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RESEARCH ARTICLE

Oxygenation properties of hemoglobin and the evolutionary origins of isoform multiplicity in an amphibious air-breathing fish, the blue-spotted mudskipper (*Boleophthalmus pectinirostris*)

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ABSTRACT

Among the numerous lineages of teleost fish that have independently transitioned from obligate water breathing to facultative air breathing, evolved properties of hemoglobin (Hb)–O_2 transport may have been shaped by the prevalence and severity of aquatic hypoxia (which influences the extent to which fish are compelled to switch to aerial respiration) as well as the anatomical design of air-breathing structures and the cardiovascular system. Here, we examined the structure and function of Hbs in an amphibious, facultative air-breathing fish, the blue-spotted mudskipper (*Boleophthalmus pectinirostris*). We also characterized the genomic organization of the globin gene clusters of the species and we integrated phylogenetic and comparative genomic analyses to unravel the duplicative history of the genes that encode the subunits of structurally distinct mudskipper Hb isoforms (isoHbs). The *B. pectinirostris* isoHbs exhibit high intrinsic O_2 affinities, similar to those of hypoxia-tolerant, water-breathing teleosts, and remarkably large Bohr effects. Genomic analysis of conserved synteny revealed that the genes that encode the α-type subunits of the two main adult isoHbs are members of paralogous gene clusters that represent products of the teleost-specific whole-genome duplication. Experiments revealed no appreciable difference in the oxygenation properties of co-expressed isoHbs in spite of extensive amino acid divergence between the alternative α-chain subunit isoforms. It therefore appears that the ability to switch between aquatic and aerial respiration does not necessarily require a division of labor between functionally distinct isoHbs with specialized oxygenation properties.

KEY WORDS: Air-breathing fish, Bimodal breathing, Bohr effect, Blood–oxygen transport, Hemoglobin, Whole-genome duplication

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INTRODUCTION

Facultative air-breathing fishes have always been of interest to comparative physiologists because of the versatility required to switch between aquatic and aerial respiration (Graham, 1997; Graham and Wegner, 2010; Bayley et al., 2019). Moreover, the physiological features that distinguish air-breathing fishes from their obligate water-breathing kin may provide clues about the types of phenotypic changes that facilitated the invasion of land by the shallow-water progenitors of modern tetrapods. In addition to evolutionary changes in branchial and cardiovascular function in fish that have evolved the capacity for aerial respiration, there has been much interest in associated changes in hemoglobin (Hb) function and respiratory gas transport (Damsgaard et al., 2014, 2015; Bayley et al., 2019).

Regardless of breathing mode, the physiologically optimal Hb–O_2 affinity is dictated by the trade-off between O_2 loading at the respiratory surfaces (gills, lungs or other air-breathing structures) and O_2 unloading to respiring tissues (Brauner and Wang, 1997; Wang and Malte, 2011; Storz, 2019), and there are reasons to expect that this trade-off may be especially profound in facultative air-breathing fishes. Because air has a much higher O_2 content than water, aerial breathing should generally place a lower premium on O_2 uptake at the respiratory surfaces (as arterial O_2 saturation is typically not a limiting factor in tissue O_2 delivery). This has led some authors to suggest that the evolutionary transition from water to air breathing generally entailed a reduction in Hb–O_2 affinity that permitted a concomitant increase in circulatory O_2 delivery and aerobic metabolism (Johansen and Lenfant, 1972; Johansen et al., 1978; Powers et al., 1979). However, among amphibious fish that alternate between breathing modes, a high Hb–O_2 affinity retains much utility under conditions of aquatic hypoxia because it improves O_2 uptake at the gills, thereby enhancing blood O_2 capacitance [the quantity of O_2 unloaded to the tissues for a given difference in arterial and venous partial pressures of O_2 (P_{O_2})]. Indeed, among water-breathing and facultative air-breathing fish alike, increased Hb–O_2 affinities are generally associated with adaptation to aquatic hypoxia (Powers, 1980; Jensen, 2004; Mandic et al., 2009; Wells, 2009; Fago, 2017; Harter and Brauner, 2017).

In addition to considerations related to differences in O_2 capacitance between air and water and the challenges posed by aquatic hypoxia, the optimal Hb–O_2 affinity in air-breathing fish may also relate to the diffusive conductance of air-breathing structures and design features of the cardiovascular system. Among most air-breathing teleosts, extrabranchial gas exchange occurs across the vascularized epithelia of the buccopharyngeal cavity, esophagus, intestine, stomach or swim bladder (Graham, 1997; Graham and Wegner, 2010). Such air-breathing structures generally have much lower diffusion capacities and lower gas exchange

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efficiencies than normal gills, so an elevated Hb–O_2 affinity can promote O_2 uptake by maintaining a steep P_{O_2} gradient across the respiratory epithelium (Hlastala and Berger, 2001; Damsgaard et al., 2014). Finally, aerial breathing generally increases the P_{O_2} of arterial blood in the ventral aorta that flows towards the gills and – in the absence of a non-respiratory branchial shunt – O_2-rich arterial blood could desaturate during branchial passage (Randall et al., 1981; Olson, 1994; Ishimatsu, 2012; Bayley et al., 2019). Thus, a high Hb–O_2 affinity may also be beneficial in facultative air breathers because it helps prevent transbranchial O_2 loss, thereby ensuring adequate O_2 delivery to metabolizing tissues. For this reason, Damsgaard et al. (2014) suggested that facultative air-breathing fish species that do not possess transbranchial shunts could be expected to have evolved higher Hb–O_2 affinities than obligate air breathers with reduced gills.

Most teleost fish co-express multiple, structurally distinct Hb isoforms (isoHbs) in adult red blood cells, which are conventionally classified as 'anodic' or 'cathodic' on the basis of electrophoretic mobility (Weber, 1990, 2000). Anodic isoHbs tend to have relatively low O_2 affinities and a large Bohr effect (reduced Hb–O_2 affinity at low pH), whereas cathodic isoHbs tend to have relatively high O_2 affinities, a heightened sensitivity to the affinity-reducing effects of organic phosphates (e.g. ATP and GTP), and a negligible Bohr effect in the presence of organic phosphates (Weber and Jensen, 1988; Weber, 1990, 2000; Jensen et al., 1998; Weber et al., 2000; Wells, 2009). According to the classification scheme of Weber (1990), 'class I' species such as plaice and carp express multiple anodal isoHbs with very similar O_2-binding properties, whereas 'class II' species such as anguillid eels, salmonids and catfish additionally express one or more cathodal isoHbs that broaden the spectrum of O_2 affinities and allosteric regulatory capacities. The isoHb multiplicity in class II species may permit a physiological division of labor whereby pH-insensitive cathodal isoHbs provide a reserve capacity for blood–O_2 transport under conditions of environmental hypoxia or metabolic acidosis (Weber, 1990, 2000). In some teleost species, evidence suggests that regulatory switches in isoHb expression may play a role in the acclimatization response to environmental hypoxia (Rutjes et al., 2007), and in amphibious fish such as mudskippers it seems plausible that a physiological division of labor between isoHbs with different oxygenation properties could contribute to the versatility required to reversibly switch between aquatic and aerial respiration.

