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REVIEW

Hemoglobin–oxygen affinity in high-altitude vertebrates: is there evidence for an adaptive trend?

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ABSTRACT

In air-breathing vertebrates at high altitude, fine-tuned adjustments in hemoglobin (Hb)–O_2 affinity provide an energetically efficient means of mitigating the effects of arterial hypoxemia. However, it is not always clear whether an increased or decreased Hb–O_2 affinity should be expected to improve tissue O_2 delivery under different degrees of hypoxia, due to the inherent trade-off between arterial O_2 loading and peripheral O_2 unloading. Theoretical results indicate that the optimal Hb–O_2 affinity varies as a non-linear function of environmental O_2 availability, and the threshold elevation at which an increased Hb–O_2 affinity becomes advantageous depends on the magnitude of diffusion limitation (the extent to which O_2 equilibration at the blood–gas interface is limited by the kinetics of O_2 exchange). This body of theory provides a framework for interpreting the possible adaptive significance of evolved changes in Hb–O_2 affinity in vertebrates that have colonized high-altitude environments. To evaluate the evidence for an empirical generalization and to test theoretical predictions, I synthesized comparative data in a phylogenetic framework to assess the strength of the relationship between Hb–O_2 affinity and native elevation in mammals and birds. Evidence for a general trend in mammals is equivocal, but there is a remarkably strong positive relationship between Hb–O_2 affinity and native elevation in birds. Evolved changes in Hb function in high-altitude birds provide one of the most compelling examples of convergent biochemical adaptation in vertebrates.

KEY WORDS: Biochemical adaptation, Blood oxygen transport, Hemoglobin, High-altitude adaptation, Hypoxia, Physiological adaptation

Introduction

An adaptive trend in phenotypic evolution is indicated when genetically based trait variation is consistently associated with the same environmental factors in multiple taxa, and when the pattern of co-variation exhibits a regularity that cannot be explained by chance alone. Such generalizations or 'ecogeographic rules' are instructive about relationships between form and function, and they motivate the development of mathematical models to describe particular features that organisms might be expected to possess in particular environments. These models can be used to explain evolved trait differences among organisms that inhabit different environments, or to predict features of organisms based on information about their habitats.

In comparative physiology, a fairly well-accepted empirical generalization is that vertebrate taxa that are native to high-altitude environments tend to have elevated hemoglobin (Hb)–O_2

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affinities in comparison with lowland relatives (Hall et al., 1936; Bullard, 1972; Lenfant, 1973; Bunn, 1980; Monge and León-Velarde, 1991; Weber, 1995, 2007; Storz, 2007; Powell and Hopkins, 2010; Storz et al., 2010b). However, this putative trend is based on a relatively small number of case studies, and comparative data have not always been interpreted in a well-informed phylogenetic framework. There are also reasons to question whether an elevational trend should generally be expected, as theory predicts that the optimal Hb–O_2 affinity is a non-monotonic function of the ambient partial pressure of O_2 (P_{O_2}). Thus, if a given lowland species colonizes a high-altitude environment, it is not always clear whether an increased or decreased Hb–O_2 affinity should be expected to improve tissue O_2 delivery, and this has been the subject of considerable debate (Barcroft et al., 1923; Aste-Salazar and Hurtado, 1944; Lenfant et al., 1968, 1969, 1971; Eaton et al., 1969; Torrance et al., 1970/71; Bullard, 1972; Turek et al., 1973; Eaton et al., 1974; Dempsey et al., 1975; Frisancho, 1975; West and Wagner, 1980; Bencowitz et al., 1982; Willford et al., 1982; Samaja et al., 1986, 2003; Mairbäurl, 1994).

The purpose of this Review is to evaluate the evidence for an empirical generalization about the relationship between Hb–O_2 affinity and native elevation in terrestrial vertebrates. I start by providing an overview of Hb function and allosteric regulatory control (see Glossary). I then review theoretical and experimental results that demonstrate how the optimal Hb–O_2 affinity varies as a function of environmental O_2 availability. Finally, I synthesize comparative data in a phylogenetic framework to assess the strength of the relationship between Hb–O_2 affinity and native elevation in mammals and birds, the vertebrate groups for which the most data are available.

Challenges to respiratory gas transport under hypoxia

In order for air-breathing vertebrates to cope with the low ambient P_{O_2} at high altitude, blood O_2 transport capacity must be increased to sustain O_2 flux to the tissue mitochondria in support of aerobic ATP synthesis (Mairbäurl, 1994; Samaja et al., 2003; Storz et al., 2010b; Scott, 2011; Mairbäurl and Weber, 2012). Such changes complement physiological adjustments in other convective and diffusive steps in the O_2 transport pathway (Bouverot, 1985; Scott and Milsom, 2006). Although an increased Hb–O_2 affinity helps to safeguard arterial O_2 saturation under environmental hypoxia, it can hinder O_2 unloading in the systemic circulation. For this reason, vertebrates at high altitude face the physiological challenge of optimizing the trade-off between O_2 loading in the pulmonary capillaries and O_2 unloading in the tissue capillaries.

The oxygenation properties of blood reflect inherent properties of the Hb protein as well as the physicochemical operating conditions for Hb in the red blood cell. Below, I briefly describe the intrinsic O_2-binding properties of the Hb protein, and I then explain how these properties are modulated by metabolically induced changes in the erythrocytic microenvironment.

List of symbols and abbreviations

βb_{O_2}	blood O_2 capacitance coefficient
Ca_{O_2}	arterial O_2 content
Cv_{O_2}	venous O_2 content
DPG	2,3-diphosphoglycerate
Hb	hemoglobin
IHP	inositol hexaphosphate
IPP	inositol pentaphosphate
n	Hill coefficient
P_{50}	partial pressure of O_2 at which Hb is half-saturated
P_{O_2}	partial pressure of O_2
Pa_{O_2}	arterial O_2 pressure
Pv_{O_2}	venous O_2 pressure
$\dot{Q}$	cardiac output
S_{O_2}	fraction of O_2-saturated Hb relative to total Hb (unsaturated +saturated) in the blood
Sa_{O_2}	arterial O_2 saturation
Sv_{O_2}	venous O_2 saturation
$\dot{V}_{O_2}$	rate of O_2 consumption

Hb function and allosteric regulatory control

The Hbs of jawed vertebrates are heterotetramers, composed of two α-type and two β-type subunits. Each of the subunit polypeptides contains a covalently bound heme group (see Glossary) that can reversibly bind a single O_2 molecule when the iron atom is in the ferrous (Fe^{2+}) state; thus tetrameric Hb binds up to four O_2 molecules. The Hb tetramer, $\alpha_2\beta_2$, consists of paired, semi-rigid αβ dimers that undergo a symmetrical rotation during the oxygenation-linked transition in quaternary structure between the high-affinity (oxygenated) 'R' state and the low-affinity (deoxygenated) 'T' state (Fig. 1A). The oxygenation-linked shift in the T↔R conformational equilibrium is central to homotropic allostery (cooperative O_2-binding that stems from subunit–subunit interaction) and heterotropic allostery (regulation of heme reactivity by ligands that bind at sites remote from the heme pocket) (Perutz, 1970; Baldwin and Chothia, 1979).

Glossary

Allosteric regulation
Regulation of protein activity by binding a cofactor molecule at a site other than the protein's active site; the binding of allosteric cofactors typically induces a change in protein conformation.

Bohr effect
The modulation of Hb–O_2 affinity by changes in pH and CO_2 content. In the physiological range, the O_2 affinity of vertebrate Hb is inversely related to the acidity and CO_2 concentration of the blood.

Donnan equilibrium
The ionic equilibrium attained in an electrolyte solution when diffusible and non-diffusible ions are separated by a semi-permeable membrane.

Heme group
A porphyrin ring that coordinates an iron atom at the centre; it serves as a prosthetic group for Hb and other hemoproteins.

Homotropic allostery
Modulation of protein activity by binding a ligand that serves as a substrate for the protein (O_2 in the case of Hb), but which also serves as a regulatory molecule. For example, heme–O_2 binding at one Hb subunit alters the heme reactivity of other subunits in the same tetrameric assembly.

Heterotropic allostery
Modulation of protein activity by binding a cofactor that is not a substrate for the protein; hydrogen ions, chloride ions and organic phosphates are examples of heterotropic regulators of Hb function.

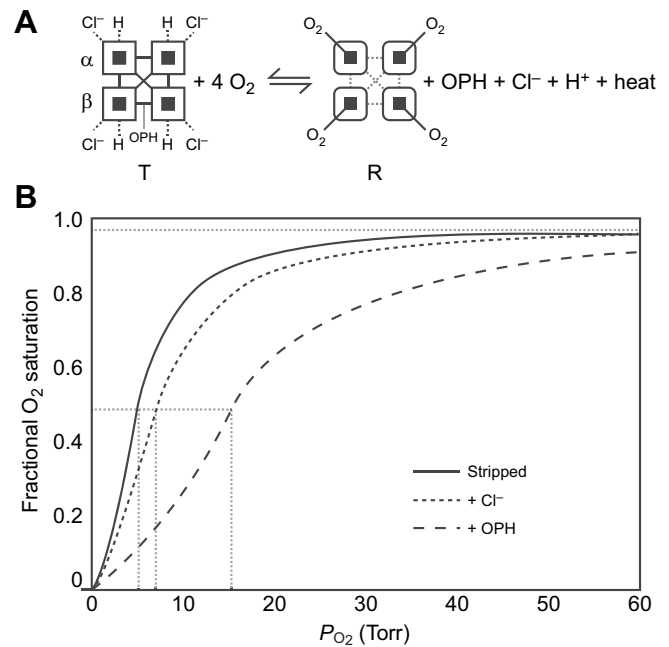**Hypoxemia**
Reduced P_{O_2} in arterial blood.

