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The evolution of Root effect hemoglobins in the absence of intracellular pH protection of the red blood cell: insights from primitive fishes

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Abstract The Root effect, a reduction in blood oxygen (O_2) carrying capacity at low pH, is used by many fish species to maximize O_2 delivery to the eye and swimbladder. It is believed to have evolved in the basal actinopterygian lineage of fishes, species that lack the intracellular pH (pH_i) protection mechanism of more derived species' red blood cells (i.e., adrenergically activated Na^+/H^+ exchangers; βNHE). These basal actinopterygians may consequently experience a reduction in blood O_2 carrying capacity, and thus O_2 uptake at the gills, during hypoxia- and exercise-induced generalized blood acidoses. We analyzed the hemoglobins (Hbs) of seven species within this group [American paddlefish (*Polyodon spathula*), white sturgeon (*Acipenser transmontanus*), spotted gar (*Lepisosteus oculatus*), alligator gar (*Atractosteus spatula*), bowfin (*Amia calva*), mooneye (*Hiodon tergisus*), and pirarucu (*Arapaima gigas*)] for their Root effect characteristics so as to test the hypothesis of the Root effect onset pH value being lower than those pH values expected during a generalized acidosis in vivo. Analysis of the haemolysates revealed that, although each of the seven species displayed Root effects (ranging from 7.3 to 40.5% desaturation of Hb with O_2, i.e., Hb O_2 desaturation), the Root effect onset pH values of all species are considerably lower (ranging from pH 5.94 to 7.04) than the maximum blood acidoses that would be expected following hypoxia or exercise (pH_i 7.15–7.3). Thus, although these primitive fishes possess Hbs with large Root effects and lack any significant red blood cell βNHE activity, it is unlikely that the possession of a Root effect would impair O_2 uptake at the gills following a generalized acidosis of the blood. As well, it was shown that both maximal Root effect and Root effect onset pH values increased significantly in bowfin over those of the more basal species, toward values of similar magnitude to those of most of the more derived teleosts studied to date. This is paralleled by the initial appearance of the choroid rete in bowfin, as well as a significant decrease in Hb buffer value and an increase in Bohr/Haldane effects, together suggesting bowfin as the most basal species capable of utilizing its Root effect to maximize O_2 delivery to the eye.

Keywords Fish physiology · Primitive fish · Hemoglobin evolution · Root effect · Bohr effect

Abbreviations

βNHE	Adrenergically activated sodium/proton exchanger
GTP	Guanosine triphosphate
Hb	Hemoglobin
NTP	Nucleoside triphosphate
pH_e	Extracellular pH
pH_i	Intracellular pH
pK	Negative logarithm of dissociation constant
RBC	Red blood cell

Introduction

Positioned at the interface of the organism and its environment, hemoglobin (Hb) has been the subject of countless

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selective pressures over its 1.8 billion year history (Powers 1980; Berenbrink 2006). This has resulted in a remarkable radiation of Hb form and function within each of the five kingdoms of life (Weber and Vinogradov 2001), from Hb's familiar role in oxygen (O_2) and carbon dioxide (CO_2) transport in the blood of vertebrates, to its role as a signal molecule in the metabolic strategy of plants (Appleby et al. 1988; Bogusz et al. 1988), to its "digestive" role in hydrothermal-dwelling giant tube worms (Arp et al. 1987; Weber and Vinogradov 2001). In some cases, these varied characteristics of Hb are believed to have contributed to the relative success of whole classes of species. One such example is the Root effect, a dramatic reduction in oxygen carrying capacity with a reduction in pH. This unique characteristic of fish Hbs allows for enhanced O_2 delivery to the eye and swimbladder, and is believed to have played a major role in the explosive adaptive radiation of the teleosts, a tremendously speciose group of fishes which comprises half of all extant vertebrates (Weber 2000; Bonaventura et al. 2004; Berenbrink et al. 2005; Berenbrink 2007).

The Root effect

Like all Hb molecules, the Root effect Hbs of teleost fishes are capable of binding protons. When protons bind to particular sites (Root groups) on the protein, however, the deoxygenated T(ense)-state of the Root Hb becomes stabilized to such an extent that complete saturation with O_2 is unattainable (Root 1931; Root and Irving 1943; Verde et al. 2002; Pelster and Decker 2004), even at oxygen tensions of up to 140 atmospheres (Scholander and Van Dam 1954). Thus, proton binding by the Root groups results in the dissociation of O_2 from the protein and allows the Root Hbs to function as proton-triggered O_2 pumps at low pH (Pelster and Randall 1998). In conjunction with an acid-producing tissue (gas gland) and a counter-current capillary network (rete), teleost fishes utilize the Root effect to generate oxygen partial pressures (PO_2s) in the blood that are orders of magnitude above atmospheric. This allows O_2 to be driven across the large diffusion distances of the fish eye (Wittenberg and Wittenberg 1974; Nicol 1989; Pelster and Randall 1998), as well as into the gas filled, hydrostatically pressurized swimbladder (Kuhn et al. 1963; Pelster and Randall 1998). The highly metabolic retinal cells thereby remain well-oxygenated despite the greatly reduced vascularization of the fish eye relative to other vertebrates (Bridges et al. 1998; Pelster 2001), and the fish's buoyancy in the water column can be regulated via swimbladder volume (Brittain 1987; Pelster and Randall 1998). However, the Root effect comes with potentially unfavorable consequences. Certain conditions, including hypoxia and exercise, are capable of producing generalized blood acidoses through the production of CO_2 and metabolic acid which, in conjunction with pH-sensitive Root Hbs, may jeopardize O_2 uptake at the gill. To compensate, teleost fishes have the ability to regulate red blood cell (RBC) intracellular pH (pH_i) through the use of adrenergically stimulated Na^+/H^+ exchangers (βNHE) on the RBC membrane. These exchangers are activated following binding of catecholamines released to the blood under stressful conditions, and with the resulting proton extrusion, are capable of keeping RBC pH_i high (Nikinmaa 1983; Thomas and Perry 1992; Nikinmaa and Salama 1998). In this way, teleosts preserve O_2 uptake during generalized blood acidoses despite possessing proton-sensitive Root Hbs. However, this may not be the case in more basal actinopterygians, the group of fishes among which the Root effect is believed to have evolved.

