBCs, decreases pH, and drives oxygen from the Hb into the swim bladder (Pelster and Randall, 1998). The situation in the eye is not as clear. In rainbow trout, perfusion of the eye alone through the ophthalmic artery with rainbow trout blood is sufficient to generate high ocular oxygen levels, which are absent when perfused with mammalian blood lacking a Root effect (Waser and Heisler, 2005). The fish eye is poorly vascularised, yet it is a very active tissue with high rates of metabolic CO_2 production (Herbert et al., 2002; Wittenberg and Haedrich, 1974; Wittenberg and Wittenberg, 1962; Wittenberg and Wittenberg, 1974), including some via the pentose phosphate shunt (Bridges et al., 1998). Thus, CO_2 levels in the eye are probably high and, as the blood enters ocular circulation, a 'Root-off' shift may elevate retinal P_{O_2}. We suggest that oxygen delivery to muscle and perhaps other tissues may be enhanced in a similar way.

Carbonic anhydrase distribution, RBC pH and the Jacobs–Stewart cycle

Protons (H^+) entering the plasma of most vertebrates distribute passively across the RBC membrane via the Jacobs–Stewart cycle. Protons combine with plasma bicarbonate and, in the presence of carbonic anhydrase (CA), are rapidly dehydrated to form CO_2. Once formed, the CO_2 freely enters the RBCs, where it is rapidly hydrated via CA to form bicarbonate and protons; bicarbonate then leaves the cell in exchange for chloride (via Band III). CA is one of the fastest enzymes known, with an average half time ($t_{1/2}$) of 5×10^{-3} to 1×10^{-3} s, approximately 6000 times faster than the uncatalyzed reaction (Cardenas et al., 1998; Geers and Gros, 2000; Henry and Swenson, 2000) and is in high concentration inside the RBCs and in many other tissues (Decker et al., 1996; Effros and Weissman, 1979; Geers and Gros, 2000; Gervais and Tufts, 1998; Gilmour et al., 1997; Henry et al., 1997; Henry and Swenson, 2000; Sender et al., 1994; Siffert and Gros, 1982; Yamamoto et al., 1985). Teleost fish possess an assortment of CA isoforms, including both soluble and membrane-bound, and for many of these isoforms, tissue distribution and molecular structure are known (see reviews by Esbaugh and Tufts, 2006; Gilmour and Perry, 2009). However, less is known of the exact cellular distribution and function of each isoform. CA has been reported in the swim bladder of bowfin (Gervais and Tufts, 1998) and in fish muscle (for review, see Henry and Swenson, 2000). CAs are important in acid-base regulation in many sites including muscle, gills and kidney (Geers and Gros, 2000; Gilmour and Perry, 2009).

Teleost fish RBCs have sodium/proton exchange (NHE) isoforms and a Band III anion exchange (AE) protein that take up sodium and chloride and excrete protons and bicarbonate, respectively (Fig. 3) and are involved in volume regulation (Heming et al., 1986; Claiborne et al., 1999; Cossins and Gibson, 1997; Rummer and Brauner, 2011; Rummer et al., 2010; Weaver et al., 1999). Many teleost fishes also possess a β-adrenergically stimulated Na^+/H^+ exchanger (βNHE) on the RBC membrane (Motais et al., 1992; Nikinmaa, 1992). Upon activation, the βNHE rapidly excretes protons from the RBC (Heming et al., 1987; Berenbrink and Bridges, 1994). CA activity is not available to plasma flowing through the gills and venous system of some, perhaps all, teleost fishes (Rahim et al., 1988; Gilmour et al., 1994; Perry et al., 1997). The absence of CA activity in the plasma will slow the Jacobs–Stewart cycle, because plasma bicarbonate dehydration will occur at an uncatalyzed slow rate ($t_{1/2}$ ~25–90 s) (Cardenas et al., 1998; Geers and Gros, 2000; Henry and Swenson, 2000). Without plasma-accessible CA, the rate at which protons move from the plasma back into the RBC via the Jacobs–Stewart cycle will be reduced and the volume-regulating RBC NHE and AE systems will, therefore, be both pH- and volume-regulating systems (Fig. 3). Development of RBC pH regulation (e.g. via βNHE or NHE) in the absence of plasma CA uncouples plasma from RBC pH, thus reducing the effects of a large plasma acidosis on oxygen transport (Primmett et al., 1986).

