J Comp Physiol B (2010) 180:521–530
DOI 10.1007/s00360-009-0438-1

ORIGINAL PAPER

The transition in hemoglobin proton-binding characteristics within the basal actinopterygian fishes

Matthew Daniel Regan · Colin J. Brauner

Received: 21 July 2009 / Revised: 5 December 2009 / Accepted: 8 December 2009 / Published online: 1 January 2010

Abstract Carbon dioxide (CO_2) transport in the blood of fishes is aided by the proton-binding properties of hemoglobin (Hb) through either a high-intrinsic buffer value and small oxylabile proton binding (Haldane effect), or a low buffer value and large Haldane effect. Primitive species, such as elasmobranchs and sarcopterygians have been shown to rely on the former, while derived species, such as teleosts rely on the latter. Both strategies are effective in the transport of CO_2 in the blood. However, there is a paucity of information on the nature of the transition between these two strategies that appears to occur within the intermediate group of fishes, the basal actinopterygians. The objective of the present study was to simultaneously assess the intrinsic Hb buffer values and Haldane effects of species within the basal actinopterygian lineage to characterize the transition in Hb-proton-binding strategy seen among the fishes. Expressed in order of most basal to most derived, the species investigated included American paddlefish (*Polyodon spathula*), white sturgeon (*Acipenser transmontanus*), spotted gar (*Lepisosteus oculatus*), alligator gar (*Atractosteus spatula*), bowfin (*Amia calva*), and mooneye (*Hiodon tergisus*). Hemolysates from these species were prepared and Hb titrations (under oxygenated and deoxygenated conditions) were performed in both the presence and absence of saturating levels of organic phosphates (GTP). The findings suggest that the nature of the Hb-proton-binding transition may have been punctuated rather than gradual, with the Hb buffer value decreasing and the Haldane effect increasing significantly in bowfin from fairly steady ancestral levels in the four more basal species. This change is coupled with the initial appearance of the choroid rete, as well as an increase in the magnitude and onset pH of the Root effect in bowfin, suggesting that the change in Hb-proton-binding strategy may be associated with the evolution of enhanced O_2 delivery to the eye and an in vivo operational Root effect.

Keywords Primitive fish · Hemoglobin · Buffer values · Haldane effect · Root effect · Hemoglobin evolution

Communicated by I. D. Hume.

M. D. Regan (✉) · C. J. Brauner (✉)
Department of Zoology, University of British Columbia,
6270 University Boulevard, Vancouver, BC, Canada
e-mail: regan@zoology.ubc.ca

C. J. Brauner
e-mail: brauner@zoology.ubc.ca

Introduction

Although known primarily for its role in oxygen (O_2) transport in the blood of virtually all vertebrate species, hemoglobin (Hb) is also crucial in the transporting of carbon dioxide (CO_2). Through its ability to bind protons produced by the hydration of CO_2 to bicarbonate, Hb significantly increases the blood's carrying capacity for CO_2. These dual roles of Hb in O_2 and CO_2 transport are tightly coupled in the blood of teleost fishes through the Bohr/Haldane effect (Brauner and Randall 1996, 1998), where protons produced by CO_2 hydration in the red blood cell (RBC) bind to specific groups on the oxygenated R-state Hb that tend to stabilize its deoxygenated T-state (the Bohr effect). This decreases the affinity of Hb for O_2, and facilitates O_2 delivery to the tissue (Riggs 1988; Jensen et al. 1998; Jensen 2004). The R→T transition also increases the *p*K values of

specific histidine (His) residues on the surface of the protein, resulting in additional Hb-proton binding (the Haldane effect) and, subsequently, an increased capacity for CO_2 transport in the blood (Brauner and Randall 1998).

Variation in Hb–H^+ binding among fishes

In the fish species studied to date, there appears to exist an inverse relationship between intrinsic Hb buffer value and the magnitude of the Haldane effect—primitive fishes, such as elasmobranchs and sarcopterygians show Hbs of high buffer values and small Haldane effects, while the more derived teleosts show Hbs of some of the lowest buffer values and largest Haldane effects seen among all vertebrate species (Jensen 1989; Brauner and Weber 1998; Jensen et al. 1998; Jensen 2001; Berenbrink et al. 2005). Thus, there exist two different mechanisms of intracellular proton binding and subsequent CO_2 transport in the blood of fishes. It is possible that the transition between these two strategies occurred among the group of species intermediate to the sarcopterygians and the teleosts on the vertebrate phylogeny, the basal actinopterygian fishes. What is more, the Root effect, a reduction in blood O_2 carrying capacity with a reduction in blood pH, has been shown to be correlated with a low Hb buffer value (Jensen 1989), and is believed to have evolved within the basal actinopterygians (Berenbrink et al. 2005). Given these, and assuming the Hb properties of extant primitive species to be representative of their respective ancestral states (Janvier 2007; McKenzie et al. 2007), it is possible that measuring the Hb buffer values and Haldane effects of basal actinopterygian species may shed light on the evolution of these Hb-proton-binding strategies.

The Hb buffer values for some species within this lineage have recently been reported by Berenbrink et al. (2005). Their results suggest that the Hb buffer values gradually decreased among these fishes towards an asymptotal low in the teleosts. However, the buffer values presented were those of strictly deoxygenated Hbs at a single pH in the absence of allosterically modifying organic phosphates. Although informative, these results highlight the importance of further investigations into these most interesting and under-studied species (Brauner and Berenbrink 2007). By accounting for Hb oxygenation state, pH, and allosteric influence of organic phosphates of species within the basal actinopterygian lineage, as well as determining their Haldane effects, we could provide a more resolute picture of the evolution of Hb-proton binding in the fishes. Both the intrinsic buffer value of Hb and the Haldane effect can be simultaneously assessed through the acid–base titrations of oxygenated and deoxygenated Hbs. In the present study, these titrations were performed on isolated Hbs of the following basal actinopterygian species, expressed in order of most basal to most derived: American paddlefish (*Polyodon spathula*), white sturgeon (*Acipenser transmontanus*), spotted gar (*Lepisosteus oculatus*), alligator gar (*Atractosteus spatula*), bowfin (*Amia calva*), and mooneye (*Hiodon tergisus*). These species are all intermediate to the ancestral sarcopterygians and the derived teleosts on the vertebrate phylogeny (Janvier 2007), and very little is known about their Hb characteristics. As well, the representative species from the acipenseriformes (paddlefish and white sturgeon) and the lepisosteids (spotted gar and alligator gar) are believed to represent the most basal and derived within their respective intra-order phylogenies (Wiley 1976; Stock et al. 1991; Nelson 1994; Gottfried and Krause 1998; Janvier 2007; Krieger et al. 2008), allowing for insight into the variation of these particular Hb traits within these groups. What is more, these six species straddle the original appearance of the choroid rete in the amiiformes (Wittenberg and Haedrich 1974; Berenbrink et al. 2005), with paddlefish, white sturgeon, spotted gar, and alligator gar lacking retia, and bowfin and mooneye possessing retia. The choroid rete, a counter-current network of capillaries, is believed to play a key role in the generation of high O_2 tensions at the eye by maximizing the acidosis in the capillaries supplying the retinal cells, resulting in the substantial release of O_2 from Hb via the Root effect (Wittenberg and Wittenberg 1974; Bridges et al. 1998). As a reduced Hb buffer value is also believed to optimize the Root effect (Brauner and Randall 1998), the presence of a choroid rete may be associated with changes in the proton-binding properties of Hb as well.