Fig. 1. The allosteric regulation of hemoglobin (Hb)–O_2 affinity. (A) The oxygenation reaction of tetrameric Hb ($\alpha_2\beta_2$) involves an allosteric transition in quaternary structure from the low-affinity T-state to the high-affinity R-state. The oxygenation-induced T→R transition entails a breakage of salt bridges and hydrogen bonds within and between subunits (open squares), dissociation of allosterically bound organic phosphates (OPHs), Cl^- ions and protons, and the release of heat (heme oxygenation is an exothermic reaction). Oxygenation-linked proton binding occurs at multiple residues in the α- and β-chains, Cl^- binding mainly occurs at the N-terminal α-amino groups of the α- and β-chains in addition to other residues in both chains, and phosphate binding occurs between the β-chains in the central cavity of the Hb tetramer. (B) O_2 equilibrium curves for purified Hb in the absence of allosteric effectors (Stripped) and in the presence of chloride ions (+Cl^-) and organic phosphates (+OPH). The preferential binding of allosteric effectors to deoxyHb stabilizes the T-state, thereby shifting the allosteric equilibrium in favour of the low-affinity quaternary structure. The O_2 equilibrium curves are therefore right-shifted (Hb–O_2 affinity is reduced) in the presence of such effectors. Hb–O_2 affinity is indexed by the P_{50} value (dashed grey lines) – the P_{O_2} at which Hb is half-saturated. The sigmoidal shape of the O_2 equilibrium curves reflects cooperative O_2 binding, involving a P_{O_2}-dependent shift from low- to high-affinity conformations.

Homotropic allostery

The cooperativity of Hb–O_2 binding stems from an interaction between subunits of the tetrameric protein. The binding of O_2 to the heme iron of each subunit produces a localized change in tertiary structure that is transmitted to each of the other unliganded heme-bearing subunits, triggering the T→R shift in quaternary structure [Perutz, 1970, 1979; Baldwin and Chothia, 1979; Gelin et al., 1983; Perutz et al., 1987; Liddington et al., 1988; extensions of this two-state allosteric mechanism are discussed by Yonetani and Tsuneshige (2003) and Eaton et al. (2007)]. The binding of O_2 to each heme therefore increases the O_2 affinity of the remaining unliganded hemes in the same Hb tetramer and, conversely, O_2 released by each heme reduces the O_2 affinity of the remaining liganded hemes. The physiological significance of cooperativity is that it permits efficient O_2 unloading over a relatively narrow range of blood P_{O_2}. This property is manifest in the sigmoidal shape of the O_2 equilibrium curve (Fig. 1), which describes how the fractional saturation of Hb varies as a function of P_{O_2}. This relationship is quantified by

the Hill equation (Hill, 1910):

$$S_{O_2} = \frac{P_{O_2}^n}{P_{O_2}^n + P_{50}^n}, \tag{1}$$

where S_{O_2} is the fractional saturation, P_{O_2} is the partial pressure of O_2 in Torr, P_{50} is the P_{O_2} at which Hb is 50% saturated, and n is the cooperativity coefficient. The Hill equation is extended by the more complex Adair equation (Adair, 1925), which expresses S_{O_2} as a function of P_{O_2} and association constants for binding each of the four O_2 molecules per Hb tetramer.

Heterotropic allostery

Heterotropic mechanisms of regulating Hb–O_2 affinity involve the oxygenation-linked binding of non-heme ligands such as H^+, Cl^-, CO_2, lactate and organic phosphates. Binding sites for these allosteric effectors are mainly located at the N- and C-termini of the globin subunits and in the positively charged central cavity of the Hb tetramer (Arnone, 1972; Arnone and Perutz, 1974; O'Donnell et al., 1979; Nigen et al., 1980). Different organic phosphates serve as allosteric effectors in the definitive red blood cells of different groups of terrestrial vertebrates: 2,3-diphosphoglycerate (2,3-DPG) in mammals, inositol pentaphosphate (IPP) in birds, adenosine triphosphate (ATP) and guanosine triphosphate (GTP) in non-avian reptiles, and ATP and DPG in amphibians (Rapoport and Guest, 1941; Benesch and Benesch, 1967; Bartlett, 1980; Hazard and Hutchison, 1982; Weber, 1995; Weber and Fago, 2004). These polyphosphate molecules electrostatically bind to a constellation of cationic residues lining the cleft between the β-chains of deoxyHb, thereby stabilizing the T-state through the formation of salt bridges within and between the α- and β-chain subunits (Arnone, 1972; Arnone and Perutz, 1974). Phosphate binding has the effect of reducing Hb–O_2 affinity by shifting the conformational equilibrium in favour of the low-affinity T-state; this allosteric transition in quaternary structure promotes O_2 unloading to the cells of respiring tissues. As organic phosphates are non-diffusible anions, changes in their intracellular concentration also exert an indirect effect on O_2 affinity by perturbing the Donnan equilibrium (see Glossary) of protons across the red cell membrane, as changes in cellular pH modulate Hb–O_2 binding via the Bohr effect (see Glossary).

Whereas cooperativity accounts for the shape of the O_2 equilibrium curve, the O_2 affinity of Hb determines the position of the curve along the x-axis. As shown in Fig. 1B, the curve is shifted to the right [corresponding to a reduction in Hb–O_2 affinity (increased P_{50})] or to the left [corresponding to an increased Hb–O_2 affinity (decreased P_{50})] in response to changes in temperature, pH and erythrocytic concentrations of Cl^- ions and organic phosphates.

Changes in Hb–O_2 affinity
Evolutionary changes in Hb–O_2 affinity

Evolutionary changes in Hb–O_2 affinity can involve changes in the intrinsic O_2 affinity of Hb and/or changes in the responsiveness to allosteric effectors. The former mechanism can involve changes in the equilibrium constants of heme–O_2 binding in the R- or T-state, or changes in the allosteric equilibrium constants for the R↔T transition. These genetically based modifications of Hb function are attributable to amino acid replacements in the α- and/or β-type subunits (Weber, 1995, 2007; Bellelli et al., 2006; Storz and Moriyama, 2008).

Reversible changes in Hb–O_2 affinity

Reversible changes in Hb–O_2 affinity can be achieved by modulating intraerythrocytic pH and/or the concentration of organic phosphates or other allosteric effectors (Nikinmaa, 2001; Jensen, 2004, 2009). These changes in the chemical milieu of the red blood cell alter the operating conditions for Hb, but are not associated with structural changes in the Hb protein itself.

In principle, reversible changes can also be produced by cellular changes in Hb isoform composition. All vertebrates possess multiple α- and β-type globin genes, and therefore express multiple, structurally distinct Hb isoforms during different stages of prenatal development and postnatal life (Hoffmann et al., 2010, 2012; Storz et al., 2011a, 2013; Storz, 2016b). In this Review, I focus on postnatally expressed Hb isoforms and blood O_2 transport during adulthood [see Brittain (2002) for a discussion of prenatally expressed Hbs and their functional properties]. Whereas adult mammals typically express a single Hb isoform or multiple isoforms that have very similar functional properties, other terrestrial vertebrates generally express two or more Hb isoforms that are functionally distinct (Weber and Jensen, 1988; Weber, 1990; Storz, 2016b). For example, birds typically express two main Hb isoforms in definitive red blood cells – a major isoform, HbA, which incorporates α-type products of the α^A-globin gene, and a minor isoform, HbD, which incorporates α-type products of the α^D-globin gene; the two isoforms share the same β-type subunits (Hoffmann and Storz, 2007; Grispo et al., 2012; Opazo et al., 2015). In all bird species that have been examined to date, the minor HbD isoform has an appreciably higher O_2 affinity than the major HbA isoform in the presence of allosteric effectors (Grispo et al., 2012; Projecto-Garcia et al., 2013; Cheviron et al., 2014; Galen et al., 2015; Natarajan et al., 2015b, 2016). Other sauropsid taxa also tend to co-express multiple, structurally distinct Hb isoforms in definitive erythrocytes (Weber and Jensen, 1988; Storz et al., 2011b, 2015b; Damsgaard et al., 2013). The Hb multiplicity that has been documented in birds and non-avian reptiles suggests a potential mechanism for modulating blood–O_2 affinity via changes in the relative abundance of distinct Hb isoforms with different O_2-binding properties (Hiebl et al., 1988; Weber et al., 1988a; Grispo et al., 2012; Opazo et al., 2015).

In summary, reversible modulation of Hb–O_2 affinity via metabolically induced changes in the red cell microenvironment and regulatory changes in Hb isoform expression represent potential mechanisms of phenotypic plasticity. These mechanisms could complement genetically based changes in Hb function, or they could obviate the need for such changes in the first place.

Is it physiologically advantageous to have an increased Hb–O_2 affinity at high altitude?

Having explored functional mechanisms for altering Hb–O_2 affinity, let us now address the question of whether the optimal Hb–O_2 affinity varies as a function of atmospheric P_{O_2}. This is central to the question of whether we should generally expect natural selection to favour different Hb–O_2 affinities in populations or species that inhabit different elevational zones.

Theoretical results

At high altitude, the reduced P_{O_2} of inspired air generally results in a concomitant reduction in the P_{O_2} of arterial blood (Pa_{O_2}). Under such conditions, changes in the oxygenation properties of red blood cells can limit the reduction in O_2 flux while simultaneously preserving an adequate pressure gradient for O_2 diffusion from the capillary blood to the cells of perfused tissue.