The basal actinopterygian fishes: a transitional group in Root effect evolution

The evolutionary relationships of the Root effect and its associated traits have recently been investigated by Berenbrink et al. (2005) in a comparative study across a broad range of fishes. Their results indicate that βNHE activity did not evolve until long after the appearance of the Root effect Hb and the choroid rete (Berenbrink et al. 2005). From this, it can be hypothesized that those species possessing a Root effect but lacking RBC βNHE could experience significant impairment of O_2 uptake at the gills during exposure to a generalized blood acidosis resulting from hypoxia or exercise. According to Berenbrink et al. (2005), the extant species that possess a considerable Root effect in the absence of RBC βNHE include the Polypteriformes (bichirs and redfish), Acipenseriformes (sturgeons and paddlefish), Lepisosteids (gars), Amiiformes (bowfin), and Osteoglossiformes (the bony-tongues; the basal teleosts). That these species are some of the very few "primitive" fishes to have successfully survived to the present day (Janvier 2007) is likely a testament to their ability to compensate in such situations. How they are accomplishing this, however, is unresolved, as relatively little is known about the blood properties of this most interesting group (Brauner and Berenbrink 2007).

We hypothesize that despite a significant Root effect and lack of RBC βNHE, O_2 binding at the gills of these transitional species during a generalized blood acidosis must be maintained. This would occur only if RBC pH_i did not get low enough to activate their Root effects in the general circulation, and could result from either high intrinsic Hb buffer capacities, or Root effect onset pH values below those which could occur in the general circulation following hypoxia or exercise. While the former appears unlikely (i.e., Hb buffer values for species with Root effects and lacking βNHE are no higher than those of species lacking Root effects altogether; Berenbrink et al. 2005; Regan and Brauner 2010), the latter has yet to be investigated and is the subject of the present study. The species investigated include: American paddlefish (*Polyodon spathula*), white sturgeon (*Acipenser transmontanus*), spotted gar (*Lepisosteus oculatus*), alligator gar (*Atractosteus spatula*), bowfin (*Amia calva*), mooneye (*Hiodon tergisus*), and pirarucu (*Arapaima gigas*), the relationships of which are shown in Fig. 1. These are all species that, for either themselves or closely related species, a Root effect has been shown to be present, while RBC βNHE activity absent (Berenbrink et al. 2005). These species were chosen in part because they are believed to represent the more basal and derived species within their respective intra-order phylogenies (Acipenseriformes: paddlefish and white sturgeon; Lepisosteids: spotted gar and alligator gar; Osteoglossiformes: mooneye and pirarucu) (Wiley 1976; Stock et al. 1991; Nelson 1994; Gottfried and Krause 1998; Lavoué and Sullivan 2004; Janvier 2007; Krieger et al. 2008), allowing for a more representative mean value for a given order. These species also straddle the original appearance of the choroid rete, a countercurrent network of capillaries that, in conjunction with the Root effect, plays a key role in the generation of high O_2 tensions in the eye (Wittenberg and Wittenberg 1974; Wittenberg and Haedrich 1974; Bridges et al. 1998; Pelster and Randall 1998). The analysis of Root effect characteristics in species that both lack (paddlefish, white sturgeon, spotted gar, and alligator gar) and possess [bowfin, mooneye (inferred from presence in goldeye, the only other Hiodontidae species) and pirarucu] choroid retia may shed light on the relationship between the rete and these Hb properties.

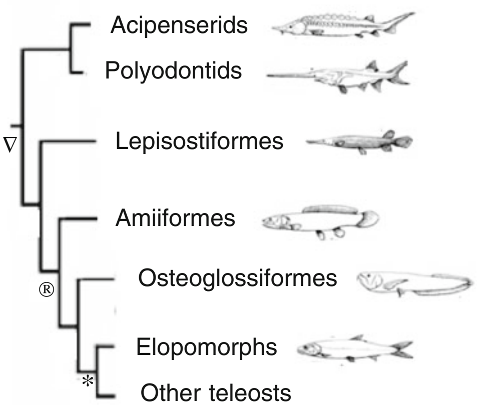

Fig. 1 Phylogeny of the actinopterygian fishes, depicting topology that is most widely accepted by morphologists and paleontologists. *Inverted triangle* the original appearance of the Root effect, *registered sign* the original appearance of the choroid rete, and *asterisk* indicates the original appearance of RBC βNHE activity (Berenbrink et al. 2005). Figure adapted from Janvier (2007), and illustrations for terminal taxa from Janvier (1996)

The objective of this study was to measure the onset pH of the Root effect in the haemolysates of these species in the absence and presence of organic phosphates (GTP). Assuming the Hb properties of these seven species to be representative of their ancestral states (Janvier 2007; McKenzie et al. 2007), this may allow insight into how a large Root effect could have evolved in this group of fishes prior to the evolution of RBC βNHE.

Materials and methods

Animal acquisition

White sturgeon (*Acipenser transmontanus*; 1–2 kg) was kept in large flow-through outdoor tanks (dechlorinated city water; $P_wO_2 > 130$ torr; $P_wCO_2 < 0.1$ torr; $T =$ 11–17°C; fish density $<$ 25 kg fish per m^3 water) prior to sampling. Pirarucu (*Arapaima gigas*; 1–2 kg) was maintained in outdoor static tanks at the National Institute for Research of the Amazon (INPA). American paddlefish (*Polyodon spathula*), spotted gar (*Lepisosteus oculatus*), alligator gar (*Atractosteus spatula*), bowfin (*Amia calva*), and mooneye (*Hiodon tergisus*) were all sampled immediately after being caught in their respective natural habitats [paddlefish (8.6–35.9 kg): Yellowstone river, Fairview, Montana, USA; spotted gar (0.7 kg): Bayou Chevreuil, Louisiana, USA; alligator gar (8.1–17.0 kg): Bayou Dularge, Louisiana, USA; bowfin (0.4–1.1 kg): Lake Erie (Long Point), Ontario, Canada; mooneye (0.3–0.5 kg): Lac du Bonnet watershed, Manitoba, Canada]. Samples for each species consisted of individual blood samples from at least three adult fish of either sex, with the exception of spotted gar, which for reasons related to species availability, consisted of the blood of a single adult.