The objectives of this study were (1) to perform acid–base titrations on the hemolysates of these understudied, phylogenetically interesting species, and (2) to gain insight from these data as to the transition in Hb-proton-binding strategy seen among fishes, from that of a high buffer value/small Haldane effect to a low buffer value/large Haldane effect. Through these analyses, we may be able to more clearly understand the reasons for this shift in proton-binding strategy, and its association with the evolution of the Root effect.

Materials and methods

Animal acquisition

White sturgeon (*Acipenser transmontanus*; 1–2 kg) were 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 <2.5 kg fish per m^3 water) prior to sampling. 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, LA, USA; alligator gar (8.1–17.0 kg): Bayou Dularge, LA, USA; bowfin (0.4–1.1 kg): Lake Erie (Long Point), ON, Canada; mooneye (0.3–0.5 kg): Lac du Bonnet watershed, MB, Canada]. Samples for each species consisted of blood from at least three adult fish of either sex, with the exception of spotted gar, which consisted of the blood of a single adult.

Hemolysate preparation

Whole blood was drawn from the caudal vein of anaesthetized animals (1 ml of 200 mmol l^{-1} benzocaine per liter of water) into heparinized syringes, where red cells were then separated by centrifugation and washed thrice in cold Cortland's physiological saline (Wolf 1963). The RBCs were lysed by the addition of two times volume of cold deionized water and subsequent freezing, and cell debris removed by 10 min of chilled (4°C) centrifugation at 14,000 rpm (Thermo Electron Corporation 21000R, Waltham, MA, USA). The hemolysates 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 use.

Hemoglobin titrations

Hemoglobin titrations were conducted on concentrated stripped hemolysates that were thawed and diluted to a final concentration of 40 mmol l^{-1} Hb_4 and 0.1 mol l^{-1} KCl according to the methods of Jensen (1989). Hb concentration was confirmed after conversion to cyanomethemoglobin using a millimolar extinction coefficient of 11 at 540 nm, 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. A 2 ml volume of the hemolysate was then transferred to a chilled (12°C), magnetically stirred glass titration vessel where the sample was equilibrated with humidified oxygen (100%) for 90 min. After reaching a stable pH, hydrogen ion titrations were performed with an automated radiometer (Copenhagen, Denmark) TitraLab 90 titration apparatus, where 0.01 mol l^{-1} NaOH (from J.T. Baker, Phillipsburg, NJ, USA) was added in 10 µl increments to raise pH (more than 5–10 min) from the isoionic value to approximately pH 9.2. After the pH reading stabilized for 5 min at pH 9.2 (with no further NaOH addition), titration with 0.01 mol l^{-1} HCl (in 10 µl increments: J.T. Baker, Phillipsburg, NJ, USA) was initiated and continued until pH 5.2 was reached, allowing 7 min of equilibration time between each injection. The total amount of NaOH and HCl to reach these end points was recorded. The same procedure was performed on a separate 2 ml sample from the same stock hemolysate, this time equilibrated in humidified nitrogen (100%). Three to four separate H^+ titrations were performed on the hemolysates of each species, yielding reproducible results.

Hemoglobin titrations were performed on hemolysates in both the absence and presence of GTP. Although ATP and GTP are the predominate organic phosphates (NTPs) of fish RBCs (Jensen et al. 1998), GTP was chosen as the representative NTP for this study owing to its higher average concentration in the blood of freshwater fishes (Val 2000), and 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 GTPs allosteric influence. Although slightly higher than the average concentration found in fish RBCs (2.16:1, averaged over 25 freshwater species; Val 2000), 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). Finally, the buffer value of GTP was subtracted from all Hb titration curves conducted in the presence of GTP.

Data analysis

The Hb buffer values were derived from the negative slope between adjacent points on the oxygenated and deoxygenated Hb titration curves for each species. Owing to the variation in proton binding between oxygenated and deoxygenated Hb at a constant pH (Jensen et al. 1998), buffer values were determined for oxygenated and deoxygenated Hbs, as well as an average of the two (referred to as the Hb P_{50} buffer value), within the known RBC physiological pH range (pH 7.2–7.4) for species in this group (extrapolated from existing whole blood pH measurements; Smatresk and Cameron 1982; Crocker and Cech 1998; Brauner and Berenbrink 2007). Although Hb's ability as a buffer is normally represented by its deoxygenated conformation (Jensen et al. 1998), we felt the inclusion of the oxygenated Hb buffer values was important, as the blood of fish in vivo is never completely deoxygenated, and under resting conditions saturation is often in the upper 50% of the oxygen equilibrium curve (Brauner and Randall 1996, 1998). Therefore, oxygenated buffer values are of in vivo relevance. The P_{50} Hb buffer value, an average of the oxygenated and deoxygenated buffer values, estimates the buffer value of the fish at half Hb-O_2 saturation and is fairly representative of conditions in the venous supply. However, as proton uptake upon deoxygenation is non-linear (Brauner and Randall 1996), the P_{50} Hb buffer value is purely an estimation of the circulatory conditions in vivo,

where blood contains a mixture of O_2 bound and unbound Hbs.

The fixed-acid Haldane effects of these species were determined by calculating the vertical distances between their oxygenated and deoxygenated titration curves (ΔZ_H, mol H^+ taken up per mol Hb_4 upon deoxygenation at constant pH). By plotting the inverse negative slope ($-\Delta pH/\Delta Z_H$) of the titration curves as a function of Z_H, the inflection points on the titration curves become localized at two peaks. Quantification of the ΔZ_H between these peaks (Z_H being a molar ratio of protons to Hb_4) gives an accurate measurement of the number of groups being titrated in the physiological pH range (De Bruin and van Os 1968; Jensen 1989) and was determined for bowfin and mooneye. The peaks on the differentiated curves of the other four species could not be accurately resolved within their titration curves.

