

RESEARCH ARTICLE

Genetically based low oxygen affinities of felid hemoglobins: lack of biochemical adaptation to high-altitude hypoxia in the snow leopard

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ABSTRACT

Genetically based modifications of hemoglobin (Hb) function that increase blood–O₂ affinity are hallmarks of hypoxia adaptation in vertebrates. Among mammals, felid Hbs are unusual in that they have low intrinsic O₂ affinities and reduced sensitivities to the allosteric cofactor 2,3-diphosphoglycerate (DPG). This combination of features compromises the acclimatization capacity of blood–O₂ affinity and has led to the hypothesis that felids have a restricted physiological niche breadth relative to other mammals. In seeming defiance of this conjecture, the snow leopard (*Panthera uncia*) has an extraordinarily broad elevational distribution and occurs at elevations above 6000 m in the Himalayas. Here, we characterized structural and functional variation of big cat Hbs and investigated molecular mechanisms of Hb adaptation and allosteric regulation that may contribute to the extreme hypoxia tolerance of the snow leopard. Experiments revealed that purified Hbs from snow leopard and African lion exhibited equally low O₂ affinities and DPG sensitivities. Both properties are primarily attributable to a single amino acid substitution, β2His→Phe, which occurred in the common ancestor of Felidae. Given the low O₂ affinity and reduced regulatory capacity of feline Hbs, the extreme hypoxia tolerance of snow leopards must be attributable to compensatory modifications of other steps in the O₂-transport pathway.

KEY WORDS: Felidae, Oxygen affinity, Amino acid substitution, Blood-oxygen transport, Allosteric regulation

INTRODUCTION

The O₂-transport properties of vertebrate red blood cells exhibit a high degree of plasticity in adjusting to changes in metabolic demands and/or environmental O₂ availability (Nikinmaa, 1990; Weber and Fago, 2004). At high altitude, O₂ uptake may be compromised by the low ambient O₂ tension (P_{O_2}) (Bouverot, 1985; Weber, 2007). As a general rule, the tolerance to low P_{O_2} at high altitude is associated with an increase in hemoglobin (Hb)–O₂ affinity that raises arterial O₂ saturation (Storz et al., 2010a; Weber, 2007). In most mammals, reversible changes in Hb–O₂ affinity are mediated by regulatory adjustments in the erythrocytic

concentration of the organic phosphate 2,3-diphosphoglycerate (DPG) (Mairbaurl and Weber, 2012; Weber, 2007), a potent allosteric regulator of Hb–O₂ affinity (Benesch and Benesch, 1967; Bunn, 1980, 1971). In human Hb, negatively charged DPG electrostatically binds in the symmetric cationic cleft between the paired β-subunits, including strong interactions with positively charged β82Lys and β2His and weaker interactions with the β-chain N-termini and β143His (Richard et al., 1993). As this positively charged cleft is accessible for DPG binding in the low-affinity T quaternary structure of the tetrameric (α₂β₂) Hb but not in the high-affinity R structure, changes in red blood cell DPG levels alter Hb–O₂ affinity by shifting the T–R allosteric equilibrium of the Hb (Weber, 2007). Although Hb–O₂ affinity is also allosterically regulated by chloride ions (Cl[−]), the Cl[−] concentration does not change appreciably in red blood cells (Nikinmaa, 1990). In addition to changes in the concentration of allosteric cofactors, adaptation of mammalian species to high altitude often involves genetically based modifications of the Hb structure that increase Hb's intrinsic O₂ affinity or hamper the protein's ability to bind DPG or Cl[−] (Storz and Moriyama, 2008; Weber and Fago, 2004; Weber, 2007).

Intriguingly, the red blood cells of two mammalian lineages, ruminant artiodactyls and carnivores in the suborder Feliformia (cats, hyenas, mongooses, civets and allies) contain exceedingly low concentrations of DPG (<1.0 mmol l^{−1} versus 4–10 mmol l^{−1} in other mammals) (Bunn et al., 1974; Scott et al., 1977). Moreover, previous studies indicate that the adult Hbs of both groups have independently evolved dramatically reduced O₂ affinities and suppressed sensitivities to DPG (Bunn et al., 1974; Bunn, 1980, 1971; Scott et al., 1976, 1977; Taketa, 1973), features that may limit the capacity to regulate Hb–O₂ affinity.

In mammals with DPG-insensitive Hbs, the reduced plasticity in blood–O₂ affinity may limit physiological niche breadth (Bunn, 1980; Kay, 1977; Scott et al., 1976, 1977). Those taxa that have secondarily lost the capacity to regulate Hb–O₂ affinity through changes in erythrocytic DPG concentration '...are restricted to a much narrower range of possible habitats and/or life-styles', according to Kay (1977). Consistent with this idea, domestic cats are notoriously intolerant of environmental hypoxia (Campbell, 1927; Reeves et al., 1963).

As a denizen of the Himalayan alpine, the snow leopard, *Panthera uncia*, defies conventional wisdom about restricted physiological tolerances among cats in general, and dispels the notion that susceptibility to hypoxic stress is a characteristic feline trait. Snow leopards are most common above the tree line at elevations between 3500 and 5000 m (Sunquist and Sunquist, 2002), although they have been recorded at elevations above 6000 m (where P_{O_2} is less than 50% of the sea level value). However, they are not restricted to these zones; in parts of their range including the Gobi Desert of Mongolia,

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List of abbreviations	
DPG	2,3-diphosphoglycerate
Hb	hemoglobin
IEF	isoelectric focusing
<i>n</i>	cooperativity (Hill) coefficient
P_{50}	O ₂ tension at half-saturation
P_{O_2}	O ₂ tension

they also occur at low elevations (1500–2000 m) (Janečka et al., 2011). In the Himalaya, the home ranges of individual snow leopards can span >2000 m in elevation. The remarkably broad elevational distribution of snow leopards and their ability to tolerate severe ambient hypoxia prompt questions about possible mechanisms of biochemical adaptation in O₂ transport. Here, we addressed these questions by determining the amino acid sequences of the α- and β-subunits of adult Hbs from a number of species of the cat family

(Felidae) and by comparing functional properties of snow leopard Hbs with those of a representative lowland species of the same genus, the African lion, *Panthera leo*, to investigate possible mechanisms of biochemical adaptation to high-altitude hypoxia.