Haemolysate preparation

Whole blood was drawn from the caudal vein of anaesthetized animals (1 ml of 200 mM benzocaine per litre of water; Sigma E1501) into heparinized syringes, where RBCs were then separated by centrifugation and washed three times in cold Cortland's physiological saline (Wolf 1963). The red cells were lysed by addition of two-times volume cold deionized water and subsequent freezing, and cell debris was removed by 10 min of chilled (4°C) centrifugation at 14,000 rpm (Thermo Electron Corporation 21000R, Waltham, MA, USA). The haemolysates were purified by removing cell solutes and organic phosphates by repeated passage through mixed-bed ion exchange columns (Amberlite IRN-150 mixed-bed ion exchange resin), and were then divided into 0.5 ml aliquots and frozen at −80°C until used. Upon thawing, Hb concentration was determined after conversion to cyanomethemoglobin using a millimolar extinction coefficient of 11 at 540 nm (derived particularly for human Hb, but also applicable to fish Hbs), and methemoglobin content was assessed on identical subsamples using the spectrophotometric method of Benesch et al. (1973). Samples where methemoglobin exceeded 10% of total Hb were discarded.

Root effect quantification

The magnitude of the Root effect was determined by measuring oxygen saturation of Hb spectrophotometrically at atmospheric PO_2 (approximately 157 mmHg) in Tris buffers (50 mmol l^{-1}; Trizma base, Sigma T6066) ranging in pH from 5.5 to 8.5. Air-equilibrated haemolysates, diluted to a final concentration of 160 mmol l^{-1} Hb_4 and 0.1 mol l^{-1} KCl, were then mixed with the buffers in a 1 ml cuvette according to Pelster and Weber (1990). Absorption at wavelengths of 540, 560 and 576 nm were measured and recorded using a Shimadzu (Columbia, MD, USA) UV-160 spectrophotometer, and were used to calculate percent Hb O_2 saturation according to Benesch et al. (1973) as described below. All analyses were conducted at 12°C. Although pH is temperature dependant and although some of the study species (pirarucu in particular) experience temperatures greater than 12°C in their natural environments, this value was chosen as the representative temperature due to the relative instability of the proteins at warm temperatures when present in dilute, stripped solutions.

Root effect analyses were performed on haemolysates in both the absence and presence of GTP. ATP and GTP are both the predominate organic phosphates (NTPs) of fish RBCs (Jensen et al. 1998), but GTP was chosen as the representative NTP for this study owing to its greater allosteric effect on Hb (Val 2000). A saturating GTP:Hb_4 ratio of 3:1 was used to ensure all Hb molecules came under GTP's allosteric influence. Although slightly higher than the average concentration found in fish RBCs, the allosteric effect of a 2:1 versus a 3:1 GTP:Hb_4 ratio does not appear significantly different (Pelster and Weber 1990; Cooper et al., unpublished).

Data analysis

Percent Hb O_2 saturation at each pH value was calculated using the following equations according to Benesch et al. (1973),

$$[\text{Oxy Hb}] = (1.4747\,A_{576} - 0.6820\,A_{560} - 0.5329\,A_{540}) \tag{1}$$

$$[\text{Deoxy Hb}] = (1.4749\,A_{560} + 0.2141\,A_{576} - 1.1042\,A_{540}) \tag{2}$$

where A is the optical density at the peak absorption wavelength for oxygenated Hb (540 and 576 nm) and for deoxygenated Hb (560 nm). Equations 1 and 2 were then added together to give total Hb in solution, by which the oxygenated Hb concentration was divided to yield the percent oxygenation status of the haemolysate at each pH.

Root effect pH onset quantification

The pH of Root effect onset (i.e., that pH value at which acid-induced Hb O_2 desaturation begins to occur) was determined for four different degrees of Hb O_2 desaturation: 5, 10, 15 and 20%. These levels of desaturation were subtracted from the average fully oxygenated percentage (between pH 7.5 and 8.5, where no Root effect is present), and the pH at the respective level of Hb O_2 desaturation was determined from the sigmoidal line of best fit describing the relationship between pH and Hb O_2 saturation for each individual sample (e.g., if the average fully oxygenated percentage between pHs 7.5 and 8.5 was 98%, a 10% Root effect onset pH would be that pH value at which Hb O_2 saturation was at 88% according to the Root effect curve).

Statistical analysis

One-way analyses of variance were conducted across the four orders comprising the seven species for Hb buffer value, Bohr/Haldane effect, maximal Root effect, and Root effect onset pH. As well, statistical t tests were performed when comparing the mean values of each species' Root effect onset pH values in the presence and absence of GTP. In all cases, statistical significance was indicated by $P < 0.05$. All statistical tests were done using SigmaStat 3.0.

Results

Percent Hb O_2 saturation as a function of pH for the stripped haemolysates in aerated Tris buffer of American paddlefish, white sturgeon, spotted gar, alligator gar, bowfin, mooneye, and pirarucu is shown in Fig. 2. The Hb O_2 saturation for each species decreased with a reduction in pH, attributable to the Root effect, and the magnitude of the Root effect varied among the seven species. When averaged among their respective orders, the mean maximal Hb O_2 desaturation percentages of the rete-bearing species (Amiiformes and Osteoglossiformes; 38.3 and 35.6%, respectively; Figs. 2, 4, 5) were significantly higher than those of the non-rete species (Acipenseriformes and Lepisosteids; 10.4 and 13.9%, respectively; Figs. 2, 4, 5; one way ANOVA, $P < 0.001$). It should be noted that the percentage of fully oxygenated Hbs (i.e., at pHs 7.5–8.5) ranged from around 91% (paddlefish; Fig. 2a) to around 103% (alligator gar; Fig. 2d). This may be due to slight differences in the Hb extinction coefficients among the different species, or to slight variation in metHb concentrations among the different samples (although, as previously mentioned, all samples contained less than 10% metHb content). In any case, the relative deoxygenated-to-oxygenated percentages for each sample should not have been affected by this.