Statistical analysis

As the intraspecific differences in Hb-proton binding were not of concern, one-way analyses of variance were conducted across the four orders comprising six species for oxygenated Hb, deoxygenated Hb, and Hb P_{50} buffer values in the physiological pH range (pH 7.2–7.4). Samples were derived from three to four individuals of each species, with the exception of a single specimen for spotted gar (due to the limited availability of this species). In all cases, statistical significance was indicated by $P < 0.05$. Statistical tests were done using SigmaStat 3.0.

Results

Hemoglobin titrations

Representative H^+ titration curves of oxygenated and deoxygenated Hb from American paddlefish, white sturgeon, spotted gar, alligator gar, bowfin, and mooneye are drawn to the same scale in Fig. 1, showing how net proton charge (Z_H, mol H^+ mol^{-1} Hb_4) of stripped Hbs in 0.1 M KCl changes as a function of pH in the absence and presence of GTP (3:1 M ratio of GTP:Hb_4). Zero net proton charge refers to the isoionic pH and is used as the reference point (Tanford 1962). Those curves of the four more basal species lacking retia are relatively linear, with discreet inflection points and minimal differences between the oxygenated and deoxygenated Hbs (Fig. 1). The curves of the rete-bearing bowfin and mooneye, however, show more pronounced inflection points indicative of increased variation in proton-binding ability as a function of pH, as well as greater variation between oxygenated and deoxygenated Hbs (Fig. 1). As well, the overall slopes of the titration curves of bowfin and mooneye are qualitatively shallower than those of the four more basal species (Fig. 1). Titrations on GTP-laden samples yielded steeper curves than those of non-GTP samples.

Hemoglobin buffer values

The slope of each Hb titration curve in Fig. 1 indicates the Hb buffer value at that pH (mol H^+ mol^{-1} Hb_4 pH $unit^{-1}$).

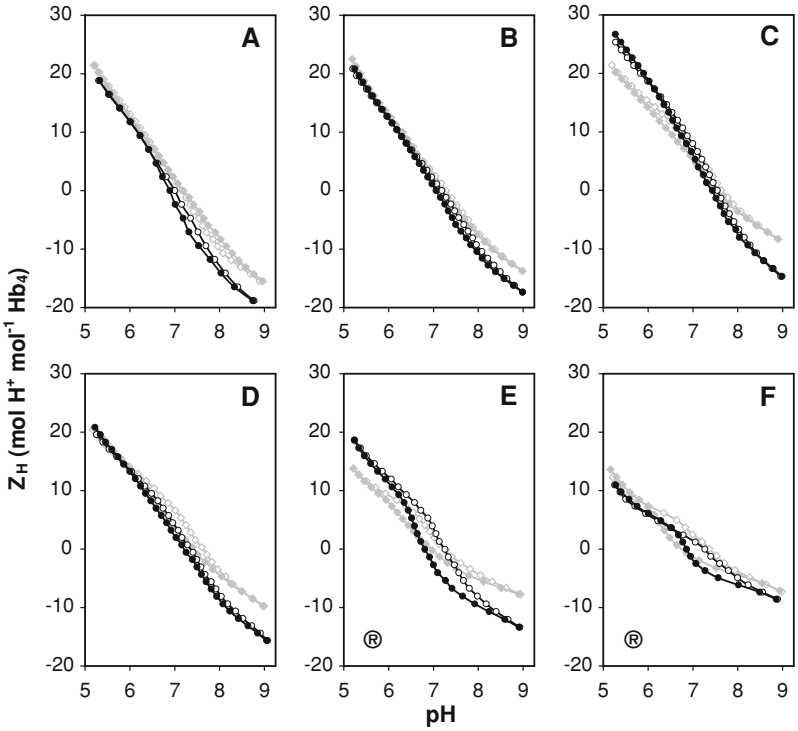

Fig. 1 Hb H^+ titration curves, Z_H (mol H^+ mol^{-1} Hb_4) as a function of pH, for oxygenated (*filled symbols*) and deoxygenated (*open symbols*) Hbs of American paddlefish (**a**), white sturgeon (**b**), spotted gar (**c**), alligator gar (**d**), bowfin (**e**), and mooneye (**f**), in the presence (*black circles*) and absence (*gray diamonds*) of organic phosphates (3:1 GTP:Hb_4). Titrations were performed on hemolysates at a [Hb_4] of 0.04 mM and a [KCl] of 0.1 M. The presence of a choroid rete within a species is indicated by ®

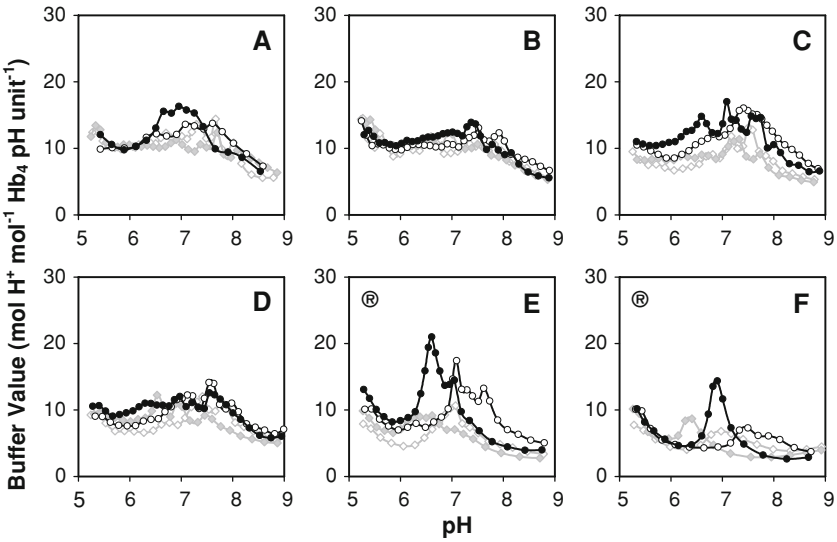

Fig. 2 Buffer values ($-\Delta Z_H/\Delta pH$) as a function of pH for oxygenated (*filled symbols*) and deoxygenated (*open symbols*) Hbs of American paddlefish (**a**), white sturgeon (**b**), spotted gar (**c**), alligator gar (**d**), bowfin (**e**), and mooneye (**f**) in the presence (*black circles*) and absence (*gray diamonds*) of organic phosphates (3:1 GTP:Hb_4). Titrations were performed on hemolysates at a [Hb_4] of 0.04 mM and a [KCl] of 0.1 M. The presence of a choroid rete within a species is indicated by ®