Here, we examine the structure and function of Hbs in the blue-spotted mudskipper (*Boleophthalmus pectinirostris*), an amphibious, facultative air-breathing fish that is distributed along the coastlines of China, Taiwan, the Korean peninsula and Japan in the northwest Pacific. This species routinely switches between breathing modes when shuttling between tidepools and mudflats in the intertidal zone (Martin and Bridges, 1999) and it uses an extensive respiratory surface for aerial breathing, including the buccal, pharyngeal, branchial and opercular cavities, in addition to the gills and integument (Graham, 1997). We also report sequence data for the full complement of α- and β-type globin genes from *B. pectinirostris*,

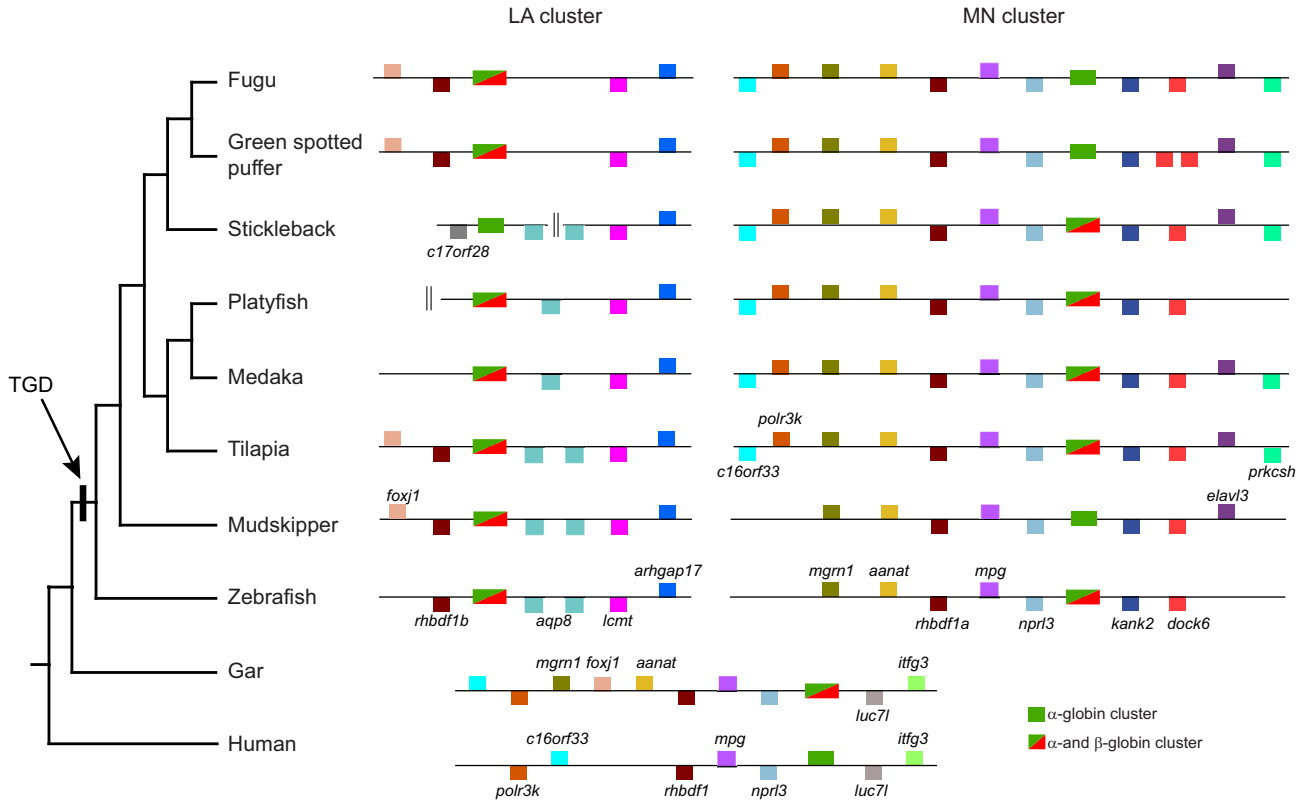

Fig. 1. Unscaled representation of the genomic organization of the LA and MN globin gene clusters in the blue-spotted mudskipper, *Boleophthalmus pectinirostris*, and other representative ray-finned fish. The α-globin gene cluster of human is shown for comparison. The LA and MN gene clusters represent paralogous chromosomal segments that are products of the teleost-specific genome duplication (TGD) (Opazo et al., 2013) that occurred ∼350 million years ago (Meyer and Van de Peer, 2005). The 'LA' and 'MN' abbreviations refer to flanking genes that demarcate the 5′ and 3′ boundaries of each set of tandemly linked globin genes. Genes in the sense orientation are shown above the chromosome, those in the anti-sense orientation are shown below, and the globin gene clusters (which include genes in both orientations) are vertically aligned along the mid-line. The globin gene clusters of each species are shown in the same orientation as those in the zebrafish genome.

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we characterize the isoHb composition of adult red cells, and we integrate comparative genomic and phylogenetic analyses to shed light on the evolutionary origins of isoHb multiplicity in mudskippers.

MATERIALS AND METHODS

Sequence data and phylogenetic analyses

We annotated the full complement of α- and β-type globin genes in the genome assembly of the blue-spotted mudskipper, *Boleophthalmus pectinirostris* (Linnaeus 1758) (GenBank accession number: JACK00000000.1). For analyses of conserved synteny and phylogenetic relationships, we used genomic sequence data from a diverse set of bony fish and cartilaginous fish, including globin gene sequences that we annotated previously (Hoffmann et al., 2012; Opazo et al., 2013, 2015).

Protein coding sequences were translated and aligned using L-INS-i strategies in Mafft v7.3 (Katoh and Standley, 2013) and were then reverse-translated so that coding sequences were codon-aligned. We annotated several genes with truncated coding sequences [*Hbb3-MN* in platyfish (*Xiphophorus maculatus*), *Hbb2-MN* and *Hbb6-MN* in Nile tilapia (*Oreochromis niloticus*) and *Hba1* in spotted gar (*Lepisosteus oculatus*)], but we did not include such sequences in the phylogenetic analyses. We reconstructed separate phylogenies for the α- and β-globin genes using maximum likelihood (ML). After partitioning the alignment into codon positions, we performed the ML analyses in RAxML v 8.1.3 (Stamatakis, 2006) using the GTRGAMMA substitution model. Support for each node in the best tree was based on 1000 bootstrap replicates.

Blood sampling and characterization of red cell isoHb composition

Prior to blood sampling, the fish were maintained in plastic aquaria with artificial seawater (15% salinity) at 27°C for 24 h. Two individual specimens of *B. pectinirostris* were anesthetized with 0.01% MS222 (Sigma-Aldrich, St Louis, MO, USA) and blood was collected via caudal venipuncture with a heparinized syringe. The animals were handled in accordance with a protocol approved by the Animal Ethics Committee and the Institutional Review Board on Bioethics and Biosafety of BGI (certification number FT15029).

Each blood sample was incubated for 5 min in a 5-fold volume of ice-cold buffer (10 mmol l^{-1} Hepes, 0.5 mmol l^{-1} EDTA, pH 7.75). Hemolysates were centrifuged to remove cell debris (30 min at 15,000 ***g***, 4°C) and KCl was then added to the clear supernatant to a final concentration of 0.2 mol l^{-1} (Jendroszek et al., 2018). The sample was then passed through a 5 ml PD-10 (GE Healthcare) desalting column equilibrated with 10 mmol l^{-1} Hepes, 0.5 mmol l^{-1} EDTA, pH 7.7. This desalting step removes endogenous organic phosphates, which are mainly ATP and GTP in fish red cells.