Both Root effect magnitude and onset pH were elevated in the presence of allosterically modifying GTP (Figs. 2, 3), with the onset pH values of the rete-bearing species, in particular, increasing significantly over those of their stripped haemolysates [at 10% desaturation: bowfin (pH 6.28 ± 0.09 vs. pH 6.63 ± 0.07; $P = 0.024$); mooneye (pH 6.41 ± 0.09 vs. pH 6.97 ± 0.03; $P = 0.002$); pirarucu (pH 6.43 ± 0.09 vs. pH 6.71 ± 0.07; $P = 0.04$)].

The pH at which four different levels of Hb O_2 desaturation (5, 10, 15 and 20% of total blood Hb) were observed for each species' stripped haemolysates in the presence and absence of GTP is shown in Fig. 3. Due to relatively small Root effects, the pH values at which 20% (and even 15% in the case of paddlefish) of total blood Hb was desaturated were indeterminable for three of the four more basal, non-rete-bearing species (a single spotted gar sample excepted). The relatively modest desaturation levels that were reached in these species were observed only at very low pH values, even for a 5% decrease in oxygen carrying capacity (Fig. 3). The three rete-bearing species, however, all exhibited large Root effects, and, when averaged among their respective orders, the mean pH values at which each of 5, 10, and 15% desaturation (20% not achieved in non-rete species) were observed were significantly higher than those of the four more basal, non-rete species (e.g., 5% desaturation with GTP: one-way ANOVA, $P < 0.001$; Figs. 3, 5). All seven species' Root effect onset pH values were considerably lower than the lowest average RBC pH_i that might be expected (based upon literature values) during hypoxia and exercise. Furthermore, the variation in pH onset between the four different levels of desaturation was less for each of the rete-bearing species than for the more basal species (Fig. 3), evident by the shallower slopes of their Root effect curves (Fig. 2).

Mean Root effect onset pH value (5% Hb O_2 desaturation) was positively correlated with maximal Root effect ($P < 0.05$; Fig. 4), where those species that exhibited relatively large Root effects generally saw them elicited at lower pH values than those with less significant Root effects.

A synthesis of four major Root effect-related Hb characteristics in the four orders of fishes among which the Root effect is believed to have evolved is shown in Fig. 5. Hemoglobin buffer value (from Regan and Brauner 2010), Bohr/Haldane effect (from Regan and Brauner 2010), maximal Root effect, and Root effect onset pH are all shown to be significantly different in species possessing choroid retia (Acipenseriformes and Lepisosteids) than those lacking retia (Amiiformes and Osteoglossiformes; one-way ANOVAs, $P < 0.05$).

Discussion

It has been proposed that the Root effect evolved in the basal actinopterygian lineage of fishes in the absence of RBC βNHE activity (Berenbrink et al. 2005), a situation that comes with the potential of reducing a significant proportion of the blood's O_2 carrying capacity during generalized blood acidoses. The objective of the present study was to gain insight into how a sizeable Root effect (up to 40%) could have evolved in the absence of such RBC pH_i protection. The findings indicate that the Root effect onset pH values of the basal actinopterygian species investigated in this study are below even the lowest predicted pH values that would be elicited in the general circulation following severe exercise or exposure to hypoxia.

Evolutionary relationships of the basal actinopterygians

Although supported by both mitochondrial and nuclear DNA analyses (Inoue et al. 2003; Arnason et al. 2004; Kikugawa et al. 2004), it is the possession of certain morphological characters that is primarily responsible for the labeling of the basal actinopterygians as "primitive" (Mckenzie et al. 2007). In particular, it is the possession of those characters that occurred earlier in the fossil record than those characters that define the more dominant, modern groups. Owing to this, we have based the phylogenetic framework of this study on the topology most widely accepted by morphologists and paleontologists (Janvier 2007), which places the Amiiformes as sister group to the teleosts, collectively forming a clade (the halecostomes) that is sister group to the more basal gars (Fig. 1; Lauder and Liem 1983).

Influence of GTP

Adenosine triphosphate (ATP) and guanosine triphosphate (GTP) are the predominant organic phosphates in fish RBCs and have been shown to act as major cofactors in Hb function by reducing Hb O_2 affinity (Weber et al. 1975; Pelster and Weber 1990; Brauner and Weber 1998; Val