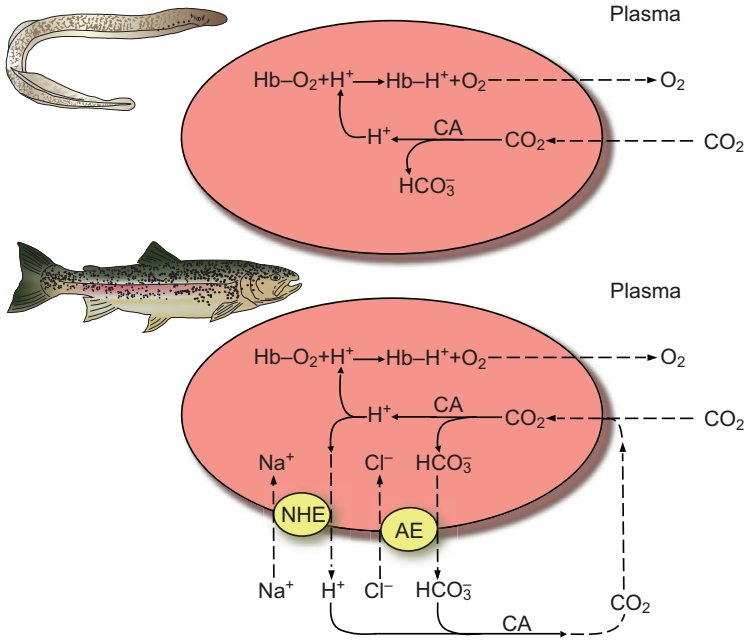

Fig. 3. Red blood cells (RBCs) release oxygen to the tissues of lamprey (top) and rainbow trout (bottom). The absence of RBC anion exchange (AE) in lamprey or plasma-accessible carbonic anhydrase (CA) in teleost fishes slows the movement of acid into the RBCs via the Jacobs–Stewart cycle. NHE refers to sodium proton exchange across the RBC membrane.

Plasma from some teleost fishes contains a CA-inhibiting factor (Haswell and Randall, 1976; Dimberg, 1994; Henry et al., 1997; Peters et al., 2000) that presumably prevents RBC pH regulation from being compromised if CA from lysed RBCs is released into the circulation (Henry et al., 1997). The inhibitor is a large molecule and has less effect on the activity of endothelial compared with cytosolic CA and probably cannot access CA bound to the sarcolemma membrane (see review by Geers and Gros, 2000). Although lysed CA will be excreted, it is possible that rapid inhibition of lysed CA is required to maintain blood capillary micro-environments involved in acid and/or bicarbonate transfer.

Nikinmaa et al. (Nikinmaa et al., 1990) reported an inhibition of the βNHE induced RBC pH increase *in vitro* when CA was added to the bathing medium whereas Motais et al. (Motais et al., 1989) reported no effect of increased plasma CA activity on βNHE-activated RBC pH. Nikinmaa et al. (Nikinmaa et al., 1990), however, still observed a drop in extracellular pH despite observing no change in RBC pH. Presumably there was a net proton efflux from the RBC, but the measurement of RBC pH was not sensitive enough to detect the change. The studies of Nikinmaa et al. (Nikinmaa et al., 1990), Motais et al. (Motais et al., 1989) and others stress the importance of slow uncatalyzed plasma bicarbonate dehydration in the regulation of RBC pH. These studies highlight the importance of the circulation of the blood in the generation of this response. The blood resides for approximately 60 s in the venous circulation with no loss or gain of CO_2. A βNHE-induced RBC pH increase in venous blood will increase oxygen binding to the Hb and lower P_{vO_2}, thus enhancing oxygen uptake as blood flows through the gills. Once blood enters the gills, CO_2 will be lost from the blood, maintaining high RBC pH.

Many factors in addition to βNHE activation may be involved in trout RBC pH regulation *in vivo*. Lessard et al. (Lessard et al., 1995) continuously infused CA into intact trout over a 6-h period but observed no short-circuiting of the catecholamine-induced RBC pH regulation (e.g. via βNHE). Changes in organic phosphates, carbamino formation or cell volume may have altered RBC membrane potential and therefore the distribution of protons between the RBC and plasma (Hemming et al., 1986). Swietach et al. (Swietach et al., 2010) showed that human RBC pH disturbances were compensated for within minutes when AE was activated in adult RBCs, with the possible involvement of NHE in immature RBCs. The teleost RBC is nucleated and regulation of intracellular pH may be similar to that of many other eukaryotic cells (Boron, 2004), where activation of NHE and AE results in acid efflux and acid-equivalent influx, respectively. A 'housekeeping' NHE has been reported in rainbow trout RBCs and may serve this purpose (Rummer and Brauner, 2011). While the exact NHE isoform is still unknown, the term 'housekeeping' will be used to refer to any fish RBC NHE other than βNHE.