Separation and purification of isoHbs

We purified individual isoHbs by means of anion-exchange fast-protein liquid chromatography (FPLC) using an Äkta Pure system (GE Healthcare). Stripped hemolysate was loaded on the prepacked HiTrap Q-HP column (GE Healthcare) and equilibrated with 10 mmol l^{-1} Hepes buffer (0.5 mmol l^{-1} EDTA, pH 7.66), and separation of individual isoHbs was achieved using a linear gradient

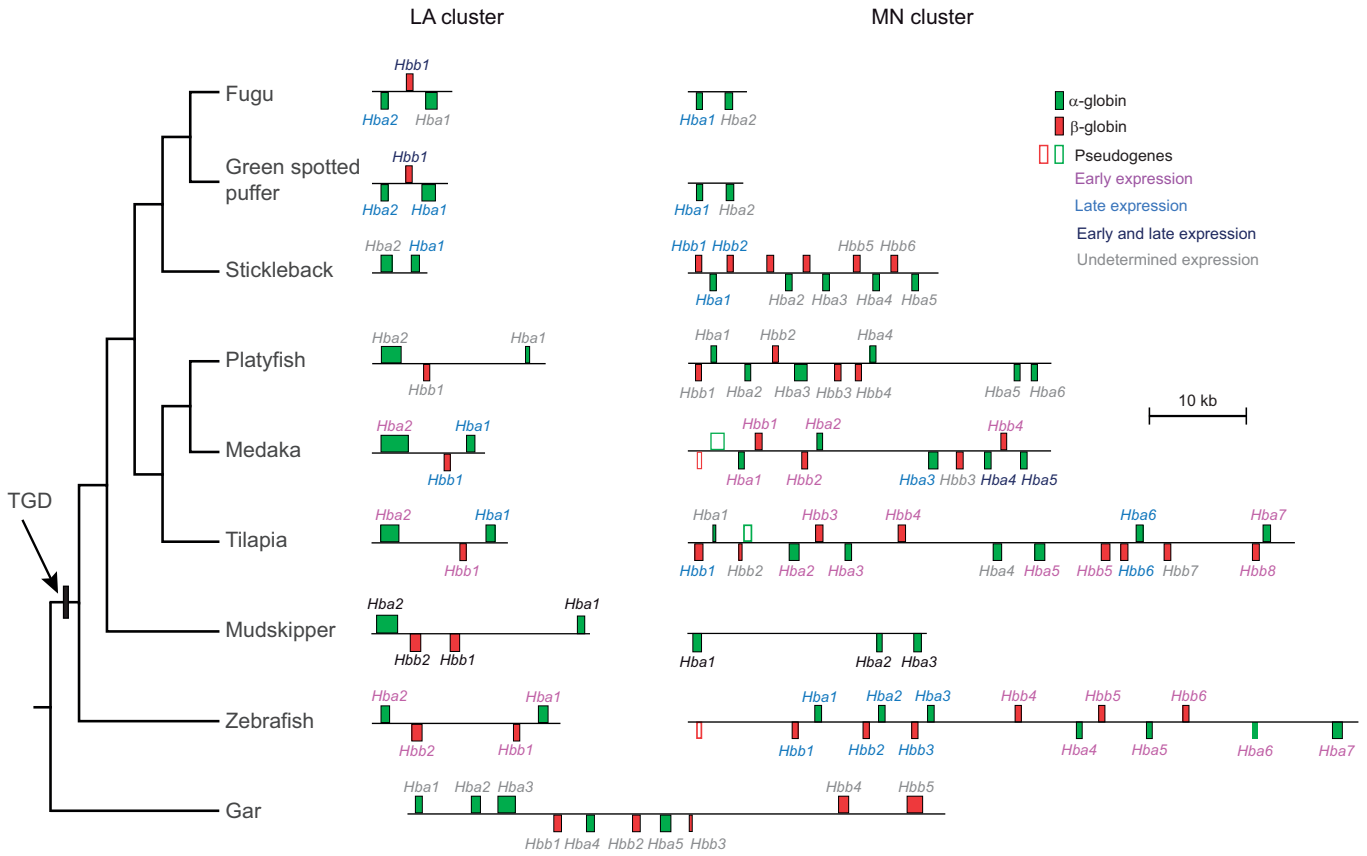

Fig. 2. Genomic organization of the MN and LA globin gene clusters of the blue-spotted mudskipper, *B. pectinirostris*, and a representative set of other ray-finned fishes. The LA and MN gene clusters represent paralogous chromosomal segments that are products of the teleost-specific genome duplication (TGD), which occurred after the ancestral line of extant teleosts diverged from the ancestor of gars (Opazo et al., 2013). Globin genes in the sense orientation are shown above the chromosome and genes in the anti-sense orientation are shown below.

of 0–80% of 0.2 mol l^{-1} NaCl, with a flow rate of 1 ml min^{-1}. Fractions were concentrated using Amicon Ultra centrifugal filters (10 kDa) and subsequently dialyzed against 10 mmol l^{-1} Hepes, 0.5 mmol l^{-1} EDTA, pH 7.7, at 4°C, to remove NaCl. Purity of the isoHb fractions was verified by means of native polyacrylamide gel electrophoresis (PAGE) using a PhastSystem (GE Healthcare) in 10–15% gradient precast gels. Purified samples were stored at −80°C at a 1 mmol l^{-1} heme concentration.

Identification of isoHb subunit composition

The subunit composition of each eluted Hb fraction was determined by tandem mass spectrometry (MS/MS). For the MS/MS analysis, eluted fractions were separated on a 20% SDS-PAGE gel and stained with Coomassie Brilliant Blue. The individual α- and β-chain subunits of the purified isoHbs were excised and digested with trypsin. Following previously described protocols (Storz et al., 2011; Revsbech et al., 2013; Storz et al., 2015), peptide mass fingerprints derived from MS/MS were queried with a custom database that included amino acid sequences from all embryonic and adult-expressed α- and β-type globin genes that we annotated from the *B. pectinirostris* genome assembly. The queries were conducted with the Mascot data search system (version 1.9.0, Matrix Science, London, UK). Search parameters included no restriction on protein molecular weight or isoelectric point, and methionine oxidation was allowed as a variable peptide modification. Mass accuracy settings were 0.15 Da for peptide mass and 0.12 Da for fragment ion masses. We identified all significant protein hits that matched more than one peptide with $P<0.05$.

Measurement of Hb–O_2 equilibria

We measured O_2-equilibrium curves of purified isoHbs at 25°C in 0.1 mol l^{-1} Hepes buffer (pH 7.40) in the absence (stripped) and presence of 0.1 mol l^{-1} KCl and ATP (0.75 mmol l^{-1}), and in the simultaneous presence of KCl and ATP. The 0.75 mmol l^{-1} ATP concentration falls within the range of values estimated for teleost red cells (Nikinmaa, 1982; Cadiz et al., 2019) and is therefore relevant to *in vivo* conditions. Heme concentration for each Hb solution was 0.3 mmol l^{-1}, yielding a 10-fold molar excess of ATP to tetrameric Hb. This ATP:Hb_4 ratio permits an assessment of the maximal possible effect of ATP on Hb–O_2 affinity. To measure the Bohr effect, we took replicate measurements of stripped isoHbs under identical conditions at pH 6.7 and 7.4. The final pH of Hb solutions used in each experiment was measured at 25°C using an InLab Micro pH electrode (Mettler Toledo). We measured O_2 equilibria of 3 µl thin-film samples in a modified diffusion chamber where absorption at 436 nm was monitored during stepwise changes in the equilibration of N_2/O_2 mixtures generated by precision Wősthoff gas-mixing pumps (Weber, 1992; Grispo et al., 2012; Weber et al., 2013; Natarajan et al., 2015, 2016). We estimated values of P_{50} and n_{50} (Hill's cooperativity coefficient at half-saturation) by fitting the Hill equation $Y = P_{O_2}^n/(P_{50}^n + P_{O_2}^n)$ to the experimental O_2 saturation data by means of nonlinear regression (where Y is the fractional O_2 saturation and n is the cooperativity coefficient). The nonlinear fitting was based on four to nine equilibration steps between 30% and 70% oxygenation, and standard errors are reported for the resultant estimates of P_{50} and n_{50}. Free Cl^- concentrations were measured with a model 926S Mark II chloride analyzer (Sherwood Scientific Ltd, Cambridge, UK).