RESULTS

Low levels of structural variation among felid Hbs

We examined structural variation in the adult-expressed Hb isoforms of five big cat species in the genus *Panthera*: snow leopard, *P. uncia* (Schreber 1775); African lion, *P. leo* (Linnaeus 1758); tiger, *P. tigris* (Linnaeus 1758); leopard, *P. pardus* (Linnaeus 1758); and jaguar, *P. onca* (Linnaeus 1758). Our sequencing results revealed that all five big cat species share the same complement of four adult-expressed Hb genes found in the domestic cat (Gaudry et al., 2014), including a tandemly linked pair of α-globin genes that are identical in amino acid sequence,

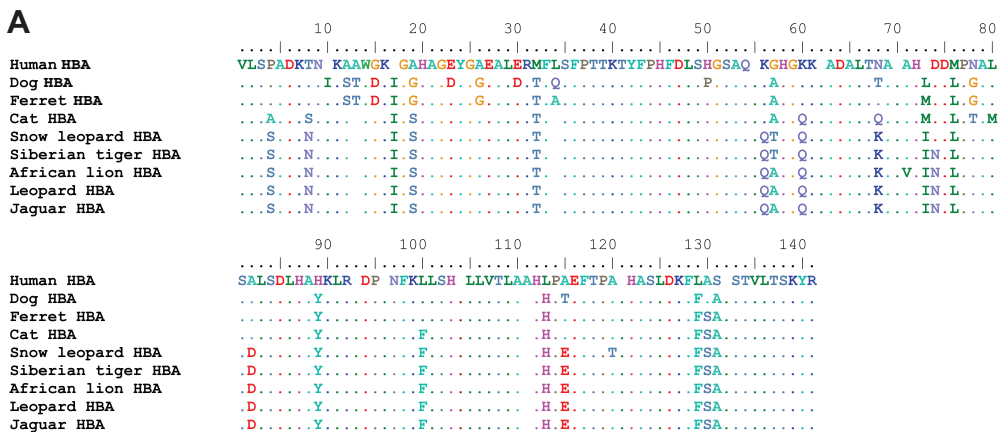
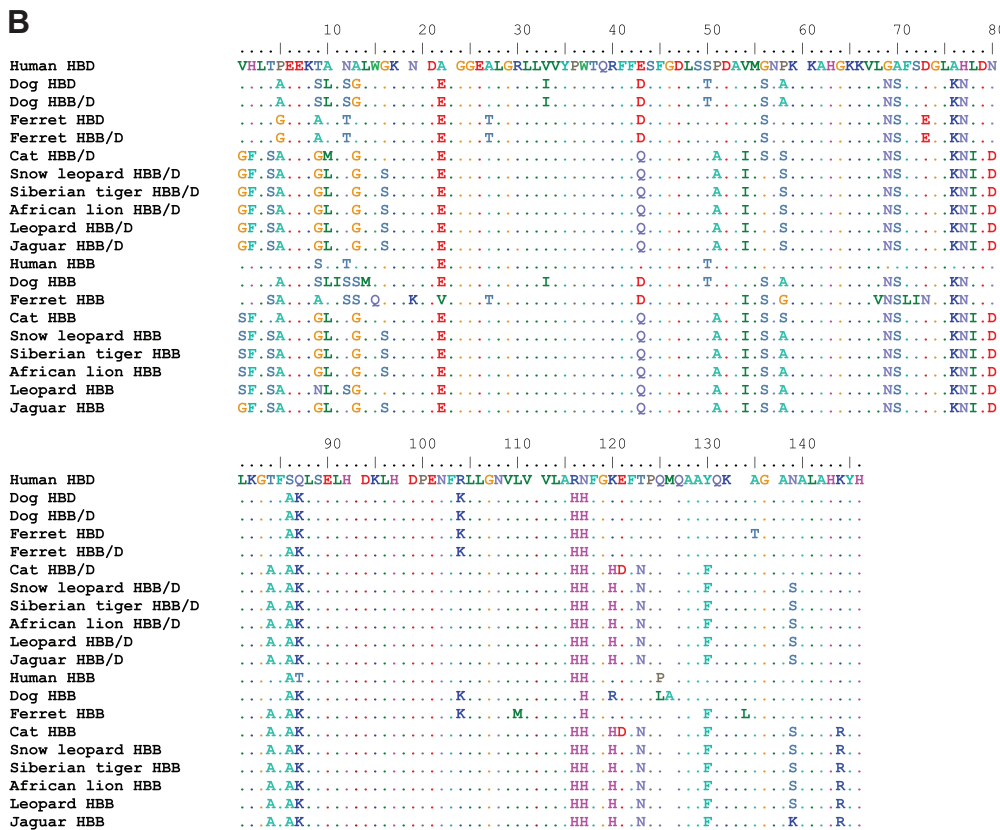


Fig. 1. Multiple sequence alignment of adult-expressed α- and β-type globins of cats and other eutherian mammals. (A) HBA α-globin genes and (B) HBB/D and HBB β-type globin genes.