At a given Pa_{O_2}, tissue O_2 supply is enhanced by increasing cardiac output ($\dot{Q}$) and/or the blood capacitance coefficient (βb_{O_2}; Fig. 2A). This latter parameter is defined as the slope of the line joining the arterial and venous points on the blood O_2 equilibrium curve:

$$\beta b_{O_2} = \frac{Ca_{O_2} - Cv_{O_2}}{Pa_{O_2} - Pv_{O_2}}, \qquad (2)$$

where $Ca_{O_2} - Cv_{O_2}$ is the arterial–venous difference in O_2 content and $Pa_{O_2} - Pv_{O_2}$ is the arterial–venous difference in P_{O_2} (Dejours et al., 1970) (Fig. 2A). The capacitance coefficient therefore quantifies the amount of O_2 unloaded to the tissues for a given arterial–venous difference in P_{O_2}.

With regard to the maintenance of an adequate pressure gradient for O_2 diffusion to the cells of perfused tissue, the venous P_{O_2} can be expressed as:

$$Pv_{O_2} = Pa_{O_2} - \frac{1}{\left(\beta b_{O_2} \times \dfrac{\dot{Q}}{\dot{V}_{O_2}} \right)}, \qquad (3)$$

where $\dot{V}_{O_2}$ is the rate of O_2 consumption and the product $\beta b_{O_2} \times (\dot{Q}/\dot{V}_{O_2})$ is the specific blood O_2 conductance (Dejours et al., 1970; Bouverot, 1985).

With the above relationships in mind, we can see that under conditions of moderate hypoxia ($Pa_{O_2} > 45$ Torr), a reduced Hb–O_2 affinity (right-shifted curve) maximizes βb_{O_2} (Fig. 2B). That is, it produces the largest increase in the slope of the line connecting the arterial and venous points, thereby maximizing tissue O_2 delivery ($Ca_{O_2} - Cv_{O_2}$) for a given difference in P_{O_2} between the sites of O_2 loading in the pulmonary capillaries and the sites of O_2 unloading in the systemic circulation ($Pa_{O_2} - Pv_{O_2}$). By contrast, under severe hypoxia, an increased Hb–O_2 affinity (left-shifted curve) produces the largest increase in βb_{O_2} because the arterial–venous difference in P_{O_2} spans a steeper portion of the curve (Fig. 2B). Figure 2B also shows that a left-shifted curve preserves a higher Pv_{O_2} under such conditions (Woodson, 1988). Under hypoxia, an increased circulatory O_2 conductance can also be achieved via increases in cardiac output, but increasing βb_{O_2} via fine-tuned adjustments in Hb–O_2 affinity is far more energetically efficient (Mairbäurl, 1994; Samaja et al., 2003).

In summary, this body of theory predicts that a reduced Hb–O_2 affinity is generally beneficial under moderate hypoxia, whereas an increased Hb–O_2 affinity is beneficial under severe hypoxia. This is consistent with theoretical investigations of tissue O_2 delivery at rest and during exercise (Turek et al., 1973; West and Wagner, 1980; Bencowitz et al., 1982; Willford et al., 1982; Samaja et al., 1986, 2003; Scott and Milsom, 2006).

Several theoretical treatments have calculated optimal values of P_{50} that maximize the arterial–venous difference in O_2 content or O_2 saturation for a given arterial–venous difference in P_{O_2} (Bencowitz et al., 1982; Willford et al., 1982; Brauner and Wang, 1997). The arterial–venous difference in O_2 saturation can be expressed as:

$$Sa_{O_2} - Sv_{O_2} = \left[\frac{Pa_{O_2}^n}{Pa_{O_2}^n - P_{50}^n} \right] - \left[\frac{Pv_{O_2}^n}{Pv_{O_2}^n - P_{50}^n} \right]. \qquad (4)$$

Taking the first derivative of the maximum arterial–venous saturation difference with respect to P_{50} indicates that the optimal P_{50} (P_{50}^*) can be expressed as:

$$P_{50}^* = (Pa_{O_2} \times Pv_{O_2})^{0.5}. \qquad (5)$$

Eqn 4 can also be solved for Pv_{O_2}, but the same conditions that maximize $Sa_{O_2} - Sv_{O_2}$ also maximize Pv_{O_2} (Willford et al., 1982).
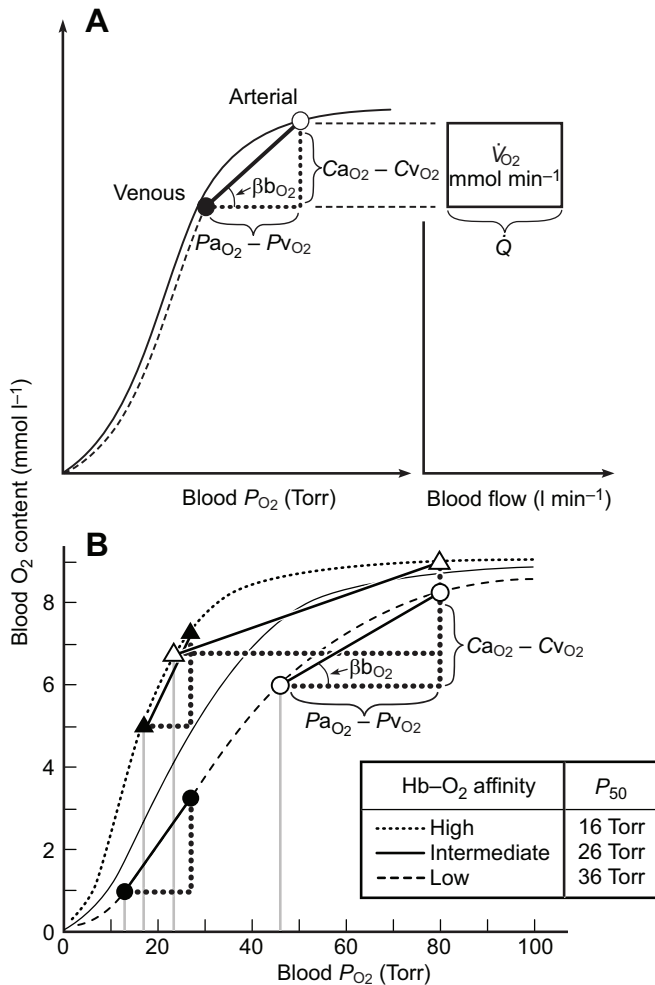

Fig. 2. Schematic illustration of blood O_2 transport. (A) An O_2 equilibrium curve under the physicochemical conditions prevailing in arterial blood (a, continuous curve, open symbol) and venous blood (v, dashed curve, closed symbol). The curve is a plot of blood O_2 content (y-axis) versus P_{O_2} (x-axis), with paired values for arterial and venous blood connected by a continuous line. $Ca_{O_2} - Cv_{O_2}$ denotes the arterial–venous difference in blood O_2 content, $Pa_{O_2} - Pv_{O_2}$ denotes the corresponding difference in P_{O_2}, βb_{O_2} denotes the blood O_2 capacitance coefficient (see text for details), Q denotes cardiac output, and $\dot{V}_{O_2}$ denotes the rate of O_2 consumption. On the right-hand side of the graph, the area of the rectangle is proportional to total O_2 consumption, which can be enhanced by increasing $\dot{Q}$ and/or by increasing βb_{O_2}. Increases in βb_{O_2} produce a corresponding increase in $Ca_{O_2} - Cv_{O_2}$ through shifts in the shape or position of the O_2 equilibrium curve. (B) O_2 equilibrium curves showing the effect of changes in Hb–O_2 affinity on tissue O_2 delivery under conditions of moderate hypoxia (open symbols) and severe hypoxia (filled symbols). For each pair of arterial and venous points, the P_{O_2} for venous blood (Pv_{O_2}) is marked by a vertical grey line that extends to the x-axis. The sigmoid O_2 equilibrium curves are shown for high, intermediate and low Hb–O_2 affinities; P_{50}, the P_{O_2} at which Hb is 50% saturated. Each change in Hb–O_2 affinity produces a shift in Pv_{O_2}, but the P_{O_2} of arterial blood (Pa_{O_2}) is assumed to remain constant. Note that under conditions of moderate hypoxia the right-shifted curve maximizes βb_{O_2} and preserves a higher Pv_{O_2} (an overall index of tissue oxygenation). Under severe hypoxia, by contrast, the left-shifted curve maximizes βb_{O_2} and preserves a higher Pv_{O_2} relative to the right-shifted curve. When the kinetics of O_2 transfer across the alveolar gas–blood barrier is a limiting step (diffusion limitation), a left-shifted O_2 equilibrium curve may also be advantageous under less severe hypoxia (Bencowitz et al., 1982).
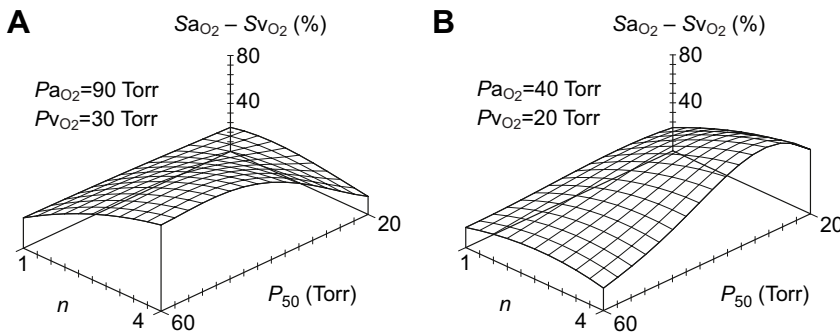
Fig. 3. Blood P_{50} and Hill's cooperativity coefficient, n, influence blood O_2 transport (indexed by the difference in arterial and venous O_2 saturation). (A) Normoxia; (B) hypoxia. In these three-dimensional plots, the difference in arterial and venous O_2 saturation ($Sa_{O_2}-Sv_{O_2}$) is indicated by the height of the projection above the reference plane. The higher the projection, the greater the difference in O_2 saturation.