RESULTS

Genomic organization of the globin gene clusters

We used a genome assembly of the blue-spotted mudskipper, *B. pectinirostris*, to characterize the physical organization of the globin gene clusters, and we integrated analyses of conserved synteny with phylogenetic reconstructions to unravel the duplicative history of the mudskipper α- and β-type globin genes. We identified a total of four α-type globin genes and three β-type globin genes in the genome of *B. pectinirostris*. As in other teleosts examined to date, these genes were distributed between two unlinked clusters, designated 'LA' and 'MN' (the abbreviations refer to flanking genes that demarcate the 5′ and 3′ boundaries of each set of tandemly

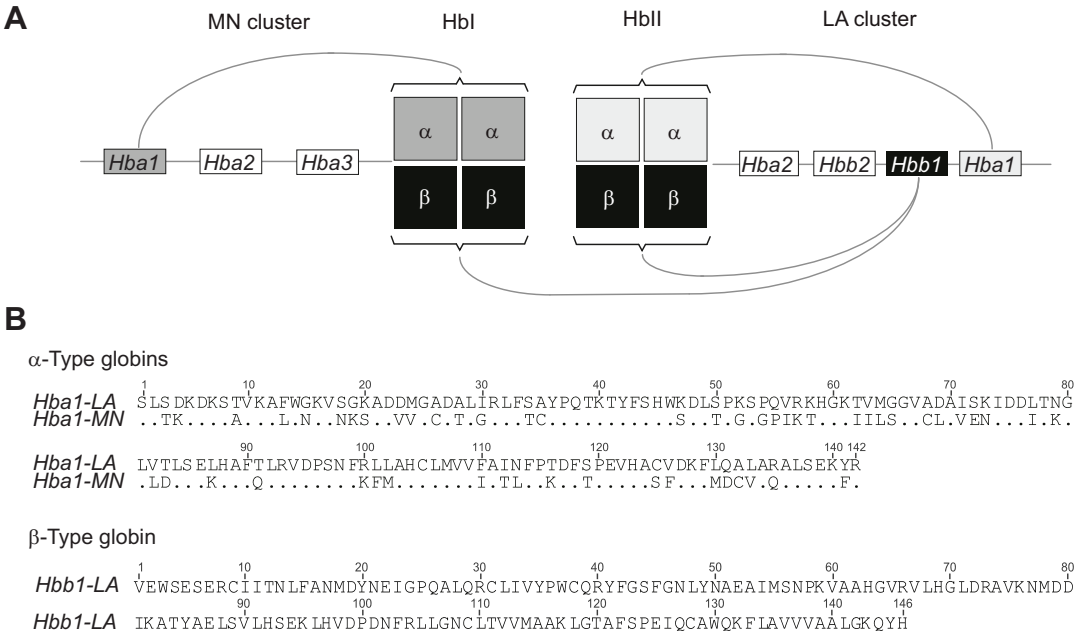

Fig. 3. IsoHb composition of mudskipper isoHbs. (A) Subunit composition of the two major isoHbs of the blue-spotted mudskipper, *B. pectinirostris*, as revealed by tandem mass spectrometry. (B) Amino acid sequences of adult α- and β-chain subunits.

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linked globin genes) (Fig. 1). The LA and MN gene clusters represent the paralogous products of the teleost-specific whole-genome duplication (TGD) (Opazo et al., 2013) that occurred ∼350 million years ago (Meyer and Van de Peer, 2005). The LA cluster of *B. pectinirostris* contains two α-type globin genes (*Hba1-LA* and *Hba2-LA*, from 5′ to 3′) with two β-type globin genes interleaved between them in the opposite orientation (*Hbb2-LA* and *Hbb1-LA*), whereas the MN cluster contains three α-type globin genes (*Hba1-MN*, *Hba2-MN* and *Hba3-MN*) (Fig. 2).

IsoHb composition of *B. pectinirostris* red cells

Red cell lysates from *B. pectinirostris* were subjected to anion-exchange FPLC, which revealed two major adult isoHbs, HbI and HbII, in approximately equal quantities and with similar anodic mobilities on native polyacrylamide gels. MS/MS analysis of the FPLC-purified isoHbs revealed that each of them incorporate the same β-chain (product of the *Hbb1-LA* gene) but different α-chains, as HbI incorporates the product of *Hba1-LA* and HbII incorporates the product of *Hba1-MN* (Fig. 3A). The two α-globin genes, *Hba1-LA* and *Hba1-MN*, are members of the two separate TGD-derived gene clusters (Fig. 2). Given the antiquity of the TGD, it is not surprising that the two α-globin paralogs are highly divergent at the amino acid sequence level, differing at 54 of 141 residue positions (Fig. 3B).

Phylogeny of α- and β-type globin genes

Consistent with the comparative genomic analysis of conserved synteny, phylogenetic analysis revealed that α- and β-type globin genes from a taxonomically diverse set of teleost species clustered into discrete LA and MN clades (Fig. 4). Within each main clade of LA- and MN-associated globin genes, the adult-expressed mudskipper globin genes were in some cases nested within subclades of genes from other species that are annotated as early-expressed embryonic globins. For example, within ‘LA Hba clade 2’ in the phylogeny of α-type globins (Fig. 4A), the adult-expressed *Hba1-LA* gene of mudskipper forms a subclade with the adult-expressed *Hba1-LA* gene from medaka (*Oryzias latipes*) and the early-expressed *Hba2-LA* gene from Nile tilapia (*O. niloticus*). Likewise, within the ‘LA Hbb clade 1’ in the phylogeny of β-type globins (Fig. 4B), the adult-expressed *Hbb1-LA* of mudskipper forms a subclade with the adult-expressed *Hbb1-LA* of medaka and the early-expressed *Hbb1-LA* of Nile tilapia, as well as the early- and late-expressed *Hbb1-LA* genes of green-spotted puffer (*Dichotomyctere nigroviridis*) and fugu (*Takifugu rubripes*).