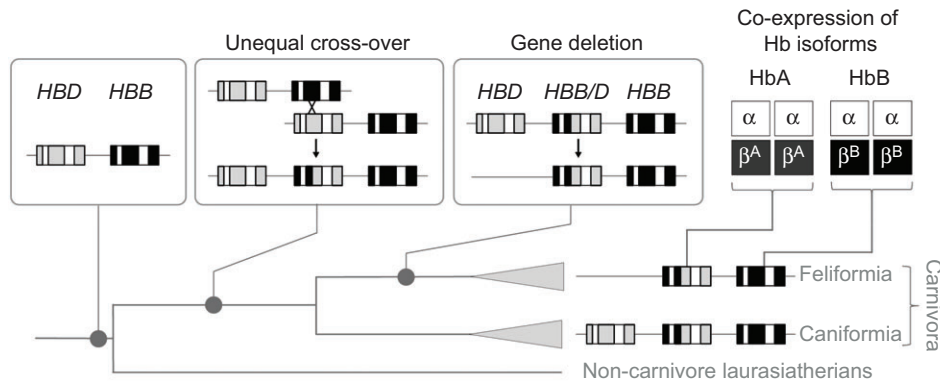


Fig. 2. History of structural changes in the adult-expressed *HBD* and *HBB* β -globin genes of feliform carnivores. The ancestor of laurasiatherian mammals [the supraordinal group that includes carnivores, as well as bats, pangolins, eulipotyphlans (shrews, moles and hedgehogs), perissodactyls and cetartiodactyls] possessed a tandemly linked pair of *HBD* and *HBB* genes. In the stem lineage of carnivores, an unequal cross-over event produced a duplication in the chromosome now containing a chimeric *HBB/D* fusion gene, flanked by the parental *HBD* and *HBB* genes on the 5' and 3' sides, respectively (Gaudry et al., 2014). In the common ancestor of felids (or possibly in the common ancestor of all feliform carnivores), the 5' *HBD* gene was deleted after divergence from the ancestor of caniform carnivores. Consequently, adult cats co-express two structurally distinct hemoglobin (Hb) isoforms: HbA (which incorporates β -chain products of the chimeric *HBB/D* gene) and HbB (which incorporates β -chain products of the *HBB* gene). The two isoforms share identical α -chain subunits.

and a tandemly linked pair of β -type globin genes (*HBB/D* and *HBB*) that are distinguished from one another by four to eight amino acid substitutions (Fig. 1). The β -type *HBB/D* gene is a chimeric fusion gene, which originated via unequal crossing over between the tandemly linked δ - and β -globin genes *HBD* and *HBB*, respectively (Gaudry et al., 2014) (Fig. 2). This recombination event occurred in the stem lineage of carnivores (Fig. 2), so the same fusion gene is shared with members of the carnivoran suborder Caniformia (Gaudry et al., 2014). The other β -globin gene is a non-chimeric *HBB* gene, which is orthologous to the adult β -globin gene of humans and most other mammals (Hoffmann et al., 2008; Opazo et al., 2008a,b, 2009). Remarkably, orthologs of *HBB/D* and *HBB* in snow leopard, tiger and African lion are completely invariant at the amino acid level (Fig. 1B). In each of these three species, the same four substitutions (at sites $\beta 1$, $\beta 56$, $\beta 58$ and $\beta 144$) distinguish the *HBB/D* and *HBB* paralogs (Fig. 1B). *HBA* orthologs of snow leopard and African lion differ at a total of four sites, $\alpha 57$, $\alpha 71$, $\alpha 74$ and $\alpha 120$, and these are the only *HBA* sites that vary among species in the genus *Panthera* (Fig. 1A).

In analogy with the domestic cat, products of each of the two β -globin genes *HBB/D* and *HBB* are incorporated into two distinct Hb isoforms, HbA and HbB, respectively, which share the same α -chain subunit encoded by the two identical *HBA* genes (Abbasi and Braunitzer, 1985; Taketa et al., 1968) (Fig. 2). The tandemly linked *HBB/D* and *HBB* genes share the same $\beta 2\text{His} \rightarrow \text{Phe}$ substitution as in the domestic cat (Fig. 1B), as a result of a history of interparalog gene conversion (Gaudry et al., 2014). Thus, the feline HbA and HbB isoforms are all missing a major residue (His $\beta 2$) involved in DPG binding. *HBB* has an additional substitution at the adjacent N-terminal residue position, $\beta 1\text{Val} \rightarrow \text{Ser}$, which is acetylated in the native HbB. Overall, our survey of sequence variation revealed very little structural variation among the adult-expressed Hbs of big cat species in the genus *Panthera* (Fig. 1).

Hb isoform composition of big cat red blood cells

We characterized the Hb isoform composition of red blood cells from the five species of big cats that comprise the genus *Panthera* (snow leopard, tiger, African lion, leopard and jaguar). Isoelectric focusing (IEF) analysis resolved the hemolysates of each species

into three major bands and one minor (more cathodic) band with identical mobilities across species (Fig. 3A). Similar to the case of the domestic cat (Hamilton and Edelstein, 1974), ion-exchange chromatography separated lion and snow leopard hemolysates into two distinct peaks, representing native HbA and HbB isoforms in an almost equimolar ratio (49:51 and 47:53, for lion and snow leopard, respectively). On IEF gels, each of these two peaks resolved into two bands with mobilities corresponding to two of the three major bands of the hemolysate (Fig. 3B).

The fact that the *HBB/D* and *HBB* paralogs differ by only four amino acid residues (at positions $\beta 1$, $\beta 56$, $\beta 58$ and $\beta 144$) suggests that the β -chain products of both genes may be incorporated in the same tetrameric Hb. This is supported by the finding of three major bands of each feline hemolysate on IEF gels (Fig. 3). Each band may represent symmetric $\alpha_2\beta^A\beta^A$ and $\alpha_2\beta^B\beta^B$ Hb tetramers along with a stable asymmetric hybrid $\alpha_2\beta^A\beta^B$ tetramer of intermediate mobility (where β^A and β^B indicate the products of genes *HBB/D* and *HBB*, respectively) (Fig. 3). Thus, feline red blood cells appear to contain the two parental symmetric isoforms HbA and HbB as well as their hybrid molecule. Feline Hbs are characterized by a relatively high tetramer–dimer dissociation constant (Hamilton and Edelstein, 1974), which may further favor hybrid tetrameric Hb assemblies in the red blood cell. Overall, the possession of structurally similar