Plotting these slopes as a function of pH (Fig. 2), it is clear that Hb buffer value varies according to pH, oxygenation status, GTP concentration and species. The values for oxygenated, deoxygenated, and P_{50} Hb buffer values in the physiological pH range (pH 7.2–7.4) and in the presence of GTP are shown in Fig. 3. There is a decreasing trend with phylogenetic progression among the four represented orders for each of the three analyzed buffer values. Oxygenated and P_{50} Hb buffer values are significantly less in the choroid rete-bearing bowfin and mooneye relative to those of the more basal, non-rete species ($P < 0.05$; Fig. 3). The same is generally true for the deoxygenated Hb buffer values, although those of the acipenseriformes (sturgeon and paddlefish) are more similar to the deoxygenated Hb buffer values of bowfin than they are to the lepisosteids (Fig. 3). These same general trends were apparent in the stripped Hbs, with lower overall buffer values due to a lack of allosteric modification through GTP binding (data not shown).

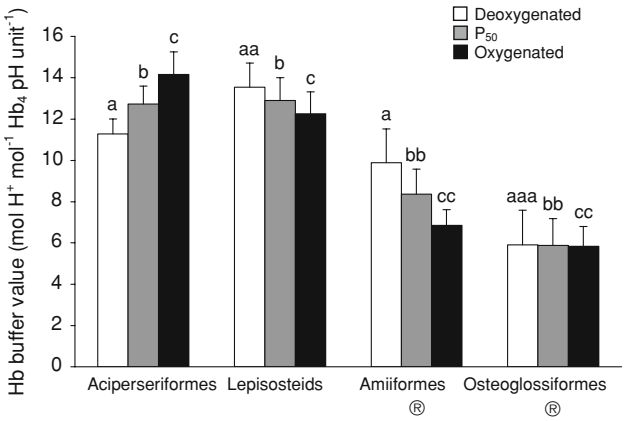

Fig. 3 Mean Hb buffer values (deoxygenated, P_{50}, and oxygenated) of four orders of basal actinopterygian fishes, with *error bars* representing SEM. Acipenseriformes includes paddlefish and white sturgeon, lepisosteids includes spotted gar and alligator gar, amiiformes includes bowfin, and osteoglossiformes includes mooneye. Values were determined by averaging all points along the oxygenated and deoxygenated buffer curves (Fig. 2) within the physiological pH range (pH 7.2–7.4) for each species. Significantly different values ($P < 0.05$) between orders for each of the deoxygenated ('*a*' labels), P_{50} ('*b*' labels), and oxygenated ('*c*' labels) Hbs are indicated by dissimilar labels above the respective columns (see text for further details). The presence of a choroid rete within a species is indicated by ®

Haldane effects

The vertical distance, or ΔZ_H, between the oxygenated and deoxygenated titration curves indicates the fixed-acid Haldane effect (ΔZ_H, mol H^+ taken up per mol Hb_4 upon deoxygenation at constant pH), which also varies according to pH, GTP concentration, and species (Fig. 4). The magnitude of oxylabile proton binding varied among the studied species, with the two rete-bearing species (bowfin, mooneye) exhibiting markedly larger maximum Haldane effects than the four more primitive species (paddlefish, sturgeon, spotted gar, and alligator gar), particularly in the presence of GTP (Fig. 4b). When averaged for the species' respective orders, these maxima were negatively correlated with Hb buffer values (Fig. 5), particularly for oxygenated and P_{50} Hb buffer values (Fig. 5b, c; $P < 0.05$ for oxygenated Hb). As well, the maxima for each species were slightly less in the absence of GTP in addition to being left shifted on the pH axis by approximately 0.2–0.4 pH units (Fig. 4a).

Titratable groups

Owing to a lack of conspicuous inflection points on the Hb titration curves of paddlefish, sturgeon, spotted gar, and alligator gar, this analysis could only be objectively performed for the Hbs of bowfin and mooneye. The number of groups titrated on the surfaces of their Hbs was 16 and 14,

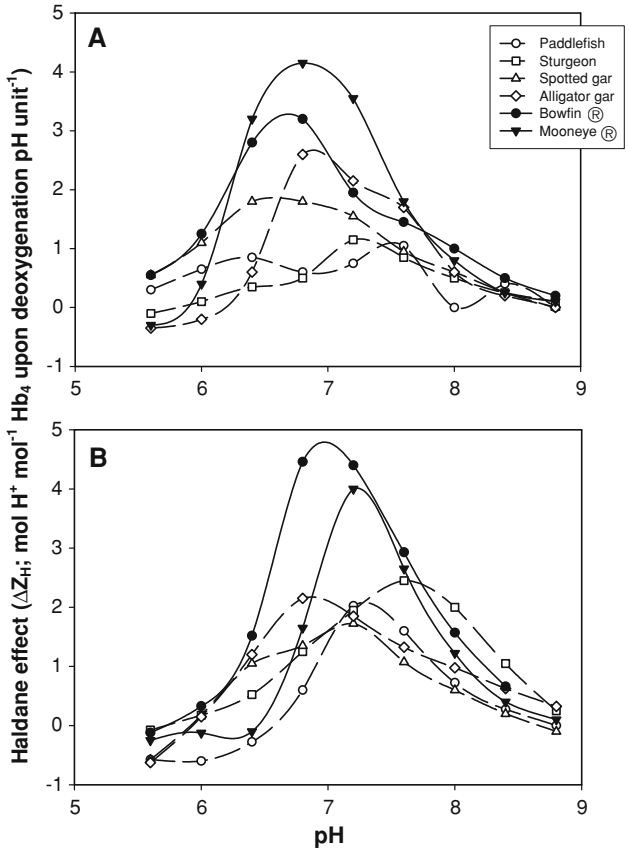

Fig. 4 Fixed-acid Haldane effects (ΔZ_H; the number of protons taken up per Hb_4 upon deoxygenation at constant pH) as a function of pH for six species of basal actinopterygian fishes, in the absence (**a**) and presence (**b**) of GTP (3:1 GTP:Hb_4). ΔZ_H was calculated from the vertical distance between the oxygenated and deoxygenated titration curves (Fig. 1) for a given species. The presence of a choroid rete within a species is indicated by ®

respectively (Fig. 6), and these numbers were determined from the titration curves of deoxygenated, stripped Hbs in accordance with the methods of Jensen (1989).