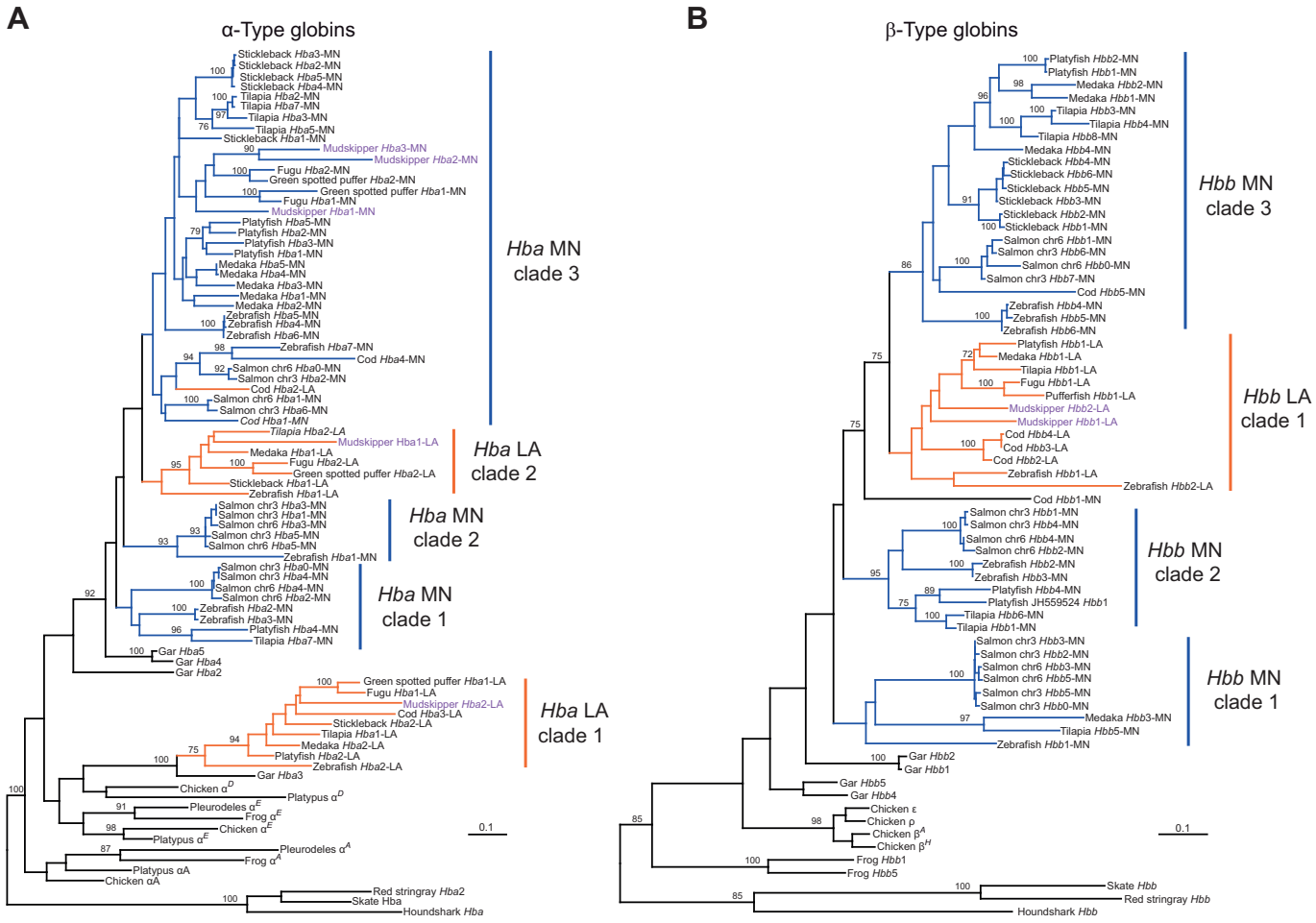

Fig. 4. Maximum likelihood phylogenetic reconstruction depicting relationships among globin sequences of the blue-spotted mudskipper, *B. pectinirostris*, and a representative set of other ray-finned fish, cartilaginous fish and tetrapods. Reconstructions were based on the coding sequences of α- and β-type globin genes (A and B, respectively). Only nodes with bootstrap support values greater than 70 are labeled. Branches are color-coded according to the location of the associated genes: MN-linked genes are shown in blue and LA-linked genes are shown in orange. The names of the mudskipper genes are written in purple. Scale bars for branch lengths denote the estimated number of nucleotide substitutions per site.

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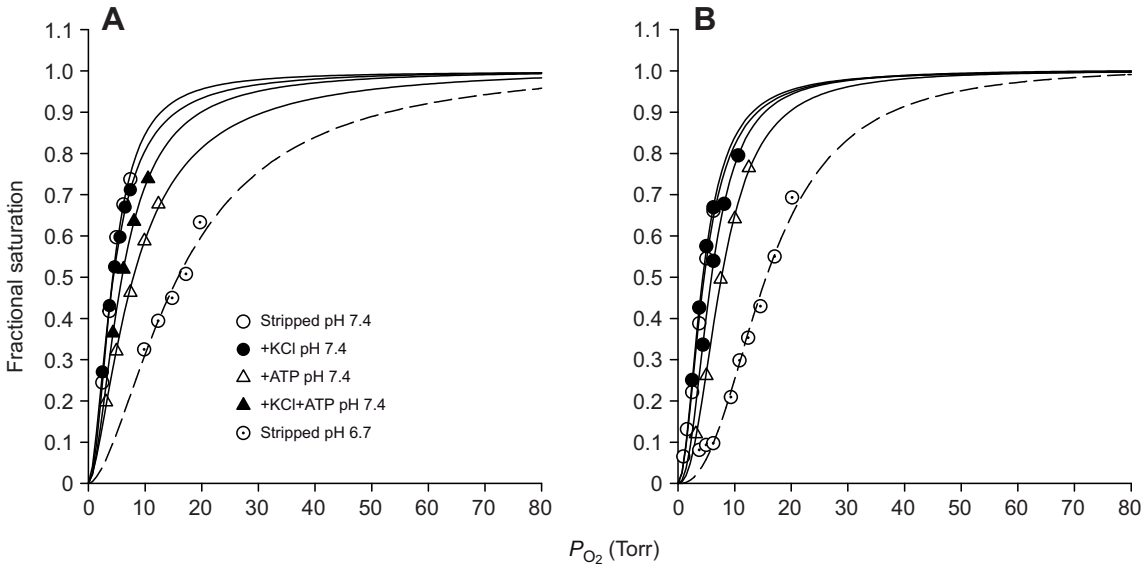

Fig. 5. O_2-equilibrium curves of purified mudskipper isoHbs. Curves for HbI (A) and HbII (B) were measured at 25°C in 0.1 mol l^{-1} Hepes buffer in the absence (stripped) and presence of allosteric cofactors (Cl^- ion, added as KCl) and ATP (see Materials and Methods for details). To measure the Bohr effect, curves were measured under stripped conditions at pH 7.40 (open circles, solid lines) and at pH 6.67 (HbI) or 6.74 (HbII) (circles with dots, dashed lines).

O_2-binding properties of mudskipper Hbs

Under identical experimental conditions, O_2-equilibrium curves revealed that the two isoHbs exhibited virtually identical O_2 affinities (as measured by P_{50}, the P_{O_2} at which Hb is 50% saturated) and cooperativities (as measured by n_{50}, Hill's cooperativity coefficient at half-saturation) (Fig. 5, Table 1). O_2 affinities of the two isoHbs were reduced (i.e. P_{50}s were increased) in the presence of ATP but not in the presence of Cl^- ions alone (added as 0.1 mol l^{-1} KCl) (Table 1, Fig. 5). Curves for both isoHbs measured in the joint presence of KCl and ATP revealed a slight decrease in P_{50} compared with curves measured with ATP alone, indicating that the binding of monovalent Cl^- ions interferes with the allosteric binding of ATP in the central cavity of T-state deoxyHb. The O_2 affinities of both isoHbs exhibited pronounced sensitivities to changes in pH: Bohr factors ($\Delta\log P_{50}/\Delta$pH) were −0.79 and −0.81 for HbI and HbII, respectively.

DISCUSSION

Oxygenation properties of mudskipper Hbs

Boleophthalmus pectinirostris expresses multiple anodic isoHbs, each with a relatively high intrinsic O_2 affinity, moderate sensitivity to organic phosphates, and a large Bohr effect – a 'class I' profile, according to the classification scheme of Weber (1990). This same basic pattern of isoHb multiplicity has been well documented in other teleosts, including facultative air breathers and obligate water breathers alike. The Hb system of *B. pectinirostris* appears functionally similar to that of the swamp eel (*Monopterus albus*), a facultative air breather in which gas exchange is largely restricted to the buccopharyngeal cavity (Damsgaard et al., 2014, 2015). Similar to *B. pectinirostris*, *M. albus* expresses multiple anodic isoHbs and no cathodic components. The two major isoHbs of *M. albus* exhibit high intrinsic O_2 affinities [$P_{50(\text{stripped})}$=4.8–5.2 torr (25°C, pH 7.7)], similar to values measured for the two isoHbs of *B. pectinirostris* (Table 1), but the mudskipper isoHbs exhibited a higher sensitivity to ATP and estimated Bohr coefficients were 2- to 3-fold larger when measured under identical buffer conditions. The mudskipper isoHbs have P_{50} values similar to those reported for the anodic isoHbs of hypoxia-tolerant, water-breathing teleosts such as carp (*Cyprinus carpio*) (Gillen and Riggs, 1972; Weber and Lykkeboe, 1978; Jensen et al., 2017) and European eel (*Anguilla anguilla*) (Fago et al., 1997). In contrast to the mudskippers, another facultative air-breathing fish, the Amazonian armored catfish (*Hoplosternum littorale*), exhibits a far more pronounced level of isoHb differentiation, with anodic and cathodic isoHbs that differ substantially in intrinsic O_2 affinity, phosphate sensitivity and Bohr effect (Weber et al., 2000).

The high Hb–O_2 affinity of the mudskipper should promote branchial O_2 uptake under conditions of aquatic hypoxia and – during terrestrial excursions – transepithelial O_2 uptake in the pharyngeal air-breathing structures. The potential drawback of an increased Hb–O_2 affinity is that it hinders O_2 unloading in the tissue capillaries, but this can be mitigated to some extent by the Bohr effect. The large Bohr effect of *B. pectinirostris* and some other facultative air-breathing fish may enhance O_2 delivery to tissues

Table 1. O_2-binding properties of the two major isoHbs of the blue-spotted mudskipper, *Boleophthalmus pectinirostris* (means±s.e.)