Under normoxia (Pa_{O_2}=90 Torr, Pv_{O_2}=30 Torr), and assuming that pH, partial pressure of CO_2 (P_{CO_2}), cardiac output and Hb concentration remain constant, Eqn 4 predicts that tissue O_2 delivery increases as n increases and as P_{50} increases to its optimum (Fig. 3A). According to Eqn 5, the optimal P_{50} under these conditions is $(90\times30)^{0.5}$=52.0 Torr. This is the point on the plot where the slope with respect to P_{50} is zero. By contrast, under severe hypoxia (Pa_{O_2}=40 Torr, Pv_{O_2}=20 Torr), with other assumptions as above, the optimal P_{50} is predicted to be $(40\times20)^{0.5}$=28.3 Torr, indicating that tissue O_2 delivery is maximized at a far higher Hb–O_2 affinity (Fig. 3B). In both cases, tissue O_2 delivery generally increases as a positive function of n, demonstrating the adaptive significance of cooperative O_2 binding. The only exception occurs under extremely severe hypoxia, when the venous point on the O_2 equilibrium curve drops down to the lower curvilinear asymptote.

Figure 4 illustrates the relationships between P_{50}, Pa_{O_2} and Pv_{O_2}, while keeping P_{50} constant. Assuming 50% tissue O_2 extraction (as might occur during exercise), the figure shows that a higher P_{50} maintains a higher Pv_{O_2} under normoxia (Pa_{O_2}=~90 Torr), resulting in improved tissue oxygenation as discussed above. By contrast, a lower P_{50} maintains a higher Pv_{O_2} under severe hypoxia (Pa_{O_2}=~40 Torr). If the 'critical Pv_{O_2}' for tissue oxygenation is, for example, 10 Torr, then a blood P_{50} of 20 Torr would allow Pa_{O_2} to fall as low as 25 Torr, whereas a blood P_{50} of 50 Torr would not allow Pa_{O_2} to fall below 52 Torr. Results are qualitatively similar under the assumption of 25% tissue O_2 extraction, corresponding to the situation at rest (Willford et al., 1982).
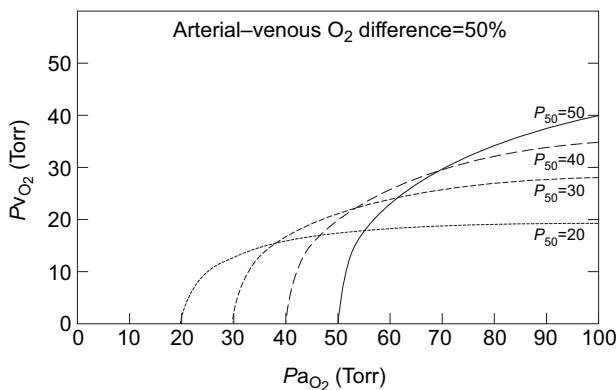

Fig. 4. Isobars showing predicted values of venous P_{O_2} (an index of tissue oxygenation) as a function of arterial P_{O_2} at different values of blood P_{50}, assuming 50% tissue O_2 extraction and constant cardiac output. At normal or moderately reduced Pa_{O_2}, a higher P_{50} results in a higher Pv_{O_2} (and hence improved tissue oxygenation). Under more severe hypoxemia, by contrast, a lower P_{50} results in a higher Pv_{O_2} while still maintaining constant O_2 extraction.

Experimental results

The most direct means of testing theoretical predictions about how the optimal Hb–O_2 affinity varies in relation to ambient P_{O_2} is to experimentally evaluate how titrated changes in blood P_{50} affect relevant measures of physiological performance under normoxia and varying degrees of hypoxia. Experiments on rats with pharmacologically manipulated Hb–O_2 affinities have confirmed theoretical predictions that an increased P_{50} improves tissue O_2 delivery under normoxia and moderate hypoxia, and that a reduced P_{50} is beneficial under severe hypoxia (Turek et al., 1978a,b). Similarly, reciprocal-transplant experiments involving wild-derived strains of deer mice (*Peromyscus maniculatus*) revealed that high-altitude natives with high Hb–O_2 affinities have higher capacities for thermogenesis and aerobic exercise under severe hypoxia, whereas lowland natives with lower Hb–O_2 affinities exhibit superior performance under normoxia (Chappell and Snyder, 1984). Experiments involving other mammals have reported qualitatively similar findings, suggesting that a reduced Hb–O_2 affinity improves tissue O_2 delivery under normoxia or moderate hypoxia, whereas an increased O_2 affinity provides the greatest improvement under more severe hypoxia (Dawson and Evans, 1966; Banchero and Grover, 1972).

Experiments involving rats with pharmacologically manipulated Hb–O_2 affinities have also demonstrated that reductions in blood P_{50} significantly increase the survival of animals subjected to acute, severe hypoxia (Eaton et al., 1974; Penney and Thomas, 1975). Similar results were reported in studies of physiological performance under hypoxia in other mammals with naturally occurring variation in Hb–O_2 affinity (Dawson and Evans, 1966; Hall, 1966; Hebbel et al., 1978). In addition to studies of survival and whole-animal physiological performance, *ex vivo* studies of microvascular O_2 transport and tissue perfusion have also demonstrated that an increased Hb–O_2 affinity enhances O_2 delivery under severe hypoxia (Bakker et al., 1976; Stein and Ellsworth, 1993; Yalcin and Cabrales, 2012).

Threshold altitude

The theoretical and experimental results reviewed above indicate that the optimal Hb–O_2 affinity varies according to ambient P_{O_2}. The relationship is non-linear and depends critically on the magnitude of diffusion limitation (Bencowitz et al., 1982). For any given species, theory predicts that there must be some threshold altitude at which it becomes beneficial to have an increased Hb–O_2 affinity. However, the threshold altitude at which this becomes beneficial varies from one species to the next due to variation in Pa_{O_2} (mainly determined by ventilation) and numerous other diffusive and convective steps in the O_2 transport pathway (Bencowitz et al., 1982; Scott and Milsom, 2006). The key question is whether a given species inhabits elevations above that

critical threshold. In a comparison between sister species with contrasting elevational ranges, the species with the higher elevational range limit would not necessarily be expected to have evolved a higher Hb–O_2 affinity unless an appreciable fraction of its native range exceeded the upper critical threshold.

In the case of humans living at high altitude, modelling results suggest that an increased Hb–O_2 affinity only confers a benefit to tissue O_2 delivery at elevations >5000–5400 m (Samaja et al., 1986, 2003). At 5400 m above sea level, which is roughly the elevation of the South Everest base camp in Nepal, the standard barometric pressure is 53 kPa (399 Torr), meaning that the ambient P_{O_2} is 52% of that at sea level. The highest human settlements in the Himalayas and the Andes are generally at elevations of <4900 m (the Peruvian mining town, La Rinconada, is situated 5100 m above sea level, and most mine workers have homes at lower elevation). The highest permanent settlements in the Ethiopian highlands are <3500 m above sea level. The fact that humans do not live at elevations above the theoretically predicted 5000–5400 m threshold provides a possible explanation for why increased Hb–O_2 affinities have not evolved in indigenous mountain dwellers. Whether the same may be true of non-human mammals and birds at high altitude is an open question.

Insights from comparative studies

Since natural selection is 'the ultimate arbiter of what constitutes an adaptation' (Snyder, 1982, p. 92), a systematic survey of altitude-related changes in Hb–O_2 affinity – as revealed by comparisons among extant species – can provide insights into the possible adaptive significance of such changes. If high-altitude taxa have generally evolved increased Hb–O_2 affinities relative to lowland sister taxa – and if the elevational pattern is too consistent to be ascribed to chance (i.e. genetic drift) – this would be consistent with the hypothesis that the elevational differences reflect a history of natural selection. Ideally, comparative studies that exploit the outcomes of natural experiments (e.g. the independent colonization of high-altitude environments by multiple species) complement insights derived from controlled laboratory experiments. For example, comparative studies have documented that high-altitude rodents often have higher Hb and/or blood O_2 affinities than their lowland relatives (Hall et al., 1936; Bullard et al., 1966; Ostojic et al., 2002; Storz, 2007; Storz et al., 2009, 2010a; Natarajan et al., 2013, 2015a; Jensen et al., 2016), and these observations complement the results of experiments demonstrating that increases in blood-O_2 affinity enhance tissue O_2 delivery and measures of physiological performance in rodents subjected to environmental hypoxia (Eaton et al., 1974; Turek et al., 1978a,b; Chappell and Snyder, 1984). Such consilience of evidence from comparative and experimental studies can greatly strengthen conclusions about the adaptive significance of evolutionary changes in Hb–O_2 affinity.

The importance of accounting for phylogenetic history

In comparative analyses of phenotypic variation it is important to account for the fact that trait values from different species are not statistically independent because the sampled species did not evolve independently of one another; the phylogenetic history of any set of species is represented by a hierarchically nested pattern of relationships (Garland et al., 2005). If a phylogeny is available for a given set of species, then phylogenetically independent contrasts (PIC) (Felsenstein, 1985) can be used to test for a relationship between native elevation and Hb–O_2 affinity. The PIC approach uses phylogenetic information and a model of trait evolution

(typically a stochastic, Brownian motion-like model) to transform the data for the set of surveyed species into values that are statistically independent and identically distributed. This approach was used to document a strong positive correlation between Hb–O_2 affinity and native elevation in Andean hummingbirds (Projecto-Garcia et al., 2013).

