The Journal of Experimental Biology 215, 518-525

doi:10.1242/jeb.063669

RESEARCH ARTICLE

Origin and mechanism of thermal insensitivity in mole hemoglobins: a test of the 'additional' chloride binding site hypothesis

Anthony V. Signore[1], Jörg Stetefeld[2], Roy E. Weber[3] and Kevin L. Campbell[1,*]

[1]Department of Biological Sciences, University of Manitoba, 50 Sifton Road, Winnipeg, Manitoba R3T 2N2, Canada, [2]Department of Chemistry, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada and [3]Zoophysiology, Department for Bioscience, University of Aarhus, DK-8000 Aarhus, Denmark

*Author for correspondence (campbelk@cc.umanitoba.ca)

Accepted 5 October 2011

SUMMARY

The structural and evolutionary origins underlying the effect of temperature on the O_2 binding properties of mammalian hemoglobins (Hbs) are poorly understood, despite their potential physiological importance. Previous work has shown that the O_2 affinities of the blood of the coast mole (*Scapanus orarius*) and the eastern mole (*Scalopus aquaticus*) are significantly less sensitive to temperature changes than that of the star-nosed mole (*Condylura cristata*). It was suggested that this difference may arise from the binding of 'additional' chloride ions within a cationic pocket between residues 8His, 76Lys and 77Asn on the β-like δ-globin chains of coast and eastern mole Hbs. To test this hypothesis, we deduced the primary sequences of star-nosed mole and American shrew mole (*Neurotrichus gibbsii*) Hb, measured the sensitivity of these respiratory proteins to allosteric effector molecules and temperature, and calculated their overall oxygenation enthalpies ($\Delta H'$). Here we show that the variability in $\Delta H'$ seen among mole Hbs cannot be attributed to differential Cl^- binding at δ8, δ76 and δ77, as the Cl^- sensitivity of mole Hbs is unaffected by amino acid changes at this site (i.e. the proposed 'additional' Cl^- binding site is not operational in mole Hbs). Rather, we demonstrate that the numerically low $\Delta H'$ of coast and eastern mole Hbs results from heightened proton binding relative to other mole Hbs. Comparative sequence analysis and molecular modelling moreover suggest that this attribute evolved in a common ancestor of these two fossorial lineages and arises from the development of a salt bridge between a pair of amino acid residues (δ125His and α34Glu/Asp) that are not present in other mole Hbs.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/215/3/518/DC1

Key words: enthalpy, hemoglobin, molecular evolution, oxygen binding, shrew mole, star-nosed mole, Talpidae, temperature effect.

INTRODUCTION

Mammalian hemoglobin (Hb) is composed of two α-type and two β-type polypeptide strands, each of which bears an iron-containing heme group (the site of reversible O_2 binding). To optimize the uptake and delivery of O_2, this metalloprotein switches its quaternary structure between high O_2 affinity [predominantly oxygenated, relaxed (R)] and low O_2 affinity [predominantly deoxygenated, tense (T)] states (Perutz, 1983). Although this general mechanism is perfectly conserved among mammalian Hbs, the physicochemical properties of the protein can vary substantially both within (e.g. ontogenically expressed Hb isoforms) and between species (Brittain, 2002; Weber, 2007). Some of this variability has been attributed to residue replacements along the α- or β-type globin chains that perturb or strengthen the R or T state, hence modifying the inherent oxygenation properties of the protein (Perutz, 1983; Weber, 2007). Alternatively, substitutions may trigger structural changes in the protein that alter its sensitivity to naturally occurring heterotropic (i.e. non-oxygen) ligands – e.g. 2,3-diphosphoglycerate (DPG), chloride ions (Cl^-), protons (H^+) and CO_2 – thereby shifting the R↔T equilibrium (Perutz, 1983; Weber, 2007).

Although heterotropic ligands are known to be important modulators of Hb–O_2 affinity *in vivo*, the molecular mechanisms underlying the effect of temperature on Hb–O_2 affinity are much less understood (Weber and Campbell, 2011). This is surprising given that the temperature dependence of blood–O_2 affinity (whereby Hb–O_2 affinity tends to increase with decreasing temperature) was first observed more than 100 years ago (Barcroft and King, 1909). As with blood–O_2 affinity, temperature sensitivity varies substantially among species, with the magnitude of this effect resulting from a symphony of oxygenation-linked chemical processes that together determine the overall enthalpy of oxygenation ($\Delta H'$) of the protein. Briefly, this can be formulated as: $\Delta H' = \Delta H^{O2} + \Delta H^{H2O} + \Delta H^{T\rightarrow R} + \Delta H^{Effector}$, where ΔH^{O2} is the intrinsic heat of heme oxygenation (approximately $-59\,kJ\,mol^{-1}\,O_2$ bound), ΔH^{H2O} is the heat of solution ($-12.55\,kJ\,mol^{-1}\,O_2$), $\Delta H^{T\rightarrow R}$ is the heat of the T→R transition and $\Delta H^{Effector}$ is the heat of effector (e.g. DPG, Cl^-, CO_2 and H^+) dissociation (Weber and Campbell, 2011). Because ΔH^{O2} and ΔH^{H2O} are virtually invariant, variations in $\Delta H'$ appear to be primarily driven by changes in endothermic contributions from oxygenation-linked effector release and/or possibly by changes in $\Delta H^{T\rightarrow R}$ (Weber and Campbell, 2011). Notably, numerical reductions in $\Delta H'$ – whereby Hb–O_2 affinity is less affected by changes in temperature – may have had important evolutionary consequences for a number of mammalian lineages (Campbell et al., 2010a; Weber and Campbell, 2011). Indeed, a numerically low negative $\Delta H'$ Hb phenotype (approximately –14 to $-19\,kJ\,mol^{-1}\,O_2$) appears to be important in allowing regionally heterothermic (e.g. arctic and aquatic) endotherms to balance O_2

delivery at the peripheral tissues in the face of temperature-induced changes in O_2 demand at these sites (De Rosa et al., 2004; Weber and Campbell, 2011).