	HbI			HbII		
	pH	P_{50} (Torr)	n_{50}	pH	P_{50} (Torr)	n_{50}
Stripped	7.40	4.27±0.11	2.03±0.11	7.40	4.60±0.14	1.93±0.11
KCl	7.40	4.39±0.03	1.73±0.02	7.40	4.35±0.03	1.98±0.04
ATP	7.40	8.02±0.17	1.55±0.05	7.40	7.67±0.12	2.32±0.07
KCl+ATP	7.40	5.92±0.01	1.79±0.01	7.40	5.87±0.02	2.29±0.02
Stripped	6.67	16.22±1.06	1.69±0.28	6.74	15.77±0.59	2.41±0.19

P_{50} and n_{50} values were estimated from O_2-equilibrium curves fitted by four to nine saturation points (r^2=0.992–0.999).

during metabolic or respiratory acidosis upon switching from aquatic to aerial respiration, where O_2 uptake may be less impaired. Likewise, liberation of Bohr protons upon Hb oxygenation would greatly facilitate CO_2 excretion. Measurements on whole blood have revealed numerous facultative air-breathing fish species that exhibit pronounced Bohr effects, but there is extensive interspecific variation (Shartau and Brauner, 2014; Bayley et al., 2019; Mendez-Sanchez and Burggren, 2019). Given the especially large Bohr effects of the mudskipper isoHbs, it is worth noting that the β-chain C-termini (HC3) have His (Fig. 3B), which is known to play a major role in deoxygenation-linked proton-binding, whereas the cathodic isoHbs of other teleosts such as *Hoplosternum* and *Anguilla* (which exhibit little or no Bohr effect, or even a reverse Bohr effect in the absence of organic phosphates) have non-ionizable Phe at the same residue position (Fago et al., 1995; Weber et al., 2000).

In addition to the hypothesis that obligate and facultative air-breathing fishes have evolved reduced Hb–O_2 affinities compared with obligate water breathers (Johansen and Lenfant, 1972; Johansen et al., 1978; Powers et al., 1979), it has been suggested that facultative air-breathing fishes may have evolved Hbs with reduced Bohr effects to cope with CO_2 retention and/or exposure to hypercarbic water (Carter, 1931). Available data do not appear to support either hypothesis. Among ray-finned fish, lobe-finned fish and amphibians, facultative air and water breathers tend to have higher blood–O_2 affinities than obligate air breathers, and facultative air breathers tend to have slightly larger Bohr effects than members of the other two groups (Bayley et al., 2019). The high intrinsic O_2 affinities and large Bohr effects that we measured for the purified mudskipper isoHbs appear to be consistent with both trends.

Genomic insights into the origins of Hb multiplicity in mudskippers

Given that the two adult-expressed isoHbs of *B. pectinirostris* are present at approximately equimolar concentrations in the red cell, it seemed reasonable to expect that the two distinct α-chain subunits would be encoded by a tandemly linked pair of genes under the transcriptional control of the same *cis*-acting regulatory elements. Such a linkage arrangement could also help explain why the two isoHbs exhibit such similar O_2-binding properties, because tandem duplicates often have similar coding sequences owing to interparalog gene conversion (Hoffmann et al., 2008 a,b; Runck et al., 2009; Storz et al., 2010; Gaudry et al., 2014; Natarajan et al., 2015; Signore et al., 2019). Contrary to this seemingly reasonable expectation, the analysis of conserved synteny revealed that the genes that encode the α-type subunits of HbI and HbII are members of unlinked, paralogous gene clusters that represent the duplicated products of the teleost-specific genome duplication (Figs 1 and 2).

In the phylogenies of α- and β-type globins, the fact that adult-expressed mudskipper genes clustered with some embryonically expressed globin genes of other teleosts is not too surprising, as evolutionary changes in stage-specific expression during ontogeny have been well documented in teleosts (Opazo et al., 2013) and in many tetrapods as well (Opazo et al., 2008; Hoffmann et al., 2010, 2018; Storz, 2016, 2019).

IsoHb multiplicity and lack of functional differentiation

In the case of mudskippers, there is no appreciable difference in the functional properties of co-expressed isoHbs in spite of extensive amino acid divergence between the alternative α-chain subunit isoforms (Fig. 3B). It therefore appears that the ability to switch between aquatic and aerial respiration does not necessarily require a division of labor between functionally distinct isoHbs that are specialized for O_2 transport under different conditions. The adult-expressed isoHbs of many obligate water-breathing fish are known to exhibit much higher levels of functional differentiation than those of mudskippers, including quantitative differences in O_2 affinity and qualitative differences in the mode of allosteric regulation, especially in relation to the magnitude of the Bohr effect and sensitivity to organic phosphates (Weber, 1990, 2000). In many teleost species, the presence of specific isoHbs with functional specializations such as the Root effect (an extreme form of pH sensitivity that plays a key role in oxygen secretion and general tissue O_2 delivery) exert an important influence on physiological capacities (Berenbrink, 2007; Rummer et al., 2013; Randall et al., 2014). However, the absence of functional isoHb differentiation in amphibious mudskippers and other facultative air breathers, and the often pronounced functional heterogeneity in the isoHbs of obligate water breathers, supports the conclusions of several authors (Fyhn et al., 1979; Ingermann, 1997; Wells, 2009; Storz, 2019) that the overall diversity of co-expressed isoHbs in fish red cells is not generally a strong determinant of physiological versatility or ecological niche breadth. In mudskippers, any changes in blood–O_2 affinity that are associated with transitions between aerial and aquatic breathing are likely caused by changes in red cell pH and/or red cell concentrations of nucleotide triphosphates.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.F.S., A.F.; Methodology: C.N., M.K.G., M.V., F.G.H., A.F.; Formal analysis: J.S., C.N., M.V., F.G.H., A.F.; Investigation: C.N., M.K.G., M.V., F.G.H., A.F.; Resources: J.F.S., X.Y., B.V.; Writing - original draft: J.F.S.; Writing - review & editing: C.N., M.V., F.G.H., X.Y., B.V., A.F.; Supervision: J.F.S., A.F.; Project administration: J.F.S.; Funding acquisition: J.F.S.

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References

Bayley, M., Damsgaard, C., Thomsen, M., Malte, H. and Wang, T. (2019). Learning to air-breathe: the first steps. *Physiology* **34**, 14-29. doi:10.1152/physiol.00028.2018

Berenbrink, M. (2007). Historical reconstructions of evolving physiological complexity: O_2 secretion in the eye and swimbladder of fishes. *J. Exp. Biol.* **210**, 1641-1652. doi:10.1242/jeb.003319

Brauner, C. J. and Wang, T. (1997). The optimal oxygen equilibrium curve: a comparison between environmental hypoxia and anemia. *Am. Zool.* **37**, 101-108. doi:10.1093/icb/37.1.101

Cadiz, L., Bundgaard, A., Malte, H. and Fago, A. (2019). Hypoxia enhances blood O_2 affinity and depresses skeletal muscle O_2 consumption in zebrafish (*Danio rerio*). *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* **234**, 18-25. doi:10.1016/j.cbpb.2019.05.003

Carter, G. S. (1931). Aquatic and aerial respiration in animals. *Biol. Rev. Biol. Proc. Camb. Philos. Soc.* **6**, 1-35. doi:10.1111/j.1469-185X.1931.tb01020.x

Damsgaard, C., Findorf, I., Helbo, S., Kocagoz, Y., Buchanan, R., Do Thi Thanh, H., Weber, R. E., Fago, A., Bayley, M. and Wang, T. (2014). High blood oxygen affinity in the air-breathing swamp eel *Monopterus albus*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **178**, 102-108. doi:10.1016/j.cbpa.2014.08.001