An alternative to using PIC is the paired-lineage test, which restricts comparisons to phylogenetically replicated pairs of taxa that are chosen so that there is no overlap in evolutionary paths of descent (see Fig. 5). A non-random association between Hb–O_2 affinity and native elevation can then be assessed using a sign test (a non-parametric test that contrasts matched pairs of samples with respect to a continuous outcome). If the comparative analysis includes a phylogenetically diverse range of taxa, an advantage of the paired-lineage test is that comparisons can be restricted to closely related species by excluding pairs with long paths between them. To determine whether there is a relationship between Hb–O_2 affinity and native elevation, we want to make comparisons between close relatives so that we can minimize the number of potentially confounding differences in other aspects of their biology. For example, a comparison of Hb–O_2 affinity between a pair of high- and low-altitude hummingbirds is more physiologically informative than a comparison involving a high-altitude hummingbird and a low-altitude duck. In the latter case, an evolved difference in Hb function may be related to the difference in native elevation, but it may also be related to differences in metabolic rate or any number of other physiological differences between the two taxa.

There are two additional issues to consider in comparative studies of Hb function in relation to native elevation. The first relates to the effect of environmentally induced variation, which can obscure phylogenetic signal in trait values (Garland et al., 2005; Storz et al., 2015a). Measurements of an environmentally labile trait (like the O_2 affinity of whole blood, which is influenced by red cell metabolism and acid–base status) may prevent an accurate assessment of the extent to which phenotypic similarity between a given pair of species is attributable to shared phylogenetic heritage versus a shared, plastic response to similar environmental conditions (i.e. exposure to hypoxia). This problem can be avoided if the trait is measured under common-garden conditions to control for environmentally induced variation, or if measurements are restricted to genetically based trait variation (e.g. O_2 affinity of purified Hb rather than O_2 affinity of blood).

Another issue concerns genealogical discordance between the phylogeny of the examined species and the phylogenies of the genes that underlie the measured trait (Hahn and Nakhleh, 2016; Storz, 2016a). In comparative studies of Hb evolution involving orthologous genes from a diversity of species, it may often be the case that phylogenies of the α- and β-globin genes are not congruent with one another or with the assumed species tree (Storz et al., 2007; Hoffmann et al., 2008a,b; Opazo et al., 2008a,b, 2009; Runck et al., 2009, 2010; Gaudry et al., 2014; Natarajan et al., 2015a). This genealogical discordance can have multiple biological causes, including ectopic gene conversion (a form of non-reciprocal recombinational exchange between duplicated genes), introgressive hybridization (incorporation of allelic variants from one species into the gene pool of another species by means of hybridization and repeated back-crossing) and incomplete lineage sorting (the retention of ancestral polymorphism from one split between populations to the next, followed by stochastic sorting of allelic lineages among the descendant species). A given amino acid substitution may have occurred a single time on an internal branch of the gene tree, but it can present the appearance of having occurred
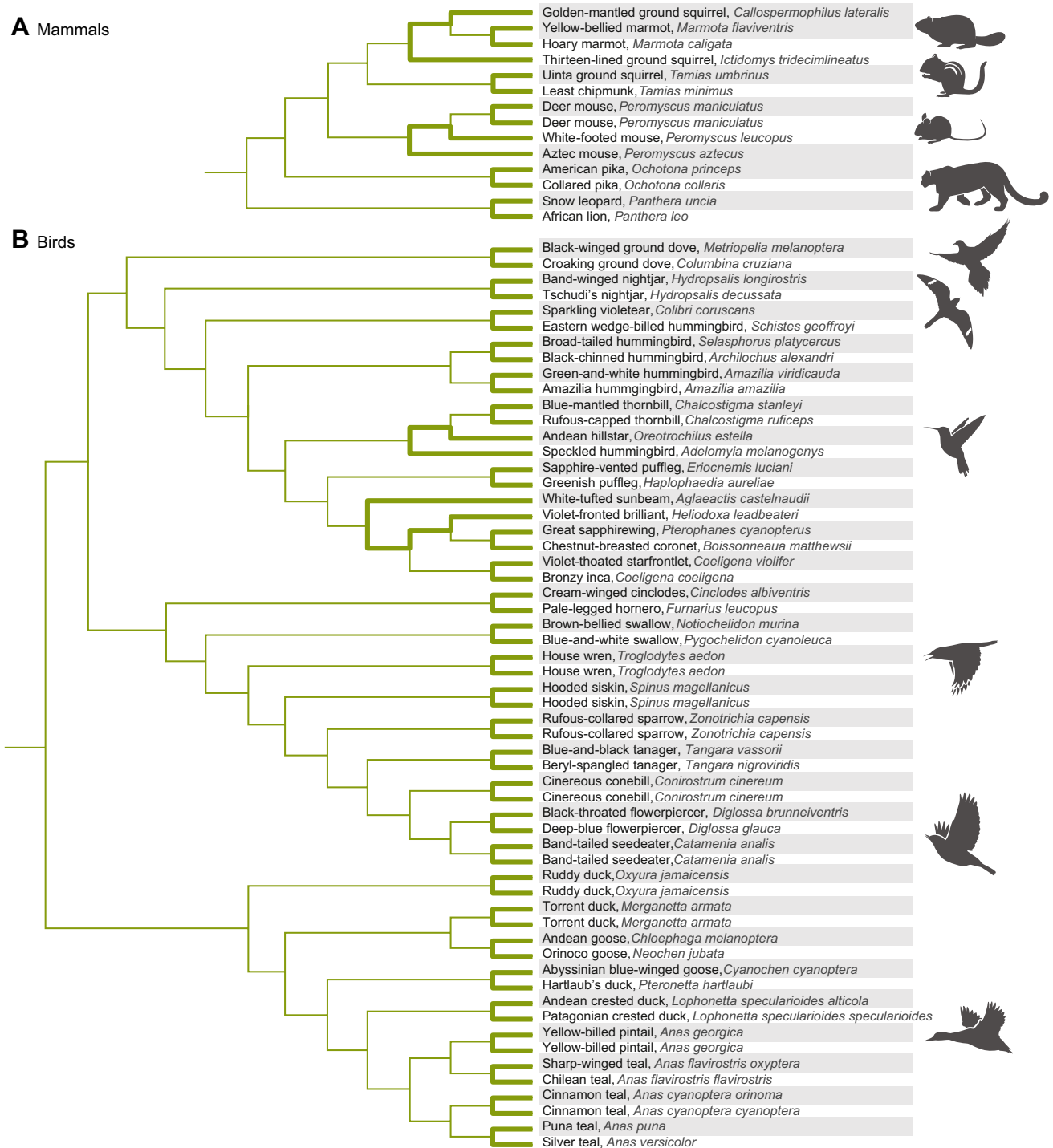
Fig. 5. Phylogenetic relationships of 14 mammalian taxa and 58 avian taxa used in comparative analyses of Hb function. Rows corresponding to high-altitude taxa are shaded. (A) In the mammalian phylogeny, branches in bold connect pairs of high- and low-altitude taxa that were used to test for a relationship between Hb–O$_2$ affinity and native elevation. As there are no overlaps in the paths of descent connecting each designated pair of high- and low-altitude taxa, the seven pairwise comparisons are statistically independent. For information regarding elevational ranges, see Storz et al. (2009, 2010a), Revsbech et al. (2013), Janecka et al. (2015), Natarajan et al. (2015a) and Tufts et al. (2015). (B) In the avian phylogeny, branches in bold connect pairs of high- and low-altitude taxa that were used to test for a relationship between Hb–O$_2$ affinity and native elevation. As in the case with the mammals, the 29 pairwise comparisons are phylogenetically independent. For information regarding elevational ranges, see Projecto-Garcia et al. (2013), Cheviron et al. (2014), Galen et al. (2015) and Natarajan et al. (2015b, 2016).

twice independently when mapped onto a discordant species tree, resulting in spurious inferences. Hahn and Nakhleh (2016) discuss possible solutions to the problem of species tree/gene tree discordance in comparative studies of trait evolution.

Considerations of zoogeographic history

In addition to making comparisons between high- and low-altitude taxa that are as closely related as possible, it is also important to consider the evolutionary histories of study species with regard to their current elevational distributions. Many alpine and subalpine natives may have predominantly lowland ancestries, possibly reflecting post-glacial range shifts. Alternatively, residence at high altitude may represent the ancestral condition for members of groups that diversified in mountainous regions. For example, Andean hummingbirds in the Brilliants/Coquettes clade diversified during a period of rapid uplift of the Andean massif in the period between ~10 and ~6 million years ago (McGuire et al., 2014). Within this group, many species with lowland distributions may have descended from highland ancestors. In such cases, it is important to consider that any altitude-related species differences in Hb–O_2 affinity could be attributable to derived increases in O_2 affinity in highland species and/or secondarily derived reductions in O_2 affinity in lowland species (Projecto-Garcia et al., 2013).

Evaluating evidence for an empirical generalization regarding the relationship between native altitude and Hb–O_2 affinity

The theoretical and experimental results reviewed above suggest that it is generally beneficial to have an increased Hb–O_2 affinity under conditions of severe hypoxia. An obvious prediction is that derived increases in Hb–O_2 affinity will have evolved repeatedly in disparate vertebrate taxa that have independently colonized extreme altitudes (provided that their range limits exceed the elevational threshold at which an increased Hb–O_2 affinity becomes beneficial). Let us now test this prediction using available comparative data for mammals and birds (Fig. 5).

I restrict the analysis to data based on standardized measurements of purified Hbs, so the variation in P_{50} values is purely genetic, reflecting evolved changes in the amino acid sequences of the α- and/or β-chain subunits. This focus on purified Hbs avoids problems associated with the confounding effects of environmentally induced variation. However, an analysis based on in vitro measures of protein function involves its own interpretative challenges because evolved changes in the inherent properties of Hb are physiologically relevant to circulatory O_2 transport only to the extent that such changes affect the oxygenation properties of blood (Berenbrink, 2006). I have opted to focus on data for purified Hbs while recognizing that species differences in Hb–O_2 affinity may not perfectly reflect in vivo differences in blood–O_2 affinity.