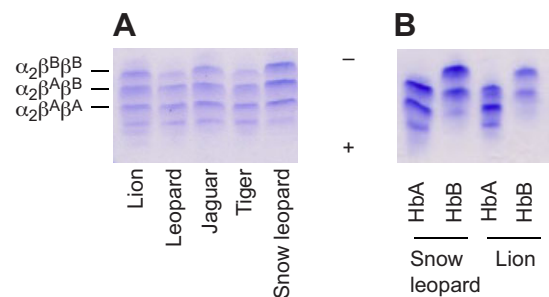


Fig. 3. Isoelectric focusing gels (pH 3–9) and isoHb multiplicity in big cats. (A) Red blood cell lysates from lion, leopard, jaguar, tiger and snow leopard, and (B) purified HbA and HbB components from snow leopard and lion, showing highly similar patterns. The putative α - and β -subunit composition of each of the three major bands is indicated, with β^A and β^B corresponding to the products of genes *HBB/D* and *HBB*, respectively. Plus sign, cathode; minus sign, anode.

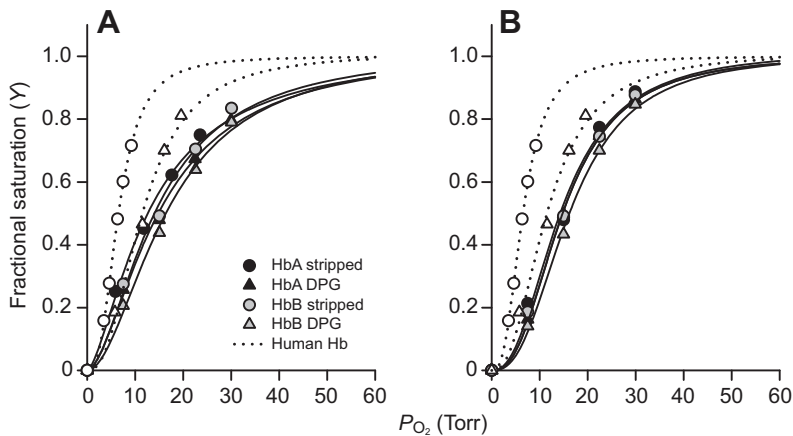


Fig. 4. Hb–O₂ equilibrium curves of big cats. Hb–O₂ equilibrium curves of African lion (A) and snow leopard (B) HbA and HbB are insensitive to 2,3-diphosphoglycerate (DPG) and are right-shifted compared with those of human adult Hb (open symbols, dotted lines). Data were obtained at a heme concentration of 0.3 mmol l⁻¹, 37°C, in 0.1 mol l⁻¹ Hepes buffer, pH 7.4, in the absence (circles) and presence of DPG (triangles), at a 2-fold molar excess of DPG over tetrameric Hb. Non-linear regression of the O₂-saturation data according to the sigmoidal Hill equation is shown for each of the conditions used. 1 Torr ≈ 133 Pa.

isoHb components among the *Panthera* species, as evident from the IEF patterns (Fig. 3), is consistent with the remarkably low level of interspecific variation in α - and β -globin primary structures (Fig. 1).

Oxygenation properties of big cat Hbs

To examine possible differences in the oxygenation properties of big cat Hbs, we measured O₂-equilibrium curves for purified HbA and HbB isoforms of lion and snow leopard under standardized conditions of pH and anion concentrations. For comparison, O₂-equilibrium curves were also measured for human Hb under identical buffer conditions (Fig. 4). Under cofactor-free (stripped) conditions, these experiments revealed uniformly low Hb–O₂ affinities (i.e. high P_{50} , the O₂ tension at half-saturation) for both isoforms compared with human Hb (Fig. 4, Table 1). Hill coefficients of both lion and snow leopard isoforms (Table 1) indicated normal cooperative O₂ binding. O₂-equilibrium curves of HbA and HbB isoforms from lion (Fig. 4A) and snow leopard (Fig. 4B) were highly similar and were almost completely unresponsive to the addition of DPG, in contrast to those of human Hb, as shown by the similar P_{50} values obtained in the absence and presence of DPG (Fig. 4, Table 1). However, in both lion and snow leopard, the O₂ affinities of HbA and HbB were markedly reduced in the presence of 0.1 mol l⁻¹ Cl⁻, indicating that these Hbs are sensitive to Cl⁻ (Fig. 5, Table 1).

DISCUSSION

Structural and functional variation of feline Hbs

Consistent with the low level of structural variation among big cat Hbs, O₂-equilibrium experiments revealed no appreciable

differences in oxygenation properties between the purified HbA and HbB isoforms isolated from either snow leopard or African lion (Table 1), demonstrating that the four amino acid substitutions that distinguish the *HBB/D* and *HBB* paralogs do not have any functionally important net effect. This finding also indicates that, if present, hybrid Hb tetramers of the type $\alpha_2\beta^A\beta^B$ would have the same O₂-binding properties as the parental $\alpha_2\beta^A\beta^A$ and $\alpha_2\beta^B\beta^B$ Hbs. Moreover, the experiments did not reveal any appreciable differences in Hb–O₂ affinity between the high-altitude, hypoxia-adapted snow leopard and the predominantly lowland African lion, as isoform-specific P_{50} values for the two species were very similar across all treatments (Table 1). As shown in Fig. 1, the amino acid sites that differ between the Hbs of snow leopard and African lion are among the only sites that vary in the Hbs of all big cats. Thus, our examination of structural and functional variation of feline Hbs indicates that all members of the genus *Panthera* possess functionally similar Hbs.