Discussion

In fish Hbs, an inverse relationship has been shown to exist between the magnitudes of Hb buffer values and oxylabile Haldane effects, such that primitive species (elasmobranchs and sarcopterygians) tend to have high Hb buffer values and small Haldane effects, while derived species (teleosts) tend to have low Hb buffer values and large Haldane effects (Jensen 1989; Jensen et al. 1998). Therefore, it is likely that this transition in Hb-proton-binding strategy occurred among the fishes intermediate to these two groups, the basal actinopterygians, and the elucidation of the proton-binding

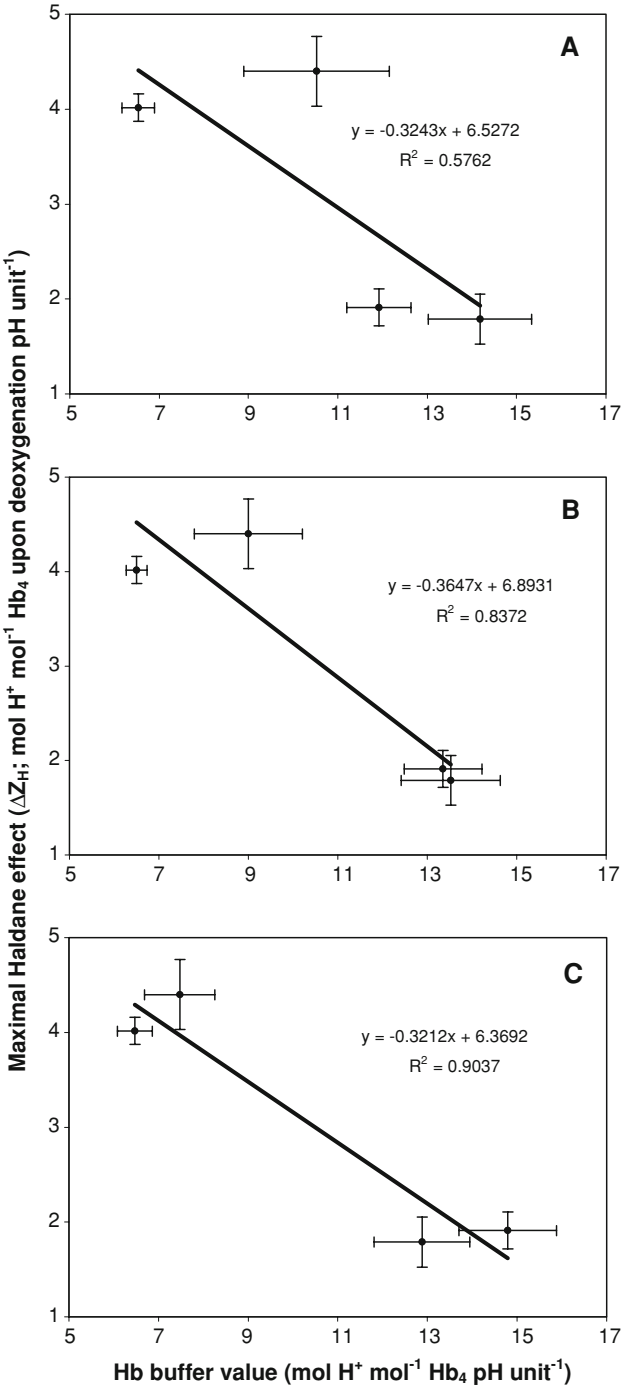

Fig. 5 Mean maximal fixed-acid Haldane effect (ΔZ_H; the number of protons taken up per Hb_4 upon deoxygenation at constant pH) as a function of mean deoxygenated (**a**), P_{50} (**b**), and oxygenated (**c**) Hb buffer values for four orders of basal actinopterygian fishes, with *error bars* representing SEM. Acipenseriformes includes paddlefish and white sturgeon, lepisosteids includes spotted gar and alligator gar, amiiformes includes bowfin, and osteoglossiformes includes mooneye. The data points dichotomize according to the presence (*top left*; amiiformes, osteoglossiformes) and absence (*bottom right*; acipenseriformes, lepisosteids) of choroid retia. $P < 0.05$ for oxygenated Hb (**c**)

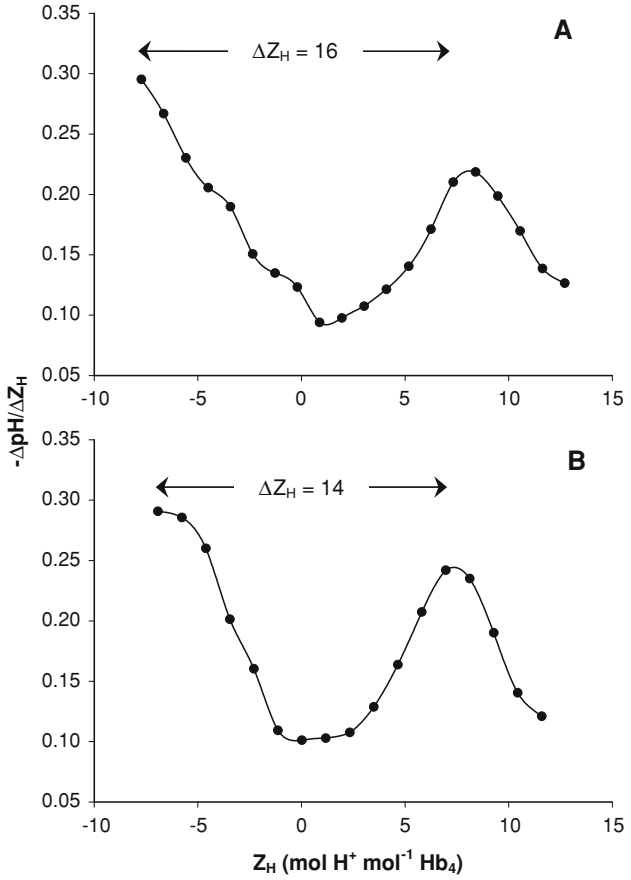

Fig. 6 Differential titration curves ($-\Delta pH/\Delta Z_H$; the inverse of buffer value) as a function of Z_H, taken from the deoxygenated titration curves of bowfin (**a**) and mooneye (**b**) in the absence of GTP. The horizontal distance between the inflection points indicates the number of titratable groups in the neutral pH range (approx. pH 6.0–9.0)

characteristics of these species' Hbs was the objective of this study. The findings suggest that the nature of the transition may not have been completely gradual, but rather punctuated, with Hb buffer value decreasing and Haldane effect increasing significantly starting with bowfin from the fairly steady ancestral values observed in the four basal species. That bowfin is the most basal of the extant species known to possess a choroid rete (Wittenberg and Haedrich 1974; Berenbrink et al. 2005) suggests the transition in Hb-proton-binding strategy observed in this study may be associated with the evolution of enhanced oxygen delivery to the eye via the Root effect, as both a rete and a low Hb buffer value are believed to optimize the functioning of the Root effect.