In the case of mammals, I have summarized data from 14 taxa representing seven high- versus low-altitude pairwise comparisons (Fig. 5A). These comparisons include rodents (marmotine ground squirrels and Peromyscus mice), lagomorphs (pikas) and carnivores (Storz et al., 2009, 2010a; Revsbech et al., 2013; Janecka et al., 2015; Natarajan et al., 2015a; Tufts et al., 2015). Six of the comparisons involve closely related species with contrasting elevational ranges, and one comparison involves high- and low-altitude populations of the broadly distributed deer mouse, P. maniculatus. Each of these pairwise comparisons involves a pronounced elevational contrast between an alpine or subalpine taxon and a closely related lowland taxon. For example, the high-altitude ground squirrels, deer mice and pikas occur at elevations >4300 m (the highest elevations that occur

within the limits of their geographical distributions in North America). Since an increased Hb–O_2 affinity is only expected to be physiologically beneficial above a given threshold elevation, potentially adaptive differences in Hb–O_2 affinity can only be detected if the high- and low-altitude members of each taxon pair have range limits on opposite sides of that threshold.

In each taxon, O_2 affinities of purified Hbs were measured in the presence and absence of Cl⁻ ions (added as KCl) and DPG (see Fig. 6 legend for experimental details). The 'KCl+DPG' treatment is most relevant to in vivo conditions in mammalian red blood cells, and I therefore focus primarily on measures of $P_{50(KCl+DPG)}$. However, measurements under each of the experimental treatments are valuable because they can provide insights into the functional mechanism responsible for observed differences in Hb–O_2 affinity.

In the presence of anionic effectors, high-altitude taxa have higher Hb–O_2 affinities than their lowland counterparts in some cases (e.g. deer mice and pikas; Fig. 6A,B), but in other cases there are no appreciable differences (e.g. marmots and big cats; Fig. 6C,D). In comparisons involving deer mice, pikas and some ground squirrels, integrated analyses of Hb function and sequence divergence revealed that the high-altitude member of each pair evolved a derived increase in Hb–O_2 affinity (i.e. the phenotype of the lowland taxon represents the ancestral condition). In the comparisons between conspecific populations of deer mice and between the golden-mantled ground squirrel (Callospermophilus lateralis) and thirteen-lined ground squirrel (Ictidomys tridecemlineatus), the evolved changes in Hb function involved an increase in intrinsic O_2 affinity in combination with a suppressed sensitivity to anionic effectors (Storz et al., 2009, 2010a; Natarajan et al., 2013; Revsbech et al., 2013; Natarajan et al., 2015a). In the case of the deer mice, this is indicated by the fact that the high-altitude Hb variant exhibits a slightly lower P_{50} in the absence of anions ('stripped') and the P_{50} difference is further augmented in the presence of Cl⁻ and DPG (Fig. 6A). By contrast, in the comparison between the two pika species (Ochotona princeps and O. collaris), the difference in Hb function was exclusively attributable to an evolved change in intrinsic O_2 affinity (Tufts et al., 2015) (Fig. 6B).

Phylogenetically independent comparisons involving the full set of mammalian taxa revealed no significant association between Hb–O_2 affinity and elevation (Wilcoxon's signed-rank test, W=6.5, P>0.05, N=7; Fig. 7). Previous studies involving experimental measurements on whole blood indicate that high-altitude mammals have lower P_{50} values than their lowland relatives in some cases (Chiodi, 1970/71; León-Velarde et al., 1996; Ostojic et al., 2002) but not in others (Lechner, 1976). Unless measures of blood–O_2 affinities are integrated with measurements on purified Hbs (Petschow et al., 1977; Campbell et al., 2010), components of environmental and genetic variation are confounded and it is unclear to what extent the measured differences represent evolved changes in Hb function and/or red cell metabolism. Overall, evidence for an altitudinal trend in Hb–O_2 affinity in mammals is equivocal; data from additional taxa may eventually reveal a clearer relationship.

In the case of birds, I have summarized data from 58 taxa representing 29 matched pairs of high- versus low-altitude taxa (Fig. 5B). These taxa include ground doves, nightjars, hummingbirds, passerines and waterfowl (Projecto-Garcia et al., 2013; Cheviron et al., 2014; Galen et al., 2015; Natarajan et al., 2015b, 2016). In the case of the passerines and waterfowl, we can make comparisons between closely related species as well as conspecific populations. All pairwise comparisons involved dramatic elevational contrasts; high-altitude taxa native to very high elevations (3500–5000 m above sea level) were paired with
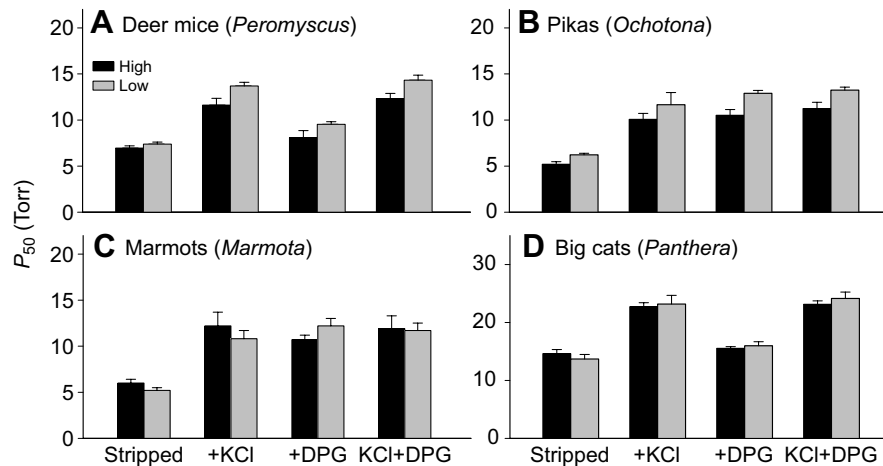
Fig. 6. O₂ affinities of purified Hbs from representative pairs of high- and low-altitude mammals. O_2 equilibria were measured at pH 7.40, 37°C, in the presence and absence of allosteric effectors ([Cl⁻], 0.10 mol l⁻¹; [Hepes], 0.1 mol l⁻¹; DPG:tetrameric Hb ratio, 2.0: [heme], 0.2–0.3 mmol l⁻¹). For each taxon, P_{50} values (±s.e.m.) are reported for stripped Hbs in the absence of added anions, in the presence of Cl⁻ alone (added as KCl), in the presence of DPG alone, and in the presence of both anions combined. This latter 'KCl+DPG' treatment is most relevant to *in vivo* conditions in mammalian red blood cells, but measurements of O_2 affinity under each of the four standardized treatments can provide insights into the functional mechanism responsible for observed differences in $P_{50(KCl+DPG)}$. (A) Comparison between Hb variants of high- and low-altitude deer mice, *Peromyscus maniculatus*, from the Rocky Mountains and Great Plains, respectively [data from Natarajan et al., 2015a; for additional details, see Storz et al. (2009, 2010a); Jensen et al. (2016)]. (B) Comparison between Hbs of the high-altitude American pika (*Ochotona princeps*) and the low-altitude collared pika (*O. collaris*) (data from Tufts et al., 2015). (C) Comparison between Hbs of the high-altitude yellow-bellied marmot (*Marmota flaviventris*) and the low-altitude hoary marmot (*M. caligata*) (data from Revsbech et al., 2013). (D) Comparison between Hbs of the high-altitude snow leopard (*Panthera uncia*) and the low-altitude African lion (*P. leo*) (data from Janecka et al., 2015). For both cat species, P_{50} is shown as the mean value for two co-expressed isoforms, HbA and HbB, which are present at roughly equimolar concentrations (Janecka et al., 2015).

close relatives that typically occur at or near sea level. As with the analysis of mammalian Hbs, O₂ affinities of purified avian Hbs were measured under standardized conditions in the presence and absence of anionic effectors (see legend for Fig. 8). However, to obtain measurements that are physiologically relevant to *in vivo* conditions in avian red cells, inositol hexaphosphate (IHP; a chemical analogue of IPP) was used instead of DPG. In species that expressed both HbA and HbD, the O₂-binding properties of isolated isoforms were measured separately.
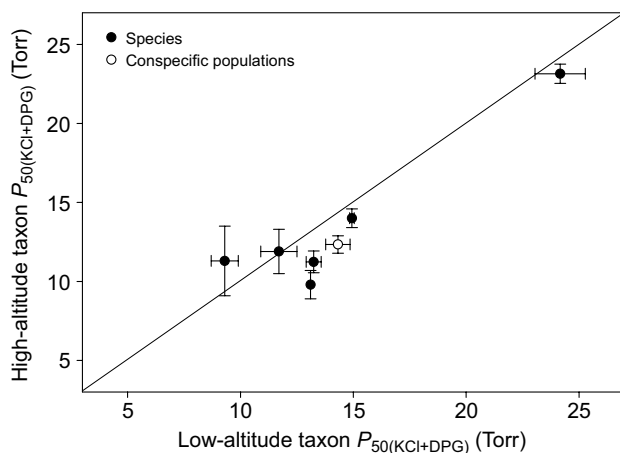

Fig. 7. There is no evidence for a significant elevational trend in the Hb–O₂ affinities of mammals. The plot shows measures of Hb–O₂ affinity in the presence of anionic effectors [$P_{50(KCl+IHP)}$ (±s.e.m.)] for seven matched pairs of high- and low-altitude taxa. Data points that fall below the diagonal ($x=y$) denote cases in which the high-altitude member of a given taxon pair possesses a higher Hb–O₂ affinity (lower P_{50}). The paired-lineage design ensures that all data points are statistically independent (see text for details). Filled symbols denote comparisons between species, whereas the open symbol denotes a comparison between high- versus low-altitude populations of the same species (*P. maniculatus*).

In the overwhelming majority of pairwise comparisons, the high-altitude taxon exhibited a higher Hb–O₂ affinity across all treatments, as illustrated by representative examples for the HbA isoform (Fig. 8). Phylogenetically independent comparisons revealed that highland natives generally have an increased Hb–O₂ affinity relative to their lowland counterparts, a pattern consistent for both HbA (Wilcoxon's signed-rank test, $Z=-4.314$, $P<0.0001$, $N=29$; Fig. 9A) and HbD ($Z=-2.798$, $P=0.0051$, $N=21$; Fig. 9B). In all pairwise comparisons in which the high-altitude taxa exhibited significantly higher Hb–O₂ affinities relative to the lowland taxa ($N=23$ taxon pairs for HbA, $N=15$ for HbD), the measured differences were almost entirely attributable to differences in intrinsic O₂ affinity rather than differences in sensitivity to Cl⁻ ions or IHP (Natarajan et al., 2015b, 2016). Comparisons between the high-flying bar-headed goose (*Anser indicus*) and lowland congeners based on O₂ affinity measurements of whole blood or purified hemolysates (Petschow et al., 1977; Black and Tenney, 1980; Jessen et al., 1991) are also consistent with the relationship shown in Fig. 9A,B.

The role of Hb isoform switching in hypoxia adaptation
In principle, regulatory changes in the expression of Hb isoforms with different oxygenation properties could provide an effective means of reversibly modulating blood–O₂ affinity in response to changes in O₂ availability or metabolic demand. This regulatory mechanism could potentially complement genetically based changes in the O₂ affinity of individual isoforms. Due to differences in the nature of isoform differentiation in birds and mammals, I will discuss relevant data for each of these taxa in turn.

In birds, consistent differences in O₂ affinity between HbA and HbD suggest that an increased blood–O₂ affinity could be achieved by up-regulating HbD (Hiebl et al., 1988; Weber et al., 1988a; Grispo et al., 2012; Opazo et al., 2015). A comparison involving 26 closely related pairs of high- and low-altitude taxa revealed that regulatory changes in Hb isoform abundance do not represent an important general mechanism of adaptation to chronic hypoxia
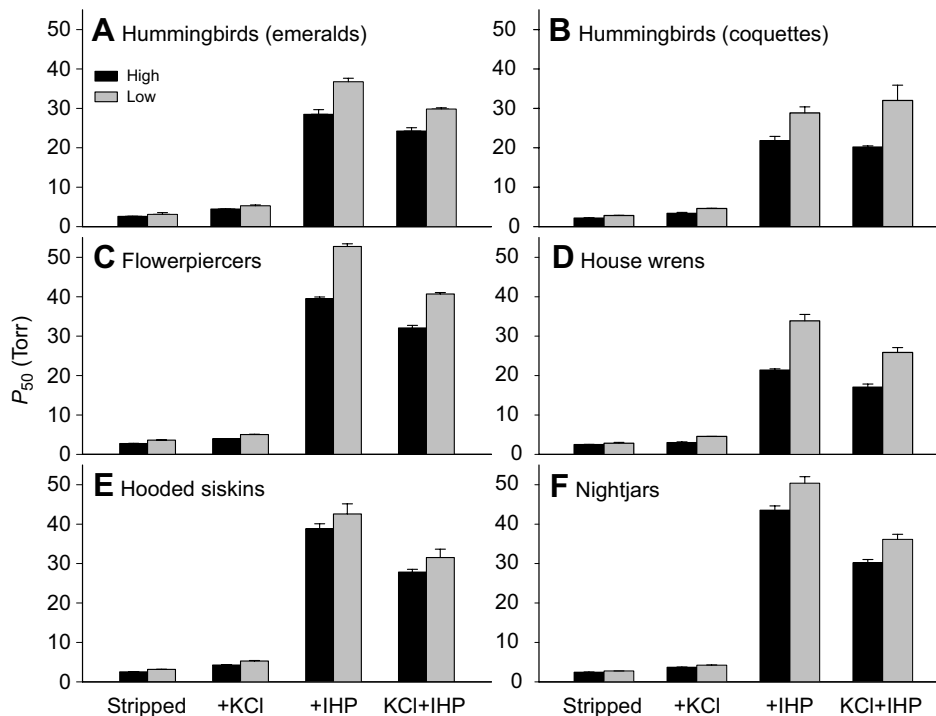
Fig. 8. Comparison of oxygenation properties of the major Hb isoform (HbA) between pairs of high- and low-altitude birds in the Andes. O_2 equilibria were measured at pH 7.40, 37°C in the presence and absence of allosteric effectors ([Cl⁻], 0.10 mol l⁻¹; [Hepes], 0.1 mol l⁻¹; IHP:tetrameric Hb ratio, 2.0: [heme], 0.3 mmol l⁻¹). For each taxon, P_{50} values (±s.e.m.) are reported for stripped Hbs in the absence of added anions, in the presence of Cl⁻ alone (added as KCl), in the presence of IHP alone, and in the presence of both anions combined. As explained in the main text, the 'KCl+IHP' treatment is most relevant to *in vivo* conditions in avian red blood cells, but measurements of O_2 affinity under each of the four standardized treatments provide insights into the functional mechanism responsible for observed differences in $P_{50(KCl+IHP)}$. In each pairwise comparison shown here, slight differences in intrinsic Hb–O_2 affinity (reflected by stripped P_{50} values) become more pronounced in the presence of IHP. (A) Comparison of HbA O_2 affinities between high- and low-altitude hummingbirds in the Emeralds clade (Trochilidae: Apodiformes): the green-and-white hummingbird, *Amazilia viridicauda*, and the amazilia hummingbird, *A. amazilia*, respectively. (B) Comparison of HbA O_2 affinities between high- and low-altitude hummingbirds in the Coquettes clade (Trochilidae: Apodiformes): the Andean hillstar, *Oreotrochilus estella*, and the speckled hummingbird, *Adelomyia melanogenys*, respectively. (C) Comparison of HbA O_2 affinities between high- and low-altitude flowerpiercers (Thraupidae: Passeriformes): the black-throated flowerpiercer, *Diglossa brunneiventris*, and deep-blue flowerpiercer, *D. glauca*, respectively. (D) Comparison of HbA O_2 affinities between high- and low-altitude populations of the house wren, *Troglodytes aedon* (Troglodytidae: Passeriformes). (E) Comparison of HbA O_2 affinities between high- and low-altitude populations of the hooded siskin, *Spinus magellanica* (Fringillidae: Passeriformes). (F) Comparison of HbA O_2 affinities between high- and low-altitude nightjars (Caprimulgidae: Caprimulgiformes): the band-winged nightjar, *Hydropsalis longirostris*, and Tschudi's nightjar, *H. decussata*, respectively. Data from Projecto-Garcia et al. (2013), Galen et al. (2015), and Natarajan et al. (2016).

(Fig. 10) (Natarajan et al., 2016). It remains to be seen whether isoform switching plays a role in the seasonal acclimatization to acute hypoxia, for example in species that undergo trans-Himalayan migratory flights like bar-headed geese, ruddy shelducks (*Tadoma ferruginea*) and demoiselle cranes (*Anthropoides virgo*).

In contrast to birds and other sauropsid taxa, most mammals do not co-express functionally distinct Hb isoforms during postnatal life. Since prenatally expressed Hb isoforms often have higher O_2 affinities than normal adult Hbs, it is possible that the continued expression of such isoforms into postnatal life could contribute to an enhanced blood–O_2 affinity in response to environmental hypoxia (a form of biochemical paedomorphosis). Among eutherian mammals, stage-specific expression of fetal Hb isoforms evolved independently in simian primates (New World monkeys, Old World monkeys, apes and humans) and in bovid artiodactyls (cattle, antelope, sheep and goats) (Storz, 2016b). In humans, retention of fetal Hb expression into adulthood is known to ameliorate the pathological effects of sickle-cell anaemia (Akinsheye et al., 2011; Pack-Mabien and Imran, 2013), but there is no evidence for the existence of a similar expression pattern in indigenous high-altitude populations. In yaks, high-affinity fetal Hb isoforms are expressed at high levels in one-month-old calves (Weber et al., 1988b), but it is

not known whether high expression is retained into adulthood. There is one dubious report claiming that fetal Hb accounts for 55% of total Hb in adult alpacas (*Vicugna pacos*) that were acclimatized at an elevation of 4200 m (Reynafarje et al., 1975). In this study, the putative fetal Hb isoform was identified as such only because it exhibited a resistance to alkaline denaturation similar to that of human fetal Hb (HbF). Thus, identification of the isoform as 'fetal Hb' was based on functional analogy rather than true homology. Moreover, there is no evidence that camelids express a true fetal Hb isoform in the first place. Comparative genomic data indicate that alpacas and other camelids do not share orthologues of the fetally expressed β-type globin gene in bovid artiodactyls [the bovid-specific duplication event that gave rise to the fetally expressed β-globin gene occurred long after the ancestors of bovids split from the ancestors of camelids (Gaudry et al., 2014)].

In summary, there is currently no compelling evidence to suggest that Hb isoform switching represents an important mechanism of physiological acclimatization to hypoxia in mammals or birds (Storz, 2016b). However, aside from recent studies of Andean birds (Natarajan et al., 2015b, 2016), it is also true that hypoxia-induced isoform switching has not been systematically investigated as a mechanism of phenotypic plasticity in terrestrial vertebrates.
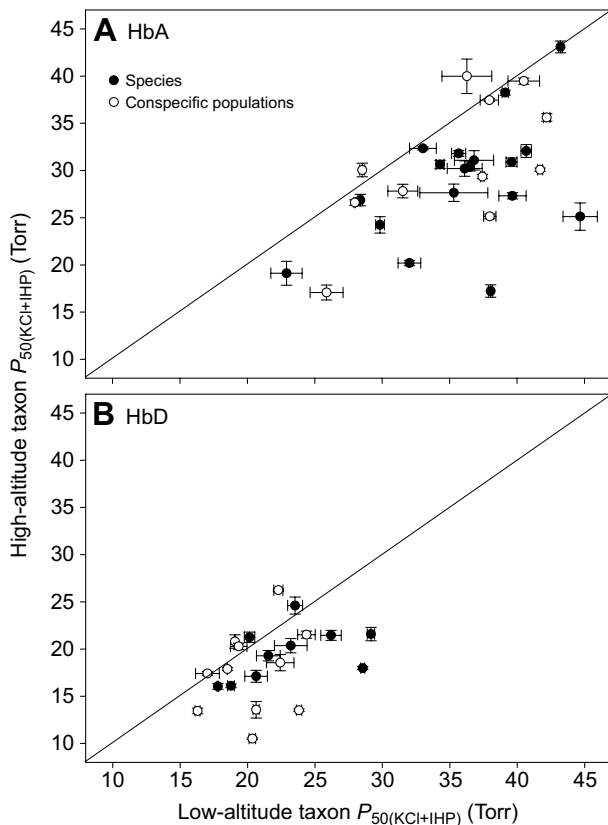
Fig. 10. There is no evidence for altitude-related differences in the relative abundance of HbA and HbD isoforms in Andean birds. Phylogenetically independent comparisons involving 26 pairs of high- and low-altitude taxa revealed no systematic difference in the relative expression level of the minor HbD isoform (Wilcoxon signed-ranks test, Z=−0.775, P=0.441). The diagonal represents the line of equality (x=y). Filled symbols denote comparisons between species, whereas open symbols denote comparisons between high- versus low-altitude populations of the same species. Data from Natarajan et al. (2016).

Fig. 9. Convergent increases in Hb–O₂ affinity in high-altitude Andean birds. (A) Plot of $P_{50(KCl+IHP)}$ (±s.e.m.) for HbA in 29 matched pairs of high- and low-altitude taxa. Data points that fall below the diagonal (x=y) denote cases in which the high-altitude member of a given taxon pair possesses a higher Hb–O₂ affinity (lower P_{50}). Comparisons involve phylogenetically replicated pairs of taxa, so all data points are statistically independent. (B) Plot of $P_{50(KCl+IHP)}$ (±s.e.m.) for the minor HbD isoform in a subset of the same taxon pairs in which both members of the pair express HbD. Sample sizes are larger for HbA than for HbD because the two ground dove species (*Metriopelia melanoptera* and *Columbina cruziana*) expressed no trace of HbD, and several hummingbird species expressed HbD at exceedingly low levels (Projecto-Garcia et al., 2013; Natarajan et al., 2016). In such cases, sufficient quantities of HbD could not be purified for measures of O₂ equilibria. Filled symbols denote comparisons between species, whereas open symbols denote comparisons between high- versus low-altitude populations of the same species. Data from Natarajan et al. (2015b, 2016).

Maladaptive plasticity and hypoxia acclimatization

I have reviewed theoretical and experimental evidence suggesting that an increased Hb–O₂ affinity is beneficial under severe hypoxia, and I have synthesized available comparative data which suggest that natural selection has favoured increased Hb–O₂ affinities in numerous high-altitude mammals and birds. It therefore seems paradoxical that the acclimatization response to environmental hypoxia in humans and other lowland mammals typically involves a reduction in blood–O₂ affinity (Mairbäurl et al., 1993; Mairbäurl, 1994; Samaja et al., 2003; Storz et al., 2010b). This is largely attributable to an increase in red cell DPG concentration (Torrance et al., 1970/71; Lenfant et al., 1971; Mairbäurl et al., 1986, 1993; Mairbäurl, 1994), or – more specifically – an increase in the relative concentration of Hb liganded with DPG and other anions that make smaller contributions. The increased concentration of DPG-liganded Hb at high altitude is largely attributable to a hypoxia-induced increase in ventilation; the resultant respiratory alkalosis stimulates red cell glycolytic activity which, in turn, increases DPG
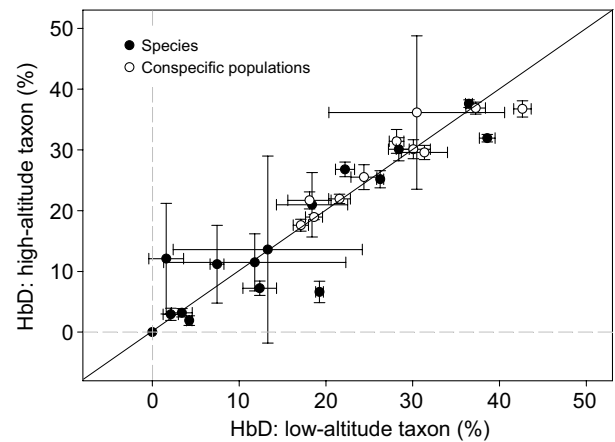
synthesis (Rapoport et al., 1977). At the whole-blood level, the hypoxia-induced increase in [DPG] is also attributable to the stimulation of erythropoiesis because this produces a downward shift in the mean age of circulating red blood cells, and newly produced red cells have higher [DPG] than older cells (Mairbäurl, 1994; Samaja et al., 2003). A number of previous authors interpreted the hypoxia-induced increase in red cell [DPG] (and the associated reduction in blood O₂ affinity) as an adaptive response (Aste-Salazar and Hurtado, 1944; Lenfant et al., 1968, 1969, 1971; Eaton et al., 1969; Frisancho, 1975). The theoretical and empirical results reviewed above suggest that such a response may be beneficial under moderate hypoxia. However, in humans and other mammals, the hypoxia-induced increase in red cell [DPG] continues at elevations well above the threshold at which further reductions in Hb–O₂ affinity become counterproductive due to arterial desaturation (Winslow et al., 1984; Samaja et al., 2003). Even when all Hb is fully liganded with DPG (at a DPG:Hb ratio of ≥2–3), further increases in [DPG] continue to indirectly reduce Hb–O₂ affinity because the increased erythrocytic concentration of non-diffusible anions reduces cellular pH, thereby reducing Hb–O₂ affinity via the Bohr effect (Duhm, 1971; Samaja and Winslow, 1979; Mairbäurl, 1994). Consequently, the increase in plasma pH caused by respiratory alkalosis has offsetting effects in mammalian red cells: the Bohr effect promotes an increased Hb–O₂ affinity, but this is counterbalanced by the increase in intracellular [DPG] (Winslow et al., 1984; Samaja et al., 1997).

In mammals that have acclimatized to chronic hypoxia, the seemingly maladaptive increase in red cell [DPG] may represent a miscued response to environmental hypoxia in species whose ancestors evolved in a lowland environment (Storz et al., 2010b; Tufts et al., 2013). In contrast to mammals, teleost fishes typically respond to environmental hypoxia by reducing red cell concentrations of nucleotide triphosphates such as ATP and GTP, thereby increasing Hb–O₂ affinity to enhance O₂ uptake in the gills (Weber, 1996; Jensen et al., 1998; Val, 2000; Nikinmaa, 2001). Whereas the enucleated red cells of mammals rely on a metabolite of anaerobic glycolysis to regulate Hb–O₂ affinity, the aerobically metabolizing red cells of fish contain high levels of ATP, so the

synthesis of allosteric effectors that reduce Hb–O_2 affinity is directly dependent on blood P_{O_2}, providing a means of positive feedback control. In mammalian red cells, DPG is a far more potent allosteric regulator of Hb–O_2 affinity than ATP, partly because ATP is ~90% complexed with Mg^{2+} (Bunn, 1971; Mairbäurl, 1994). It remains to be seen whether birds and other terrestrial vertebrates with nucleated red cells acclimatize to environmental hypoxia via changes in red cell organic phosphate concentrations, but available evidence indicates that [IPP] in avian red cells is highly constant and is unresponsive to changes in temperature or P_{O_2} (Jaeger and McGrath, 1974; Lutz, 1980).

Conclusions

Theoretical results indicate that it is generally beneficial to have an increased Hb–O_2 affinity under conditions of severe hypoxia. This prediction is supported by experimental studies of survival and whole-animal physiological performance and by *ex vivo* studies of microvascular O_2 transport and tissue perfusion. A number of detailed case studies involving mammals and birds have provided evidence for adaptive increases in Hb–O_2 affinity in high-altitude natives. Evolutionary changes in Hb–O_2 affinity involve a variety of functional mechanisms. In mammals, evolved increases in Hb–O_2 affinity in high-altitude populations or species involve changes in the intrinsic O_2 affinity of Hb and, in some cases, suppressed sensitivities to anionic effectors. In birds, evolved increases in Hb–O_2 affinity are consistently attributable to changes in intrinsic affinity that do not compromise allosteric regulatory capacity (Natarajan et al., 2015b, 2016). Available evidence suggests that regulatory changes in Hb isoform composition do not play a general role in adaptation to high-altitude hypoxia in birds or mammals.

In mammals, the evidence for a positive relationship between Hb–O_2 affinity and native elevation is equivocal. In birds, by contrast, there is a remarkably strong positive relationship between Hb–O_2 affinity and native elevation. In fact, the data for high-altitude birds provide one of the most compelling examples of convergent biochemical adaptation in vertebrates. An important question for future work concerns the reason for this apparent difference between mammals and birds, two amniote lineages that independently evolved endothermy and which diversified in parallel during periods of increasing atmospheric O_2 in the late Mesozoic Era. In comparisons within and among different vertebrate groups, it will also be important to determine whether it is possible to predict the threshold values of Pa_{O_2} at which it becomes beneficial to have an increased or decreased Hb–O_2 affinity.

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