A single amino acid substitution is associated with suppressed DPG sensitivity and reduced O₂ affinity of big cat Hbs

The low intrinsic O₂ affinities, suppressed DPG sensitivities and large Cl⁻ effects of the big cat Hbs (Table 1) relative to human Hb can be considered characteristic features of feline Hbs, as they are consistent with previously described properties of cat Hbs (Baumann and Haller, 1975; Hamilton and Edelstein, 1974; León-Velarde et al., 1996; Parer et al., 1970; Taketa et al., 1971; Taketa, 1973). Specifically, the suppressed DPG sensitivities of snow leopard and African lion HbA and HbB isoforms (Figs 4, 5)

Table 1. O₂ affinity (P_{50}) and cooperativity (Hill) coefficients (n) of African lion and snow leopard HbA and HbB and of human Hb

	Stripped		+KCl		+DPG		+KCl+DPG	
	P_{50}	n	P_{50}	n	P_{50}	n	P_{50}	n
African lion								
HbA	13.03±0.59	1.52±0.11	20.73±1.55	1.74±0.23	15.21±0.71	1.68±0.13	21.38±0.85	1.41±0.08
HbB	14.39±0.98	1.75±0.20	25.64±1.44	1.98±0.21	16.72±0.72	1.87±0.15	26.95±1.38	1.93±0.17
Snow leopard								
HbA	14.47±0.93	2.36±0.31	23.17±0.83	2.48±0.23	14.81±0.20	2.50±0.07	22.99±0.75	2.46±0.20
HbB	14.74±0.51	2.41±0.17	22.30±0.56	2.53±0.16	16.27±0.40	2.55±0.14	23.30±0.46	2.66±0.20
Human								
Hb	6.48±0.05	2.70±0.05	12.98±0.25	2.60±0.17	11.58±0.62	2.41±0.28	13.27±0.53	2.46±0.22

Values (means±s.e.m.) were calculated from non-linear regression of four to six saturation steps using the sigmoidal Hill equation. P_{50} values are in Torr (where 1 Torr ≈ 133 Pa).

Measurements were made at 37°C in 0.1 mol l⁻¹ Hepes buffer, pH 7.4, in the absence (stripped) and presence of 0.1 mol l⁻¹ KCl and 2,3-diphosphoglycerate (DPG) (2-fold molar excess over tetrameric hemoglobin, Hb) added individually and in combination.

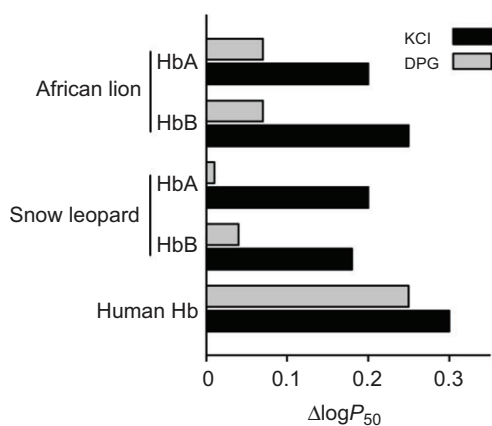


Fig. 5. Allosteric regulation of Hb–O₂ affinity (P_{50}) by DPG and Cl⁻ ions in African lion and snow leopard HbA and HbB. Sensitivity to DPG and Cl⁻ anions is indexed by the difference in log-transformed P_{50} values (reported in Table 1) measured in the presence and absence (stripped) of each ionic cofactor. Human Hb–O₂ affinity with a high sensitivity to both DPG and Cl⁻ is shown as a comparison.

are chiefly attributable to the single $\beta 2\text{His} \rightarrow \text{Phe}$ substitution shared by both *HBB/D* and *HBB* genes (and also shared by the corresponding orthologs of domestic cat and other big cat species) (Fig. 1B) (Gaudry et al., 2014). As the $\beta 1\text{Val} \rightarrow \text{Ser}$ substitution in the *HBB* gene only affects the HbB isoform, and as our experiments revealed no appreciable differences in DPG sensitivity between the HbA and HbB isoforms of snow leopard or lion (Table 1, Fig. 5), it appears that replacing $\beta 1\text{Val}$ with acetylated Ser does not impair DPG binding beyond that induced by $\beta 2\text{His} \rightarrow \text{Phe}$ alone. This interpretation agrees with the minor role of the β -subunit N-terminus in DPG binding (Richard et al., 1993) compared with the original model for DPG binding proposed by Arnone (1972). In addition to impairing DPG binding, the $\beta 2\text{His} \rightarrow \text{Phe}$ substitution that exchanges a basic residue with an apolar one also contributes to the reduced intrinsic O₂ affinity typical of feline Hb isoforms by moving the β -chain N-termini towards the center of the Hb molecule, thereby stabilizing the T-state conformation (Perutz et al., 1993; Safo and Abraham, 2001) even when the heme is fully ligated, as found for domestic cat Hb (Balasubramanian et al., 2014). Similarly, the low O₂ affinity and DPG insensitivity of bovine Hb is also attributable to amino acid substitutions in the β -subunit N-termini ($\beta 1\text{Val}$ deleted and $\beta 2\text{His} \rightarrow \text{Met}$), which stabilize the low-affinity T state and prevent DPG access to the central cavity (Perutz et al., 1993; Safo and Abraham, 2001). Although amino acid substitutions replacing the key $\beta 2\text{His}$ with uncharged residues may cause a high Hb–O₂ affinity as a result of weakened DPG binding in species adapted to high altitudes (Weber and Fago, 2004; Weber, 2007), we find the opposite functional effect of the same substitution (i.e. the Hb–O₂ affinity is reduced) when it is combined with a more hydrophobic N-terminal segment of the β -subunit.

Allosteric regulatory control of feline Hbs

Although feline Hbs have severely reduced capacities for allosteric regulatory control by DPG (Fig. 5), HbA and HbB are both strongly modulated by Cl⁻ ions, as indicated by the increased Hb P_{50} in the presence of 0.1 mol l⁻¹ Cl⁻ (Table 1, Fig. 5). Although Cl⁻ ions present in red blood cells will affect P_{50} *in vivo*, this may not fully compensate for the reduced DPG regulatory capacity, as the Cl⁻ concentration typically exhibits only minor fluctuations mainly

caused by the influx and efflux of bicarbonate ions (Nikinmaa, 1990).