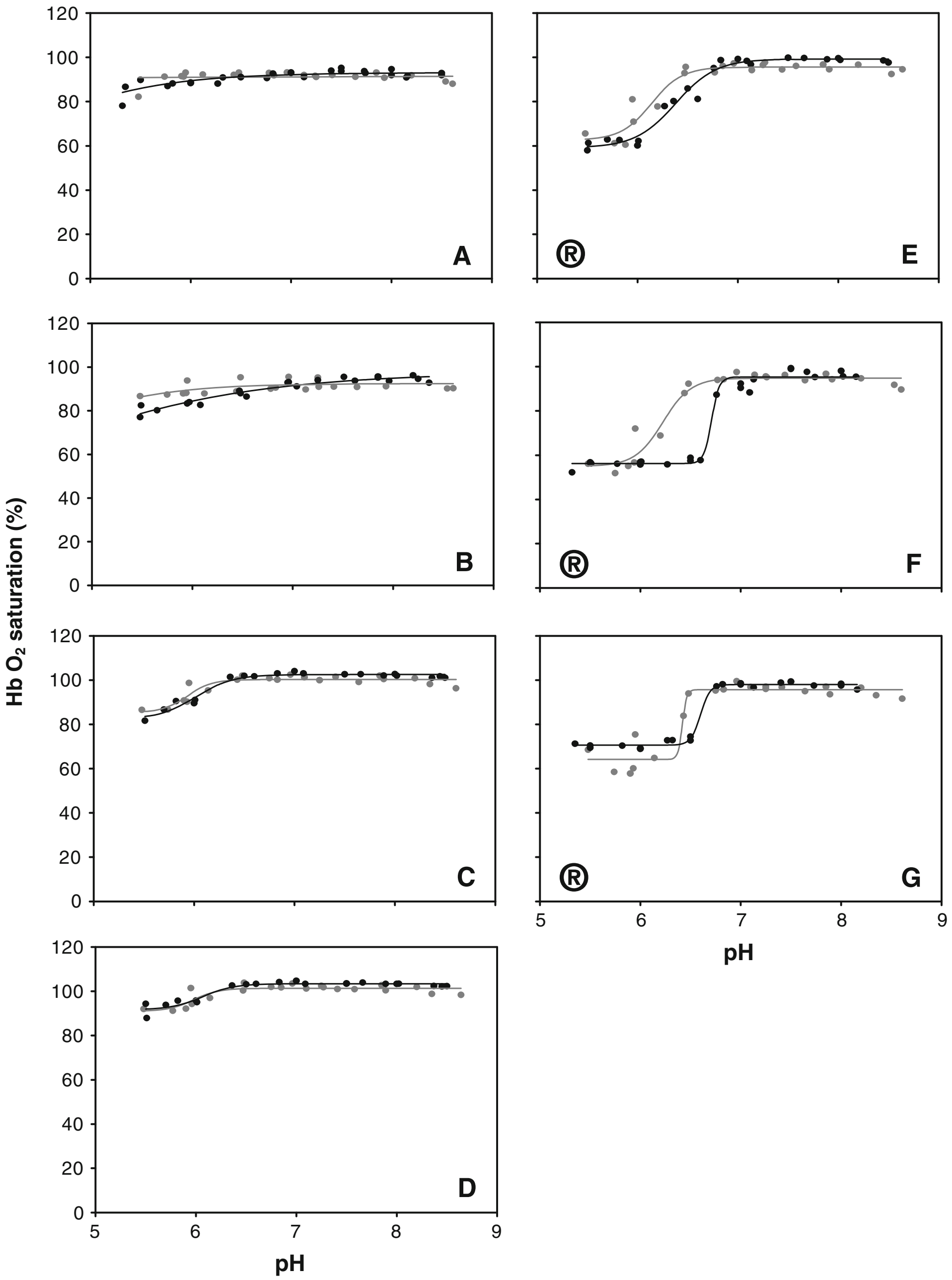

Fig. 2 The magnitude of the Root effect at different pHs in the presence (*darkly filled circle*) and absence (*lightly filled circle*) of 3:1 GTP:Hb_4 in American paddlefish (**a**), white sturgeon (**b**), spotted gar (**c**), alligator gar (**d**), bowfin (**e**), mooneye (**f**), and pirarucu (**g**). Values were determined spectrophotometrically on heamolysates at a [Hb_4] of 0.16 mmol l^{-1} and a [KCl] of 0.1 mol l^{-1}. The presence of a choroid rete within the species is indicated by *registered sign*

2000). Of these, GTP exerts the stronger effect on Hb O_2 saturation due to an additional stabilizing bond within the T-state of the Hb molecule (Pelster and Weber 1990; Val 2000). As well as reducing Hb O_2 affinity, GTP increases the pK values of the Root groups (Pelster and Weber 1990; Jensen et al. 1998) resulting in an increase in both the magnitude of the Root effect and its onset pH (Figs. 2, 3), although the degree to which GTP influences these Hb characteristics is species-specific. For instance, the Hbs of carp and eel each show a Root effect of 40% in the presence of saturating levels of organic phosphates, but no Root effect in their absence (Pelster and Weber 1990). Conversely, trout Hbs display Root effects of 55% in the presence of organic phosphates, but still exhibit large Root effects of 40% in their absence (Pelster and Weber 1990). In the present study, the influence of GTP on these Hb characteristics was also shown to vary interspecifically. Although modest, Root effect magnitude was generally increased upon GTP binding, ranging from very slight (e.g., alligator gar and pirarucu; Fig. 2) up to a 7% increase in overall Hb O_2 desaturation (sturgeon; Fig. 2). Onset pH was more influenced by the presence of GTP, increasing in all species by up to 0.5 pH units (mooneye; Figs. 2, 3).

The remainder of the discussion will focus primarily on those experiments conducted in the presence of GTP, as we believe these to be more representative of in vivo conditions.