Evolutionary relationships of the basal actinopterygians

Within the piscine phylogeny, the lepisosteid–amiiformes–teleost node remains controversial depending on which features of the animals are used to elucidate the relationships. Although exceptions exist, there are some general trends among these. Current morphological data place the amiiformes as sister group to the teleosts, collectively forming a clade (the halecostomes) that is sister group to the more basal gars (Lauder and Liem 1983; Janvier 2007). Relationships based on the mitochondrial DNA are less resolute, with the acipenseriformes, lepisosteids, and amiiformes forming an unresolved group basal to the monophyletic teleosts (Inoue et al. 2003; Arnason et al. 2004). Finally, nuclear DNA evidence suggests the holosteans (lepisosteids and amiiformes) as a monophyletic group, sister to the more derived teleosts (Kikugawa et al. 2004). There are of course exceptions to these trends, but in any case, the inter-order relationships of the basal actinopterygians are distant, with the acipenseriformes, lepisosteids, amiiformes, and osteoglossiformes all bifurcating from one another between 200 and 250 million years ago (Nelson 1994; Berenbrink et al. 2005; Janvier 2007).

The intra-order relationships of the species studied here also indicate long divergence times. Paddlefish and white sturgeon (acipenseriformes) are believed to have shared a last common ancestor approximately 170–200 million years ago (Krieger et al. 2008), while spotted gar and alligator gar (lepisosteids) are thought to have split between 65 and 100 million years ago (Janvier 2007). Although more closely related than those species occupying different orders, the species within the same orders are still quite distantly related.

Influence of GTP

Organic phosphates have been shown to influence Hb function by reducing Hb O_2 affinity through the stabilization of the protein's T-state (Weber et al. 1975; Pelster and Weber 1990; Brauner and Weber 1998). Adenosine triphosphate and GTP are the predominant organic phosphates in the RBCs of fishes (Val 2000), with GTP exerting a greater effect on Hb O_2 affinity due to the formation of an additional T-state stabilizing bond (Pelster and Weber 1990; Val 2000). For this reason, all of the experiments in the present study were performed in both the presence and absence of saturating levels of GTP (3:1 GTP:Hb_4). As well as reducing Hb O_2 affinity, the allosteric effects of GTP expose more proton-binding groups (His residues) on the surface of the Hb molecule, and alter the molecular microenvironments of existing His residues in such a way that increases their *p*K values (Pelster and Weber 1990; Jensen et al. 1998). This results in a respective increase in overall proton-binding ability and a right shift of the titration curve (and resulting buffer curve) along the pH scale in the presence of GTP (Figs. 1, 2). The magnitude of these effects varied among species. For instance, bowfin Hbs showed much greater GTP-dependent variation in buffer value than

did sturgeon Hbs. This is likely the result of more additional His residues being exposed on the surface of bowfin Hb as a direct result of GTP binding relative to sturgeon Hb.

The Haldane effect is also influenced by GTP in that it is manifest to a greater degree and over a narrower, higher pH range when GTP is bound to Hb. Again, this effect varies interspecifically, but among the species studied here it is particularly apparent in the two most derived species with the largest Haldane effects—bowfin and mooneye (Figs. 1, 4).

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

Hemoglobin buffer values and titratable histidines

The capacity of Hb as a buffer is a function of the type and number of amino acid residues capable of binding protons over a given pH range, as well as the molecular microenvironments in which these residues are located. These titratable amino acid residues are divided into three pH-dependent classes (Tanford 1962): the guanidyl group of arginine and the amino group of lysine are titrated at basic pH values (>pH 9.0); the carboxyl groups of glutamic acid and aspartic acid are titrated at acidic pH ranges (<pH 6.0); and the imidazole group of His residues and the terminal α-amino groups are titrated in the "physiological" pH range (pH 6.0–9.0). It is the disparity in *p*K values of the His residues on the Hb molecule that leads to the variation in proton-binding ability within this "physiological" pH range (Fig. 2). Furthermore, the T- and R-states of an Hb molecule come with changes in the protein conformation, leading to variation in the exposed titratable groups and their microenvironments. This results in intra-molecular differences in oxygenated and deoxygenated Hb buffer curves (Fig. 2), a phenomenon that becomes more apparent with phylogenetic progression due to increases in the Haldane effect and, subsequently, variation in the oxygenated and deoxygenated Hb titration curves for a given species (Fig. 1). Similarly, the allosteric effects of GTP influence the proton-binding abilities of Hb for the reasons outlined above (Figs. 1, 2).