The absence of plasma-accessible CA in the gills and veins of teleost fishes (Rahim et al., 1988, Gilmour et al., 1994; Perry et al., 1997) also results in nearly all bicarbonate dehydration associated with CO_2 excretion occurring inside the RBCs, where CA activity is high. Hb oxygenation produces the protons that drive bicarbonate dehydration inside the RBCs, resulting in tight coupling between O_2 and CO_2 transfer (Brauner et al., 2000). This tight coupling is further enhanced by a reduction in Hb buffering capacity due to a reduction in the number of histidine residues associated with teleost Hb compared with other vertebrate Hbs. Thus, fewer protons are bound to histidine residues and more are available to couple O_2 and CO_2 transfer. Berenbrink et al. (Berenbrink et al., 2005) presented an elegant review of the evolution of vertebrate Hbs, concentrating on the relationship between the number of histidine side chains, specific buffer values and the magnitude of the Bohr coefficient. They point out that lamprey and many teleosts have a low Hb buffer value when compared with other vertebrates, which is related to a reduction in the number of histidine residues. The absence of either plasma-accessible CA (teleosts) or Band III AE protein [lamprey (Nikinmaa, 1997; Tufts, 1992)] aids in uncoupling RBC and plasma pH by slowing the Jacobs–Stewart cycle, and both have a low Hb buffering capacity.

Bohr/Root effect Hbs

Most teleost fishes possess Root effect Hbs, where a reduction in blood pH not only decreases the Hb–O_2 affinity (Bohr effect) but also dramatically reduces Hb–O_2 carrying capacity (Root effect). Because of the high pH sensitivity of Root effect Hbs, a pH change could increase blood P_{O_2} in fish by 2.5–25 times that possible in terrestrial vertebrates with Hbs that only possess a Bohr effect (Rummer and Brauner, 2011; Rummer et al., 2013). The general model in mammalian systems for pH- and/or CO_2-induced enhancement of O_2 delivery involves CO_2 diffusing quickly into the RBCs and producing protons via the CA-mediated CO_2 hydration reaction. In mammalian RBCs, both CO_2 and H^+ will bind to the Hb, which will reduce Hb–O_2 affinity and enhance O_2 delivery to the tissues (Bohr effect). Lapennas (Lapennas, 1983) suggested an optimal Bohr coefficient (change in log P_{O_2} with a change in pH) for O_2 delivery, but the general conclusion was that the only organisms that actually possessed 'optimal Bohr coefficients' were air-breathing vertebrates. Furthermore, under Lapennas' analysis, it appeared that fish blood is not optimized for general O_2 delivery, and the large Bohr coefficient associated with the Root effect might even impair oxygen delivery. However, Lapennas' analysis assumed steady-state conditions within the blood (Lapennas, 1983). This is not the case in teleost blood. We suggest that this large Bohr coefficient associated with the Root effect Hb is important in oxygen delivery to the tissues in teleost fish.

CO_2 excretion and tissue oxygenation

The majority of CO_2 excreted across the gills originates as bicarbonate in the plasma that enters the RBCs, where it is dehydrated by CA to form CO_2 (Brauner et al., 2000). Therefore, the rate-limiting step for CO_2 excretion across the gills is bicarbonate entry into the RBCs (Gilmour, 1998; Perry and Gilmour, 1993). Transit time for blood flowing through the gills is of the order of ~1 s, which is insufficient for bicarbonate dehydration in the plasma at an uncatalyzed rate ($t_{1/2}$ ~25–90 s) (Cardenas et al., 1998; Geers and Gros, 2000; Henry and Swenson, 2000). Excess bicarbonate, therefore, will be left behind in the plasma leaving the gills. In addition, CO_2 released from the tissues will end up as bicarbonate in the venous plasma while protons are bound to Hb. Even if CA is available to plasma perfusing capillaries (see below), bicarbonate disequilibria can still develop within the venous system. This will occur as the rate of RBC bicarbonate transport (via Band III) is slower than capillary transit time, and CA is not plasma-accessible within the venous system, as is the situation in teleost fishes. Perry et al. (Perry et al., 1997) observed bicarbonate disequilibrium states in the venous circulation of unstressed fish that were less than that observed in the arterial blood, which may have been a reflection of venous residence time (~60 s, sufficient time for significant bicarbonate dehydration at an uncatalysed rate) compared with that in the arterial system (~10 s). Because an extracorporeal loop was used for this analysis, the reported magnitudes of both venous and arterial disequilibria are probably underestimates. Finally, venous disequilibrium states may be further enhanced in stressed fish when

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both bicarbonate and H^+ are actively excreted from the RBCs via βNHE. Perry et al. (Perry et al., 1997) eliminated bicarbonate disequilibria in both arterial and venous plasma by infusion of CA, confirming that the disequilibria were related to the absence of accessible CA.