We previously determined that the individual Hb components of the coast mole (*Scapanus orarius*) and the eastern mole (*Scalopus aquaticus*) likewise possess numerically low enthalpies of oxygenation (between –8 and –14 kJ mol^{-1} O_2) in the presence of allosteric effectors, a trait that was hypothesized to minimize the impairment of O_2 uptake at the lungs while burrowing in hypoxic tunnels (Campbell et al., 2010b). Interestingly, the effects of temperature on the whole-blood–O_2 affinity of coast, eastern and star-nosed (*Condylura cristata*) moles indicated that the blood of the first two species is considerably less sensitive to temperature than that of the latter (Campbell et al., 2010b). This finding is surprising given that the star-nosed mole is not only semi-aquatic, but is distributed substantially farther north than the thermally buffered, strictly fossorial coast and eastern moles (Petersen and Yates, 1980). In fact, star-nosed moles have been observed tunnelling in snow and even diving beneath ice during the winter (Merriam, 1884; Hamilton, 1931). Results of Campbell and coworkers (Campbell et al., 2010b) further suggested that coast and eastern mole Hbs possess an extra (relative to human HbA) Cl^- binding site that does not overlap with DPG binding. Like that of coast and eastern moles, bovine Hb possesses a specific and 'additional' (with respect to human HbA) oxygenation-linked Cl^- binding site (De Rosa et al., 2004). Based on functional data obtained from human site-directed mutants, it was proposed that this Cl^- binding site resides between three cationic residues on the β-globin chain: β8Lys, β76Lys and β77His (Fronticelli et al., 1995). Later work revealed that Hbs possessing β8Lys and β76Lys together with His or Asn at β77 display numerically reduced $\Delta H'$ values in the presence of 0.1 mol l^{-1} Cl^- relative to human HbA (–41 kJ mol^{-1}); HbA possesses a neutral Ala residue at β76, which presumably prevents Cl^- from bridging this positively charged cavity (De Rosa et al., 2004). Notably, and consistent with the 'additional' Cl^- binding site hypothesis, the β-like δ-globin chains of coast and eastern mole Hbs have Lys at positions 8 and 76 together with Asn at position 77 (Campbell et al., 2010b).

Taken together, these results raise the possibility that a differential ability to bind chloride ions may underlie the observed differences in the whole-blood $\Delta H'$ of coast (–1.0 kJ mol^{-1}) and eastern moles (–8.3 kJ mol^{-1}) relative to that of the star-nosed mole (–29.9 kJ mol^{-1}) (Campbell et al., 2010b). We thus hypothesized that the higher thermal sensitivity of star-nosed mole blood may be attributed to residue variations at positions 8, 76 and 77 of the β-type chain, creating an unfavourable environment for chloride binding. To examine this possibility, we amplified and sequenced the expressed α- and β-type globin genes of the star-nosed mole, measured the O_2-binding properties of its Hb components at two temperatures (25 and 37°C) in the presence and absence of allosteric effectors, and calculated their overall oxygenation enthalpies. To provide insight into the mechanism, origin and potential adaptive significance of this trait in moles, we additionally collected comparable data from the Hb of the American shrew mole, *Neurotrichus gibbsii*. This diminutive insectivore is basal to the clade containing star-nosed, coast and eastern moles (Shinohara et al., 2004; Sánchez-Villagra et al., 2006), thus allowing us to deduce whether a numerically low $\Delta H'$ is an ancestral or derived trait in talpid moles. Moreover, as the semi-fossorial American shrew mole digs shallow tunnels and primarily scavenges on the forest floor (Dalquest and Orcutt, 1942), it presumably evolved under relaxed hypoxic selection pressures relative to the strictly fossorial (coast and eastern moles) and semi-aquatic (star-nosed) moles. Hence, it was hoped that assessment of the inherent oxygenation characteristics and effector sensitivity of its Hb might further our understanding of the mechanisms underlying hypoxic adaptation in the more derived mole species.

MATERIALS AND METHODS

Sample collection

Two American shrew moles, *Neurotrichus gibbsii* (Baird 1858), were live captured in pitfall traps, one from Blaine, Whatcom County, WA, USA, and the other from Vancouver, British Columbia, Canada. Three star-nosed moles, *Condylura cristata* (Linnaeus 1758), were captured in Sherman live traps, two in southeast Manitoba, Canada (Caddy Lake and Piney), and one in Potter County, PA, USA. The lack of discernable intraspecific differences in gene sequences and/or Hb–O_2 binding characteristics (see below) of individuals from these geographically separated populations suggested that our findings are a good representation for each species. Blood and tissue samples were obtained soon after anaesthesia-induced euthanization following both University of British Columbia and University of Manitoba approved protocols and with adherence to the guidelines of the Canadian Council on Animal Care. In all cases, blood samples were stored in sealed vials at –70°C. Fresh liver and spleen samples were excised immediately and transferred to vials containing 95% ethanol and RNAlater (Ambion, Austin, TX, USA), respectively, and stored similarly.

Hb–O_2 affinity

The Hb components from two star-nosed moles (Manitoba and PA) and a single American shrew mole (Vancouver) were isolated by isoelectric focusing in a 110 ml LBK sucrose density gradient column following the procedure of Campbell et al. (Campbell et al., 2010b). Measurements of the partial pressure of oxygen required to saturate 50% of Hb molecules (P_{50}) dissolved in 0.1 mol l^{-1} HEPES buffer and in the presence of varying concentrations of H^+, Cl^- and DPG were collected for the individual Hb components of each species at 25 and 37°C using the thin-film technique (Weber, 1992). P_{50} values, interpolated from Hill plots (log–log plots of [percent oxyhemoglobin/percent deoxyhemoglobin] *vs* P_{O_2}), were calculated from at least four O_2 equilibration steps between 30 and 70% saturation. These data sets were compared with those previously collected for coast and eastern mole Hbs (Campbell et al., 2010b). For each Hb component, the overall enthalpy of oxygenation (kJ mol^{-1} O_2), adjusted for the heat of solution of O_2 (12.55 kJ mol^{-1}), was calculated over a range of pHs (~6.0–8.5) using a modified version of the integrated van't Hoff isochore (Wyman, 1964):

$$\Delta H' = \{[2.303R\Delta\log P_{50} / \Delta(1 / T)] / 1000 \times 4.1868\} + 12.55, \quad (1)$$

where R is the gas constant (J K^{-1} mol^{-1}), $\Delta\log P_{50}$ is the change in $\log P_{50}$ (mmHg) observed between temperatures T_1 and T_2 (K) and 1000×4.1868 converts the formula from cal mol^{-1} O_2 to kJ mol^{-1} O_2.

DNA sequencing

Total RNA was extracted from star-nosed mole and American shrew mole spleen samples using the TRIZOL® method, as per the manufacturer's directions (Invitrogen, Carlsbad, CA, USA). The purified mRNA was subsequently used to synthesize cDNA using SuperScript™ II Reverse Transcriptase and Invitrogen Adapter Primer (supplementary material Table S1), following the manufacturer's protocol (Invitrogen). For each sample, 1 µl aliquots of cDNA were polyC tailed in a 25 µl reaction consisting of 1× terminal deoxynucleotidyl transferase (TdT) buffer, 200 µmol l^{-1}

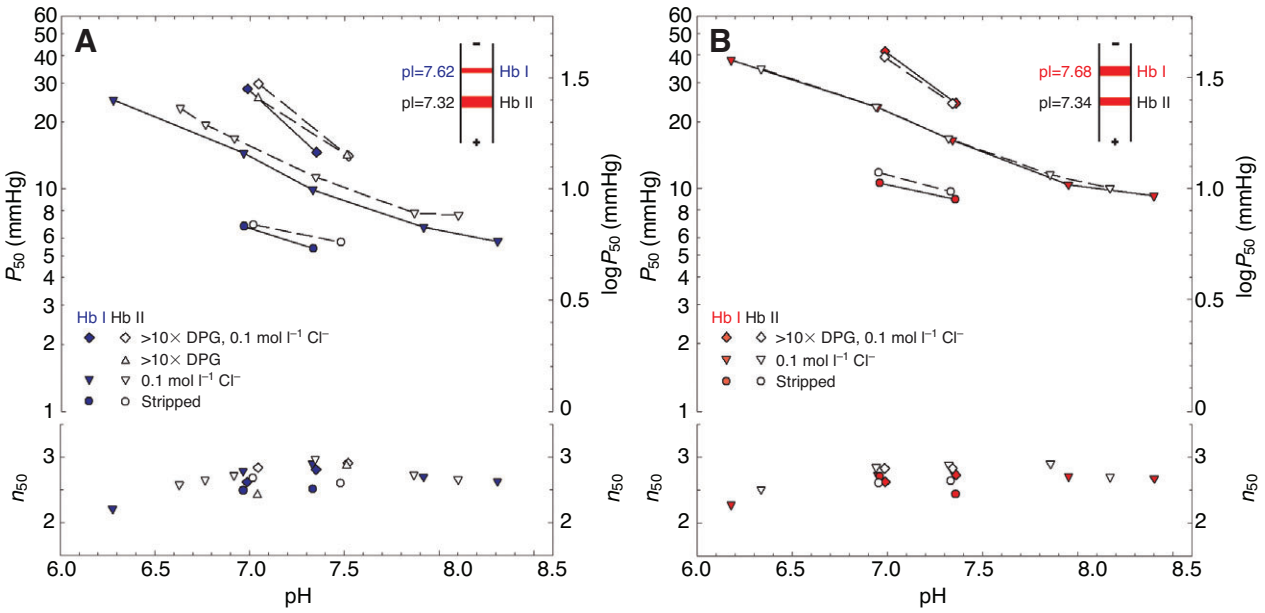

Fig. 1. Values of P_{O_2} and Hill's cooperativity coefficient at half oxygen saturation (P_{50} and n_{50}, respectively) for the individual hemoglobin components ([Hb_4]=0.05 mmol l^{-1}) of (A) star-nosed moles and (B) American shrew moles at 37°C, and their pH dependence in the absence ('Stripped') and presence of added Cl^- and 2,3-diphosphoglycerate (DPG). Insets: diagrams of an isoelectric focusing column at the end of focusing, illustrating the relative abundance of the individual hemoglobin components and their isoelectric point (pI) values at 5°C.

dCTP and 15 units of TdT (Invitrogen). Reaction mixtures were incubated at 37°C for 10 min, followed by heat inactivation of the enzyme at 65°C for 10 min.

Aliquots of polyC-tailed cDNA were subsequently used in rapid amplification of cDNA ends (RACE) PCRs to amplify the coding sequences of the α-like and β-like globin mRNA transcripts of *C. cristata* and *N. gibbsii*. RACE PCRs were conducted using gene-specific primers that were designed from highly conserved regions among mole Hbs using Primer Premier 5.0 (PREMIER Biosoft International, Palo Alta, CA, USA; supplementary material Table S1). 3′ RACE PCRs utilized the polyA tail at the end of the cDNA transcript as a universal priming site, whereas 5′ experiments utilized the TdT-generated polyC tag (above). PCR reactions were conducted using 1 µl of polyC-tailed cDNA template in a 20 µl reaction comprising: 200 µmol l^{-1} dNTPs, 1× *Taq* Reaction Buffer, 1.5 mmol l^{-1} $MgCl_2$, 0.5 µmol l^{-1} of each primer and two units of *Taq* DNA polymerase (New England Biolabs, Ipswich, MA, USA). The reactions were amplified in an Eppendorf Mastercycler® Gradient thermocycler (Hamburg, Germany) with the following thermal cycling profile: 3 min at 95°C, followed by 30 cycles of 95°C for 30 s, 55–65°C (depending on the primer) for 15 s, 68°C for 45 s, ending with a final extension period of 10 min at 68°C. Reaction products were electrophoresed on an agarose gel and bands of expected size were excised and purified with an Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Life Sciences, Piscataway, NJ, USA). Purified target bands were either ligated into pDrive vectors (Qiagen, Valencia, CA, USA) or used as template in a hemi-nested PCR (in cases where the initial PCR amplified multiple products) with the same conditions as above, except using a nested gene-specific primer (supplementary material Table S1). Hemi-nested PCR products were treated as described above.

Ligations were transformed into Qiagen® EZ Competent Cells. Transformed cells were plated on Luria-Bertani (LB) agar plates containing 100 µg ml^{-1} ampicillin, 40 µg ml^{-1} X-Gal and 0.04 mmol l^{-1} isopropyl β-D-1-thiogalactopyranoside, and then incubated for 18 h at 37°C. Positive clones were transferred to 8 ml of LB culture medium containing 100 µg ml^{-1} ampicillin and incubated for 18 h at 37°C while shaking at 225 r.p.m. A 3 ml sample of each culture was pelleted by centrifugation for 3 min at 6800 ***g*** and the plasmid DNA was purified using a QIAprep® Spin Miniprep Kit (Qiagen).

Sequencing reactions were performed on 250 ng of purified plasmid DNA using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the universal sequencing primers M-13(F)-40 or M-13(R) (supplementary material Table S1). Reaction mixtures were sequenced using a four-capillary Applied Biosystems 3130 Genetic Analyzer. Consensus alignments for each gene were constructed from between four and eight clones amplified from two to three separate PCR reactions, respectively, using Sequencher™ software (Version 4.6, Gene Codes Corp., Ann Arbor, MI, USA), and the amino acid complement of each globin chain was deduced.

In order to verify the cDNA sequencing results, genomic DNA was extracted from star-nosed mole and American shrew mole liver samples using a DNeasy Blood & Tissue Kit (Qiagen). Primers were designed to target individual α-like and β-like globin genes from the star-nosed and American shrew moles. Fifty nanograms of DNA were used in 20 µl PCR reactions, which had the same composition as described above. Reactions were amplified with the following thermal cycling profile: 3 min at 95°C, followed by 30 cycles of 95°C for 30 s, 55–65°C (depending on the primer) for 15 s, 68°C for 1 min 30 s, ending with a final extension period of 10 min at 68°C. The reactions were verified by gel electrophoresis, cloned and sequenced as described above. Sequence data were deposited in GenBank (accession numbers JN208865–JN208876, JQ336982–JQ336984).

RESULTS

Isolation of hemoglobin components

Blood of the star-nosed mole and American shrew mole possesses two major Hb components (insets of Fig. 1A,B). The two

Table 1. Intrinsic oxygen affinity [expressed as half-saturation oxygen tension, P_{50} (mmHg)] of mole hemoglobins ([Hb_4]=0.05 mmol l^{-1}) and their sensitivity to allosteric effectors at 37°C and pH 7.2 in 0.1 mol l^{-1} HEPES buffer

	Shrew mole		Star-nosed mole			Coast mole[a]	
	Hb I	Hb II	Hb I – MB	Hb II – MB	Hb II – PA	Hb I	Hb II
P_{50} stripped	9.54	10.33	5.87	6.42	6.53	5.37	5.16
Cl^- effect[b]	0.29	0.26	0.29	0.30	0.28	0.31	0.33
DPG effect[c]	0.22	0.20	0.23	0.25	0.28	0.26	0.23
Bohr effect[d]							
Stripped	–0.18	–0.23	–0.27	–0.17	–0.22	–0.32	–0.44
0.1 mol l^{-1} Cl^-	–0.38	–0.38	–0.45	–0.41	–0.34	–0.54	–0.59
0.1 mol l^{-1} Cl^- + 2.5 mol l^{-1} DPG	–0.63	–0.59	–0.78	–0.68	–0.68	–0.86	–0.78
$\Delta H'$[e]	–25.2	–20.3	–24.4	–24.2	–24.1	–9.7	–7.6
$\Delta \log P_{50}/\Delta T$[e]	0.0213	0.0185	0.0209	0.0208	0.0207	0.0126	0.0114

The temperature sensitivity of each Hb component expressed as overall enthalpy of oxygenation ($\Delta H'$; kJ mol^{-1} O_2) and as $\Delta \log P_{50}/\Delta T$ for the temperature range 25–37°C is also presented. The major Hb component (Hb II) was analyzed from two different two star-nosed moles, one from Manitoba (MB) and the other from Pennsylvania (PA).

DPG, 2,3-diphosphoglycerate; stripped, co-factor free solution.

[a]Data taken from Campbell et al. (Campbell et al., 2010b).

[b]$\log P_{50}$ (0.1 mol l^{-1} Cl^-) – $\log P_{50}$ (stripped).

[c]$\log P_{50}$ (2.5 mmol l^{-1} DPG+0.1 mol l^{-1} Cl^-) – $\log P_{50}$ (0.1 mol l^{-1} Cl^-).

[d]$\Delta \log P_{50}/\Delta$pH; over the pH range 7.0–7.4.

[e]In 0.1 mol l^{-1} Cl^-.

components (Hb I and Hb II) of the star-nosed mole (Piney, Manitoba) have isoelectric points (pI) at 5°C of 7.62 and 7.32, respectively, and account for ~30 and 70% of the hemolysate, respectively (Fig. 1A). Only the major component (Hb II) from the Pennsylvanian star-nosed mole was analyzed, and was found to possess a pI (7.29) similar to that of the Manitoban mole. The two major Hb components isolated from American shrew mole blood have pIs of 7.68 (Hb I) and 7.34 (Hb II), with Hb I comprising ~55% of the hemolysate (Fig. 1B).

Oxygen binding characteristics

The intrinsic Hb–O_2 affinities of both American shrew mole Hb components at pH 7.2 were ~40–50% lower (i.e. P_{50} values were ~1.6–2.0 times higher) than those of the star-nosed mole (Table 1). Conversely, expressed in terms of the shift in $\log P_{50}$, the effects on Hb–O_2 affinity of 0.1 mol l^{-1} Cl^- (0.26–0.30), and of saturating concentrations of DPG in the presence of 0.1 mol l^{-1} Cl^- (0.20–0.28), varied little between the Hb components of each species (Table 1). Notably, when both of these cofactors were present together, the P_{50} of the major component in star-nosed mole blood (Hb II) at a pH of 7.2 (23.23 mmHg) was higher than that of DPG alone (20.89 mmHg; Fig. 1A). The major Hb component of the Pennsylvanian mole exhibited a nearly identical response (23.76 *vs* 21.81 mmHg, respectively; supplementary material Fig. S1). The observed Bohr effects ($\Delta \log P_{50}/\Delta$pH) of star-nosed and American shrew mole Hbs were appreciably lower than those found for coast and eastern moles (Table 1) (Campbell et al., 2010b).

Thermal sensitivity

The $\Delta H'$ of both Hb components of the American shrew mole at pH 7.2 and in the presence of 0.1 mol l^{-1} Cl^- (Hb I=–25.2 kJ mol^{-1} O_2, Hb II=–20.3 kJ mol^{-1}; Table 1) were similar to those of star-nosed moles collected in Manitoba (Hb I=–24.4 kJ mol^{-1}, Hb II=–24.2 kJ mol^{-1}) and Pennsylvania (Hb II=–24.1 kJ mol^{-1}). These values are numerically twofold to threefold higher than the major Hb components of coast (–7.6 to –9.7 kJ mol^{-1}) and eastern moles (–10.3 to –13.7 kJ mol^{-1}; Table 1) measured under identical conditions (Campbell et al., 2010b). A similar trend is seen amongst the temperature effect ($\Delta \log P_{50}/\Delta T$) calculated for the Hb components of these species, with the $\log P_{50}$ of American shrew and star-nosed mole Hb increasing nearly twice as much per 1°C decrease in temperature compared with that of coast and eastern moles (Table 1).

Primary sequences of the α- and β-type globin chains

One α-like and two β-like genes were amplified and sequenced from the American shrew mole, whereas two α-like and two β-like genes were obtained from the star-nosed mole (Fig. 2). Phylogenetic analyses of intron 2 sequences confirmed that these β-like genes are orthologous to the *HBD* (δ) globin genes of eutherian mammals (data not shown), as found for other eulipotyphlan (e.g. shrews, moles and hedgehogs) mammals (Opazo et al., 2008; Campbell et al., 2010b). The deduced δ-globin chains of the star-nosed mole differed from one another at two residue positions (δ75Ile→Val and δ76Lys→Asn), whereas the two δ chains of the American shrew mole differed at five sites (δ9Gly→Ser, δ22Glu→Asp, δ23Val→Ile, δ58Ala→Pro and δ61Lys→Met).

Notably, there was a single charge-altering substitution within the δ chains of each species (Fig. 2). Consequently, the polypeptide with the higher net charge was designated δ^I (corresponding to isoHb I with the higher pI; insets of Fig. 1A,B) whereas the more negatively charged chain was designated δ^{II} (corresponding to isoHb II exhibiting the lower pI). The two α-like chains of the star-nosed mole differed at only one (uncharged) residue position (α55Val→Ile) and were thus arbitrarily designated α^I and α^{II} (Fig. 2).

With respect to the proposed 'additional' Cl^- binding site (δ8, δ76 and δ77), a charge altering residue substitution (Lys→Asn) was detected at residue 76 of the star-nosed mole δ^{II}-globin chain. No changes were found within this motif in the δ^I-globin chain of this species or in either of the δ-globin chains of the American shrew mole (Fig. 2). Similarly, no other amino acid substitutions along the α- or δ-globin chains of star-nosed or American shrew moles were detected at residue positions implicated in Cl^- binding (Perutz et al., 1993; Weber, 2007).

α-type chains

```
Amino acid  1        10        20        30        40        50        60        70
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
T. europaea α   VLSGTDKSNIKAAWDKVGAHAGEYGAEALERTFTSFPTTKTYFPHFDLSHGSAQVKAHGKKVADALTNAVGHLDD
S. orarius α    ..YD.........Q...T......G.....M.E.............K..........N..........A....
S. aquaticus α^I  ..YD.........Q...T......G.....M.DC............KP.........S...E...K..D....
S. aquaticus α^II ..YD.........Q...T......G.....M.DC............M.P.........S...E...K..D....
C. cristata α^I   ...A........F.G.I.D..............AA.....................G.............N....
C. cristata α^II  ...A........F.G.I.D..............AA.....................I.G.............N....
N. gibbsii α    ...AA..T..........S...D..........A..............NP......G.............A....

            76  80        90        100       110       120       130       140
            ....|....|....|....|....|....|....|....|....|....|....|....|....|.
T. europaea α   LPGAMSALSDLHAHKLRVDPVNFKLLSHCLLVTLACHHPNDFTPAVHASLDKFLATVSTVLTSKYR
S. orarius α    ....L NT.............................S...A.....M.....................
S. aquaticus α^I  ....L NV.............................S.LSA.....M.......F............
S. aquaticus α^II ....L NV.............................S.LSA.............F............
C. cristata α^I   ....L...............L..................S.IS.......................
C. cristata α^II  ....L...............L..................S.IS.......................
N. gibbsii α    ..............................S..........A.........................
```

β-type chains

```
Amino acid  1        10        20        30        40        50        60        70
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
T. europaea 'β'   VHLSGEEKGLVTGMWGKVNVDEVGGEALGRLLVVYPWTQRFFDSFGDLSSASAIMGNAKVKAHGKKVANSITDGV
S. orarius δ    ....A........L.......D..A...........................G.....P.............S..I
S. aquaticus δ  ..M.A....I..S........DI.A.........................PA.....P..........H..S..I
C. cristata δ^I   ............S.....................................................H.....I
C. cristata δ^II  ............S.....................................................H......
N. gibbsii δ^I    ...T.D..S....L.......DI.A.....................................................S...
N. gibbsii δ^II   ...T.D.......L..........A..............................P..M..........S...

                80        90        100       110       120       130       140
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
T. europaea 'β'   KNLDNLKGTYAKLSELHCDKLHVDPENFRLLGNVLVCVLARNLGKEFTPQAQAAFQKVVLGVATALAHKYH
S. orarius δ    ....................................M..T.......H.......M.............
S. aquaticus δ  ..............................................H..T....M..E..A.......
C. cristata δ^I   ....................................................................
C. cristata δ^II  N...................................................................
N. gibbsii δ^I    ....................................................................
N. gibbsii δ^II   ....................................................................
```

Fig. 2. Amino acid sequences of the (A) α- and (B) β-type (δ) globin chains of five mole species. Sequences for star-nosed moles (*Condylura cristata*) and American shrew moles (*Neurotrichus gibbsii*) were determined in the present study, whereas those of the European mole (*Talpa europaea*), coast mole (*Scapanus orarius*) and eastern mole (*Scalopus aquaticus*) were published previously (Kleinschmidt et al., 1981; Campbell et al., 2010b). Dots indicate sequence identity with *T. europaea*.

DISCUSSION

Thermal sensitivity

Precisely mirroring the pattern found for whole blood (Campbell et al., 2010b), we found that the major Hb components of the star-nosed mole are more sensitive to changes in temperature than those of coast and eastern moles (Table 1). However, it is of note that star-nosed mole Hbs can still be considered to have a relatively low thermal sensitivity, as their $\Delta H'$s in the presence of 0.1 mol l^{-1} Cl^- and at pH 7.2 (–24.1 to –24.4 kJ mol^{-1} O_2) are nearly half of that reported for human HbA (–41.0 kJ mol^{-1}) (De Rosa et al., 2004). In fact, the $\Delta H'$s of star-nosed and American shrew mole Hbs (–20.3 to –25.2 kJ mol^{-1} O_2) are comparable to that of bovine (–27.2 kJ mol^{-1}) and woolly mammoth Hbs (–26.0 kJ mol^{-1}) in the presence of 0.1 mol l^{-1} Cl^- at pH 7.4 (De Rosa et al., 2004; Campbell et al., 2010a). In contrast, the $\Delta H'$s of coast (7.6–9.7 kJ mol^{-1}) and eastern mole (10.3–13.7 kJ mol^{-1}) Hbs (Campbell et al., 2010b) are even lower than those of some of the most cold-tolerant mammalian species known, e.g. reindeer (–14.0 kJ mol^{-1}) and musk ox (–15.0 kJ mol^{-1}) (De Rosa et al., 2004). Is the reduced thermal sensitivity encountered in mole Hbs attributable to the cationic pocket between residues 8, 76 and 77 of their β-type globin chains that are hypothesized to constitute an 'additional' Cl^- binding site in ruminant Hbs (Fronticelli et al., 1995; De Rosa et al., 2004)? Although Campbell et al. (Campbell et al., 2010b) found that Cl^- and DPG exert a synergistic effect on the P_{50} of coast (and presumably eastern) mole Hb (consistent with the binding of extra chloride ions to Hb), the blood and Hb components of coast and eastern moles possess numerically low oxygenation enthalpies, and the β-type chains of both species possess residues consistent with the 'additional' Cl^- binding site hypothesis (De Rosa et al., 2004), we found no evidence to suggest that this cluster of residues is responsible for the variable thermal sensitivity among mole Hbs. This assertion is well supported by several lines of evidence.

The first lies in a non-synonymous nucleotide replacement (AA<u>A</u>→AA<u>C</u>) found in the codon encoding residue 76 of the δ^{II}-globin chain of star-nosed Hb II, which causes a neutral Asn residue to be translated rather than the cationic Lys characteristic of other Hbs with reduced thermal sensitivity (De Rosa et al., 2004). Hence, if the putative 'additional' Cl^- binding site was operational in mole Hbs, the presence of a second neutral charge within this cleft would be expected to markedly reduce Cl^- binding to this star-nosed mole

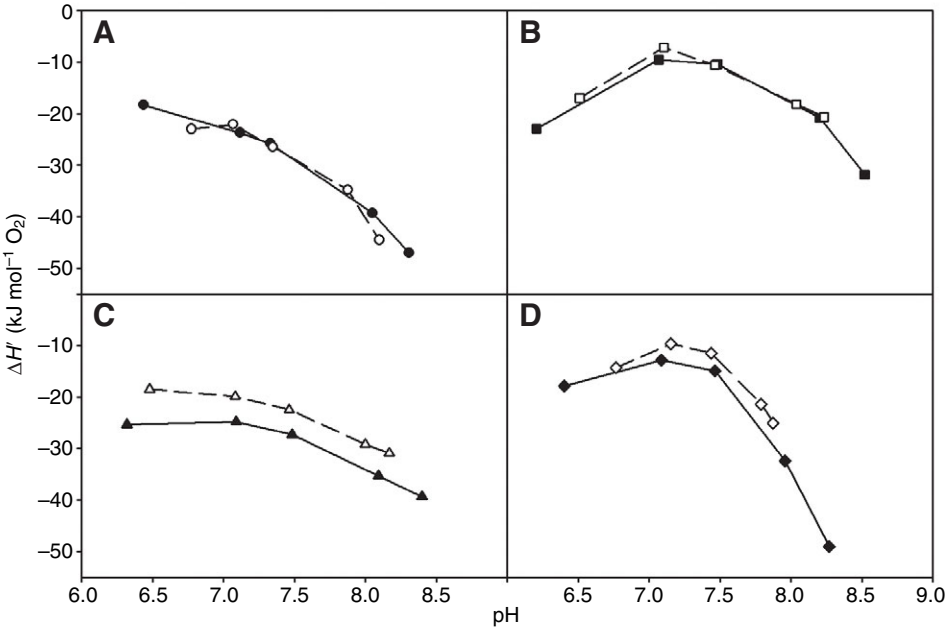

Fig. 3. Effect of pH on the enthalpy of oxygenation ($\Delta H'$) of hemoglobin components I (solid line) and II (dashed line) from (A) star-nosed, (B) coast, (C) American shrew and (D) eastern moles. $\Delta H'$ was calculated from P_{50} data collected at 37 and 25°C in the presence of 0.1 mol l^{-1} Cl^- using the integrated van't Hoff isochore, and corrected for the heat of oxygen solution (see Materials and methods for details). Data for coast and eastern moles were analyzed from the raw data set of Campbell et al. (Campbell et al., 2010b).

Hb isoform. In contrast, the Cl^- sensitivities of the two Hb components of this species were nearly indistinguishable (Table 1). Moreover, both Hb components exhibited nearly identical $\Delta H'$s and associated thermal sensitivities (Table 1), thus arguing against a differential number of Cl^- binding sites between the two star-nosed mole isoforms. Although β8Lys, β76Lys and β76Asn may constitute an additional Cl^- site in brown bear Hb (De Rosa et al., 2004), our results suggest that this site is not operational in star-nosed mole Hbs.

Further evidence against the 'additional' Cl^- binding site hypothesis is provided by data collected from American shrew mole Hb (Fig. 1B). The genes encoding both δ-globin chains of this species were observed to possess amino acid complements at residue positions 8, 76 and 77 identical to those found in coast and eastern moles (Fig. 2). However, the O_2 affinities of both American shrew mole Hb isoforms are more strongly governed by temperature than those of the two fossorial species, despite possessing similar Cl^- sensitivities (Table 1). Thus, although this site may be operational in bovine Hb (Fronticelli et al., 1995), our results suggest that these findings cannot be extrapolated to other species based on the amino acid complement at positions 8, 76 and 77 of β-type globin chains. Indeed, despite possessing the identical trio of residues as bovine, reindeer and musk ox Hb, O_2 binding data indicate that Cl^- is also unable to bridge this cationic cluster in woolly mammoth and Asian elephant Hbs (Campbell et al., 2010a).

As noted above, we previously demonstrated that coast mole Hb possesses an extra Cl^- binding site relative to human HbA, as treatments with 0.1 mol l^{-1} Cl^- and saturating levels of DPG lowered Hb–O_2 affinity more than DPG alone (Campbell et al., 2010b). However, it is clear that this site is not the reason for the differential temperature sensitivities seen among mole Hbs, as the major Hb components (Hb II) of star-nosed moles from both Manitoba and Pennsylvania were also found to possess an additional Cl^- binding site (Fig. 1A, supplementary material Fig. S1). Furthermore, the Cl^- sensitivity of Hbs from coast, star-nosed and American shrew moles are nearly identical (Table 1). [Eastern mole Hb possesses a central cavity δ136Gly→Glu replacement that not only abolishes DPG binding, but also lowers the sensitivity of its Hb components to Cl^- and H^+ (Campbell et al., 2010b), hence precluding its inclusion here.] This striking similarity in Cl^- sensitivity argues for a non-DPG-overlapping Cl^- binding site operating in the Hbs of these species. Although this site is yet to be identified, the synergistic effect of Cl^- and DPG is abolished above pH 7.5 in the star-nosed mole (Fig. 1A), indicating that it may be proton-linked, similar to that observed in Eskimo dog Hb (Bårdgard et al., 1997). Although it is likely that differential Cl^- binding is at least partly responsible for the numerically lower $\Delta H'$ values of mole Hbs compared with human HbA, it is important to stress that the variable thermal sensitivity among mole Hbs does not appear to be due to a differential ability to bind Cl^-.

As with chloride ions, coast, star-nosed and American shrew mole Hbs exhibit similar sensitivities to the red cell organophosphate DPG (Table 1). Thus, we find no evidence to suggest that DPG plays a role in the variable thermal sensitivity of the Hbs of these species. Notably, however, coast and eastern mole Hbs (Campbell et al., 2010b) exhibited appreciably larger Bohr coefficients than those of star-nosed and American shrew moles under all experimental conditions (Table 1). Moreover, the $\Delta H'$s of coast and eastern mole Hbs show greater pH-dependent variation than those of star-nosed and American shrew moles (Fig. 3). These results provide strong evidence that a differential ability to bind H^+ underlies the variable thermal sensitivity among the Hbs of these species. Given that the acid dissociation constant (pK_a) of His residues tend to be near physiological pH (Berenbrink, 2006), we therefore speculated that coast and eastern mole Hbs may contain one or more His residues that are not present in the Hbs other mole species.

Consistent with this prediction, the Hbs of both species were found to possess a Gln→His replacement at position 125 of their δ-globin chains (Fig. 2). Moreover, molecular modelling illustrates that residue δ125His is located on the surface of the deoxy-state protein (Fig. 4), and is thus accessible to H^+. However, it should be stressed that protonation of the imidazole side chain (resulting from an increase in the pK_a of this group in the T state) is in itself not enough to help stabilize the deoxy-state protein (i.e. contribute to the Bohr effect). In this regard, it is of note that residue 34 of the adjacent α chain is not only negatively charged in both coast

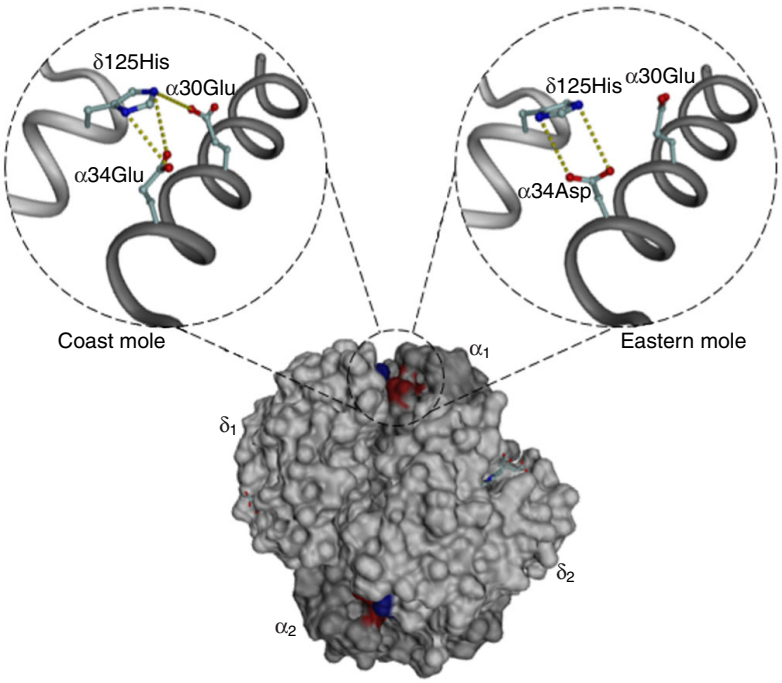

Fig. 4. Surface model of coast mole hemoglobin with residues Glu30 and Glu34 (red) on the H helix of the α-globin chains and His125 (blue) on the B helix of the δ-globin chains highlighted. Structural models were constructed for both coast and eastern moles by inserting species-specific amino-acid substitutions (coast mole: δ125Gln→His and α34Thr→Glu; eastern mole: δ125Gln→His and α34Thr→Asp) into models previously prepared for these species (Campbell et al., 2010b) using the MODELLER function of the Insight II program package version 97.2 (Biosym Technologies, San Diego, CA). For each model, strain energy minimization precisely followed the procedures outlined in Campbell et al. (Campbell et al., 2010b). Optimized electrostatic interactions (i.e. salt bridges) between neighbouring residues were evaluated using CHARMM (Chemistry at HARvard Macromolecular Mechanics; http://www.charmm.org) potentials. Inter-atomic distances of 4 Å were set as a minimal criterion for salt-bridge formation. Our results suggest that δ125His of coast mole Hb interacts closely with two Glu residues (30 and 34) of the α-chain (top left), whereas δ125His of the eastern mole protein forms a stable salt bridge with α34Asp of the neighbouring chain (top right). These salt bridges presumably stabilize the T-state protein and are likely responsible for the elevated Bohr effect of these two proteins relative to other mole species (see Discussion for details). Surface and ribbon representations were visualized with DINO version 0.9.1 (Philippsen, 2002).

and eastern moles – Glu and Asp, respectively – but lies within salt-bridge distance (<4 Å) of δ125His (i.e. close enough to interact with and alter the pK_a of this residue). Indeed, our computational models suggest that α34Asp is able to form a stable bond with δ125His of eastern mole Hb, whereas δ125His of coast mole Hb can electrostatically interact with both of the slightly longer side chains of α34Glu and α30Glu (Fig. 4). Notably, other mole species possess a neutral residue (Ala or Thr) at α34 (Fig. 2), signifying that this is the ancestral state and suggesting the following pattern of nucleotide substitutions at this codon: GCG[Ala] (star-nosed/shrew mole) → G<u>A</u>G[Glu] (coast mole) → GA<u>C</u>[Asp] (eastern mole). These observations suggest that both a titratable buffer group at δ125 (Gln→His) and an anionic amino acid at α34 (Ala→Glu) evolved in a common ancestor of coast and eastern moles, which together conferred an elevated Bohr effect of its Hb relative to that found for other moles (Table 1, Fig. 3). Consequently, our results indicate that a Hb phenotype with a relatively low thermal sensitivity ($\Delta H' \approx -24$ kJ mol^{-1}) is the ancestral condition in moles, with further reductions (in absolute terms) in $\Delta H'$ being a shared derived trait that evolved in a common ancestor of the two subterranean forms.

Oxygen binding properties

It has been suggested that the high O_2 affinity of European mole blood (P_{50}=20–24 mmHg) may be advantageous in loading O_2 in chronically hypoxic burrows (Bartels et al., 1969; Quilliam et al., 1971; Jelkmann et al., 1981). However, the same may not be expected for small (10–12 g) semi-fossorial species such as the American shrew mole, which not only constructs shallow tunnel galleries but also frequently forages above ground (Dalquest and Orcutt, 1942). In fact, an elevated blood–O_2 affinity would potentially be maladaptive for fuelling the high mass-specific metabolic rate of this species, which is ~2.3 times the value predicted by allometry for eutherian mammals (Campbell and Hochachka, 2000). Consistent with this suggestion, the intrinsic O_2 affinity of American shrew mole Hb (P_{50}=9.5–10.3 mmHg at pH 7.2 and 37°C; Table 1) is markedly lower than that of star-nosed and coast moles (5.2–6.5 mmHg). Hence, because the sensitivity of American shrew mole Hbs to DPG and Cl^- is nearly identical to that of star-nosed (Table 1), coast (Campbell et al., 2010b) and European mole Hbs (Jelkmann et al., 1981), the P_{50} of American shrew mole blood is likely ~10 mmHg higher than these fossorial and semi-aquatic forms. The similarity in DPG sensitivities among mole species is also noteworthy, as it suggests that a reduced sensitivity to DPG may not be a specific adaptation to subterranean life as previously presumed (Jelkmann et al., 1981).

Regardless of the mechanism, the heightened blood–O_2 affinity of subterranean moles is presumably advantageous as it safeguards O_2 uptake under periods of hypoxia. However, this trait is also expected to markedly reduce their ability to unload O_2 from arterial blood. The elevated Bohr coefficients of coast and eastern mole Hbs in the presence of allosteric effectors (–0.63 to –0.86) relative to less fossorial star-nosed and American shrew moles are thus notable, and potentially adaptive in this regard, as they would increase the P_{O_2} at which maximal O_2 offloading occurs. Importantly, our results show that increased proton binding also numerically lowers the $\Delta H'$ of coast and eastern mole blood (Fig. 3), a trait that may minimize impairment of O_2 loading at the lungs while burrowing, i.e. during exercise-induced hyperthermia (Campbell et al., 2010b). Given that both traits are potentially 'adaptive', we are (unfortunately) unable to speculate which of these two features was selected upon by evolution. Indeed, given that changes in $\Delta H'$ tend to be accompanied by changes in blood–O_2 affinity, care must be taken to consider both of these attributes together rather than in isolation.

LIST OF SYMBOLS AND ABBREVIATIONS

DPG	2,3-diphosphoglycerate
Hb	hemoglobin
n_{50}	Hill's cooperative coefficient
P_{50}	half-saturation pressure of oxygen
pI	isoelectric point
pK_a	acid dissociation constant
RACE	rapid amplification of cDNA ends
$\Delta H'$	overall enthalpy of oxygenation

ACKNOWLEDGEMENTS

We thank K. Catania for providing the blood sample from the Pennsylvania collected star-nosed mole, R. Gusztak and S.-I. Kawada for helping trap star-nosed and American shrew moles, and A. Sloan and J. Howatt for assistance with gene sequencing.

FUNDING

This research was supported by grants from the Natural Sciences and Engineering Research Council (NSERC) of Canada (to K.L.C. and J.S.), the Danish Natural Science Research Council (to R.E.W.), and the Carlsberg Foundation (to R.E.W.). A.V.S. received support from an NSERC Undergraduate Student Research Award and an NSERC Alexander Graham Bell Canada Graduate Scholarship.

REFERENCES

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

Bårdgard, A. J., Strand, I., Nuutinen, M., Jul, E. and Brix, O. (1997). Functional characterisation of eskimo dog hemoglobin: I. Interaction of Cl^- and 2,3-DPG and its importance to oxygen unloading at low temperature. *Comp. Biochem. Physiol.* **117A**, 367-373.

Bartels, H., Schmelzle, R. and Ulrich, S. (1969). Comparative studies of the respiratory function of mammalian blood. V. Insectivora: shrew, mole and nonhibernating and hibernating hedgehog. *Respir. Physiol.* **7**, 278-286.

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

Brittain, T. (2002). Molecular aspects of embryonic hemoglobin function. *Mol. Aspects Med.* **23**, 293-342.

Campbell, K. L. and Hochachka, P. W. (2000). Thermal biology and metabolism of the American shrew-mole, *Neurotrichus gibbsii*. *J. Mammal.* **8**, 578-585.

Campbell, K. L., Roberts, J. E. E., Watson, L. N., Stetefeld, J., Sloan, A. M., Signore, A. V., Howatt, J. W., Tame, J. W. R., Rohland, N., Shen, T.-J. et al. (2010a). Substitutions in woolly mammoth hemoglobin confer biochemical properties adaptive for cold tolerance. *Nat. Genet.* **42**, 536-540.

Campbell, K. L., Storz, J. F., Signore, A. V., Moriyama, H., Catania, K. C., Payson, A., Bonaventura, J., Stetefeld, J. and Weber, R. E. (2010b). Molecular basis of a novel adaptation to hypoxic-hypercapnia in a strictly fossorial mole. *BMC Evol. Biol.* **10**, 214.

Dalquest, W. W. and Orcutt, D. R. (1942). The biology of the least shrew-mole, *Neurotrichus gibbsii* minor. *Am. Midl. Nat.* **27**, 387-401.

De Rosa, M. C., Castagnola, M., Bertonati, C., Galtieri, A. and 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.

Fronticelli, C., Sanna, M. T., Perez-Alvarada, G. C., Karavitis, M., Lu, A. and 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.

Hamilton, W. J. (1931). Habits of the star-nosed mole, *Condylura cristata*. *J. Mammal.* **12**, 345-355.

Jelkmann W., Oberthür W., Kleinschmidt T. and Braunitzer G. (1981). Adaptation of hemoglobin function to subterranean life in the mole, *Talpa europaea*. *Respir. Physiol.* **46**, 7-16.

Kleinschmidt, T., Jelkmann, W. and Braunitzer, G. (1981). Die Primärstruktur des Hämoglobins des Maulwurfs (*Talpa europaea*). *Hoppe-Seyler's Z. Physiol. Chem.* **362**, 1263-1272.

Merriam, C. H. (1884). The star-nosed mole amphibious. *Science* **4**, 429.

Opazo, J. C., Hoffmann, F. G. and Storz, J. F. (2008). Differential loss of embryonic globin genes during the radiation of placental mammals. *Proc. Natl. Acad. Sci. USA* **105**, 12950-12955.

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

Perutz, M. F., Fermi, G., Poyart, C., Pagnier, J. and Kister, J. (1993). A novel allosteric mechanism in haemoglobin: structure of bovine deoxyhaemoglobin, absence of chloride-binding sites and origin of the chloride-linked Bohr effect in bovine and human haemoglobin. *J. Mol. Biol.* **233**, 536-545.

Petersen, K. E. and Yates, T. L. (1980). *Condylura cristata*. *Mamm. Spec.* **129**, 1-4.

Philippsen, A. (2002). *DINO: Visualizing Structural Biology*. Basel, Switzerland: University of Basel, www.dino3d.org.

Quilliam, T. A., Clarke, A., and Salsbury, A. J. (1971). The ecological significance of certain new haematological findings in the mole and hedgehog. *Comp. Biochem. Physiol.* **40A**, 89-102.

Sánchez-Villagra, M., Horovitz, I. and Motokawa, M. (2006). A comprehensive morphological analysis of talpid moles (Mammalia) phylogenetic relationships. *Cladistics* **22**, 59-88.

Shinohara, A., Suzuki, H., Tsuchiya, K., Zhang, Y., Luo, J., Jiang, X., Wang, Y. and Campbell, K. L. (2004). Evolution and biogeography of talpid moles from Continental East Asia and the Japanese Islands inferred from mitochondrial and nuclear gene sequences. *Zool. Sci.* **21**, 1177-1185.

Weber, R. E. (1992). Use of ionic and zwitterionic (Tris/BisTris and HEPES) buffers in studies on hemoglobin function. *J. Appl. Physiol.* **72**, 1611-1615.

Weber, R. E. (2007). High-altitude adaptations in vertebrate hemoglobins. *Resp. Physiol. Neurobiol.* **158**, 132-142.

Weber, R. E. and Campbell, K. L. (2011). Temperature dependence of haemoglobin-oxygen affinity in heterothermic vertebrates: mechanisms and biological significance. *Acta Physiol.* **202**, 549-562.

Wyman, J. (1964). Linked functions and reciprocal effects in hemoglobin: a second look. *Adv. Prot. Chem.* **19**, 223-286.