It is clear that the loss of DPG sensitivity caused by the $\beta 2\text{His} \rightarrow \text{Phe}$ substitution effectively eliminates a mechanism of phenotypic plasticity that plays a well-documented role in the acclimatization response to hypoxia in many other eutherian mammals (MacArthur, 1984; Tufts et al., 2013; Weber and Fago, 2004). The reduced intrinsic O₂ affinity of feline Hb clearly compensates for the loss of DPG-mediated regulation of O₂ binding, otherwise the constitutively elevated blood–O₂ affinity would impair O₂ unloading to the cells of respiring tissues. This is evidenced by the fact that human Hb mutants with suppressed DPG sensitivity are invariably associated with erythrocytosis (often at clinically pathological levels) caused by inadequate tissue oxygenation (Percy et al., 2009).

Hypoxia tolerance of snow leopards is not associated with specialized Hb adaptations

The results of our experiments clearly demonstrate that the ability of snow leopards to tolerate high-altitude hypoxia cannot be explained by the evolution of Hbs with specialized oxygenation properties. Snow leopards possess typical feline Hbs, characterized by a low intrinsic O₂ affinity and a restricted capacity for allosteric regulatory control. In fact, Hbs of all felid species appear to have remarkably similar respiratory properties, despite extensive variation in body size, from 2 kg domestic cats to 220 kg African lions.

Studies of Hb function in high-altitude birds and mammals typically reveal distinctive increases in Hb–O₂ affinity relative to closely related, similar-sized lowland taxa (Natarajan et al., 2013; Projecto-Garcia et al., 2013; Storz et al., 2009, 2010b; Storz and Moriyama, 2008; Tufts et al., 2015; Weber, 2007). In a few cases there is no evidence for differentiation in Hb function between species or conspecific populations that are native to different elevations (Cheviron et al., 2014; Natarajan et al., 2015; Revsbech et al., 2013; Storz et al., 2007). The snow leopard represents one more example of a high-altitude species that tolerates chronic altitudinal hypoxia in spite of the fact that it possesses Hbs that are no different from those of its closest lowland relatives. In parts of Nepal, India and Bhutan the distribution of snow leopards, leopards and tigers is partitioned by altitude. Leopards and tigers are known to occur at elevations up to 3000 m, but the snow leopard is the only big cat species that is a permanent resident of the high alpine. Given the low O₂ affinity and reduced regulatory capacity of feline Hbs, the ability of snow leopards to tolerate the severe hypoxia of their high-altitude environment must be attributable to compensatory modifications of other convective and conductive steps in the O₂-transport pathway.

In humans, theoretical analyses indicate that changes in ventilation, pulmonary diffusion capacity and tissue diffusion capacity exert strong influences on O₂ consumption and exercise performance at high altitude (Wagner, 1996). Under severe hypoxia, tissue diffusion capacity is a limiting factor for O₂ consumption when blood P_{50} is low (e.g. in species with high Hb–O₂ affinity). By contrast, in snow leopards and other species that have a low Hb–O₂ affinity, venous blood would be largely desaturated under conditions of severe hypoxia compared with humans (Cambier et al., 2004), so an increase in tissue diffusion capacity would lead to comparatively smaller improvements in the overall level of tissue oxygenation. However, an increase in lung size (which increases the surface area for pulmonary O₂ diffusion) and an enhanced hypoxic ventilatory response could increase arterial P_{O_2} and thus improve exercise performance under hypoxia

regardless of blood P_{50} , as in some high-altitude birds (Scott and Milsom, 2006). Because hypoxia-induced hyperventilation can cause respiratory hypocapnia/alkalosis, it is typically beneficial for high-altitude vertebrates to maintain a higher ventilation rate in spite of the reduced blood P_{CO_2} and increased pH (Scott and Milsom, 2007). Thus, an enhanced capacity to increase ventilation under severe hypoxia could help compensate for the low Hb–O₂ affinity of snow leopards living at high altitude. Similarly, humans that are indigenous to the Tibetan Plateau do not possess Hbs with specialized oxygenation properties, and their ability to tolerate severe hypoxia appears to be partly attributable to the maintenance of high resting ventilation and a brisk hypoxic ventilatory response (Beall et al., 1997; Hackett et al., 1980; Zhuang et al., 1993).

MATERIALS AND METHODS

Blood sample collection

The collection of blood samples was approved by the Clinical Research and Review Committee of the College of Veterinary Medicine and Biomedical Science, Texas A&M University (permit CRRC no. 10-44). We obtained blood samples from captive, adult animals representing five big cat species, including four snow leopards (*P. uncia*, San Francisco Zoo, USA and Philadelphia Zoo, USA), two African lions (*P. leo*, San Francisco Zoo), one tiger (*P. tigris*, Center for Animal Research and Education, Bridgeport, TX, USA), one common leopard (*P. pardus*, Feline Conservation Center, Rosamond, CA, USA) and one jaguar (*P. onca*, Feline Conservation Center). The individual snow leopards that we examined (studbook numbers 1650, 1850, 2662, and 2573) were descendants of wild-caught animals derived from high-altitude regions of Kazakhstan, Kyrgyzstan, China and Russia (Blomqvist, 2008). For each individual animal, approximately 7 ml of blood was collected by means of standard venipuncture. All blood samples were collected by qualified veterinarians during the course of routine medical examinations.

Processing of blood samples

Following blood collection, samples were shipped to the laboratory on an ice pack and were processed immediately after being received. Red blood cells were isolated using the following protocol. A 20 μ l volume of blood was suspended in 80 μ l of 0.85% NaCl saline solution, gently mixed, and centrifuged at 3000 *g* for 5 min. The supernatant was removed and 100 μ l of the saline solution was added and samples were incubated on ice for 15 min, followed by centrifugation at 10,000 *g* for 30 min. Isolated red blood cells were stored at -80°C until analysis. DNA was extracted from blood samples using the Qiagen Genra PureGene Blood Kit (Qiagen, Venlo, The Netherlands) following the manufacturer's recommendations.