Unlike the situation in the venous and gill circulations, there is evidence for endothelial-bound, plasma-accessible CA embedded in the peripheral circulation (for general review, see Geers and Gros, 2000). Henry et al. (Henry et al., 1997) and Wang et al. (Wang et al., 1998) reported that there was extracellular CA activity in trout white muscle, and concluded, based on the earlier work of Geers et al. (Geers et al., 1985), that it was associated with the sarcolemma membrane rather than the vascular endothelium. Despite this location, it has been shown that plasma bicarbonate has access to this CA, at least in mammals (see review by Geers and Gros, 2000). CA-like immunoreactivity has been located in the arteries of the glass catfish (Fig. 4) using a CA (Abcam ab6621-5) polyclonal antibody raised against whole-molecule human erythrocyte CAII. The supplier states that this antibody has negative cross-reactivity with human CAI, but other isoforms have not been tested. Although positive cross-reactivity has been demonstrated with green spotted pufferfish and zebrafish CA, the specific isoform cross-reactivity was not determined (Tang and Lee, 2007; J.M.W., unpublished). The data presented in Fig. 4 are preliminary, and indeed, a higher-resolution technique such as immunogold would help to identify the exact sub-cellular localization of the CA-like staining and its plasma accessibility. Additionally, isoform-specific antibodies should also be used (i.e. CAIV) to determine the molecular identity of the dorsal aortic CA isoform. Gilmour et al. (Gilmour et al., 1994) reported marked bicarbonate disequilibria in blood withdrawn from the coeliac artery of rainbow trout, indicating the absence of plasma-accessible CA activity in this vessel and the dorsal aorta, at least between the gills and the point of origin of the coeliac artery. Endothelial surface area to plasma volume ratios increase as vessels get smaller, and thus the effects of endothelial-bound, plasma-accessible CA may become apparent only in smaller vessels. We

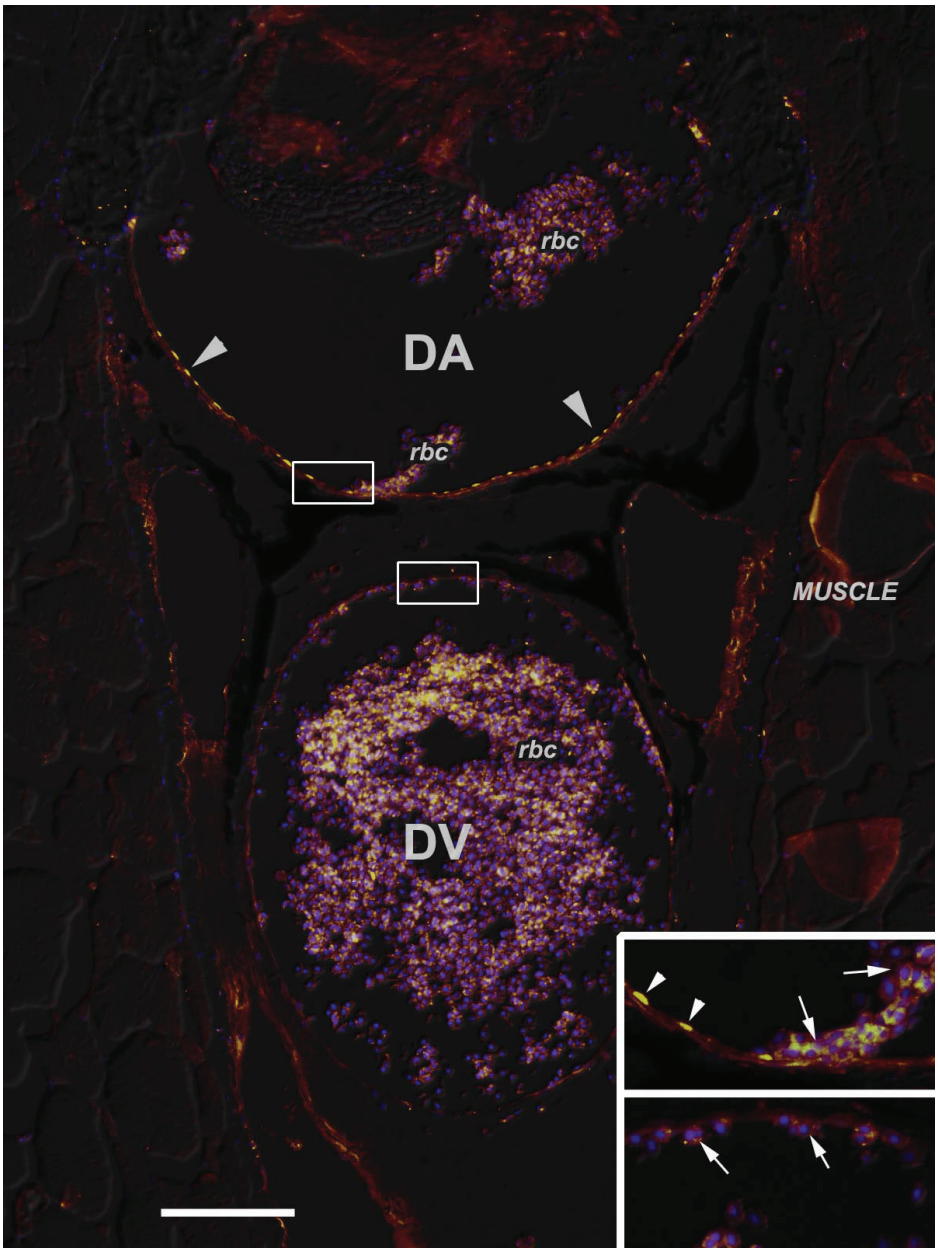

Fig. 4. Discontinuous immunofluorescent staining of CA in the dorsal aortic (DA) endothelium (arrowheads) is not apparent in the dorsal vein (DV) endothelium of the glass catfish. Red blood cells are indicated by arrows or labeled 'rbc'. Higher magnification (4×) insets are provided of DA and DV. Sections were indirectly probed with a heterologous CAII antibody and counter-stained with DAPI (blue) for labelling nuclei and the differential interference contrast image overlaid for tissue orientation. Scale bar, 100 µm and 400 µm for insets.