Damsgaard, C., Phuong, L. M., Huong, D. T. T., Jensen, F. B., Wang, T. and Bayley, M. (2015). High affinity and temperature sensitivity of blood oxygen binding in *Pangasianodon hypophthalmus* due to lack of chloride-hemoglobin

allosteric interaction. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **308**, R907-R915. doi:10.1152/ajpregu.00470.2014
Fago, A. (2017). Functional roles of globin proteins in hypoxia-tolerant ectothermic vertebrates. *J. Appl. Physiol.* **123**, 926-934. doi:10.1152/japplphysiol.00104.2017
Fago, A., Carratore, V., di Prisco, G., Feuerlein, R. J., Sottrup-Jensen, L. and Weber, R. E. (1995). The cathodic hemoglobin of *Anguilla anguilla*. amino acid sequence and oxygen equilibria of a reverse Bohr effect hemoglobin with high oxygen affinity and high phosphate sensitivity. *J. Biol. Chem.* **270**, 18897-18902. doi:10.1074/jbc.270.32.18897
Fago, A., Bendixen, E., Malte, H. and Weber, R. E. (1997). The anodic hemoglobin of *Anguilla anguilla*. molecular basis for allosteric effects in a root-effect hemoglobin. *J. Biol. Chem.* **272**, 15628-15635. doi:10.1074/jbc.272.25.15628
Fyhn, U. E. H., Fyhn, H. J., Davis, B. J., Powers, D. A., Fink, W. L. and Garlick, R. L. (1979). Hemoglobin heterogeneity in Amazonian fishes. *Comp. Biochem. Physiol. A Physiol.* **62**, 39-66. doi:10.1016/0300-9629(79)90740-0
Gaudry, M. J., Storz, J. F., Butts, G. T., Campbell, K. L. and Hoffman, F. G. (2014). Repeated evolution of chimeric fusion genes in the β-globin gene family of laurasiatherian mammals. *Genome Biol. Evol.* **6**, 1219-1233. doi:10.1093/gbe/evu097
Gillen, R. G. and Riggs, A. (1972). Structure and function of hemoglobins of carp, *Cyprinus carpio*. *J. Biol. Chem.* **247**, 6039-6046.
Graham, J. B. (1997). Breathing air in water and in air: the air-breathing fishes. In: *Air-Breathing Fishes: Evolution, Diversity, and Adaptation*. San Diego: Academic Press.
Graham, J. B. and Wegner, N. C. (2010). *Respiratory Physiology of Vertebrates: Life With and Without Oxygen* (ed. G. E. Nilsson), pp. 174-221. Cambridge: Cambridge University Press.
Grispo, M. T., Natarajan, C., Projecto-Garcia, J., Moriyama, H., Weber, R. E. and Storz, J. F. (2012). Gene duplication and the evolution of hemoglobin isoform differentiation in birds. *J. Biol. Chem.* **287**, 37647-37658. doi:10.1074/jbc.M112.375600
Harter, T. S. and Brauner, C. J. (2017). The O_2 and CO_2 transport system in teleosts and the specialized mechanisms that enhance Hb–O_2 unloading to tissues. **36**, 1-106. doi:10.1016/bs.fp.2017.09.001
Hlastala, M. and Berger, A. (2001). *Physiology of Respiration*. Oxford: Oxford University Press.
Hoffmann, F. G., Opazo, J. C. and Storz, J. F. (2008a). New genes originated via multiple recombinational pathways in the β-globin gene family of rodents. *Mol. Biol. Evol.* **25**, 2589-2600. doi:10.1093/molbev/msn200
Hoffmann, F. G., Opazo, J. C. and Storz, J. F. (2008b). Rapid rates of lineage-specific gene duplication and deletion in the α-globin gene family. *Mol. Biol. Evol.* **25**, 591-602. doi:10.1093/molbev/msn004
Hoffmann, F. G., Storz, J. F., Gorr, T. A. and Opazo, J. C. (2010). Lineage-specific patterns of functional diversification in the α- and β-globin gene families of tetrapod vertebrates. *Mol. Biol. Evol.* **27**, 1126-1138. doi:10.1093/molbev/msp325
Hoffmann, F. G., Opazo, J. C. and Storz, J. F. (2012). Whole-genome duplications spurred the functional diversification of the globin gene superfamily in vertebrates. *Mol. Biol. Evol.* **29**, 303-312. doi:10.1093/molbev/msr207
Hoffmann, F. G., Vandewege, M. W., Storz, J. F. and Opazo, J. C. (2018). Gene turnover and diversification of the α- and β-globin gene families in sauropsid vertebrates. *Genome Biol. Evol.* **10**, 344-358. doi:10.1093/gbe/evy001
Ingermann, R. L. (1997). Vertebrate hemoglobins. *Handb. Physiol. Comp. Physiol.* **30**, 357-408.
Ishimatsu, A. (2012). Evolution of the cardiorespiratory system in air-breathing fishes. *Aqua Biosci. Monogr.* **5**, 1-28. doi:10.5047/absm.2012.00501.0001
Jendroszek, A., Malte, H., Overgaard, C. B., Beedholm, K., Natarajan, C., Weber, R. E., Storz, J. F. and Fago, A. (2018). Allosteric mechanisms underlying the adaptive increase in hemoglobin-oxygen affinity of the bar-headed goose. *J. Exp. Biol.* **221**, jeb185470. doi:10.1242/jeb.185470
Jensen, F. B. (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. doi:10.1111/j.1365-201X.2004.01361.x
Jensen, F. B., Fago, A. and Weber, R. E. (1998). Hemoglobin structure and function. *Fish Physiol.* **17**, 1-40. doi:10.1016/S1546-5098(08)60257-5
Jensen, F. B., Kolind, R. A. H., Jensen, N. S., Montesanti, G. and Wang, T. (2017). Interspecific variation and plasticity in hemoglobin nitrite reductase activity and its correlation with oxygen affinity in vertebrates. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **206**, 47-53. doi:10.1016/j.cbpa.2017.01.010
Johansen, K. and Lenfant, C. (1972). A comparative approach to the adaptability of O_2-Hb affinity. In *Oxygen Affinity of Hemoglobin and Red Cell Acid-Base Status* (ed. P. Astrup and M. Rørth), pp. 750-780. Academic Press.
Johansen, K., Mangum, C. P. and Weber, R. E. (1978). Reduced blood O_2 affinity associated with air breathing in osteoglossid fishes. *Can. J. Zool.* **56**, 891-897. doi:10.1139/z78-124
Katoh, K. and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* **30**, 772-780. doi:10.1093/molbev/mst010
Mandic, M., Todgham, A. E. and Richards, J. G. (2009). Mechanisms and evolution of hypoxia tolerance in fish. *Proc. R. Soc. B Biol. Sci.* **276**, 735-744. doi:10.1098/rspb.2008.1235
Martin, K. L. M. and Bridges, C. R. (1999). Respiration in water and air. In *Intertidal Fishes. Life in Two Worlds* (ed. M. Horn, K. Martin and M. Chotkowski), pp. 54-78. Academic Press. doi:10.1016/B978-012356040-7/50005-6
Mendez-Sanchez, J. F. and Burggren, W. W. (2019). Very high blood oxygen affinity and large Bohr shift differentiates the air-breathing siamese fighting fish (*Betta splendens*) from the closely related anabantoid the blue gourami (*Trichopodus trichopterus*). *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **229**, 45-51. doi:10.1016/j.cbpa.2018.11.014
Meyer, A. and Van de Peer, Y. (2005). From 2R to 3R: evidence for a fish-specific genome duplication (FSGD). *BioEssays* **27**, 937-945. doi:10.1002/bies.20293
Natarajan, C., Hoffmann, F. G., Lanier, H. C., Wolf, C. J., Cheviron, Z. A., Spangler, M. L., Weber, R. E., Fago, A. and Storz, J. F. (2015). Intraspecific polymorphism, interspecific divergence, and the origins of function-altering mutations in deer mouse hemoglobin. *Mol. Biol. Evol.* **32**, 978-997. doi:10.1093/molbev/msu403
Natarajan, C., Hoffmann, F. G., Weber, R. E., Fago, A., Witt, C. C. and Storz, J. F. (2016). Predictable convergence in hemoglobin function has unpredictable molecular underpinnings. *Science* **354**, 336-339. doi:10.1126/science.aaf9070
Nikinmaa, M. (1982). *Vertebrate Red Blood Cells.* Berlin: Springer Verlag.
Olson, K. R. (1994). Circulatory anatomy in bimodally breathing fish. *Am. Zool.* **34**, 280-288. doi:10.1093/icb/34.2.280
Opazo, J. C., Hoffmann, F. G. and Storz, J. F. (2008). Genomic evidence for independent origins of β-like globin genes in monotremes and therian mammals. *Proc. Natl. Acad. Sci. USA* **105**, 1590-1595. doi:10.1073/pnas.0710531105
Opazo, J. C., Butts, G. T., Nery, M. F., Storz, J. F. and Hoffmann, F. G. (2013). Whole-genome duplication and the functional diversification of teleost fish hemoglobins. *Mol. Biol. Evol.* **30**, 140-153. doi:10.1093/molbev/mss212
Opazo, J. C., Lee, A. P., Hoffmann, F. G., Toloza-Villalobos, J., Burmester, T., Venkatesh, B. and Storz, J. F. (2015). Ancient duplications and expression divergence in the globin gene superfamily of vertebrates: insights from the elephant shark genome and transcriptome. *Mol. Biol. Evol.* **32**, 1684-1694. doi:10.1093/molbev/msv054
Powers, D. A. (1980). Molecular ecology of teleost fish hemoglobins: strategies for adapting to changing environments. *Am. Zool.* **20**, 139-162. doi:10.1093/icb/20.1.139
Powers, D. A., Fyhn, H. J., Fyhn, U. E. H., Martin, J. P., Garlick, R. L. and Wood, S. C. (1979). A comparative study of the oxygen equilibria of blood from 40 genera of Amazonian fishes. *Comp. Biochem. Physiol. A Physiol.* **62**, 67-85. doi:10.1016/0300-9629(79)90741-2
Randall, D. J., Cameron, J. N., Daxboeck, C. and Smatresk, N. (1981). Aspects of bimodal gas exchange in the bowfin, *Amia calva* L. (Actinopterygii: Amiiformes). *Respir. Physiol.* **43**, 339-348. doi:10.1016/0034-5687(81)90114-6
Randall, D. J., Rummer, J. L., Wilson, J. M., Wang, S. and Brauner, C. J. (2014). A unique mode of tissue oxygenation and the adaptive radiation of teleost fishes. *J. Exp. Biol.* **217**, 1205-1214. doi:10.1242/jeb.093526
Revsbech, I. G., Tufts, D. M., Projecto-Garcia, J., Moriyama, H., Weber, R. E., Storz, J. F. and Fago, A. (2013). Hemoglobin function and allosteric regulation in semi-fossorial rodents (family Sciuridae) with different altitudinal ranges. *J. Exp. Biol.* **216**, 4264-4271. doi:10.1242/jeb.091397
Rummer, J. L., McKenzie, D. J., Innocenti, A., Supuran, C. T. and Brauner, C. J. (2013). Root effect hemoglobin may have evolved to enhance general tissue oxygen delivery. *Science* **340**, 1327-1329. doi:10.1126/science.1233692
Runck, A. M., Moriyama, H. and Storz, J. F. (2009). Evolution of duplicated β-globin genes and the structural basis of hemoglobin isoform differentiation in *Mus*. *Mol. Biol. Evol.* **26**, 2521-2532. doi:10.1093/molbev/msp165
Rutjes, H. A., Nieveen, M. C., Weber, R. E., Witte, F. and van den Thillart, G. E. E. J. M. (2007). Multiple strategies of lake Victoria cichlids to cope with lifelong hypoxia include hemoglobin switching. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **293**, R1376-R1383. doi:10.1152/ajpregu.00536.2006
Shartau, R. B. and Brauner, C. J. (2014). Acid-base and ion balance in fishes with bimodal respiration. *J. Fish Biol.* **84**, 682-704. doi:10.1111/jfb.12310
Signore, A. V., Yang, Y.-Z., Yang, Q.-Y., Qin, G., Moriyama, H., Ge, R.-L. and Storz, J. F. (2019). Adaptive changes in hemoglobin function in high-altitude Tibetan canids were derived via gene conversion and introgression. *Mol. Biol. Evol.* **36**, 2227-2237. doi:10.1093/molbev/msz097
Stamatakis, A. (2006). RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**, 2688-2690. doi:10.1093/bioinformatics/btl446
Storz, J. F. (2016). Gene duplication and evolutionary innovations in hemoglobin-oxygen transport. *Physiology* **31**, 223-232. doi:10.1152/physiol.00060.2015
Storz, J. F. (2019). *Hemoglobin: Insights into Protein Structure, Function, and Evolution*. Oxford: Oxford University Press.
Storz, J. F., Runck, A. M., Moriyama, H., Weber, R. E. and Fago, A. (2010). Genetic differences in hemoglobin function between highland and lowland deer mice. *J. Exp. Biol.* **213**, 2565-2574. doi:10.1242/jeb.042598
Storz, J. F., Hoffmann, F. G., Opazo, J. C., Sanger, T. J. and Moriyama, H. (2011). Developmental regulation of hemoglobin synthesis in the green anole lizard *Anolis carolinensis*. *J. Exp. Biol.* **214**, 575-581. doi:10.1242/jeb.050443