PCR and molecular cloning

We used the domestic cat genome reference sequence to design paralog-specific primers for the *HBA*, *HBB/D* and *HBB* genes of the five big cat species. Following DNA isolation from samples of each individual specimen, we PCR-amplified full-length *HBA*, *HBB/D* and *HBB* genes using the following paralog-specific primers: *HBA*-29F: CACCTTCTGG-TCCCGACAC, *HBA*-1032R: TGAACACGGTTCAGCACATT, *HBB/D*-264F: CATACCCTTGAAGGTGGACA, *HBB/D*-1871R: TGGCTGTCA-TCATTGAGACC, *HBB*-92F: TGGGCATAAAAGGAAGAGCA and *HBB*-1633R: GCAGGATCTGTTTCCACAT. The *HBA* gene was amplified using the following PCR conditions: 1 \times Qiagen Type-it Master Mix (HotStarTaq Plus DNA Polymerase, 3.0 mmol l⁻¹ MgCl₂, 200 μ mol l⁻¹ dNTPs), 0.480 μ mol l⁻¹ of each primer and 15 ng of DNA in a 50 μ l reaction volume. The cycling conditions included a 95 $^{\circ}\text{C}$, 5 min activation step, followed by 10 cycles of 95 $^{\circ}\text{C}$ for 30 s, 65–55 $^{\circ}\text{C}$ for 30 s (1 $^{\circ}\text{C}$ reduction per cycle) and 72 $^{\circ}\text{C}$ for 2.0 min, and 20 cycles of 95 $^{\circ}\text{C}$ for 30 s, 55 $^{\circ}\text{C}$ for 30 s and 72 $^{\circ}\text{C}$ for 2.0 min, followed by a final extension of 7 min. PCR amplicons were purified using the Qiagen QIAquick PCR Purification Kit and cloned with the NEB PCR Cloning Kit (New England Biolabs, Ipswich, MA, USA) following manufacturer recommendations. The *HBB/D* and *HBB* genes were amplified using the following PCR conditions:

Applied Biosystems AmpliTaq Gold 1 \times 360 PCR Buffer (Life Technologies, Valencia, CA, USA), 0.2 units of AmpliTaq Gold *taq*, 2.0 mmol l⁻¹ MgCl₂, 200 μ mol l⁻¹ dNTPs, 0.480 μ mol l⁻¹ of each primer and 15 ng of DNA in a 50 μ l reaction volume. The cycling conditions included a 95 $^{\circ}\text{C}$, 10 min activation step, followed by 10 cycles of 95 $^{\circ}\text{C}$ for 30 s, 65–55 $^{\circ}\text{C}$ for 30 s (1 $^{\circ}\text{C}$ reduction per cycle) and 72 $^{\circ}\text{C}$ for 1.5 min, and 20 cycles of 95 $^{\circ}\text{C}$ for 30 s, 55 $^{\circ}\text{C}$ for 30 s and 72 $^{\circ}\text{C}$ for 1.5 min, followed by a final extension of 10 min. The cloning was conducted in two independent replicates. We randomly selected a total of 16 colonies per individual for each gene (i.e. eight colonies from each cloning replicate). These were PCR amplified in 25 μ l reactions with the following conditions: NEB LongAmp 1 \times PCR Buffer (contained 2.0 mmol l⁻¹ MgSO₄), 2.5 units of LongAmp *taq*, 300 μ mol l⁻¹ dNTPs and 0.300 μ mol l⁻¹ of each primer. Cycling conditions included a 94 $^{\circ}\text{C}$, 2 min activation step, followed by 30 cycles of 94 $^{\circ}\text{C}$ for 15 s, 57 $^{\circ}\text{C}$ for 15 s and 68 $^{\circ}\text{C}$ for 1.5 min, followed by a final 68 $^{\circ}\text{C}$ extension of 5 min. All PCR products were cleaned and sequenced using ABI BigDye chemistry (Beckman Coulter, Pasadena, CA, USA). We cloned and sequenced the full complement of adult-expressed α - and β -type globin genes in each individual that was used as a subject for the experimental analysis of Hb function and isoform composition (see supplementary material Table S1). We generated data for the remaining individuals via direct sequencing of diploid PCR products. All newly generated sequences were submitted to GenBank under accession nos KR818795–KR818811 (see supplementary material Table S1).

We augmented our newly generated sequence data for the five big cat species with globin sequences annotated from genome assemblies of domestic cat and Siberian tiger (*P. tigris altaica*). For comparative purposes, we also included the full complement of adult α - and β -like genes from human and two caniform carnivores, dog (*Canis lupus familiaris*, Canidae) and ferret (*Mustela putorius furo*, Mustelidae).

Characterization of Hb isoform composition

Frozen blood samples from each specimen were lysed as previously described (Revsbech et al., 2013) and Hb multiplicity was analyzed by IEF (pH 3–9) on polyacrylamide gels (Phastgel, GE Healthcare Biosciences AB, Uppsala, Sweden). For lion and snow leopard hemolysates, separation of Hb isoforms and simultaneous removal (stripping) of endogenous DPG were achieved by anion-exchange chromatography using an Äkta Pure chromatography system and a Hi-Trap Q 5 ml column (both GE Healthcare) equilibrated with 20 mmol l⁻¹ Tris-HCl buffer pH 8.26, 0.5 mmol l⁻¹ EDTA, and eluted using a 0–0.3 mol l⁻¹ NaCl gradient over 80 min, at a flow rate of 1 ml min⁻¹. Fractions containing HbA and HbB were pooled, concentrated by ultrafiltration (heme concentration >1 mmol l⁻¹), dialyzed against 10 mmol l⁻¹ Hepes buffer pH 7.6 to remove NaCl in the elution buffer and stored at -80°C in aliquots. Heme concentration was measured spectrophotometrically using published extinction coefficients (van Assendelft and Zijlstra, 1975). The purity of separate Hb isoforms was checked by IEF. For comparison of functional properties, human Hb was prepared and stripped of DPG as described elsewhere (Weber, 1992), dialyzed against 10 mmol l⁻¹ Hepes buffer pH 7.6 and stored at -80°C .