suggest that CA might be located in smaller arteries close to tissue capillaries. Although the distribution of endothelial CA has been studied in only a few teleost species, there is some preliminary evidence of CA bound to arterial endothelial cells, oriented toward the plasma (Fig. 4). Unfortunately, a more detailed distribution pattern is not yet available. However, because the presence or absence of plasma-accessible CA in the circulation could play a dramatic role in oxygen delivery to various teleost tissues, this is an area worthy of further investigation.

As suggested earlier, elevated plasma bicarbonate encountering plasma-accessible CA will be rapidly dehydrated to form CO_2, enter the RBCs and be hydrated to form protons. The protons will bind to Hb, increase P_{aO_2} and enhance oxygen diffusion into the tissues. This has been demonstrated in an *in vitro* closed system using rainbow trout blood incubated at elevated P_{CO_2} to increase plasma bicarbonate. The whole blood was acidified, adrenergically stimulated and exposed to CA (Rummer and Brauner, 2011). The increase in P_{O_2} following the addition of CA was as much as 30 mmHg. In addition, activation of βNHE on the RBC membrane could further enhance tissue oxygenation, presumably by enhancing the plasma bicarbonate disequilibria. *In vitro*, the increase in rainbow trout blood P_{O_2} observed upon acidification, β-adrenergic stimulation and then exposure to CA was much smaller when β-adrenergic stimulation was omitted from the experimental series (Rummer and Brauner, 2011). This indicates that activation of βNHE may further enhance tissue oxygenation during stressful situations when catecholamine levels are elevated. Furthermore, non-specific inhibition of NHE completely eliminated the response (Rummer and Brauner, 2011), suggesting that a 'housekeeping' NHE may play a role under conditions when catecholamine levels are low. Rummer et al. (Rummer et al., 2013) further supported this concept when they exposed resting rainbow trout to elevated environmental CO_2, which presumably resulted in an increase in the plasma bicarbonate disequilibria in arterial blood. The presumed subsequent acidification of the RBC resulted in an increase in P_{RMO_2} of over 30 mmHg, which was completely eliminated by the addition of a membrane-impermeant CA inhibitor (C18, i.e. inhibiting only plasma-accessible isoforms; Fig. 5), thus demonstrating the importance of plasma-accessible CA to this process (Fig. 6).

Rummer et al. (Rummer et al., 2013) found that infusing a CA inhibitor that blocks only plasma-accessible CA (C18) tended to reduce P_{RMO_2} in resting rainbow trout, but the decrease was not statistically significant; that is, the effects of endothelial CA may be minimal during rest. It is also possible that plasma bicarbonate dehydration is limited by proton availability, and oxygen delivery is enhanced by plasma acidosis, e.g. following burst swimming. Under stressful conditions, the release of catecholamines via activation of RBC βNHE could enhance plasma bicarbonate disequilibria and augment oxygen delivery to the tissues. In addition, a βNHE-induced rise in venous RBC pH would increase Hb–O_2 binding and reduce P_{vO_2}, which in turn would enhance oxygen uptake at the gills.

A number of investigations other than those by Rummer and colleagues (Rummer and Brauner, 2011; Rummer et al., 2013) have looked at the effects of CA infusion in intact fish. Wood and Munger (Wood and Munger, 1994) infused resting rainbow trout with CA and observed a reduction in blood total CO_2 and P_{aCO_2} and an increase in plasma pH, consistent with an increase in CO_2 excretion from plasma. The much larger increase in P_{aCO_2} and decrease in plasma pH following exhaustive exercise in saline-infused versus CA-infused rainbow trout could reflect differences in the extent of arterial plasma bicarbonate disequilibrium and tissue oxygen delivery. Wood and Munger (Wood and Munger, 1994) observed greater increases in oxygen uptake with little change in P_{aCO_2} in saline-infused versus CA-infused fish. Wood and Munger (Wood and Munger, 1994) also reported that CA infusion did not reduce aerobic swimming performance, indicating that either oxygen delivery was not reduced by CA infusion or oxygen transfer is not the rate-limiting step in aerobic performance. However, these swimming tests lasted several hours, sufficient time for small proteins such as CA (~29 kDa) to be filtered by the glomerulus and lost in the urine. Blood oxygen content was unaffected by CA infusion in intact trout (Lessard et al., 1995), but there was an expected reduction in CO_2 content of the blood. Surprisingly, the decrease in CO_2 content and plasma pH following exhaustive exercise was reduced compared with that observed in saline-infused fish, perhaps because of reduced acid excretion from the muscle in the CA-infused exhausted fish. The work of Wood and Munger (Wood and Munger, 1994) and Lessard et al. (Lessard et al., 1995) neither support nor refute our model of tissue oxygen delivery, nor were they designed to test our hypothesis.