Storz, J. F., Natarajan, C., Moriyama, H., Hoffmann, F. G., Wang, T., Fago, A., Malte, H., Overgaard, J. and Weber, R. E. (2015). Oxygenation properties and isoform diversity of snake hemoglobins. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **309**, R1178-R1191. doi:10.1152/ajpregu.00327.2015

Wang, T. and Malte, H. (2011). O_2 uptake and transport: the optimal P_{50}. *Encyclopedia of Fish Physiology. From Genome to Environment* (ed. A. P. Farrell). **pp.** 893-898. Elsevier. doi:10.1016/B978-0-12-374553-8.00233-1

Weber, R. E. (1990). Functional significance and structural basis of multiple hemoglobins with special reference to ectothermic vertebrates. *Anim. Nutr. Transport Processes* **6**, 58-75.

Weber, R. E. (1992). Use of ionic and zwitterionic (tris/bistris and HEPES) buffers in studies on hemoglobin function. *J. Appl. Physiol.* **72**, 1611-1615. doi:10.1152/jappl.1992.72.4.1611

Weber, R. E. (2000). Adaptations for oxygen transport: lessons from fish hemoglobins. In *Hemoglobin Function in Vertebrates. Molecular Adaptation to Extreme and Temperate Environments* (ed. G. Di Prisco, B. Giardina and R. E. Weber), pp. 23-37. Berlin: Springer-Verlag.

Weber, R. E. and Jensen, F. B. (1988). Functional adaptations in hemoglobins from ectothermic vertebrates. *Annu. Rev. Physiol.* **50**, 161-179. doi:10.1146/annurev.ph.50.030188.001113

Weber, R. E. and Lykkeboe, G. (1978). Respiratory adaptations in carp blood Influences of hypoxia, red cell organic phosphates, divalent cations and CO_2 on hemoglobin-oxygen affinity. *J. Comp. Physiol. B* **128**, 127-137. doi:10.1007/BF00689476

Weber, R. E., Fago, A., Val, A. L., Bang, A., Van Hauwaert, M. L., Dewilde, S., Zal, F. and Moens, L. (2000). Isohemoglobin differentiation in the bimodal-breathing amazon catfish *Hoplosternum littorale*. *J. Biol. Chem.* **275**, 17297-17305. doi:10.1074/jbc.M001209200

Weber, R. E., Fago, A., Malte, H., Storz, J. F. and Gorr, T. A. (2013). Lack of conventional oxygen-linked proton and anion binding sites does not impair allosteric regulation of oxygen binding in dwarf caiman hemoglobin. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **305**, R300-R312. doi:10.1152/ajpregu.00014.2013

Wells, R. M. G. (2009). Blood-gas transport and hemoglobin function: adaptations for functional and environmental hypoxia. *Fish Physiol.* **27**, 255-299. doi:10.1016/S1546-5098(08)00006-X