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Altered hemoglobin co-factor sensitivity does not underlie the evolution of derived fossorial specializations in the family Talpidae

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Abstract

The high O₂ affinity of European mole (*Talpa europaea*) blood is postulated to largely arise from the presence of two β -globin chain residues (β 4 Ser and β 5 Gly) that weak the interaction of its hemoglobin (Hb) with the red cell organophosphate 2,3-diphosphoglycerate (DPG). This latter trait is generally accepted to be an ‘adaptation to subterranean life’, despite the fact that no data are available for more basal mole lineages that have no evolutionary history of fossoriality (i.e. the ambulatory, high-elevation shrew-like moles and the semi-aquatic desmans), and which may similarly benefit from an elevated blood O₂ affinity. To test whether evolution of a low DPG sensitivity phenotype is linked to derived fossorial lifestyles or represents an ancestral trait for the family, we determined the globin gene sequences and measured the intrinsic O₂ affinity and co-factor sensitivity of the major Hb component of the gracile shrew-like mole (*Uropsilus gracilus*) and the Pyrenean desman (*Galemys pyrenaicus*). Our results unequivocally demonstrate that the presence of β 4 Ser and β 5 Gly, together with a low DPG sensitivity Hb phenotype, predates the radiation of the family Talpidae, and hence did not evolve as a specific adaptation to fossorial life. By contrast, our comparative analyses suggest that variations in whole blood O₂ affinity among members of this family predominantly arose from amino acid substitutions that increase or decrease the intrinsic O₂ affinity of the protein.

1. Introduction

Whereas the hemoglobins (Hbs) of all jawed vertebrates are structurally conserved heterotetramers consisting of 2 α - and 2 β -type globin polypeptides, the binding affinity of this molecule for oxygen varies extensively across the mammalian phylogeny (Poyart et

al., 1992). The half oxygen saturation pressures ($P_{50's}$) of shrew and hedgehog blood (~37 Torr), for example, is 10-16 mm Hg higher than that of fossorial talpid moles (Bartels et al., 1969; Quilliam et al., 1971; Jelkmann et al., 1981; Jürgens et al., 1981; Campbell et al., 2010). Such variations in blood O_2 affinity are primarily considered to have arisen via selection pressures to optimize the delicate balance between the uptake of O_2 at the alveoli and its unloading at the tissues (Tenny, 1995). Accordingly, the relatively high $P_{50's}$ typically observed in the blood of small terrestrial species (e.g. shrews, mice, and voles) are considered to favour the unloading of O_2 at the tissues to meet high mass-specific metabolic demands (Schmidt-Nielsen and Larimer, 1958; Hall, 1966; Jürgens et al., 1981). Conversely, the relatively low blood $P_{50's}$ of subterranean mammals (e.g. moles, ground squirrels, and prairie dogs) are presumed to facilitate maximal oxygenation of Hb at low environmental O_2 pressures associated with underground habitats (Hall, 1966; Campbell et al., 2010; Revsbech et al., 2013). Although variations in blood O_2 affinity may arise from modifications in allosteric effector (e.g. H^+ , Cl^- , and organophosphate) concentrations in the red blood cells, long-term adaptive changes predominantly arise from residue substitutions within the component globin chains that alter the inherent O_2 affinity of the protein and/or the strength of its interaction with erythrocytic effector molecules (Weber 1995).

The high O_2 affinity of European mole (*Talpa europaea*) blood in particular has been attributed to a weak interaction of its Hb with 2,3-diphosphoglycerate (DPG) due to two amino acid substitutions relative to human HbA ($\beta 4$ Thr→Ser and $\beta 5$ Pro→Gly) that border the DPG binding site (Jelkmann et al., 1981). Notably, a low DPG sensitivity Hb phenotype (relative to human HbA) is also found in other burrowing mole species

examined to date (Campbell et al., 2010; Signore et al., 2012), which include the aquatic/fossorial star-nosed mole (*Condylura cristata*), the semi-fossorial American shrew mole (*Neurotrichus gibbsii*), and the North American coast mole (*Scapanus orarius*). This latter subterranean species is placed within a separate tribe (Scalopini) than the Eurasian fossorial species (Talpini), with both groups likely having evolved fossorial specializations independently (Shinohara et al., 2003; He et al. 2017). However, it is not known either when this low DPG sensitivity Hb phenotype arose, or if it evolved as a specific adaptation to for fossorial life, as no data is currently available for talpid mole species that do not have an evolutionary history of subterranean habitation. These include the ‘basal’ shrew-like moles (subfamily Uropsilinae) which are the earliest offshoot of the talpid family lineage (Shinohara et al., 2003; He et al., 2017) and hence sister or all other extant mole taxa, and the semi-aquatic Eurasian desmans. The small (~10 g) ambulatory shrew-like moles exhibit no specializations for burrowing and instead forage in the leaf litter of southeastern Asian montane forests at elevations up to 4500 m (Nowak, 1999; Wan et al., 2013). Members of this subfamily display striking ecological and morphological similarities with shrews (e.g. well-developed ear pinnae, a long tail, weak claws, and slender forelimbs with relatively little musculature; Nowak, 1999; Wan et al., 2013), and hence are presumably little changed from the ancestral talpid ecotype. The semi-aquatic Eurasian desmans (tribe Desmanini) represent a phylogenetically and morphologically more derived talpid lineage (He et al., 2017), though still retain undeveloped forelimbs. Both extant species use their long laterally flattened tail, large webbed hind feet, and prehensile snout to forage underwater for aquatic invertebrates (Palmeirim and Hoffman, 1983; Nowak, 1999). As exposure to hypoxia is pervasive in both semi-aquatic (i.e. while submerged) and high-