There was a general trend of decreasing Hb buffer values with phylogenetic progression among the studied groups (Fig. 3). This trend was especially apparent for the oxygenated and P_{50} Hb buffer values (Fig. 3). It has been repeatedly observed that the Hb buffer values of teleost fishes are significantly lower than those of almost all other vertebrate species (Jensen 1989; Brauner and Weber 1998; Jensen et al. 1998; Jensen 2001; Berenbrink et al. 2005; Berenbrink 2006; Brauner and Berenbrink 2007), a trait ultimately attributable to a lower number of proton-binding groups (i.e., His residues) on the molecule itself (Riggs 1970; Jensen 1989; Berenbrink et al. 2005; Berenbrink 2006). For instance, human HbA contains 38 His residues per tetramer (Braunitzer et al. 1961), while that of the elasmobranch, spiny dogfish, contains 40 His residues (Aschauer et al. 1985). In comparison, the tetramers of two teleost species, trout and carp, contain only 16 and 18 His residues, respectively (Hilse and Braunitzer 1968; Bossa et al. 1978). Appropriately, the Hb buffer values of these species reflect the number of His residues that make them up, with both human and dogfish displaying much higher Hb buffer values than those of trout and carp (Siggaard-Andersen 1975; Jensen 1989). However, the overall His content of a Hb molecule does not fully account for its ability to buffer protons, as some of these residues may have *p*K values too low to be operational in vivo, or, more regularly, be buried within the protein moiety and unavailable for titration. Thus, the true reflection of an Hb's ability to buffer protons is the number of titratable groups available on its surface. For the aforementioned Hbs of human and dogfish, these numbers are 26 and 30, respectively, while trout and carp display 7 and 6 groups on the surface of their respective Hbs that are available for titration (Berenbrink 2006). Through the differential plotting of the Hb titration curves (i.e., $-\Delta pH/\Delta Z_H$) as a function of Z_H, the inflection points on the regular titration curves become localized as two separate peaks (Fig. 6), the difference between which gives an accurate measure of the number of groups being titrated (De Bruin and van Os 1968; Jensen 1989). Owing to the linear shape of their Hb titration curves, well-resolved peaks were not observed in the differentially plotted titration curves of the more basal species (paddlefish, white sturgeon, spotted gar, and alligator gar). However, the differential plots of the deoxygenated, stripped Hbs of bowfin and mooneye indicated approximately 16 and 14 titratable groups per tetramer, respectively (Fig. 6). Although these numbers may have been slightly larger had titrations been taken up to pH 10, they agree well with the predictions of Berenbrink et al. (2005) based on a correlation of the buffer values of Hbs with known numbers of titratable groups on their surfaces. However, this is the first time they have been measured directly in these groups.

The Haldane effect

A reduced Hb buffer value, in the absence of any other changes, comes with potentially negative consequences to CO_2 transport in the blood. Teleost fishes, which possess low Hb buffer values, compensate for this by way of a large Haldane effect, a different, but equally viable strategy for accounting for the protons produced by CO_2

hydration which results in a tight interaction between O_2 and CO_2 exchange (Brauner and Randall 1996, 1998). In the present study, negative correlations between Hb buffer values (oxygenated, deoxygenated, and P_{50}) and the Haldane effect in the physiological pH range were observed (Fig. 5). Therefore, despite the lower intrinsic Hb buffer values of the more derived fishes in this study, CO_2 transport and acid–base homeostasis during blood transit is likely very dependent on their large Haldane effects.

Why the shift in strategy to low Hb buffer value?

In fishes, there has been shown to exist a relationship between a low Hb buffer value and a Root effect (Jensen 1989; Brauner and Randall 1996, 1998), where it is believed a reduced Hb buffer value may facilitate the exploitation of the Root effect by reducing the amount of acid required to decrease RBC pH_i to Root effect-activating levels (Brauner and Randall 1998). In the present study, a significant reduction in Hb buffer value is observed in bowfin (amiiformes) relative to the more ancestral species (Fig. 3). This is furthered with mooneye, whose Hb buffer values are equally as low as those of the more derived teleosts examined to date (Jensen 1989; Brauner and Randall 1996; Brauner and Weber 1998; Jensen et al. 1998; Jensen 2001; Berenbrink et al. 2005; Berenbrink 2006). The reduction in Hb buffer value among these two orders of fishes is paralleled with the significant increase in both Root effect magnitude and Root effect onset pH (from seemingly in vivo-irrelevant pH values in the lepisosteids) relative to their most immediate, basal ancestors (Regan and Brauner, submitted). As well, it is believed that the choroid rete, used to maximize the Root effect-activating acidosis at the fish eye, originally evolved in the last common ancestor of bowfin and the teleosts approximately 200–250 million years ago (Wittenberg and Haedrich 1974; Berenbrink et al. 2005). Taken together and assuming the Hb properties of extant primitive species to be representative of their respective ancestral states (Janvier 2007; McKenzie et al. 2007), it is, therefore, possible that the shift from high Hb buffer value/small Haldane effect to low Hb buffer value/large Haldane effect occurred in the last common ancestor of bowfin and the teleosts so as to facilitate O_2 delivery to the eye via the Root effect. This is further supported by the high ocular PO_2 values in bowfin (650 mmHg) relative to spotted gar and alligator gar (90 mmHg; Wittenberg and Wittenberg 1974), as well as the siluriformes order of catfish, which have secondarily lost both Root effects and retia, but have secondarily elevated their Hb buffer values to levels consistent with those of more ancestral, pre-Root effect fishes (Berenbrink et al. 2005).

Conclusions

The findings of the present study indicate that the transition in Hb-proton-binding strategy from large intrinsic buffer value/small Haldane effect to low intrinsic buffer value/large Haldane effect occurred not by gradual succession among the basal actinopterygian fishes, but rather a more punctuated manner. A significant decrease in buffer value and increase in the Haldane effect appear to have occurred in the last common ancestor of bowfin and the teleosts, and is correlated with the first appearance of the choroid rete. As the choroid rete has also been shown to be correlated with a significant increase in the magnitude and onset pH of the Root effect (Regan and Brauner, submitted), it is possible that this transition in Hb-proton binding is related to the optimization and/or utilization of the Root effect in these fishes—a low intrinsic Hb buffer value would reduce the amount of acid required to be transferred to the RBC cytosol to activate the Root effect within the choroid rete, while a large Haldane effect would ensure sufficient conditions for both acid–base homeostasis and CO_2 transport in the general circulation.

Acknowledgments We thank Cosima Ciuhandu, Dane Crossley, Allyse Ferrara, Steve Peake, and Tommy Tsui for their help in acquiring samples. We also thank the two anonymous reviewers for their valuable suggestions. This research was supported by an NSERC Discovery Grant to CJB.

References

Arnason U, Gullberg A, Janke A, Joss J, Elmrot C (2004) Mitogenomic analyses of deep gnathostome divergences: a fish is a fish. Gene 333:61–70

Aschauer H, Weber RE, Braunitzer G (1985) The primary structure of the hemoglobin of the dogfish shark *(Squalus acanthias)*. Antagonistic effects of ATP and urea on oxygen affinity of an elasmobranch hemoglobin. Biol Chem Hoppe-Seyler 366:589–599

Benesch RE, Benesch R, Yung S (1973) Equations for spectrophotometric analysis of hemoglobin mixtures. Anal Biochem 55:245–248

Berenbrink M (2006) Evolution of vertebrate hemoglobins: histidine side chains, specific buffer value and Bohr effect. Respir Physiol Neurobiol 154:165–184

Berenbrink M, Koldkjaer P, Kepp O, Cossins AR (2005) Evolution of oxygen secretion in fishes and the emergence of a complex physiological system. Science 307:1752–1757