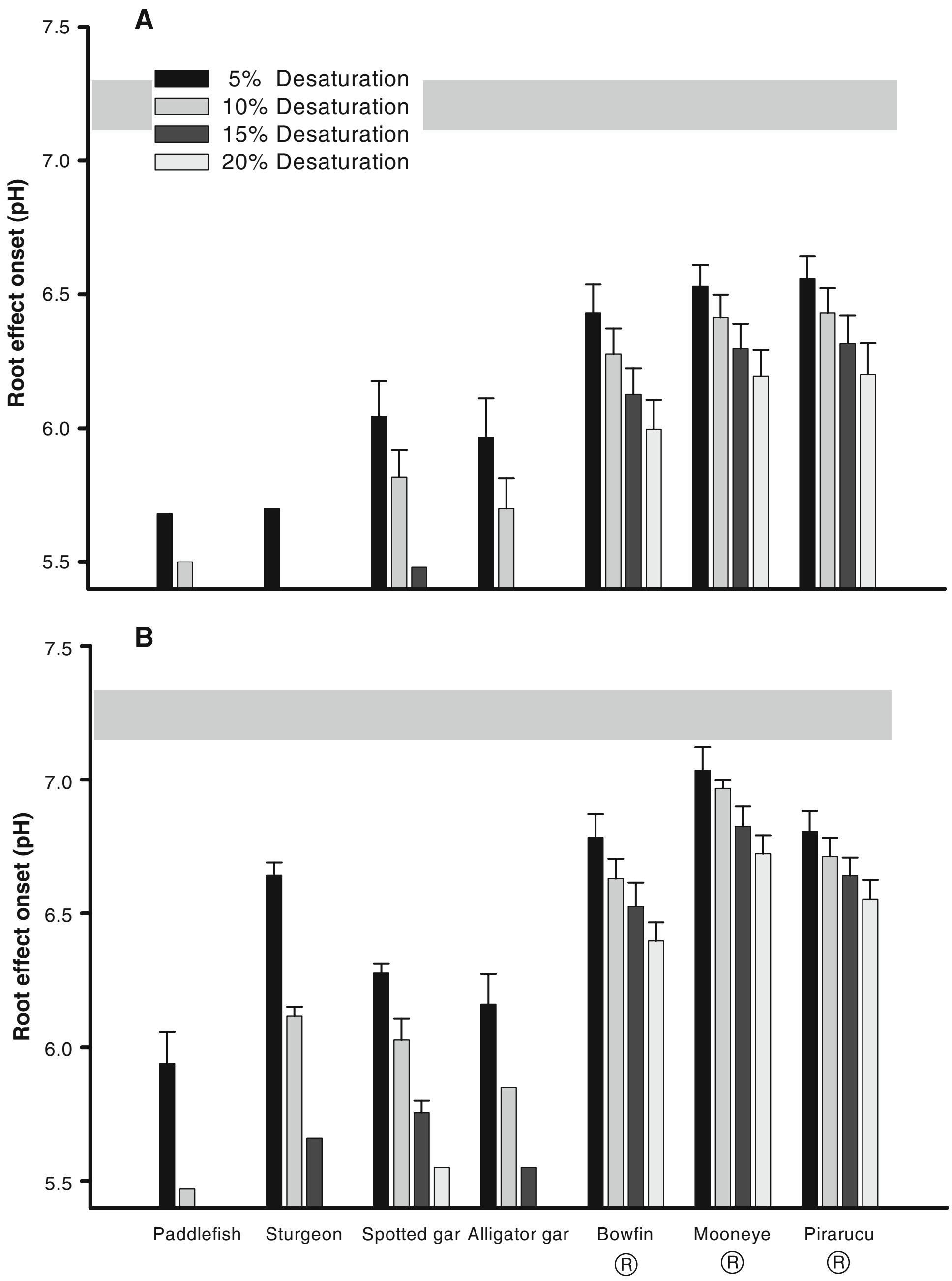

Fig. 3 Root effect onset pH values in haemolysates of seven basal actinopterygian fishes in the absence (**a**) and presence (**b**) of saturating GTP (3:1 GTP:Hb_4). Root effect onset was determined for 5, 10, 15, and 20% Hb O_2 desaturation. Data are mean values ± SEM. *Bars* lacking SEM represent the lone sample to reach the respective desaturation percentage from that group. The presence of a choroid rete within the species is indicated by *registered sign*. *Horizontal grey bars* are indicative of predicted lowest RBC pH_i that would occur during hypoxia or exercise, based upon literature values (see "Discussion"). Average onset value for the three rete-bearing species is significantly greater than that of the four non-rete species ($P < 0.001$; see "Results" for further details)

Potential influence of temperature

For reasons outlined in "Methods", these experiments all took place at 12°C. It is likely, however, that temperature plays a role in the manifestation of the Root effect. The combination of the exothermic nature of Hb oxygenation and the inverse relationship of pH and temperature (–0.01 to –0.02 pH unit $°C^{-1}$) insinuates a larger Root effect at higher temperatures. That most of these species find themselves in temperate habitats suggests that their respective Root effects may be elicited to a greater degree, and at higher relative pHs, during the warmer months, and vice versa during the colder months. No data confirming this specific idea exist to our knowledge, but it would certainly be a worthy line of investigation.

Root effect onset pH

Although the Root effect is often described as a trait almost exclusive to the Hbs of teleosts (Pelster and Randall 1998), this study supports the findings of Berenbrink et al. (2005) that it is also a characteristic of the Hbs of more primitive fishes. Each of the seven species studied here possesses Hbs that exhibit a Root effect (Fig. 2), ranging from

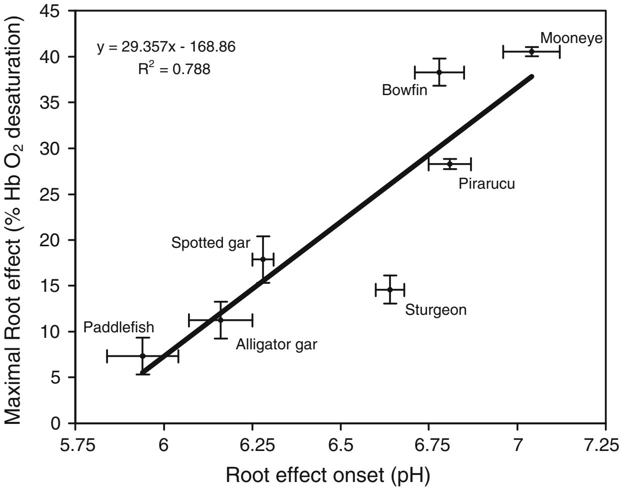

Fig. 4 Mean maximal Root effect as a function of mean Root effect onset pH (5% Hb O_2 desaturation) for the haemolysates (3:1 GTP:Hb_4) of four orders of basal actinopterygian fishes, including Acipenseriformes (paddlefish and white sturgeon), Lepisosteids (spotted gar and alligator gar), Amiiformes (bowfin), and Osteoglossiformes (mooneye and pirarucu). *Error bars* represent SEM. Rete-bearing Amiiformes and Osteoglossiformes are located in the upper right of the graph, and non-rete-bearing Acipenseriformes and Lepisosteids are located in the lower left of the graph

approximately 7% of total blood oxygen carrying capacity in paddlefish, to 40% in mooneye. Some of these values were very similar to those from other studies looking at the same (or very closely related) species. For example, our maximal Root effect magnitudes for white sturgeon (15%) were very much in line with those values of Berenbrink et al. (2005) and Clementi et al. (2001) on closely related species [sterlet (22%) and Italian sturgeon (12–17%), respectively]. However, our values for spotted gar (18%) and bowfin (39%) were lower than those measured in other studies. Berenbrink et al. (2005) measured a maximal Root effect of 43% in the haemolysate of spotted gar, while Weber et al. (1976) and Berenbrink et al. (2005) measured a maximal Root effect in bowfin between 55 and 60%. The reasons for these discrepancies may be related to variation in measurement protocols, organic phosphate concentrations, pH at which maximal values were measured, or perhaps more interestingly, intraspecific variation in Hb function. The possibility of the latter would certainly prove worthy of further investigation.

Variation also exists in the onset pH values of these species' Root effects (Figs. 2, 3). To our knowledge, few, if any, studies have looked at the mechanism underlying the variation in Root effect onset pH. However, it is likely that variation in onset pH is a function of the p*K* values of the particular amino acid residues responsible for binding the protons that bring about the $R \rightarrow T$ conformational change in the Hb (the Root groups). As it has been shown that the total mechanism by which the Root effect is elicited can vary between the Hbs of different species [i.e., different amino acid residues on different Hbs have been shown to be involved in the Root effect-associated conformational changes (Perutz and Brunori 1982; Nagai et al. 1985; Mylvaganam et al. 1996; Jensen et al. 1998; Tsuneshige et al. 2002)], one may also expect the p*K* values of these different groups to vary.

The onset pH value for each species was determined at four different levels of Hb O_2 desaturation (5, 10, 15, and 20% of total Hb), although not all seven species had Root effects capable of achieving 20% desaturation. Of the four more plesiomorphic species, only the haemolysates of spotted gar became 20% desaturated (a single sample), while those of paddlefish, white sturgeon, and alligator gar showed at most 10–15% desaturation (Fig. 3). Moreover, the pH values at which these levels of desaturation were achieved (pH 6.3; 5% desaturation in white sturgeon excepted; Fig. 3) were very low relative to possible in vivo conditions in the general circulation discussed below. Conversely, the haemolysates of the rete-bearing bowfin, mooneye, and pirarucu all displayed Root effects larger than 20%, and the pH values at which the four desaturation levels were achieved were in a more physiologically relevant range. Five percent desaturation occurred in these three species between pHs 6.8 and 7.0, while 20% was achieved between pHs 6.4 and 6.7 (Figs. 2, 3, 4).

Each of the seven species' Root effect onset pH values was quite low compared to those that would be expected during a generalized acidosis. Exercise (acute and exhaustive) and hypoxia are capable of inducing severe acidoses in the blood. However, little information on the effect of these challenges on blood–gas transport exists for these species (Brauner and Berenbrink 2007). A survey of the responses to exhaustive exercise in a phylogenetically broad range of fishes (*Squalus acanthias*; *Carcharhinus plumbeus*; *Lepisosteus osseus*; *Amia calva*; and *Oncorhynchus mykiss*) indicates that blood pH_e may be reduced to between 7.3 and 7.7 (Milligan and Wood 1986; Burleson et al. 1998; Gonzalez et al. 2001; Richards et al. 2003; Brill et al. 2008). Assuming a $\Delta pH_i/\Delta pH_e$ value of 0.47 ($pH_i = 0.47 \times pH_e + 3.67$; 0.471 in rainbow trout, Heming et al. 1986; 0.469 in white sturgeon, Baker et al. 2008), these extracellular pH values translate to RBC pH_i values between 7.1 and 7.3. These are considerably higher than those pH values that would result in even a 5% Root effect in the studied species (Fig. 3). Similarly, hypoxia acidifies the blood to pH 7.4–7.7 in a comparable range of fishes (Holeton and Randall 1967; Butler and Taylor 1975; Randall et al. 1992), translating to intracellular pH values between 7.15 and 7.3, which again are considerably higher than the Root effect onset pH values in any of the studied species (Fig. 3). Even in extreme cases where the blood could become more acidified than this (e.g., McKenzie et al. 1997), and even if the $\Delta pH_i/\Delta pH_e$ value, which varies among species, was greater than 0.47 [e.g., Tufts et al. 1994 ($pH_i = 0.667 \times pH_e + 1.974$)], the Root effect would not likely be activated. It therefore appears as though O_2 uptake is not likely to be compromised in these species following exhaustive exercise or hypoxia, despite relatively large Root effects and no known RBC βNHE activity.

There are conditions which may have posed larger threats to these species' O_2 uptake throughout evolutionary time, however. Stem actinopterygian species, which gave rise to the species studied here, date back over 250 million years (Janvier 2007) to a time when the earth's atmosphere and water systems were significantly warmer, higher in CO_2, lower in O_2, and more acidic (Algeo et al. 2001; Berner 2006; Clack 2007). Animals from this era dealt with these challenges in different ways—some secured O_2 from the air; others abandoned their aquatic habitats altogether; while still others became more robust at dealing with their harsh environments (Clack 2007; Brauner and Baker 2008). An example of the latter is found in the Acipenseriformes, whose extant sturgeon species have been shown to be among the most CO_2 tolerant of fishes (Crocker and Cech 1998; Baker et al. 2008). In conditions of hypercarbia that may be similar to those of its proposed ancestral habitats, white sturgeon have been shown to survive despite a blood acidosis resulting in a RBC pH_i of 6.8 (Baker et al. 2008; Brauner and Baker 2008). Taken as the extreme example of a potential generalized blood acidosis, pH_i of 6.8 would still not be capable of activating the Root effect in the four more plesiomorphic species, although it may be low enough to reduce blood oxygen carrying capacity by 10% in the three rete-bearing species (15% in the case of mooneye; Fig. 3b). Whether this degree of desaturation would pose a threat to these animals is not known. However, significant RBC βNHE activity first appeared in the Elopomorphs, sister group to the rest of the teleosts (Fig. 1; Berenbrink et al. 2005; Berenbrink 2007). One could therefore hypothesize that the Root effect onset pH values of the Elopomorphs are higher than those of the non-βNHE species possessing a Root effect, such as is the case with rainbow trout, a species with a Root effect and high RBC βNHE activity (Pelster and Weber 1990; Thomas and Perry 1992; Berenbrink et al. 2005).

The low Root effect onset pHs of the four more basal species in this study (paddlefish, sturgeon, spotted gar, and alligator gar) suggest Root effects that may not be operational in vivo, particularly due to these species lacking any known tissue capable of generating such acidoses (i.e., gas gland and rete; Berenbrink et al. 2005). Their possession of a seemingly in vivo-irrelevant trait may be explained by the hypothesis of the Root effect being the extreme expression of the mechanism that elicits the Bohr effect in fishes (Berenbrink et al. 2005). Once an acid-producing tissue (i.e., the rete) capable of appropriately acidifying the blood at a particularly beneficial site (i.e., the avascularized retina) evolved in the last common ancestor of bowfin and the teleosts, it is possible that only then was the Root effect capable of being utilized by the animal. This may have led to it coming under positive selection in and of itself, and may explain the significant changes in four Root effect-related Hb properties starting with bowfin (Fig. 5). Except in teleost species where the Root effect has been secondarily lost (and the Hb buffer value secondarily increased; e.g., Siluriformes, Synbranchiformes; Farmer et al. 1979; Berenbrink et al. 2005), the magnitudes of these Hb traits change very little in any of the more derived teleost fishes studied to date (Jensen 1989; Pelster and Weber 1990; Brauner and Weber 1998; Pelster and Randall 1998; Jensen 2001; Berenbrink et al. 2005; Berenbrink 2006, 2007), suggesting that these values became optimized for exploitation of the Root effect starting with the last common ancestor of bowfin and the teleosts, the species among which the choroid rete originally evolved. This idea is further supported by ocular PO_2 values in bowfin of 650 mmHg compared to 90 mmHg in spotted and alligator gar (Wittenberg and Wittenberg 1974), PO_2 values high enough to only be attributable to the Root effect (Pelster and Randall 1998). However, more work needs to be done to support this idea, particularly on more derived species that have secondarily lost one or more Root effect-related properties.

Conclusions

It was shown that the Root effect is indeed a feature of the Hbs of the basal actinopterygian fishes, in agreement with the recent findings of Berenbrink et al. (2005). However, the in vivo relevance of these Root effects appears to vary interspecifically in a manner correlated with the presence or absence of the counter-current rete. Paddlefish, white sturgeon, spotted gar, and alligator gar all lack retia, and although they possess Hbs that display Root effects, they are not likely capable of utilizing them under in vivo conditions by reason of low onset pH values and a lack of any known tissue capable of generating the appropriate acidosis. Bowfin, mooneye, and pirarucu all possess choroid retia, however, and their higher onset pH values indicate that they are likely operational in vivo at the eye. Taken together, the fact that the onset pH values of all seven species' Root effects are lower than even the lowest pH values likely to occur following exposure to hypoxia or exhaustive exercise renders it unlikely that the presence of the Root effect in the absence of βNHE would pose a threat to these species' O_2 uptake. This would allow for the positive selection of the

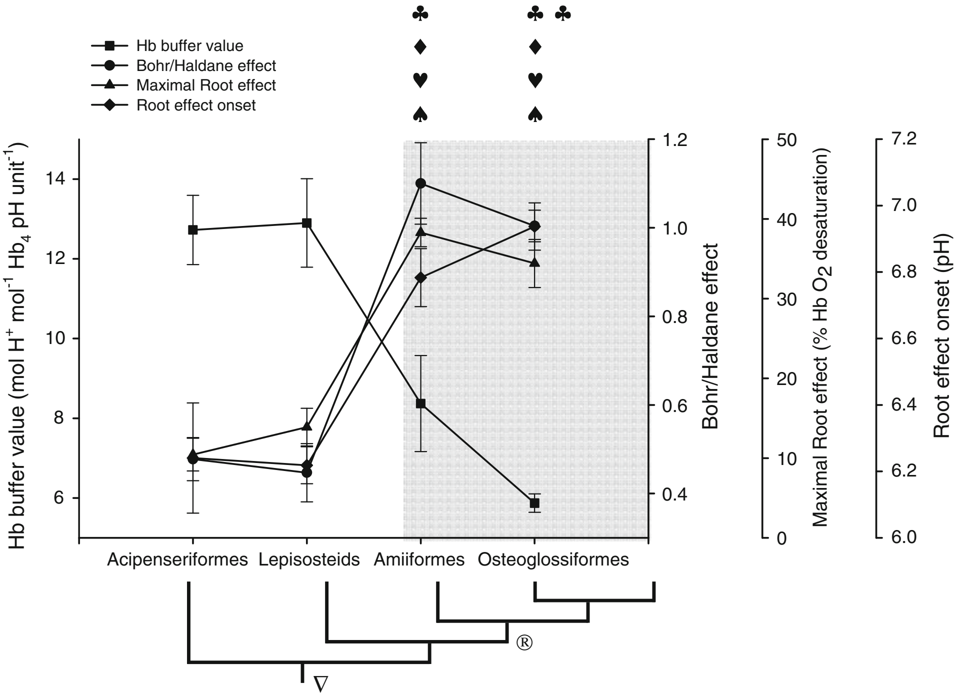

Fig. 5 Changes in mean values of hemoglobin (Hb) P_{50} buffer value [average mol H^+ mol^{-1} Hb_4 pH $unit^{-1}$ of oxygenated and deoxygenated Hbs at physiological pH (pH 7.2–7.4); from Regan and Brauner 2010], Bohr/Haldane effect ($-\Delta \log P_{50}/\Delta pH$; from Regan and Brauner 2010), maximal Root effect (% Hb O_2 desaturation at pH 5.5), and Root effect onset pH (at 5% Hb O_2 desaturation) in seven species belonging to four orders of basal actinopterygian fishes. These include: Acipenseriformes (American paddlefish; white sturgeon); Lepisosteids (spotted gar; alligator gar); Amiiformes (bowfin); Osteoglossiformes (mooneye; pirarucu). Data are mean values of all measurements taken on species within a given order ± SEM. *Grey field* indicates the presence of a choroid rete, with species belonging to the Amiiformes believed to be the first in which this capillary network evolved. All values determined from experiments performed on haemolysates at [Hb_4] of 0.04 mmol l^{-1} (buffer value and Haldane effect) or 0.16 mmol l^{-1} (Root effect maximum and onset pH) and a [KCl] of 0.1 mol l^{-1}, in the presence of organic phosphates (3:1 GTP:Hb_4). Statistically significant differences ($P < 0.05$) represented by *club symbol* (Hb buffer value), *diamond* (Bohr/Haldane effect), *heart symbol* (maximal Root effect), and *spade symbol* (Root effect onset pH). See text for further details. Phylogeny of extant actinopterygian fishes, adapted from Janvier (2007), based on morphological characters. *Inverted triangle* the original appearance of the Root effect, and *registered sign* the original appearance of the choroid rete (Berenbrink et al. 2005)

Root effect as a mechanism of O_2 delivery to override any negative selection for it brought about by its potentially adverse effects on O_2 uptake. In this fashion, the low onset pH values of the species within this transitional phase of Root effect evolution may explain the virtual ubiquity of this Hb characteristic among the more derived teleost fishes.

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