Species variations in tissue oxygenation

The model we describe for enhanced oxygen delivery is likely to vary between species and during development, and it is likely less important in those animals with a reduced Hb pH sensitivity. Hbs vary in their pH sensitivity, and the complement of Hbs can change with age of the fish (Giles and Vanstone, 1976; Rummer et al., 2010). Some teleost species have lost RBC βNHE activation

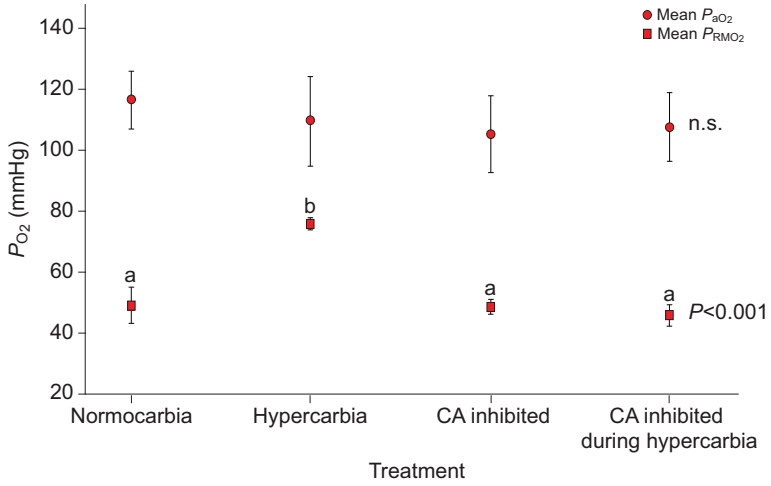

Fig. 5. Changes in P_{RMO_2} and P_{aO_2} in rainbow trout during periods of aquatic hypercarbia with and without CA inhibition. Data are means ± s.e.m., and statistically significant differences are demarcated by different letters, if applicable, otherwise n.s. (not significant), N=7–11 (for details, see Rummer et al., 2013).

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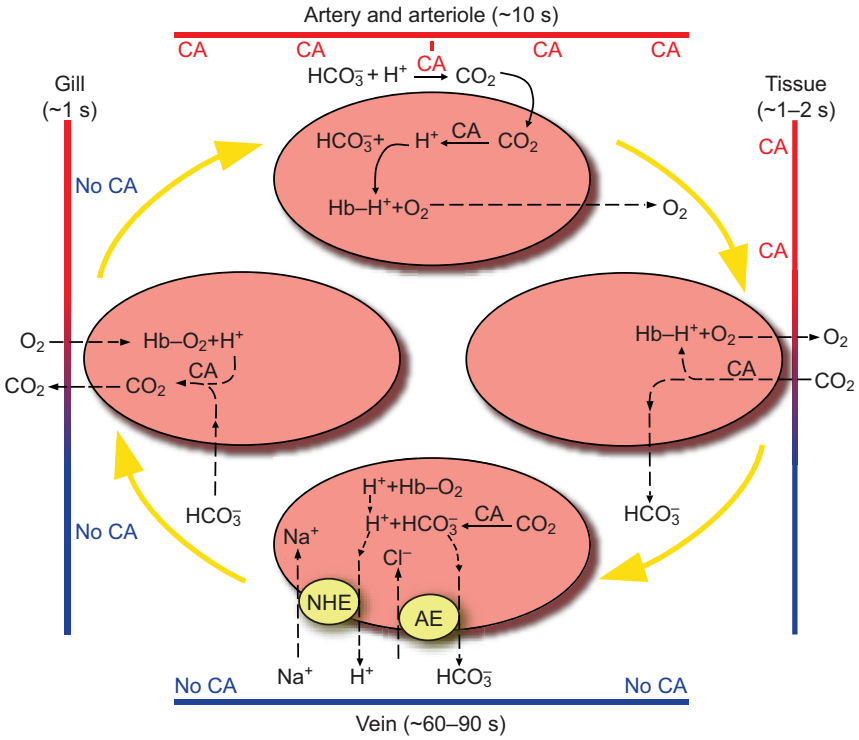

Fig. 6. The transfer of oxygen, carbon dioxide, protons and bicarbonate between the plasma and RBCs as blood flows through the gills, arteries, tissues and venous circulation. The approximate transit time (s) for blood through these portions of the circulation is in brackets. There is no CA available to plasma in the gills and veins, but CA is available in the arteries and arterioles. NHE, sodium proton exchange; AE, anion exchange.

(Romero et al., 1996; Rummer et al., 2010; Weaver et al., 1999), and this occurs in species that have lost or exhibit a minimal Root effect (Berenbrink et al., 2005; Rummer et al., 2010). The loss of RBC βNHE activation may be related to requirements for RBC volume regulation. RBC NHEs are involved in volume increase as well as pH regulation, and the response varies between species. Rainbow trout RBC NHE responds to adrenergic stimulation but not to reduced volume, whereas eel NHE responds to reduced volume, and flounder NHE shows adrenergic, hypertonic and acid activation (Weaver et al., 1999). High oxygen levels inhibit trout, carp and flounder RBC NHEs, but RBC membrane K^+Cl^- co-transporters (KCC) decrease volume and are activated by oxygenation. Teleost RBC AE has the potential via KCC activation to modulate volume as well as RBC pH. Swietach et al. (Swietach et al., 2010) showed that human RBC pH regulation is modulated by changes in RBC volume. The physiological role of these membrane ion exchangers in RBC pH-volume coupling in teleost fishes and the consequences on both blood rheology and gas transport are not clear, but are worthy of further investigation and may explain varying patterns of oxygen transfer in teleost fishes.

Tissue oxygenation in elasmobranchs, lampreys and hagfish

Elasmobranchs have CA embedded in the gill pillar cells and in the plasma, both available for plasma bicarbonate dehydration, but overall CA activity levels are less than those observed in teleosts (Swenson and Maren, 1987; Gilmour et al., 1997; Gilmour and Perry, 2009; Gilmour et al., 2001; Perry et al., 1999; Wilson et al., 2000). At the gills, CO_2 can be excreted directly from the plasma and there is no tight coupling of O_2 and CO_2 transfer through the RBCs, as in teleost fishes. Swenson and Maren (Swenson and Maren, 1987) concluded that gill CA was important in acid-base regulation rather than CO_2 excretion. As far as we know, there are no reports of changes in RBC organic phosphate levels during hypoxia, and tissue oxygen levels have not been measured in any elasmobranch. Spiny dogfish sharks show no RBC βNHE pH regulation and no plasma bicarbonate disequilibria [see discussion in Randall (Randall, 1998)]. Dogfish Hbs also have more histidine residues and therefore a greater buffer value than teleost Hbs (Berenbrink et al., 2005). Elasmobranchs do produce lactate and become acidotic following burst swimming (Holeton and Heisler, 1983). These plasma pH changes will be transferred to the RBCs, but elasmobranch Hbs have a high buffer value and reduced Bohr coefficients (Berenbrink et al., 2005). Brill et al. observed no change in RBC pH following anaerobic exercise in the sandbar shark (Brill et al., 2008). It would seem that the mechanisms of oxygen delivery to the tissues between teleosts and elasmobranchs differ, and this is related to the distribution of plasma-accessible CA and Hb buffering capacity.

Lampreys and hagfish both lack chloride/bicarbonate exchange on their RBC membranes (Fig. 3), but only lamprey RBCs have sodium/proton exchange (Nikinmaa, 1997; Tufts, 1992). In the lamprey, CO_2 is hydrated to bicarbonate and transported within the RBCs. Once reaching the gills, bicarbonate is dehydrated within the RBCs and released at the gills as CO_2. Hagfish have CA available to plasma passing through the gills, and plasma bicarbonate is excreted directly from the plasma as CO_2 (Esbaugh et al., 2009). Thus, in the lamprey, CO_2 transport is centred entirely on the RBCs, whereas elasmobranchs and hagfish can excrete plasma bicarbonate directly from plasma as CO_2 without it first passing through the RBCs.

Evolution of tissue oxygen delivery in teleosts

Approximately 400 MYA, Root effect Hbs probably evolved in basal actinopterygian fishes. It has been argued that in those ancient fish that possessed a Root effect, the onset of the ‘Root-off’ shift occurred at a pH much lower than that expected to occur in the general circulation and, therefore, Hb oxygenation would not likely be impaired (Regan and Brauner, 2010a; Regan and Brauner, 2010b; Rummer et al., 2010). These Hbs were likely selected for oxygen delivery, as their Bohr coefficients were close to the optimal values proposed by Lappenas (Berenbrink et al., 2005).