Bossa F, Barra D, Petruzzelli R, Martini F, Brunori M (1978) Primary structure of hemoglobin from trout *(Salmo irideus)*. Amino acid sequence of *a* chain of trout I. Biochim Biophys Ada 536:298–305

Brauner CJ, Berenbrink M (2007) Gas Exchange. In: McKenzie DJ, Farrell AP, Brauner CJ (eds) Fish physiology: primitive fishes, vol 26. Academic Press, San Diego, pp 213–282

Brauner CJ, Randall DJ (1996) The interaction between oxygen and carbon dioxide movements in fishes. Comp Biochem Physiol A 113:83–90

Brauner CJ, Randall DJ (1998) The linkage between O_2 and CO_2 transport. In: Perry SF, Tufts BL (eds) Fish physiology: fish respiration, vol 17. Academic Press, San Diego, pp 283–321

Brauner CJ, Weber RE (1998) Hydrogen ion titrations of the anodic and cathodic haemoglobin components of the European eel, *Anguilla anguilla*. J Exp Biol 201:2507–2514

Braunitzer G, Gehring-Muller R, Hilschmann N, Hilse K, Hobom G, Rudloff V, Wittmann-Liebold B (1961) Die konstitution des normalen adulten Humanhamoglobins. Hoppe-Seyler's Z Physiol Chem 325:283–286

Bridges CR, Berenbrink M, Muller R, Waser W (1998) Physiology and biochemistry of the pseudobranch: an unanswered question? Comp Biochem Physiol A Mol Integr Physiol 119:67–77

Crocker CE, Cech JJ Jr (1998) Effects of hypercapnia on blood-gas and acid-base status in the white sturgeon, *Acipenser transmontanus*. J Comp Physiol B 168:50–60

De Bruin SH, van Os GAJ (1968) Determination and interpretation of the differential titration curves of proteins: the differential curves of bovine methemoglobin, CO-hemoglobin and serum albumin. Rec Trav Chim 87:861–872

Gottfried MD, Krause DW (1998) First record of gars (Lepisosteidae, Actinopterygii) on Madagascar: late Cretaceous remains from the Mahajunga basin. J Vert Paleont 18:275–279

Hilse K, Braunitzer G (1968) Die Aminosauresequens der a-ketten der beiden Hauptkomponenten des Karphenhamoglobins. Hoppe-Seyler's Z Physiol Chem 349:433–450

Inoue JG, Miya M, Tsukamoto K, Nishida Y (2003) Basal actinopterygian relation-ships: a mitogenomic perspective on the phylogeny of the ''ancient fish''. Mol Biol Evol 26:110–120

Janvier P (2007) Living primitive fishes and fishes from deep time. In: McKenzie DJ, Farrell AP, Brauner CJ (eds) Fish physiology: primitive fishes, vol 26. Academic Press, San Diego, pp 2–53

Jensen FB (1989) Hydrogen ion equilibria in fish hemoglobins. J Exp Biol 143:225–234

Jensen FB (2001) Hydrogen ion binding properties of tuna haemoglobins. Comp Biochem Physiol A Mol Integr Physiol 129:511–517

Jensen FB (2004) Red blood cell pH, the Bohr effect, and other oxygenation-linked phenomena in blood O_2 and CO_2 transport. Acta Physiol Scand 182:215–227

Jensen FB, Fago A, Weber RE (1998) Red blood cell physiology and biochemistry. In: Perry SF, Tufts BL (eds) Fish physiology: fish respiration, vol 17. Academic Press, San Diego, pp 1–40

Kikugawa K, Katoh K, Kuraku S, Sakurai H, Ishida O, Iwabe N, Miyata T (2004) Basal jawed vertebrate phylogeny inferred from multiple nuclear DNA-coded genes. BMC Biol 2:1–11

Krieger J, Hett AK, Fuerst PA, Artyukhin E, Ludwig A (2008) The molecular phylogeny of the order Acipenseriformes revisited. J Appl Ichthyol 24:36–45

Lauder GV, Liem KF (1983) The evolution and interrelationships of the actinopterygian fishes. Bull Mus Comp Zool 150:95–197

McKenzie DJ, Farrell AP, Brauner CJ (2007) In: McKenzie DJ, Farrell AP, Brauner CJ (eds) Fish physiology: primitive fishes, vol 26. Academic Press, San Diego, pp xi–xiii

Nelson JS (1994) Fishes of the world, 3rd edn. Wiley, New York, pp 77–96

Pelster B, Weber RE (1990) Influence of organic phosphates on the Root effect of multiple fish hemoglobins. J Exp Biol 149:425–437

Riggs AF (1970) Properties of fish hemoglobins. In: Hoar WS, Randall DJ (eds) Fish physiology, vol 4. Academic Press, San Diego, pp 209–252

Riggs AF (1988) The Bohr effect. Annu Rev Physiol 50:181–204

Siggaard-Andersen O (1975) The acid–base status of the blood, 4th edn. Munksgaard, Copenhagen

Smatresk NJ, Cameron JN (1982) Respiration and acid–base physiology of the spotted gar, a bimodal breather. J Exp Biol 96:295–306

Stock DW, Gibbons JK, Whitt GS (1991) Strengths and limitations of molecular sequence comparisons for inferring the phylogeny of the major groups of fishes. J Fish Biol 39(Suppl A):225–236

Tanford C (1962) The interpretation of hydrogen ion titration curves of proteins. Adv Protein Chem 17:69–165

Val AL (2000) Organic phosphates in the red blood cells of fish. Comp Biochem Physiol A Mol Integr Physiol 125:417–435

Weber RE, Lykkeboe G, Johansen K (1975) Biochemical aspects of adaptation of hemoglobin oxygen affinity of eels to hypoxia. Life Sci 17:1345–1349

Wiley EO (1976) The phylogeny and biogeography of fossil and recent gars (Actinopterygii: Lepisosteidae), vol 64. University of Kansas Publications, Museum of Natural History, Miscellaneous Publication, pp 1–111

Wittenberg JB, Haedrich RL (1974) Choroid rete mirabile of fish eye.2. Distribution and relation to pseudobranch and to swimbladder rete mirabile. Biol Bull 146:137–156

Wittenberg JB, Wittenberg BA (1974) Choroid rete mirabile of fish eye. 1. Oxygen secretion and structure—comparison with swimbladder rete mirabile. Biol Bull 146:116–136

Wolf K (1963) Physiological salines of fresh water teleosts. Prog Fish Cult 25:135–140