elevation habitats (MacArthur, 1984; Storz, 2016), it is thus conceivable that evolution of a high O₂ affinity/low DPG sensitivity Hb phenotype is not an adaptation to fossorial lifestyles in the family Talpidae, but rather predates exploitation of this ecological niche.

To test this hypothesis, Hb–O₂ equilibrium curves (including their responses to temperature and the red cell ligands H⁺, DPG, and Cl[−]) were determined for the major Hb component of the gracile shrew-like mole (*Uropsilus gracilis*) and the Pyrenean desman (*Galemys pyrenaicus*). The α - and β -type chains encoding the adult-expressed Hbs of these species were also deduced to trace the evolutionary history of amino acid substitutions implicated in altering the sensitivity of the protein for DPG.

2. Material and methods

2.1. Sample collection

Blood collected from a female *Uropsilus gracilis* specimen collected at an elevation of ca. 3900 m on Mt. Laojun (N: 26°37'94", E: 99°43'52"), Lijiang District, Yunnan Province, China, was immediately stored at -80°C. The spleen was excised and placed in RNAlater (Ambion), then stored at -20°C until analysis. Animal trapping and care was approved by the Ethics Committee of the Kunming Institute of Zoology, Chinese Academy of Sciences, and the forestry bureau of Lijiang, China.

Blood samples were also obtained from the thoracic cavity of three freshly-thawed *Galemys pyrenaicus* (2♂, 1♀) stored at the Parque Nacional de Peneda-Gerês, Portugal. The specimens were collected from two rivers (Rio Sabor and Rio Paiva, respectively) in the Douro Basin, Portugal by the Instituto da Conservação da Natureza e das Florestas. Thin layer isoelectric focusing revealed no differences in banding patterns among the three

specimens, and thus blood from one of the Rio Sabor male specimens was used for subsequent analysis. A spleen sample from the female specimen (Rio Paiva) preserved in 95% ethanol was used for DNA extraction and sequencing.

2.2. Sample preparation and oxygen affinity

Prior to conducting experiments, the major Hb component was isolated from thawed blood samples by preparative isoelectric focusing (see Fig. S1), concentrated by centrifugal ultrafiltration, and stored in the CO form at -80°C as previously described (Campbell et al., 2010; Campbell et al., 2012). These Hb samples were then diluted to create 0.1 M HEPES buffer solutions over a range of pH's and containing varying concentrations of KCl and DPG; final Hb₄ concentrations for the *Galemys* trials was 0.05 mM, while those for the *Uropsilus* experiments ranged from 0.02 to 0.05 mM. Oxygen equilibration data were determined for small ($\sim 3\ \mu\text{L}$) aliquots via absorbance changes at 436 nm at both 25 and 37°C using the thin-film technique (Weber, 1992; Campbell et al., 2010). For each run, P_{50} and n_{50} (Hill's cooperativity coefficients at 50% saturation) values were interpolated from linear plots of Log (percent oxyhemoglobin/percent deoxyhemoglobin) vs. Log PO_2 using a minimum of four oxygen equilibration steps between 30 and 70% saturation. Bohr coefficients were calculated from P_{50} measurements obtained within the pH range 7.0–7.5. The enthalpy of oxygenation (ΔH , $\text{kJ mol}^{-1}\ \text{O}_2$) in the presence of 0.1 M Cl^- , corrected for the solubilization heat of O_2 ($-12.55\ \text{kJ mol}^{-1}$), was interpolated at pH 7.2 following Signore et al. (2012). The pH of each sample was measured at experimental test temperatures using a Radiometer BMS2 Mark 2 Blood Micro system and PHM 64 Research pH meter.

2.3. Globin sequence determination

To determine the α - and β -like (δ) globin coding sequences, we adopted a cross-species hybridization approach following Mason et al. (2011) and Horn (2012). Genomic DNA of tissue samples from each species were extracted using a Qiagen DNeasy kit (Qiagen, Valencia, CA, USA). The concentration of total DNA was determined using a NANODROP 2000c spectrophotometer (Thermo Fisher Scientific, