ACTA PHYSIOLOGICA

Acta Physiol 2011, **202**, 549–562

REVIEW

Temperature dependence of haemoglobin–oxygen affinity in heterothermic vertebrates: mechanisms and biological significance

R. E. Weber[1] and K. L. Campbell[2]

1 Zoophysiology, Institute of Biological Sciences, University of Aarhus, Aarhus, Denmark

2 Department of Biological Sciences, University of Manitoba, Winnipeg, MB, Canada

Received 13 July 2010, revision requested 30 August 2010, revision received 4 October 2010, accepted 8 October 2010
Correspondence: R. E. Weber, Zoophysiology, University of Aarhus, Building 1131, Universitetsparken, DK 8000 Aarhus, Denmark.
E-mail: roy.weber@biology.au.dk

Abstract

As demonstrated by August Krogh *et al.* a century ago, the oxygen-binding reaction of vertebrate haemoglobin is cooperative (described by sigmoid O_2 equilibrium curves) and modulated by CO_2 and protons (lowered pH) that – in conjunction with later discovered allosteric effectors (chloride, lactate and organic phosphate anions) – enhance O_2 unloading from blood in relatively acidic and oxygen-poor tissues. Based on the exothermic nature of the oxygenation of the haem groups, haemoglobin–O_2 affinity also decreases with rising temperature. This thermal sensitivity favours oxygen unloading in warm working muscles, but may become detrimental in regionally heterothermic animals, for example in cold-tolerant birds and mammals and warm-bodied fish, where it may perturb the balance between O_2 unloading and O_2 requirement in organs with substantially different temperatures than at the respiratory organs and thus commonly is reduced or obliterated. Given that the oxygenation of haemoglobin is linked with the endothermic release of allosteric effectors, increased effector interaction is an effective strategy that is widely exploited to achieve adaptive reductions in the temperature dependence of blood-O_2 affinity. The molecular mechanisms implicated in heterothermic vertebrates from different taxonomic groups reveal remarkable variability, both as regards the effectors implicated (protons in tunas, organic phosphates in sharks and billfish, chloride ions in ruminants and chloride and phosphate anions in the extinct woolly mammoth, etc.) and binding sites for the same effectors, indicating multiple evolutionary origins, but convergent physiological functionality (reductions in temperature dependence of O_2-binding affinity that safeguard tissue O_2 supply).

Keywords haemoglobin, heterothermy, oxygen affinity, oxygen transport, respiration, temperature.

Globins or their genes appear to occur in all living organisms (Weber & Vinogradov 2001) and possibly in every cell (Riggs & Gorr 2006). They exhibit a characteristic tertiary structure (the 'globin fold') that reflects common ancestry, but serve a wide range of functions, that include transporting, storing, sensing and scavenging respiratory gases (oxygen, carbon dioxide and nitric oxide). The circulating haemoglobins (Hbs) of vertebrate animals are exquisitely engineered to transport O_2 from the respiratory surfaces to the respiring tissues, bridging wide and independent variations in gas tension at both sites. As a direct link between ambient O_2 availability and aerobic metabolism, they exhibit striking functional adaptations to

exogenous factors (temperature, O_2 and water availability, etc.) as well as endogenous constraints (mode of life, developmental stage, activity, etc.) and are the paradigm for studying allosteric interactions of proteins. 'During the past 60 years, the study of human Hb, probably more than any other molecule, has allowed the birth and maturation of molecular medicine' (Schechter 2008).

The O_2 affinity of blood is a product of (1) Hb's intrinsic O_2 affinity and its sensitivity to red cell allosteric effectors (chiefly protons, organic phosphates and chloride ions) that modulate this affinity – both of which are dependent on the structural properties of the Hb molecule (e.g. effector-binding sites, conformational changes), and (2) the microenvironmental conditions that exist inside the red cells (e.g. the concentrations of allosteric effectors). An additional important *de facto* effector of Hb function – particularly for regionally heterothermic vertebrates – is temperature. First, the oxygenation of the haem groups is exothermic (i.e. the oxygenation enthalpy, ΔH^{O}, is negative, whereby O_2 affinity decreases with increasing temperature); secondly, the overall oxygenation enthalpy observed in blood ($\Delta H^{'}$) includes endothermic contributions from oxygenation-linked release of effector molecules (which tend to preferentially bind to the deoxy form of Hb) and thus is modulated by these reactions. Illustrated with selected examples, this treatise focuses on this relatively neglected aspect of Hb research; the variations in the temperature sensitivity of Hb–O_2 affinity caused by oxygenation-linked interactions, their biological significance and the underlying molecular mechanisms, focusing on vertebrates that exhibit regional and temporal heterothermy. It should be borne in mind that these variations in thermal sensitivity of O_2 affinity are distinct from adjustments in blood-O_2 affinity resulting from thermo-acclimatory changes in the red cell concentrations of allosteric effectors (Wood *et al.* 1978).

Phenomenological systemic- and molecular physiological background

Maintenance of blood-O_2 transport in the face of variable O_2 availability depends on a symphony of integrated organismic, cellular and molecular adaptations – as aptly formulated in the Fick equation: $\dot{V}O_2 = \dot{V}b\ (CaO_2 - CvO_2)$ (Fick 1870), where $\dot{V}O_2$ is the O_2 consumption (transport) rate, $\dot{V}b$ the cardiac output and $(CaO_2 - CvO_2)$ the difference in O_2 contents between the arterial and mixed-venous blood – which in turn equals $\beta bO_2\ (PaO_2 - PvO_2)$, where $(PaO_2 - PvO_2)$ is the arterial-mixed-venous PO_2 difference and βbO_2 is the O_2 capacitance coefficient $(CaO_2 - CvO_2)/(PaO_2 - PvO_2)$, i.e. the steepness of the functional part

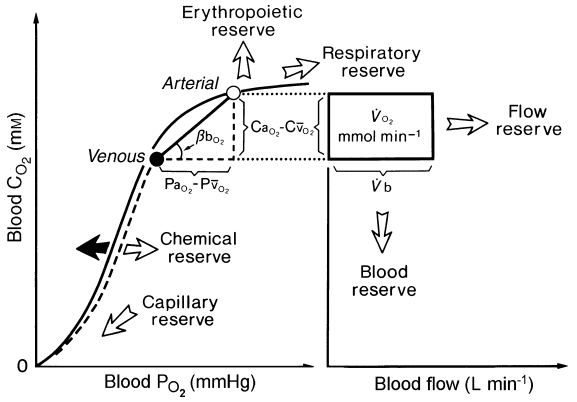

Figure 1 Schematic representation of O_2 equilibrium curves for arterial (solid curve, open circle) and venous (dashed curve, solid circle) and the interrelationships between blood oxygenation characteristics and circulatory (blood flow) rate that govern O_2 transport (aerobic metabolic) rate ($\dot{V}O_2$) [modified after (Bouverot 1985)].

of the O_2 equilibrium curve (Fig. 1). It follows that for given magnitudes of cardiac output, O_2 carrying capacity, and arterial and venous O_2 tension, blood O_2 transport depends solely on left or right shifts in the O_2 equilibrium curve (decreases or increases, respectively, in the half-saturation O_2 tension, P_{50}) that change βbO_2.

Vertebrate Hbs consist of 2α and 2β type polypeptide chains (composed of 141 and 146 amino acid residues, respectively, in humans), each comprising eight α-helices (termed A, B, etc.), interlinked by non-helical segments (AB, BC, etc.) and N- and C-terminal extensions (NA and HC respectively). Individual amino acid residues are identified by their positions in the chain and/or the helix. Thus $\alpha_1$131(H14)-Ser refers to serine occupying position 131 in the α_1 chain and position 14 in the H-helix.

Vertebrate Hbs may assume two conformational structures, the high affinity oxygenated R (relaxed) structure that predominates in the respiratory organs (lungs or gills) and the low affinity T (tense) form that is constrained by additional hydrogen bonds and salt bridges and mainly occurs in the tissues. As demonstrated by Bohr *et al.* (1904) more than a century ago, Hb–O_2 binding exhibits two fundamental functional traits that favour O_2 unloading in the (relatively hypoxic and acidic) tissues: sigmoid Hb–O_2 equilibrium curves that express cooperativity (positive, homotropic interactions) between the haem groups, and a Bohr effect (negative, heterotropic interactions between proton-binding sites and the O_2-binding haems). Moreover, half a century before the key role of organic phosphate O_2 affinity modulators [DPG (2,3-diphosphoglycerate) in mammals, IPP (inositol pentaphosphate) in birds and ATP in ectothermic vertebrates] were first documented

(Benesch *et al.* 1969, Chanutin & Hermann 1969), Krogh & Leitch (1919) explicitly predicted their existence and functional significance: 'We believe that the adaptation of the…blood must be brought about by some substance or substances present along with the Hb within the corpuscles. …By this arrangement just that chemical environment can be secured which is most suitable for the respiratory function of the Hb in that particular organism'.

The major allosteric effectors that decrease Hb–O_2 affinity in the red cells bind at (relatively few) specific amino acid residues (Perutz 1983, Riggs 1988). Thus, whereas the phosphate effectors ATP and DPG bind at four amino acid residues of the β-chains, i.e. β1(NA1)-Val, β2(NA2)-His, β82(EF6)-Lys and β143(H21)-His, Cl^- ions bind mainly at one α-chain site [between α131(H14)-Ser and α1(NA1)-Val] and one β-chain site [between β82(EF6)-Lys and β1(NA1)-Val]. Whereas the majority of 'Bohr' protons bind at β146(HC3)-His (the C-terminal residue of the β-chains), additional deoxygenation-linked proton binding also occurs at α1(NA1)-Val (the N-terminal residues of the α chains) and at solution accessible imidazole side chains of other His residues in both chains (Lukin & Ho 2004, Berenbrink 2006).

Temperature sensitivity and thermodynamics of O_2 binding

The temperature sensitivity of P_{50} can be quantified in terms of the overall (apparent) enthalpy of oxygenation ($\Delta H'$) calculated using the van't Hoff isochore: $\Delta H' = 2.303R{\cdot}\Delta\log P_{50}/\Delta(1/T)$ (Wyman 1964), where R is the gas constant and T is the absolute temperature. Thus numerically high negative and positive $\Delta H'$ values, respectively, denote high normal and reverse temperature effects. Derived in this manner $\Delta H' = \Delta H^{O_2} + \Delta H^{H_2O} + \Delta H^{P^-} + \Delta H^{H^+} + \Delta H^{Cl^-} + \Delta H^{X^-} + \Delta H^{T \rightarrow R}$, i.e. the overall enthalpy of oxygenation comprises, respectively, the intrinsic heat of haem oxygenation, the heat of solution of O_2 (-12.6 kJ mol^{-1}), the heats of dissociation of phosphates, protons, Cl^- ions and unknown effectors, and the heat of the $T \rightarrow R$ transition. Given that ΔH^{O_2}, and ΔH^{H_2O} are practically invariant for most Hbs, and that contributions from ΔH^{X^-} can be excluded in suitably prepared Hb solutions, it follows that the heat of reactions with specific allosteric effectors (protons, organic phosphates or Cl^- ions) and the heat of conformational change can be assessed from differences in enthalpies measured in the presence and absence of such ligands (cf. Weber *et al.* 1985, 2008). Thus, whereas exothermic haem oxygenation causes a normal temperature effect (decreased affinity with rising temperature), the coupled endothermic release of the major effectors (protons, organic phosphates and Cl^- ions) reduces or reverses the overall temperature effect, and the latter contributions to $\Delta H'$ can be assessed by judicious choice of experimental conditions that include or exclude specific effectors (singly or in combination).

The decrease in Hb–O_2 affinity with increasing temperature mandated by the exothermic nature of the oxygenation of haem groups is considered advantageous in that it enhances O_2 unloading from blood that perfuses warm tissues, for example exercising muscle that have increased O_2 requirements (Barcroft & King 1909) – although its contribution is presumably marginal compared to increased blood flow to exercising tissues. However, this trait may be maladaptive in regionally heterothermic animals where it can lead to a mismatch between the O_2 delivery and demand in tissues maintained at temperatures different than that at the respiratory surface. A pertinent example is fast-swimming oceanic fish like tunas, sharks and billfish (Rossi-Fanelli *et al.* 1960, Larsen & Malte 2003, Weber *et al.* 2010) that have warm swimming muscles (Carey & Gibson 1977, Larsen *et al.* 2003) and/or warm eyes and brains (Carey *et al.* 1971, Block & Carey 1985, Block 1986) (Fig. 2) – where normal thermal sensitivity would increase the risk of arterio-venous short-circuiting of O_2 in the countercurrent heat-exchangers supporting the warm organs (Larsen & Malte 2003) or cause abrupt O_2 unloading from blood perfusing the warm, red swimming muscles while inhibiting O_2 unloading in cold organs including the liver and gut (Clark *et al.* 2008). Another example is cold-tolerant Arctic mammals, where a normal temperature sensitivity (increased O_2 affinity at low temperature) would impede O_2 unloading to skin and extremities (Giardina *et al.* 1989, Brix *et al.* 1990a, Coletta *et al.* 1992, De Rosa *et al.* 2004) whose temperatures may fall to near 0 °C (Irving & Krog 1955). As observed in reindeer (Fig. 3) (Johnsen *et al.* 1985), extremity temperatures increase during activity at low ambient temperatures, indicating that normal (albeit reduced) temperature sensitivities continue to contribute in matching O_2 delivery to O_2 demand in exercising muscles under these conditions.

Adaptive mechanisms – case studies

Fish

Regional heterothermy among fish (often termed endothermy) is considered to have involved independently in different lineages, each time to permit thermal niche expansion into cool temperate waters (Block *et al.* 1993). Compared to tunas where elevated temperatures in the swimming muscles result from high metabolic rates and reduced whole body thermal conductance,

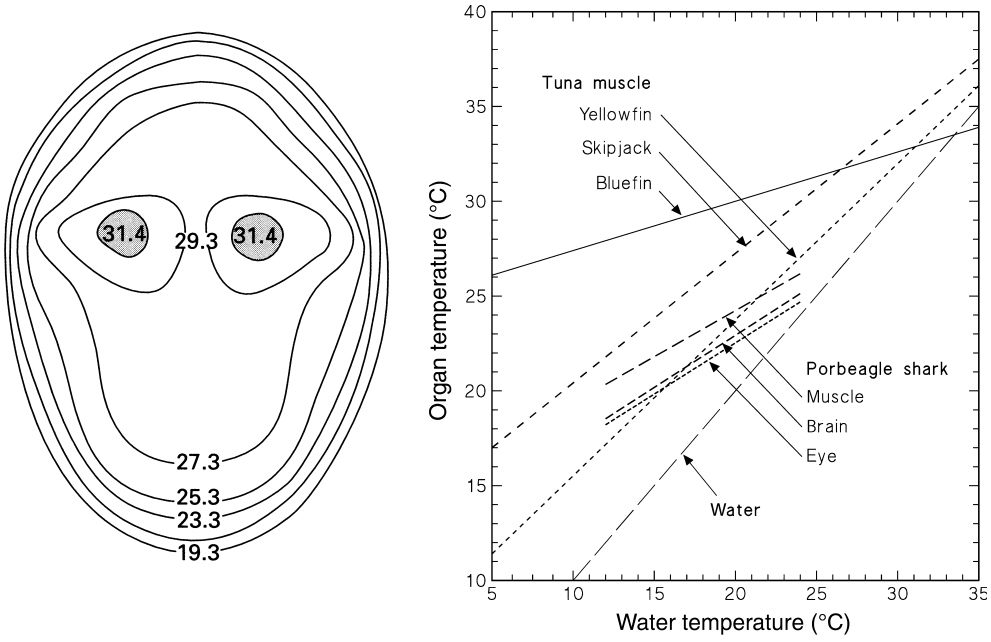

Figure 2 Temperature profile across the trunk of bluefin tuna at a water temperature of 19 °C (left) and temperatures in muscle, eyes and brain of tunas and porbeagle shark at different water temperature (right). Based on data from Carey (1973) and Block and Carey (1985).

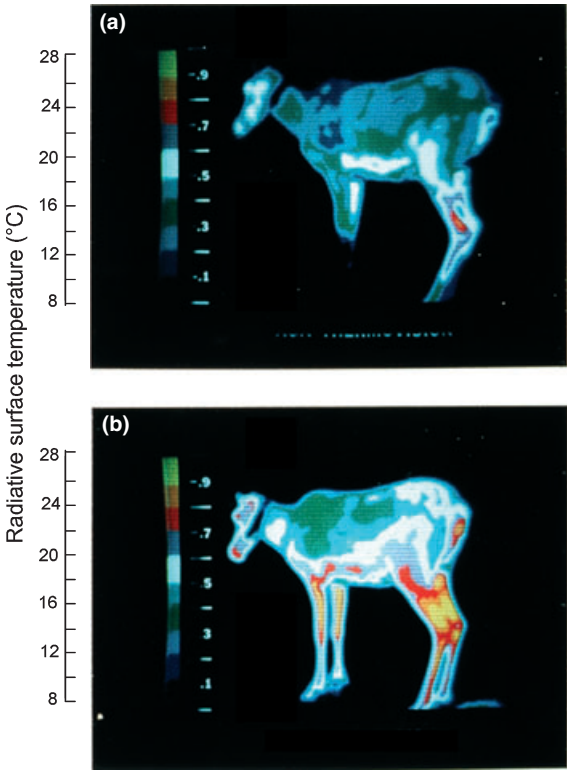

Figure 3 Radiative surface temperatures for reindeer (*Rangifer tarandus tarandus*) before (a) and after (b) 45 min running on a treadmill at an ambient temperature of 2 °C with isotherms represented by nine colours in the scales on the left. After Johnsen *et al.* (1985). The frames were kindly provided by Dr A.S. Blix, University of Tromsø, Norway.

billfish accomplish a more limited form of endothermy associated with modified muscle cells that produce heat in a futile Ca^{2+} cycle that does not generate force (Block *et al.* 1993). As normal temperature sensitivities (endothermic deoxygenation in the tissues and exothermic oxygenation in the gills) imply outward heat transport, the reduced temperature sensitivities contribute to maintaining warm organs in endothermic fish (Weber & Wells 1989).

The occurrence of low thermal dependence of blood-O_2 affinity in ectothermic fish (e.g. the chub mackerel *Scomber japonicus*) indicates that this attribute is not uniquely associated with heterothermy (Clark *et al.* 2010), but may have arisen in response to alternative evolutionary incentives. Hbs of highly active fish generally exhibit large Bohr effects, that – apart from enhancing O_2 unloading in the relative acid respiring tissues – confer reductions in oxygenation enthalpy (cf. Weber *et al.* 2010), aligning with the positive correlation between large Bohr factors and low temperature sensitivities in tuna species (Cech *et al.* 1984, Brill & Bushnell 1991, Lowe *et al.* 2000). Extremely high blood Bohr effects (φ = −1.5 at 30 °C in chub mackerel) may, moreover compensate for the lack of temperature influence on O_2 unloading in tissues (Clark *et al.* 2010).

Tunas. Assessed from the low temperature dependence of P_{50}, the overall heat of oxygenation of Hb of bluefin tuna, *Thunnus thynnus*, that exhibits large body temperature gradients (Fig. 2), is very low (−7.5 kJ mol^{-1}

at pH 6.5–8.7, including the heat of solution of O_2) (Rossi-Fanelli *et al.* 1960) compared to corresponding values in ectothermic fish (e.g. −58 kJ mol^{-1} in crucian carp Hb at pH 7.6) (Sollid *et al.* 2005). However, the temperature sensitivity of tuna Hb is saturation dependent, being normal at low O_2 saturation, and reverse (O_2 affinity increasing with rising temperature) at high saturation (Ikeda-Saito *et al.* 1983) (Fig. 4a). This suggests the endothermic release of an allosteric effector late in the oxygenation process, which correlates neatly with observations that in yellowfin tuna (Lowe *et al.* 1998) (Fig. 4b) – as in tench and trout (Jensen 1986, Brauner *et al.* 1996) – the decrease in red cell pH that accompanies deoxygenation is almost entirely limited to O_2 saturations above 50%. Indeed analysis in terms of Adair's four-step oxygenation theory reveals that in tench Hb most protons are released upon binding of the third O_2 molecule (Jensen & Weber 1987, Weber *et al.* 1987a).

The substantial enthalpic contribution of proton binding/dissociation in tuna Hb correlates with the large fixed acid Haldane effect (release of almost 4 mole protons per mole Hb upon oxygenation) (Jensen 2001). Moreover X-ray analysis of tuna Hb crystals indicates a novel mechanism of pH control involving additional proton-binding sites (at α60-His, β69-His/β72-Asp and $\alpha_1$96-Asp/$\beta_2$101-Asp) (Yokoyama *et al.* 2004) that may have different heats of ionization. That the oxygenation-linked effector interaction itself may be temperature dependent follows from the observation that blood of southern bluefin tuna lacks significant temperature effects above 23 °C, but exhibits a strong reverse sensitivity below this temperature (Clark *et al.* 2008). In contrast to the well-defined role of proton binding, the possible contributions of phosphate and chloride binding to the enthalpy of tuna Hbs seem not to have been investigated.

The temperature sensitivities of blood and Hb solutions are standardly assessed from the effects of 'open-system' temperature changes – that permit exchange of gases or proton equivalents with another (e.g. gaseous) medium – on P_{50}. However, as demonstrated for endothermic tunas, blood passing from the gills (at ambient temperature) through the vascular countercurrent heat exchangers undergoes 'closed-system' temperature shifts (i.e. without exchanging gases and acidic equivalents with another medium), whereby blood O_2 content remains constant, but O_2 and CO_2 tensions and plasma pH undergo substantial variations (Cech *et al.* 1984, Brill & Bushnell 1991, 2006, Lowe *et al.* 2000,) that may contribute to the effects of temperature *per se*. This temperature response varies broadly in tunas. Thus blood-O_2 affinity is independent of 'closed-system' temperature in skipjack tuna, normal (i.e. affinity is reduced with rising temperature) in yellowfin tuna and reversed (affinity is increased) in albacore (Cech *et al.* 1984, Brill & Bushnell 1991, 2006).

Billfish. These agile predators possess modified (non-contractile) cranial muscles that warm their brains and large eyes to temperatures of up to 15 °C above ambient water values (Block 1986, Fritsches *et al.* 2005). In the swordfish *Xiphias gladius*, retinal warming drastically increases temporal resolution, imparting 'crucial advantage over their agile, cold-blooded prey' (Fritsches *et al.* 2005).

Analysis of the temperature sensitivities of 'stripped' (purified) Hbs from blue marlin, striped marlin and

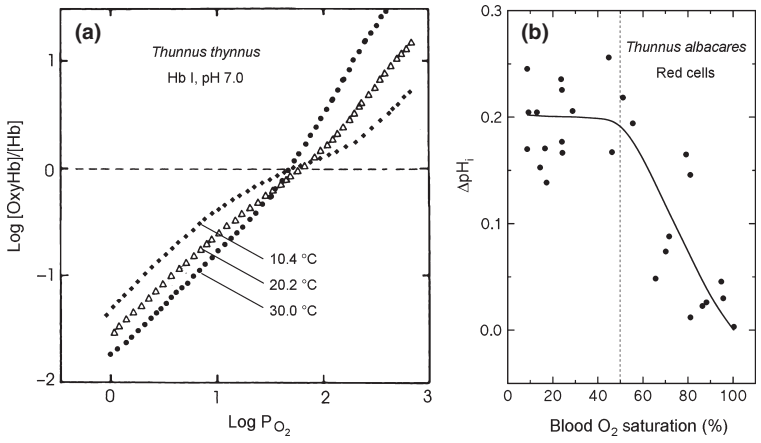

Figure 4 (a) Hill plots of the main Hb component (Hb I) of bluefin tuna (*Thunnus thynnus*) showing that the temperature independence at half-saturation results from normal temperature sensitivity at low Hb-O_2 saturation and a reverse temperature sensitivity at saturations above ~50% [modified after Ikeda-Saito *et al.* (1983)]. (b) Changes in intracellular pH accompanying deoxygenation of yellowfin tuna (*Thunnus albacares*) red cells, showing that oxygenation-linked release of protons is almost entirely restricted to saturation levels above 50%. Modified after Lowe *et al.* (1998).

shortbill spearfish over a wide pH range and in the absence and presence of a physiological ATP/Hb molar ratio (Weber *et al.* 2010) permits assessment of contributions of protonation and phosphate binding to the thermodynamics of O_2 binding in billfish. In the absence of effectors and at high pH (where the Bohr effect is absent; Fig. 5a), the measured enthalpy reflects an intrinsic heat of O_2 binding ($\Delta H^{O_2} \sim -62$ kJ mol^{-1}), which corresponds with the value for fish (e.g. eelpout *Zoarces viviparus* Hb corrected for the heat of solvation) (Weber *et al.* 2003) and moreover closely agrees with that (−59 kJ mol^{-1}) determined calorimetrically for human Hb (Atha & Ackers 1974). As illustrated for blue marlin (Fig. 5b), $\Delta H'$ at pH 7.4 decreased to −39 kJ mol^{-1}, reflecting an enthalpic contribution from proton dissociation of +23 kJ mol^{-1}. In the presence of ATP, $\Delta H'$ at this pH becomes +26 kJ mol^{-1}, indicating that substantial enthalpy of dissociation of ATP and linked ions (39 + 26 = +65 kJ mol^{-1}) obliterates the intrinsic heat of haem oxygenation (Weber *et al.* 2010). The thermodynamic contribution of Cl^- binding/dissociation to billfish Hbs needs yet to be assessed.

The different molecular mechanisms of thermal insensitivities in tuna and billfish Hbs (major enthalpic contributions from proton and ATP binding, respectively) are consistent with the independent evolutionary origins of endothermy in these two fish lineages (above).

Lamnid sharks. Porbeagle and mako sharks are active, swift fish with body core temperatures of 7–13 °C above water temperatures (Carey & Teal 1969) – a temperature difference that raises the rates of muscle force development, contraction and relaxation about twofold (Bennett 1985). As in scombroids, endothermy in lamnid sharks developed independently thereby providing evidence of adaptive convergent evolution – both have countercurrent rete and blood O_2 affinities with low thermal dependence.

In contrast to tunas, proton binding does not contribute materially in modulating the temperature sensitivity in lamnid sharks – as judged from the absence of Bohr effects in the two major Hb components of the porbeagle shark at physiological red cell pH (∼7.4) (Larsen *et al.* 2003). That ATP may play a major role (as in billfish) follows from the finding that the 'normal' temperature effects exhibited by porbeagle Hbs III and IV ($\Delta H'$ = −21 and −40 kJ mol^{-1}, respectively, at pH 7.3) are reversed ($\Delta H'$ = +12 kJ mol^{-1}) in the presence of this effector (Larsen *et al.* 2003).

Endotherms

Birds. There is a dearth of information on structure–function adaptations in Hbs from cold-tolerant heterothermic birds – although it is well-documented that temperature in their extremities/feet may approach the freezing point through countercurrent heat exchange that reduces heat loss. Adelie penguin (*Pygoscelis adeliae*) Hb dialysed and suspended in phosphate buffer displays a temperature sensitivity ($\Delta H'$ = −53 kJ mol^{-1} at pH 7.3) (R.E. Weber, unpublished data) similar to that of Hbs from non-Antarctic birds. In the absence of organic phosphates, $\Delta H'$ of emperor penguin (*Aptenodytes forsteri*) Hb approximates −40 kJ mol^{-1} and is almost pH independent even in the pH range where the Bohr effect is operative (Tamburrini *et al.* 1994).

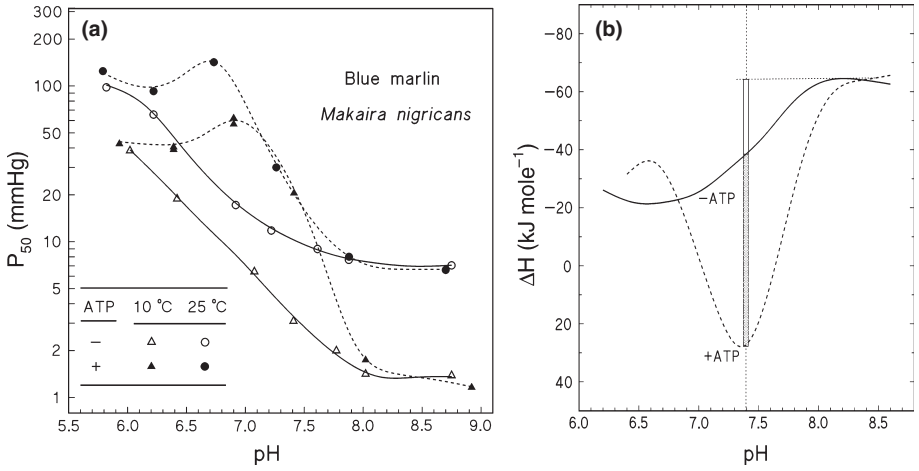

Figure 5 The pH dependence of (a) the half-saturation O_2 tensions (P_{50}) of blue marlin Hb at 10° (triangles) and 25 °C (circles), measured in the absence of added chloride (chloride concentration 4–6 mM), and in the absence (continuous curves) and presence (dashed curves) of ATP at twofold molar excess over Hb molecules, and (b) the enthalpies of oxygenation of the Hb (calculated from temperature-induced P_{50} shifts and corrected for the heat of solvation of O_2). Open and shaded vertical columns depict the contributions of oxygenation-linked binding of protons and ATP, respectively, to the overall oxygenation enthalpies at pH 7.4. Modified after Weber *et al.* (2010).

However, in the presence of IHP (inositol hexaphosphate, a structural analogue of IPP) and Cl^- ions, $\Delta H'$ decreases strongly once pH decreases below ~7.4 (almost twice as much as in pigeon Hb), indicating that O_2-linked dissociation of these anions accounts for reduced thermal sensitivity in penguin blood. The strong expression of this effect at low pH may relate to the ability of fasting (male!) penguins to incubate eggs (held by the feet against the body) while standing on ice and relying predominantly on fat metabolism that would produce significant metabolic acidosis (Tamburrini *et al.* 1994). Having the same IPP-binding amino acid residues as other bird Hbs, the molecular mechanism underlying the strong pH dependence in penguin Hb is as yet not elucidated.

Mammals. Several mammalian groups, including cetartiodactylids (whales and even-toed mammals like ruminants and pigs), carnivores (bears, felines and seals), talpid moles and elephantids possess members that exhibit prominent reductions in the thermal sensitivity of blood and Hb–O_2 affinities that may be physiologically advantageous, for example in favouring O_2 unloading in cold limbs and extremities.

Cetartiodactylids

Ruminants. Ruminant Hbs differ from Hbs of most other mammals in exhibiting low intrinsic O_2 affinities and low sensitivities to DPG (which moreover occurs at low concentrations in the red cells) that correlate with distinctive structural differences: the N-terminus of their β-chains is hydrophobic and points inwards into the protein moiety, stabilizing the Tense state – mimicking the action of DPG (Perutz & Imai 1980).

In contrast to adult human Hb, bovine and some other ungulate (e.g. horse) Hbs moreover possess a cluster of basic (His and Lys) residues at β-chain positions 8, 76 and 77 that has been suggested to form an 'additional' chloride-binding site that reduces the oxygenation enthalpy (via increased endothermic O_2-linked Cl^- dissociation). Ruminant Hbs are also unusual in that they exhibit 'synergistic' Cl^- and DPG binding – in that O_2 their affinities are depressed more by both effectors than when only one is present (De Rosa *et al.* 2004). This phenomenon, first described in bear Hb (Coletta *et al.* 1994) (see below) illustrates the presence of two distinct classes of chloride-binding sites in these species: one that overlaps (and competes) with the DPG docking site within the cationic pocket between the β-type chains, and one that is independent of DPG binding. Interestingly, a human mutant Hb constructed to possess similar hydrophobicity in the N-terminus of the β-chains as found in bovine Hb (β1-Val → Met; β2 deleted, β5-Pro → Ala) released very few chloride ions (0.8 per tetramer) upon oxygenation compared to the number (2.5 per tetramer) released when – in addition to these exchanges – a basic residue is introduced at position β76 (Ala → Lys) (Fronticelli *et al.* 1995).

Together with possible differences in the Bohr effects, these findings indicate that distinctive differences in $\Delta H'$ values between bovine and human Hbs (−27.2 and −41.0 kJ mol^{-1}, respectively, in the presence of 0.1 M Cl^- and at pH 7.4) (De Rosa *et al.* 2004) may variously result from differences in the enthalpic contributions from proton binding, Cl^- binding and structural constraints (Razynska *et al.* 1990). Indeed, measurement of the oxygenation enthalpies on both Hbs, in the absence and the presence of physiological chloride concentration and over a wide pH range (R.E. Weber, A. Fago and K.L. Campbell, unpublished data) reveal that most of the differences in temperature sensitivity between bovine and human Hbs reflect inherent structural differences (e.g. in hydrophobicity and $\Delta H^{T \rightarrow R}$) rather than differences in the enthalpies of proton and Cl^- binding.

Pigs. Low temperature dependence of O_2 affinity also characterizes pig blood ($\Delta \log P_{50}/\Delta T = 0.016$, compared to 0.022–0.024 in dog and humans), its heat of oxygenation corresponding to −16 kJ mol^{-1} when corrected for ΔH^{H20} (Willford & Hill 1986). As with cow and reindeer, the β-chains of porcine Hb possess a cluster of three positively charged amino acid residues (Lys, Lys and His) at positions 8, 76 and 77. However, unlike ruminant Hbs it has Val and His residues at positions 1 and 2 (Braunitzer *et al.* 1978). It thus lacks a hydrophobic N-terminal region and possesses the full complement of DPG-binding residues. Porcine red cells moreover contain high DPG concentrations (~1.5 times that in dog cells and 1.6–2.1 times that in human cells) (Rapoport & Guest 1941, Scott *et al.* 1977, Willford & Hill 1986). In the absence of DPG, at high pH (where proton binding is low), and at low (0.07 mM) Cl^- concentration, swine Hb exhibits high temperature sensitivity (−67 kJ mol^{-1}, corrected for ΔH^{H2O}) that is drastically reduced (to −14 kJ mol^{-1} at pH 7.4) by proton binding and the presence of DPG and Cl^- ions (Weber *et al.* 1987b). Although the temperature sensitivity of pig Hb in the presence of 0.1 M Cl^- (−34 kJ mol^{-1}) is intermediate between that of human Hb (−41 kJ mol^{-1}) and those of bovine and reindeer Hbs (−16 and −21 kJ mol^{-1}), it decreases to the same level as the ruminant Hbs upon the addition of DPG at its physiological red cell concentration (Condo *et al.* 1992). The molecular mechanism for the low ΔH in the presence of DPG may be that movement of the N-terminal residues (that are located at the exterior surface of the molecule) towards the interior of the

protein upon the dissociation of O_2, produces a distortion that moves β8(A5)Lys towards the E helix, allowing it to become part of the cluster of positive charges that binds Cl^- (Fronticelli 1990, Condo *et al.* 1992).

Given the tropical origins of this group, the biological significance of this trait is not initially clear. However, suids (both domestic and their wild ancestors) appear to be unique among placental (eutherian) mammals in that they lack both brown fat and its integral uncoupling 1 (UCP1) protein (Berg *et al.* 2006). Thus, neonatal swine are poor thermoregulators and notably sensitive to cold stress. In contrast, and despite possessing only a thin cover of bristly hair (the skin of the pig is as naked as that of man), adult pigs are notably cold tolerant (Irving 1956). This cold tolerance appears to arise from a shunting of blood away from the entire body surface, such that skin temperatures may drop by up to 30 °C relative to that in the core – hence creating a distinct thermal gradient (Irving 1956) or a form of regional heterothermy (a similar principle operates in the skin/blubber layer of seals, walruses and whales).

Baleen whales. Several species of large baleen whales forage for several months on end in the nutrient rich waters of the Arctic and Antarctic regions, where surface temperatures may drop to −1.9 °C. Although the body core is well insulated by a thick blubber layer, the fins and tail are held at much lower temperatures by countercurrent heat exchangers to minimize conductive heat loss (Scholander & Schevill 1955). Muscles in these propulsive appendages must thus be supplied with sufficient O_2 to sustain metabolic activity in face of temperature shifts during periods of rest and activity. The mechanism by which Lesser Rorqual (*Balaenoptera acutorostrata*) whales solve this problem appears to be unique in that it does not involve additional Cl^- binding, but instead is dependent upon a heightened effect of organic phosphates, together with synergistic contributions from the end products of both aerobic (CO_2) and anaerobic (lactate) metabolism in a temperature-dependent manner (Brix *et al.* 1989, 1990b). Thus, while the $\Delta H'$ of whale Hb at pH 7.4 and in the presence of 0.1 M Cl^- (−46 kJ mol^{-1}) is slightly elevated relative to human HbA under the same conditions, it is sharply reduced (−18.8 kJ mol^{-1}) – and comparable to that of Arctic ruminant Hbs (−14.0 to −15.0 kJ mol^{-1}; De Rosa *et al.* 2004) – upon addition of 3 mM DPG (Brix *et al.* 1989, 1990b). Interestingly, this larger reduction in $\Delta H'$ relative to HbA occurs despite the fact that DPG exerts a smaller allosteric effect on P_{50} in the whale Hb, the latter of which has been attributed to a β5-Pro → Ala exchange in whale Hb (Corda *et al.* 2003). Another unusual feature of whale Hb is that $\Delta H'$ is further reduced (to −8.8 kJ mol^{-1}) under high CO_2 tensions that might be expected to occur in the blood during prolonged dives. Lactate also exerts an additional allosteric effect in the presence of organic phosphates, however, like is found for CO_2, this effect disappears at 37 °C (Brix *et al.* 1990b). Consequently, temperature appears to modulate the allosteric binding of CO_2, facilitating O_2 delivery to cold tissues, but having little or no effect within the body core (Brix *et al.* 1989, 1990b). A similar interplay between organic phosphates, CO_2 and lactate is also found in the Hb of the Mediterranean whale (*B. physalus*), suggesting the same mechanism is widespread among baleen whales (Corda *et al.* 2003).

Carnivores

Bears. Brown bears (*Ursus arctos*) and polar bears (*U. maritimus*) are closely related sister species that diverged only some 150 000 years ago (Lindqvist *et al.* 2010), and perhaps unsurprisingly, the functional properties of their Hbs are very similar (Coletta *et al.* 1994, Pomponi *et al.* 2004). Unfortunately, the primary sequence of brown bear Hb is unknown; however, that of polar bears and Asiatic brown bears (*U. thibetanus*) – whose lineages diverged >2.5 Ma (Lindqvist *et al.* 2010) – are identical (Hofmann *et al.* 1986), suggesting the same may be true for brown bears. Importantly, this demonstrates that the rapid (10 000–30 000 year) morphological and ecological adaptation of polar bears to the Arctic marine biome (Lindqvist *et al.* 2010) is unrelated to modifications in their Hb. As is the case for ruminant Hbs, Cl^- and DPG modulate bear Hb–O_2 affinity synergistically, indicating the presence of two classes of Cl^--binding sites (Coletta *et al.* 1994, Pomponi *et al.* 2004). There is some debate regarding the location of the 'additional' deoxygenation-linked Cl^--binding site, with De Rosa *et al.* (2004) suggesting that, despite the His → Asn exchange at β77 of bear Hb, Cl^- is still able to bridge the cationic pocket formed by β8-Lys and β76-Lys. However, Pomponi *et al.* (2004) noted the β5-Pro → Gly substitution may alter the symmetry of DPG binding such that a single, additional Cl^- is able to form H-bonds between β82-Lys and β143-His. Nonetheless, the oxygenation enthalpy of bear Hb is reduced in the presence of Cl^- (−34.5 kJ mol^{-1} at pH 7.3), however, only to a value that is intermediate between that of human and bovine Hbs (De Rosa *et al.* 2004). Interestingly, this effect is strongly pH dependent, such that the $\Delta H'$ is reduced to −14.2 kJ mol^{-1} at pH 7.0 and −9.6 kJ mol^{-1} at pH 6.8 (Coletta *et al.* 1994). This attribute has been speculated to be beneficial for O_2 delivery during the winter months, when bears (including pregnant/lactating polar bears) enter an extended state of shallow hibernation ('winter lethargy') whereby eating and drinking completely cease, body temperature drops to ∼30 °C, and systemic tissue

acidosis develops (Coletta *et al.* 1994, Pomponi *et al.* 2002, 2004). Conversely, given that countercurrent vascular heat exchangers are observed in the limbs of both polar and Asiatic brown bears (Øritsland 1970, Daigo *et al.* 1972), it is possible that the reduced $\Delta H'$ of bear Hbs may safeguard O_2 offloading at (the much cooler) extremities, as postulated for other regionally heterothermic mammals.

Seals. Body temperatures of seals decrease during diving (Kvadsheim *et al.* 2005). In freely diving Weddell seals (*Lyptonychotes weddelli*) arterial temperature falls by ~3 °C during a 53-min dive (Kooyman *et al.* 1980). Interestingly, brain temperatures may decrease more readily – by as much 3 °C during relatively short (9–15 min) experimental dives in the hooded seal *Cystophora cristata* and the harp seal *Pagophilus groenlandicus*, where the biological advantage of 'keeping a cool head' may be a reduction in brain O_2 demand and neuroprotection against hypoxic injury (Odden *et al.* 1999). Extremity (skin and flipper) temperatures likely vary even more during swimming in cold water and basking in the sun. The low temperature sensitivity of blood-O_2 affinity in the harbour seal *Phoca vitulina* ($\Delta\log P_{50}/\Delta T = 0.014$), would insure sufficient O_2 unloading as tissue temperature decreases (Willford *et al.* 1990). Seal red cells commonly contain two major Hb components that share the same α-chain. Unlike ruminants, the β-chains of species investigated (ringed, harp, Weddell, harbour and Galapagos fur seals) have neither the cluster of three positively charged Cl^--binding residues at positions 8, 76 and 77, nor hydrophobic N-terminal residues [Swissprot, accession numbers P09909, P15166 and P68047, and Ikehara *et al.* (1996)]. Moreover, the $\Delta H'$ values (corrected for the heats of solvation of O_2) of the composite Weddell seal hemolysate at pH 7.25 (−34 kJ mol^{-1}) (Qvist *et al.* 1981) as well as the isolated 'fast' and 'slow' electrophoretic Hb components (−45 and −36 kJ mol^{-1}) (Wells & Brennan 1979) are typical for mammals Hbs under these conditions. These traits indicate that the numerically low enthalpy of oxygenation in blood that would secure O_2 unloading in cold tissues is attributable to endothermic dissociation of protons and DPG – which is consistent with the high red cell DPG levels (10.2 mM) (Scott *et al.* 1977) and the relatively large Bohr effect ($\Delta\log P_{50}/\Delta pH = -0.61$) (Willford *et al.* 1990) in harbour seal blood.

Felines. A low temperature sensitivity of blood-O_2 affinity is also encountered in cats ($\Delta\log P_{50}/\Delta T = 0.016$) (Cambier *et al.* 2004). As with ruminants, the Hbs of felines exhibit low intrinsic O_2 affinities and low sensitivities to DPG; moreover in both taxons the phosphate insensitivity correlates with the presence of low (<1 mM L^{-1} red cells) DPG concentrations (Taketa 1974, Scott *et al.* 1976, 1977, Herrmann & Haskins 2005) indicating that Cl^- ions are the major effectors of blood-O_2 affinity. However, the β-type chains of cat Hbs lack hydrophobic N-terminal amino acid residues and the Cl^--binding cluster of three negatively charged residues (at positions 8, 76 and 77) (Abbasi & Braunitzer 1985) that account, respectively, for the loss of DPG sensitivity, and additional Cl^- binding in ruminant Hb. Cats have two Hb components that differ only in their β-type chains: HbA, which is weakly DPG-responsive and has Gly and Phe at positions 1, and 2, and HbB, which is unaffected by DPG and has acetylated-Ser and Phe at these DPG-binding sites (Taketa 1974) – compared to Val and His, respectively, in human and other vertebrate DPG-sensitive mammalian Hbs. Identification of the cause of the low temperature sensitivity of feline blood must await analysis of the influences of allosteric effectors, particularly Cl^-, on the temperature dependence of feline Hbs. With no information on heterothermy in cats its biological significance is not obvious.

Talpid moles

The subterranean environment inhabited by talpid moles is thermally moderate and buffered from climatic extremes. Thus, at first sight it is surprising that the $\Delta H'$ values of whole blood (−1.0 to −8.3 kJ mol^{-1} O_2) and Hb (−7.6 to −13.7 kJ mol^{-1}) of strictly fossorial coast (*Scapanus orarius*) and eastern (*Scalopus aquaticus*) moles, respectively, are notably lower than those recorded for the blood of northerly distributed, semi-aquatic star-nosed mole (−29.3 kJ mol^{-1}) (Campbell *et al.* 2010a) and even 'cold-adapted' ruminants (−14.0 to −16.8 kJ mol^{-1}; see above). However, the coast and eastern species spend most of their life in the hypoxic/hypercapnic confines of closed burrow systems. Consequently, sharp reductions in blood-O_2 affinity with increasing temperature may be maladaptive as it could compromise O_2 uptake during burrowing induced hyperthermia (Campbell *et al.* 2010a). Additionally, Hb with a high negative $\Delta H'$ value (whereby oxygenation is strongly exothermic) could further hinder O_2 uptake potential as it increases the heat liberated upon oxygenation in the lungs (which may account for up to 9% of metabolic heat production at a $\Delta H'$ of −42 kJ mol^{-1} O_2) (Weber & Wells 1989). The mechanism(s) underlying the extremely low oxygenation enthalpy phenotype of coast and eastern mole blood is not known, although interestingly, their Hb–O_2 affinity is synergistically modulated by both Cl^- and DPG (Campbell *et al.* 2010a), as is found for bear and ruminant Hbs. In this light, it is illuminating that mole Hbs possess the same amino acid residues (Ala or Gly)

(Campbell *et al.* 2010a) as ruminant and bear Hbs at *β5* (human HbA has Pro at this position), as well as the same cluster of basic residues at positions 8, 76 and 77 of their *β*-like *δ*-globin chains to those implicated in forming the above-noted 'additional' Cl^--binding site in bears (De Rosa *et al.* 2004). Moreover, the Hb components of both these mole species also exhibit relatively high Bohr effects (−0.63 to −0.86), which may potentially contribute to the numerically low overall oxygenation enthalpies of their Hb components. Studies on shrews (close outgroup of moles) and additional mole species are required to firmly elucidate the mechanism and (potentially) evolutionary significance of this trait in these strictly fossorial mammals.

Elephantids

Extinct woolly mammoths (*Mammuthus primigenius*) offer a consummate model system to examine both the mechanistic basis and genetic origins of Hbs with a numerically reduced $\Delta H'$ as they evolved in (warm) equatorial Africa 5.8–7.7 Ma (Rohland *et al.* 2007), and only recently (1.2–2.0 Ma) invaded high-latitude environments during the Pleistocene Ice Ages (Lister *et al.* 2005). Thus, unlike their living (and extinct) tropical relatives, whose primary physiological problem revolves around heat dissipation, the chief thermal challenge facing woolly mammoths undoubtedly concerned the *conservation* of metabolic heat (that drove the development of the thick wool undercoat and reduced surface area of the ears and tail). In this regard, the ability to chronically reduce the temperature of their extremities during the harsh winter season would be expected to further lower the gradient for sensible heat loss, thereby minimizing energy intake requirements at a time when food was presumably hard to come by and of lower nutritional quality. However, this adjustment might also be expected to disrupt O_2 delivery at the extremities, thereby requiring modification in Hb function such that temperature-related reductions in tissue metabolic requirements are closely matched by oxygenation enthalpy-dictated increases in blood-O_2 affinity.

Given this apparently robust evolutionary incentive, it is somewhat surprising that the Hb of this iconic species has only acquired three amino acid changes since their divergence from Asian elephants ~6.7 Ma (Rohland *et al.* 2007); Asian and African elephants – which separated ~7.7 Ma (Rohland *et al.* 2007) – differ by four residue exchanges (Campbell *et al.* 2010b). All three replacements in the mammoth protein are found on the chimeric *β*/*δ* chain, which arose from an unequal crossover event in the ancestor of paenungulate (elephants, hyraxes and sea cows) mammals (Opazo *et al.* 2009). Functional analysis of recombinant (*E. coli*) expressed mammoth Hb demonstrated that these changes (*β*/*δ*12-Thr → Ala, *β*/*δ*86-Ala → Ser and *β*/*δ*101-Glu → Gln) significantly lower the mean $\Delta H'$ from -35.4 ± 3.4 SD kJ mol^{-1} O_2 in the absence of allosteric effectors to -19.3 ± 2.5 SD kJ mol^{-1} in the presence of Cl^- and DPG anions (Campbell *et al.* 2010b). Notably, this adaptive reduction arises from enhanced binding of both DPG and Cl^- to the mammoth protein relative to that of living elephantids, and thus originates from (at least) two separate amino acid substitutions. In elephant (and human) Hb, a hydrogen bond is formed between the polar hydroxyl side-chain of *β*/*δ*12-Thr and the carbonyl group of *β*/*δ*8-Lys. Disruption of this linkage by the mammoth *β*/*δ*12-Thr → Ala change allows *β*/*δ*8-Lys to interact closely with Asp79 of the same chain, hence drawing this anionic residue away from the positively charged DPG-binding site between the two *β*-type chains (Campbell *et al.* 2010b), strengthening DPG binding. The mammoth *β*/*δ*101-Glu → Gln replacement deletes an anionic charge along the highly conserved sliding interface between the $\alpha_1\beta_2$ subunits that perturbs the $T \rightarrow R$ transition and profoundly increases the inherent O_2 affinity of the protein. However, this mutation also creates a novel proton-linked Cl^--binding site that reverses the intrinsic increase in O_2 affinity, and further reduces the $\Delta H'$ of mammoth Hb relative to that of living elephantids (Campbell *et al.* 2010b). Consequently, in the presence of allosteric effectors, the overall oxygenation enthalpy of woolly mammoth Hb is reduced by 8.8 kJ mol^{-1} relative to Asian elephant Hb, thereby facilitating O_2 offloading at cool peripheral tissues.

It is of note that elephantids possess the same molecular motif (*β*/*δ*8-Lys, *β*/*δ*76-Lys, *β*/*δ*77-His) implicated in forming the 'additional' Cl^--binding site (relative to human HbA) in ruminant Hbs. However, surprisingly, the Hbs of living elephants exhibit a markedly reduced Cl^- sensitivity compared to human Hb, illustrating that these three residues are unable to be bridged by Cl^- in elephantid Hbs (Campbell *et al.* 2010b). This finding demonstrates that the occurrence of identical substitutions in independent evolutionary lineages may have different functional consequences on protein behaviour. Additionally, the observation that elephantid Hbs also possess reduced Bohr proton and DPG sensitivities relative to human Hb suggests that the lower $\Delta H'$ of living proboscideans' Hbs in the absence and presence of allosteric effectors arises – at least in part – from endotropic processes unrelated to ligand binding, as also appears to be the case for ruminant Hbs (see above).

Concluding remarks

The temperature dependence of blood-O_2 affinity in heterothermic vertebrates commonly show substantial reductions, exhibiting numerically low values for the

overall oxygenation enthalpy ($\Delta H'$) compared to the intrinsic values ($\Delta H^{O_2} \approx -60$ kJ mol^{-1}) observed for Hb in the absence of allosteric effectors or by calorimetric measurements. The elucidation of the structural properties of these Hbs and their effector interactions has in recent years resulted in major advances in qualitatively understanding the underlying molecular mechanisms. This review reveals a large variation in molecular strategies and in the roles of specific allosteric effectors in modulating $\Delta H'$ under specific and controlled physico-chemical conditions that, however, is associated with striking 'functional convergence': matching tissue O_2 supply with demand in the face of internal temperature gradients. Analysis of the adaptive nature of temperature insensitivity in specific animals is hampered by insufficient available data on molecular and functional traits in closely related (non-heterothermic) outgroups. However, as with heterothermia in fish (cf. Weber *et al.* 2010), the occurrence of some specific traits (dominant contributions from ΔH^{Cl^-} and elevated $\Delta H^{T \rightarrow R}$ in ruminants, from DPG and proton binding in pigs, and from DPG and CO_2/lactate binding in baleen whales) suggest that they evolved independently several times in this mammalian clade. Almost no data exist on potential differences in inherent $\Delta H^{T \rightarrow R}$ values in different taxa; its value in ruminants and elephants clearly deviates from that in other Hbs. Finally, there is a dearth of knowledge of *in vivo* pH values, O_2 and CO_2 tensions and possible variations in the levels of lactate, chloride and organic phosphate ions in blood perfusing the organs (extremities, swimming muscles, eyes, brains, etc.) whose temperatures deviate substantially from those in the respiratory organs, which would permit assessment of the specific effects of effector-induced changes in thermal sensitivity on O_2 unloading [($CaO_2 - CvO_2$) in Fig. 1], irrespective of whether the *in vivo* variations in the circulating blood represent 'open- or closed-system' temperature changes.

Conflict of interest

There is no conflict of interest in this article.

References

Abbasi, A. & Braunitzer, G. 1985. The primary structure of hemoglobins from the domestic cat (*Felis catus*, Felidae). *Biol Chem Hoppe-Seyler* **366**, 699–704.

Atha, D.H. & Ackers, G.K. 1974. Calorimetric determination of the heat of oxygenation of human hemoglobin as a function of pH and the extent of reaction. *Biochemistry* **13**, 2376–2382.

Barcroft, J. & King, W.O.R. 1909. The effect of temperature on the dissociation curve of blood. *J Physiol (Lond)* **39**, 374–384.

Benesch, R.E., Benesch, R. & Yu, C.I. 1969. The oxygenation of hemoglobin in the presence of 2,3- diphosphoglycerate. Effect of temperature, pH, ionic strength, and hemoglobin concentration. *Biochemistry* **8**, 2567–2571.

Bennett, A.F. 1985. Temperature and muscle. *J Exp Biol* **115**, 333–344.

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

Berg, F., Gustafson, U. & Andersson, L. 2006. The uncoupling protein 1 gene (*UCP1*) is disrupted in the pig lineage: a genetic explanation for poor thermoregulation in piglets. *PLoS Genet* **2**, e129.

Block, B.A. 1986. Structure of the brain and eye heater tissue in marlins, sailfish, and spearfishes. *J Morphol* **190**, 169–189.

Block, B.A. & Carey, F.G. 1985. Warm brain and eye temperatures in sharks. *J Comp Physiol [B]* **156**, 229–236.

Block, B.A., Finnerty, J.R., Stewart, A.F. & Kidd, J. 1993. Evolution of endothermy in fish: mapping physiological traits on a molecular phylogeny. *Science* **260**, 210–214.

Bohr, C., Hasselbalch, K. & Krogh, A. 1904. Über einen in biologischer Beziehung wichtigen Einfluss, den die Kohlensaurespannung des Blutes auf dessen Sauerstoffbindung ubt. *Skand Arch Physiol* **16**, 402–412.

Bouverot, P. 1985. *Adaptation to Altitude-Hypoxia in Vertebrates*. Spriger-Verlag, Berlin.

Brauner, C.J., Gilmour, K.M. & Perry, S.F. 1996. Effect of haemoglobin oxygenation on Bohr proton release and CO_2 excretion in the rainbow trout. *Respir Physiol* **106**, 65–70.

Braunitzer, G., Schrank, B., Stangl, A. & Scheithauer, U. 1978. Die Sequenzanalyse des Hämoglobins des Schweines. *Hoppe Seyler's Z Physiol Chem* **359**, 137–146.

Brill, R.W. & Bushnell, P.G. 1991. Effects of open- and closed-system temperature changes on blood oxygen dissociation curves of skipjack tuna, *Katsuwonus pelamis*, and yellowfin tuna, *Thunnus albacares*. *Can J Zool* **69**, 1814–1821.

Brill, R. & Bushnell, P. 2006. Effects of open- and closed-system temperature changes on blood O_2-binding characteristics of Atlantic bluefin tuna (*Thunnus thynnus*). *Fish Physiol Biochem* **32**, 283–294.

Brix, O., Condò, S.G., Lazzarino, G., Clementi, M.E., Scatena, R. & Giardina, B. 1989. Artic life adaptation- III. The function of whale (*Balaenoptera acutorostrata*) hemoglobin. *Comp Biochem Physiol [B]* **94B**, 139–142.

Brix, O., Bårdgard, A., Mathisen, S., Tyler, N., Nuutinen, M., Condò, S.G. & Giardina, B. 1990a. Oxygen transport in the blood of arctic mammals: adaptation to local heterothermia. *J Comp Physiol [B]* **159**, 655–660.

Brix, O., Condò, S.G., Bardgard, A., Tavazzi, B. & Giardina, B. 1990b. Temperature modulation of oxygen transport in a diving mammal (*Balaenoptera acutorostrata*). *Biochem J* **271**, 509–513.

Cambier, C., Wierinckx, M., Clerbaux, T., Detry, B., Liardet, M.P., Marville, V., Frans, A. & Gustin, P. 2004. Haemoglobin oxygen affinity and regulating factors of the blood oxygen transport in canine and feline blood. *Res Vet Sci* **77**, 83–88.

Campbell, K.L., Storz, J.F., Signore, A.V., Moriyama, H., Catania, K.C., Payson, A., Bonaventura, J., Stetefeld, J. &

Weber, R.E. 2010a. Molecular basis of a novel adaptation to hypoxic-hypercapnia in a strictly fossorial mole. *BMC Evol Biol* **10**, 214.

Campbell, K.L., Roberts, J.E.E., Watson, L.N., Stetefeld, J., Sloan, A.M., Signore, A.V., Howatt, J.W., Tame, J.R.H., Rohland, N., Shen, T.-J., Austin, J.J., Hofreiter, M., Ho, C., Weber, R.E. & Cooper, A. 2010b. Substitutions in woolly mammoth hemoglobin confer biochemical properties adaptive for cold tolerance. *Nat Genet* **42**, 536–540.

Carey, F.G. 1973. Fishes with warm bodies. *Sci Am* **228**, 36–44.

Carey, F.G. & Gibson, Q.H. 1977. Reverse temperature dependence of tuna hemoglobin oxygenation. *Biochem Biophys Res Com* **78**, 1376–1382.

Carey, F.G. & Teal, J.M. 1969. Mako and porbeagle – warm-bodied sharks. *Comp Biochem Physiol* **28**, 199.

Carey, F.G., Teal, J.M., Kanwisher, J.W., Lawson, K.D. & Beckett, J.S. 1971. Warm-bodied fish. *Am Zool* **11**, 135–143.

Cech, J.J., Laurs, R.M. Jr & Graham, J.B. 1984. Temperature-induced changes in blood gas equilibria in the albacore, *Thunnus alalunga*, a warm-bodied tuna. *J Exp Biol* **109**, 21–34.

Chanutin, A. & Hermann, E. 1969. The interaction of organic and inorganic phosphates with hemoglobin. *Arch Biochem Biophys* **131**, 180–184.

Clark, T.D., Seymour, R.S., Wells, R.M. & Frappell, P.B. 2008. Thermal effects on the blood respiratory properties of southern bluefin tuna, *Thunnus maccoyii*. *Comp Biochem Physiol A Mol Integr Physiol* **150**, 239–246.

Clark, T.D., Rummer, J.L., Sepulveda, C.A., Farrell, A.P. & Brauner, C.J. 2010. Reduced and reversed temperature dependence of blood oxygenation in an ectothermic scombrid fish: implications for the evolution of regional heterothermy? *J Comp Physiol B* **180**, 73–82.

Coletta, M., Clementi, M.E., Ascenzi, P., Petruzzelli, R., Condò, S.G. & Giardina, B. 1992. A comparative study of the temperature dependence of the oxygen-binding properties of mammalian hemoglobins. *Eur J Biochem* **204**, 1155–1157.

Coletta, M., Condò, S.G., Scatena, R., Clementi, M.E., Baroni, S., Sletten, S.N., Brix, O., Giardina, B. & Condo, S.G. 1994. Synergistic modulation by chloride and organic phosphates of hemoglobin from bear (*Ursus arctos*). *J Mol Biol* **236**, 1401–1406.

Condo, S.G., Corda, M., Sanna, M.T., Pellegrini, M.G., Ruiz, M.P., Castagnola, M. & Giardina, B. 1992. Molecular basis of low-temperature sensitivity in pig hemoglobins. *Eur J Biochem* **209**, 773–776.

Corda, M., Tamburrini, M., De Rosa, M.C., Sanna, M.T., Fais, A., Olianas, A., Pellegrini, M., Giardina, B. & Di Prisco, G. 2003. Whale (*Balaenoptera physalus*) haemoglobin: primary structure, functional characterisation and computer modelling studies. *Comp Biochem Physiol B Biochem Mol Biol* **134**, 53–62.

Daigo, M., Sato, Y., Otsuka, M., Yoshimura, T., Komyama, S. & Ogawa, Y. 1972. (Stereoroentgenographical studies on peripheral arteries of the anterior and posterior extremities of the bears) – in Japanese. *Bull Nippon Vet Zootech Coll* **21**, 32–40.

De Rosa, M.C., Castagnola, M., Bertonati, C., Galtieri, A. & Giardina, B. 2004. From the Arctic to fetal life: physiological importance and structural basis of an 'additional' chloride-binding site in haemoglobin. *Biochem J* **380**, 889–896.

Fick, A. 1870. Über die Messung des Blutquantums in den Herzventrikeln. *Sitzber Physik Med Ges, Würzburg* **2**, 16.

Fritsches, K.A., Brill, R.W. & Warrant, E.J. 2005. Warm eyes provide superior vision in swordfishes. *Curr Biol* **15**, 55–58.

Fronticelli, C. 1990. A possible new mechanism of oxygen affinity modulation in mammalian hemoglobins. *Biophys Chem* **37**, 141–146.

Fronticelli, C., Sanna, M.T., Perez-Alvarado, G.C., Karavitis, M., Lu, A.L. & Brinigar, W.S. 1995. Allosteric modulation by tertiary structure in mammalian hemoglobins. Introduction of the functional characteristics of bovine hemoglobin into human hemoglobin by five amino acid substitutions. *J Biol Chem* **270**, 30588–30592.

Giardina, B., Brix, O., Nuutinen, M., El-Sherbini, S., Bardgard, A., Lazzarino, G. & Condò, S.G. 1989. Arctic adaptation in reindeer. The energy saving of a hemoglobin. *FEBS Lett* **247**, 135–138.

Herrmann, K. & Haskins, S. 2005. Determination of P50 for feline hemoglobin. *J Vet Emerg Crit Care* **15**, 26–31.

Hofmann, O., Schreitmuller, T. & Braunitzer, G. 1986. Die Primärstruktur der Hämoglobine von Eisbär (*Ursus maritimus*, Carnivora) and Kragenbär (*Ursus tibetanus*, Carnivora). *Biol Chem Hoppe Seyler* **367**, 53–59.

Ikeda-Saito, M., Yonetani, T. & Gibson, Q.H. 1983. Oxygen equilibrium studies on hemoglobin from the bluefin tuna (*Thunnus thynnus*). *J Mol Biol* **168**, 673–686.

Ikehara, T., Eguchi, Y., Kayo, S. & Takei, H. 1996. Amino acid sequences of hemoglobin β chains of five species of pinnipeds: *Neophoca cinerea*, *Otaria byronia*, *Eumetopias jubatus*, *Pusa hispida*, and *Pagophilus groenlandica*. *J Protein Chem* **15**, 659–665.

Irving, L. 1956. Physiological insulation of swine as bare-skinned mammals. *J Appl Physiol* **9**, 414–420.

Irving, L. & Krog, J. 1955. Temperature of skin in the arctic as a regulator of heat. *J Appl Physiol* **7**, 355–364.

Jensen, F.B. 1986. Pronounced influence of Hb-O_2 saturation on red cell pH in tench blood in vivo and in vitro. *J Exp Zool* **238**, 119–124.

Jensen, F.B. 2001. Hydrogen ion binding properties of tuna haemoglobins. *Comp Biochem Physiol A Mol Integr Physiol* **129**, 511–517.

Jensen, F.B. & Weber, R.E. 1987. Thermodynamic analysis of precisely measured oxygen equilibria of tench hemoglobin and their dependence on ATP and protons. *J Comp Physiol* **157B**, 137–143.

Johnsen, H.K., Rognmo, A., Nilssen, K.J. & Blix, A.S. 1985. Seasonal changes in the relative importance of different avenues of heat loss in resting and running reindeer. *Acta Physiol Scand* **123**, 73–79.

Kooyman, G.L., Wahrenbrock, E.A., Castellini, M.A., Davis, R.W. & Sinnett, E.E. 1980. Aerobic and anaerobic metabolism during voluntary diving in Weddell seals – evidence of preferred pathways from blood-chemistry and behavior. *J Comp Physiol* **138**, 335–346.

Krogh, A. & Leitch, I. 1919. The respiratory function of the blood in fishes. *J Physiol (Lond)* **52**, 288–300.

Kvadsheim, P.H., Folkow, L.P. & Blix, A.S. 2005. Inhibition of shivering in hypothermic seals during diving. *Am J Physiol Regul Integr Comp Physiol* **289**, R326–R331.

Larsen, C. & Malte, H. 2003. Reversed temperature effect of haemoglobin oxygen binding in the porbeagle shark: potential implications for oxygen loss in the heat exchanger. *Comp Biochem Physiol* **134A**, S18.

Larsen, C., Malte, H. & Weber, R.E. 2003. ATP induced reverse temperature effect in iso-hemoglobins from the endothermic porbeagle shark (*Lamna nasus*). *J Biol Chem* **278**, 30741–30747.

Lindqvist, C., Schuster, S.C., Sun, Y.Z., Talbot, S.L., Qi, J., Ratan, A., Tomsho, L.P., Kasson, L., Zeyl, E., Aars, J., Miller, W., Ingolfsson, O., Bachmann, L. & Wiig, O. 2010. Complete mitochondrial genome of a Pleistocene jawbone unveils the origin of polar bear. *Proc Natl Acad Sci USA* **107**, 5053–5057.

Lister, A.M., Sher, A.V., van Essen, H. & Wei, G.B. 2005. The pattern and process of mammoth evolution in Eurasia. *Quat Int* **126-28**, 49–64.

Lowe, T.E., Brill, R.W. & Cousins, K.L. 1998. Responses of the red blood cells from two high-energy-demand teleosts, yellowfin tuna (*Thunnus albacares*) and skipjack tuna (*Katsuwonus pelamis*), to catecholamines. *J Comp Physiol B Biochem Syst Environ Physiol* **168**, 405–418.

Lowe, T.E., Brill, R.W. & Cousins, K.L. 2000. Blood oxygen-binding characteristics of big-eye tuna (*Thunnus obesus*), a high-energy-demand teleost that is tolerant of low ambient oxygen. *Mar Biol* **136**, 1087–1098.

Lukin, J.A. & Ho, C. 2004. The structure–function relationship of hemoglobin in solution at atomic resolution. *Chem Rev* **104**, 1219–1230.

Odden, A., Folkow, L.P., Caputa, M., Hotvedt, R. & Blix, A.S. 1999. Brain cooling in diving seals. *Acta Physiol Scand* **166**, 77–78.

Opazo, J.C., Sloan, A.M., Campbell, K.L. & Storz, J.F. 2009. Origin and ascendancy of a chimeric fusion gene: the β/δ-globin gene of paenungulate mammals. *Mol Biol Evol* **26**, 1469–1478.

Øritsland, N.A. 1970. Temperature regulation of the polar bear (*Thalarctos maritimus*). *Comp Biochem Physiol* **37**, 225–233.

Perutz, M.F. 1983. Species adaptation in a protein molecule. *Mol Biol Evol* **1**, 1–28.

Perutz, M.F. & Imai, K. 1980. Regulation of oxygen affinity of mammalian haemoglobins. *J Mol Biol* **136**, 183–191.

Pomponi, M., Bertonati, C., Patamia, M., Marta, M., Derocher, A.E., Lydersen, C., Kovacs, K.M., Wiig, O. & Bardgard, A.J. 2002. Polar bear hemoglobin and human Hb A0: same 2,3-diphosphoglycerate binding site but asymmetry of the binding? *Hemoglobin* **26**, 363–371.

Pomponi, M., Gavuzzo, E., Bertonati, C., Derocher, A.E., Lydersen, C., Wiig, O. & Kovacs, K.M. 2004. Hemoglobin, pH and DPG/chloride shifting. *Biochimie* **86**, 927–932.

Qvist, J., Weber, R.E. & Zapol, W.M. 1981. Oxygen equilibrium properties of blood and hemoglobin of fetal and adult Weddell seals. *J Appl Physiol* **50**, 999–1005.

Rapoport, S. & Guest, G.M. 1941. Distribution of acid-soluble phosphorus in the blood cells of various vertebrates. *J Biol Chem* **138**, 269–282.

Razynska, A., Fronticelli, C., Di Cera, E., Gryczynski, Z. & Bucci, E. 1990. Effect of temperature on oxygen affinity and anion binding of bovine hemoglobin. *Biophys Chem* **38**, 111–115.

Riggs, A.F. 1988. The Bohr effect. *Annu Rev Physiol* **50**, 181–204.

Riggs, A.F. & Gorr, T.A. 2006. A globin in every cell? *PNAS* **103**, 2469–2470.

Rohland, N., Malaspinas, A.S., Pollack, J.L., Slatkin, M., Matheus, P. & Hofreiter, M. 2007. Proboscidean mitogenomics: chronology and mode of elephant evolution using mastodon as outgroup. *PLoS Biol* **5**, 1663–1671.

Rossi-Fanelli, A., Antonini, E. & Giuffrè, R. 1960. Oxygen equilibrium of haemoglobin from *Thunnus thynnus*. *Nature* **186**, 895–897.

Schechter, A.N. 2008. Hemoglobin research and the origins of molecular medicine. *Blood* **112**, 3927–3938.

Scholander, P.F. & Schevill, W.E. 1955. Counter-current vascular heat exchange in the fins of whales. *J Appl Physiol* **8**, 279–282.

Scott, A.F., Bunn, H.F. & Brush, A.H. 1976. Functional aspects of hemoglobin evolution in the mammals. *J Mol Evol* **8**, 311–316.

Scott, A.F., Bunn, H.F. & Brush, A.H. 1977. The phylogenetic distribution of red cell 2,3 diphosphoglycerate and its interaction with mammalian hemoglobins. *J Exp Zool* **201**, 269–288.

Sollid, J., Weber, R.E. & Nilsson, G.E. 2005. Temperature alters the respiratory surface area of crucian carp *Carassius carassius* and goldfish *Carassius auratus*. *J Exp Biol* **208**, 1109–1116.

Taketa, F. 1974. Organic phosphates and hemoglobin structure-function relationships in the feline. *Ann N Y Acad Sci* **241**, 524–537.

Tamburrini, M., Condo, S.G., di, P.G. & Giardina, B. 1994. Adaptation to extreme environments: structure-function relationships in Emperor penguin haemoglobin. *J Mol Biol* **237**, 615–621.

Weber, R.E. & Vinogradov, S.N. 2001. Nonvertebrate hemoglobins: functions and molecular adaptations. *Physiol Rev* **81**, 569–628.

Weber, R.E. & Wells, R.M.G. (1989) Hemoglobin structure and function. In: S.C. Wood (ed.) *Lung Biology in Health and Disease. Comparative Pulmonary Physiology, Current Concepts*, pp. 279–310. Marcel Dekker, Inc., New York.

Weber, R.E., Wells, R.M.G. & Rossetti, J.E. 1985. Adaptations to neoteny in the salamander, *Necturus maculosus*. Blood respiratory properties and interactive effects of pH, temperature and ATP on hemoglobin oxygenation. *Comp Biochem Physiol* **80A**, 495–501.

Weber, R.E., Jensen, F.B. & Cox, R.P. 1987a. Analysis of teleost hemoglobin by Adair and Monod-Wyman-Changeux models. Effects of nucleoside triphosphates and pH on oxygenation of tench hemoglobin. *J Comp Physiol* **157B**, 145–152.

Weber, R.E., Kleinschmidt, T. & Braunitzer, G. 1987b. Embryonic pig hemoglobins Gower I ($\zeta_2\epsilon_2$), Gower II ($\alpha_2\epsilon_2$),

Heide I ($\zeta_2\nu_2$) and Heide II ($\alpha_2\nu_2$): oxygen-binding functions related to structure and embryonic oxygen supply. *Respir Physiol* **69**, 347–357.

Weber, R.E., Hourdez, S., Knowles, F. & Lallier, F. 2003. Hemoglobin function in deep-sea and hydrothermal-vent endemic fish: *Symenchelis parasitica* (Anguillidae) and *Thermarces cerberus* (Zoarcidae). *J Exp Biol* **206**, 2693.

Weber, R.E., Behrens, J.W., Malte, H. & Fago, A. 2008. Thermodynamics of oxygenation-linked proton and lactate binding govern the temperature sensitivity O_2 binding in crustacean (*Carcinus maenas*) haemocyanin. *J Exp Biol* **211**, 1057–1062.

Weber, R.E., Campbell, K.L., Fago, A., Malte, H. & Jensen, F.B. 2010. ATP-induced temperature independence of hemoglobin-O_2 affinity in heterothermic billfish. *J Exp Biol* **213**, 1579–1585.

Wells, R.M. & Brennan, S.O. 1979. Oxygen equilibrium properties of isolated haemoglobins from the weddell seal *Leptonychotes weddelli*. *Comp Biochem Physiol A Mol Integr Physiol* **63**, 365–368.

Willford, D.C. & Hill, E.P. 1986. Modest effect of temperature on the porcine oxygen dissociation curve. *Respir Physiol* **64**, 113–123.

Willford, D.C., Gray, A.T., Hempleman, S.C., Davis, R.W. & Hill, E.P. 1990. Temperature and the oxygen-hemoglobin dissociation curve of the harbor seal, *Phoca vitulina*. *Respir Physiol* **79**, 137–144.

Wood, S.C., Lykkeboe, G., Johansen, K., Weber, R.E. & Maloiy, G.M.O. 1978. Temperature acclimation in the pancake tortoise, *Malacochersus tornieri*: metabolic rate, blood pH, oxygen affinity and red cell organic phosphates. *Comp Biochem Physiol* **59A**, 155–160.

Wyman, J. 1964. Linked functions and reciprocal effects in hemoglobin: a second look. *Adv Protein Chem* **19**, 223–286.

Yokoyama, T., Chong, K.T., Miyazaki, G., Morimoto, H., Shih, D.T.B., Unzai, S., Tame, J.R.H. & Park, S.Y. 2004. Novel mechanisms of pH sensitivity in tuna hemoglobin: a structural explanation of the Root effect. *J Biol Chem* **279**, 28632–28640.