Measurement of Hb–O₂ equilibria

O₂ equilibrium curves of isolated Hbs from snow leopard, African lion and human were measured using 5 μ l samples in 0.1 mol l⁻¹ Hepes buffer pH 7.4, 0.5 mmol l⁻¹ EDTA using a thin-layer chamber technique described in detail elsewhere (Cheviron et al., 2014; Damsgaard et al., 2013; Storz et al., 2009; Tufts et al., 2015; Weber, 1992). For each curve, measures of O₂ affinity (P_{50} , the P_{O_2} at which Hb is half-saturated with O₂) and cooperativity (Hill) coefficients n were calculated by non-linear regression of four to six saturation steps using the sigmoidal Hill equation $Y = P_{O_2}^n / (P_{50}^n + P_{O_2}^n)$, where Y is the fractional saturation (Tufts et al., 2015). Experiments were performed at 37 $^{\circ}\text{C}$ and at a heme concentration of 0.3 mmol l⁻¹ in the absence (stripped) and presence of 0.1 mol l⁻¹ KCl and DPG (2-fold molar excess over Hb tetramer), that approximate standard physiological levels within red blood cells of most mammals. Cl⁻ and DPG were added separately or in combination, to isolate the allosteric effects of each cofactor on Hb–O₂ affinity (expressed as log P_{50} differences with and without cofactor) (Tufts et al., 2015). By contrast, previous

studies of cat Hbs (Bunn, 1971; Hamilton and Edelstein, 1974; Taketa, 1973) measured O₂ equilibria using buffer solutions that contained Cl⁻ ions and inorganic phosphates, which potentially perturb DPG binding to Hb (Weber, 1992).

Acknowledgements

We thank G. R. Scott and two anonymous reviewers for helpful suggestions that improved the manuscript. We thank R. Jenkins and J. Jencek (San Francisco Zoo), K. Hinshaw and J. Blough (Philadelphia Zoo), J. Maynard (Feline Conservation Center) and H. Krahn (Center for Animal Research and Education) for providing blood samples, and B. Palmer (Denver Zoo) for providing pedigree information for captive snow leopards. We thank E. Ellebæk Petersen (Aarhus University) for excellent technical support and R. Jackson (Snow Leopard Conservancy) for additional assistance with the snow leopard initiative.

Competing interests

The authors declare no competing or financial interests.

Author contributions

J.E.J. and T.A. carried out the cloning and sequencing; F.G.H., J.E.J., and J.F.S. analyzed the sequence data; S.S.E.N. and S.D.A. carried out the isohemoglobin separations and O₂-equilibrium experiments; S.S.E.N., S.D.A. and A.F. analyzed the functional data; F.G.H. and R.E.W. critically revised the manuscript and helped with data interpretation; J.E.J., J.F.S. and A.F. conceived the research; J.F.S. and A.F. designed the experiments, analysed the data and wrote the manuscript. All authors contributed critically to manuscript writing and approved the final version.

Funding

This work was supported by the Danish Council for Independent Research, Natural Sciences (grant no. 10-084-565 to A.F.), the Carlsberg Foundation (grant no. 2013-01-0178 to A.F.), the National Institutes of Health (NIH grant no. HL087216 to J.F.S.), the Faculty of Science and Technology, Aarhus University (to R.E.W.), the Snow Leopard Conservancy (to J.E.J.) and the Faculty Development Fund of Duquesne University (to J.E.J.). Deposited in PMC for release after 12 months.

Supplementary material

Supplementary material available online at <http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.125369/-/DC1>

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Table S1. Sequences of adult-expressed α - and β -type globin genes that were used in the reference alignment.

Species	GenBank accession number	Label
Human, <i>Homo sapiens</i>	NG_000006	Human <i>HBA</i>
	NG_000007	Human <i>HBD</i>
	NG_000007	Human <i>HBB</i>
Dog, <i>Canis lupus familiaris</i>	ENSCAFG00000029904	Dog <i>HBA</i>
	AC127473	Dog <i>HBD-T1</i>
	AC127473	Dog <i>HBD-T2</i>
	AC127473	Dog <i>HBB/D</i>
Ferret, <i>Mustela putorius furo</i>	AC232652	Ferret <i>HBA</i>
	AC231452	Ferret <i>HBD-T1</i>
	AC231452	Ferret <i>HBD-T2</i>
	AC231452	Ferret <i>HBB/D</i>
Cat, <i>Felis catus</i>	AC219132	Cat <i>HBA</i>
	AC129072	Cat <i>HBB/D</i>
	AC129072	Cat <i>HBB</i>
Siberian tiger, <i>Panthera tigris altaica</i>	ATCQ01121671	Siberian tiger <i>HBA</i>
	XXXX (pending)	Siberian Tiger <i>HBB/D</i>
	ATCQ01114550	Siberian tiger <i>HBB</i>
Snow leopard, <i>Panthera uncia</i>	XXXX (pending)	Snow leopard <i>HBA</i>
	XXXX (pending)	Snow leopard <i>HBB/D</i>
	AC129072	Snow leopard <i>HBB</i>
African lion, <i>Panthera leo</i>	P18975	African lion <i>HBA</i>
	XXXX (pending)	African lion <i>HBB/D</i>
	P68050	African lion <i>HBB</i>
Leopard, <i>Panthera pardus</i>	P63110	Leopard <i>HBA</i>
	XXXX (pending)	Leopard <i>HBB/D</i>
	1206295B	Leopard <i>HBB</i>
Jaguar, <i>Panthera onca</i>	P63109	Jaguar <i>HBA</i>
	XXXX (pending)	Jaguar <i>HBB/D</i>
	P68064	Jaguar <i>HBB</i>