Over the next 100 million years, RBC pH regulation, coupled to the absence of plasma-accessible CA, evolved and ameliorated the effects of a plasma acidosis on Hb oxygenation. This loss of plasma-accessible CA slowed the Jacobs–Stewart cycle and the transfer of acid between plasma and the RBCs via AE and 'housekeeping' NHE transport proteins on the RBC membrane. With the uncoupling of plasma and RBC pH, the importance of Hb as a buffer diminished and led to a reduction in Hb buffering (Berenbrink et al., 2005; Regan and Brauner, 2010a; Regan and Brauner, 2010b), thus enhancing the tight coupling of O_2 and CO_2 transfer through the RBCs (Brauner et al., 2000). Many extant teleosts are able to regulate RBC pH and have Root effect Hbs that operate at physiological pH. Presumably, as the capacity to regulate RBC pH evolved, the magnitude of the Root effect increased and the pH onset moved into the pH range of that in the general circulation. We stress, however, that it is the large Bohr coefficient [greater than that deemed optimal by Lapennas (Lapennas, 1983)] associated with Root effect Hbs that is important for oxygen delivery to tissues (other than the eye and swim bladder) in teleost fishes. Such Hbs appear to exist in both teleosts and bowfin, indicating that the evolution of this system occurred in a common ancestor of bowfin and teleosts, before the late Permian.

Plasma disequilibrium states for CO_2 hydration/dehydration reactions occurred resulting from the pattern of CO_2 excretion across the gills and tissues. Plasma-accessible CA embedded in arterial endothelial cells eliminated bicarbonate disequilibria and generated CO_2 that moved into the RBCs, thus elevating P_{aO2} in the blood and enhancing tissue oxygenation. Mechanisms to reduce RBC NTP during hypoxia evolved, which increased Hb–O_2 affinity without compromising tissue oxygen delivery (note: this could have occurred much later).

The absence of plasma-accessible CA activity created conditions for the subsequent evolution of RBC pH regulation via βNHE. Berenbrink et al. (Berenbrink et al., 2005) concluded that RBC pH regulation involving βNHE evolved in the late Jurassic, some 250 million years after the appearance of Root effect Hbs (Fig. 1). Oxygen delivery to the eye and swim bladder clearly depends on the Root effect (Pelster and Randall, 1998; Waser and Heisler, 2005), but cannot be related to catecholamine-stimulated RBC βNHE pH regulation, which is inhibited by high oxygen levels (Gibson et al., 2000; Weaver et al., 1999). In fact, the choroid rete in the eye evolved in the late Permian (Berenbrink et al., 2005), before the appearance of βNHE pH regulation (Berenbrink et al., 2005). Furthermore, high ocular oxygen levels have been measured in the absence of catecholamines (Waser and Heisler, 2005). Catecholamines reduce blood flow to the gas gland, therefore inhibiting oxygen secretion into the swim bladder (Pelster, 1994). Finally, eels lack RBC βNHE pH regulation but deliver high levels of oxygen to their swim bladder (Weaver et al., 1999). Thus, catecholamine-activated RBC βNHE pH regulation is not directly involved in developing the high oxygen levels seen in the eye and swim bladder. We suggest that catecholamine-activated RBC βNHE pH regulation enhances oxygenation of tissues other than the eye and swim bladder, as well as protects RBC pH from a plasma acidosis such that O_2 uptake at the gills is safeguarded.

The CA-mediated oxygen delivery system evolved further with the appearance of acid-producing gas glands that enhanced oxygen transfer into the swim bladder of some fishes. This may have been associated with the appearance of a rete mirabile at the swimbladder in the early Cretaceous (Berenbrink et al., 2005). Tuna and some other fish have evolved counter-current circulation to muscle to retain heat and warm the muscles. Given an appropriate distribution of endothelia-bound CA, this counter-current circulation could also be used to raise muscle oxygen levels; the two systems need not be mutually exclusive.

In conclusion, we suggest that blood leaving the tissues and the gills of at least some teleost fishes has elevated plasma bicarbonate levels due to a disequilibrium state. The bicarbonate is dehydrated to form CO_2 as blood encounters plasma-accessible CA bound to arterial and/or capillary endothelial cells. The CO_2 enters and acidifies the RBC and drives oxygen from Hb into the muscle and perhaps other tissues. During exercise, stress or adverse environmental conditions, this process is enhanced by RBC βNHE activation. Oxygen transfer to the tissues depends on C_{aO2} and the magnitude of the combined Bohr/Root effect to enhance the oxygen gradient. This allows for the selection of mechanisms, in particular, increased Hb–O_2 affinity, to enhance uptake from the environment to maintain C_{aO2} during hypoxia, but without impairing oxygen delivery to the tissues.

We propose that the evolution of this mode of tissue oxygen transfer facilitated teleost radiation in the Triassic and Jurassic periods, when oxygen levels were low (Fig. 1). Radiation was also associated with the evolution of novel feeding mechanisms, buoyancy control and increased swimming abilities, thus widening food sources for these active, agile hunters (Near et al., 2012; Ilves and Randall, 2007; Helfman et al., 1997). With increasing activity and mobility, muscle density also increased. We suggest that the above advances hinged on enhanced O_2 transfer from the water to the tissues via a pH-sensitive Hb and CA-mediated oxygen delivery system unique to teleost fishes.

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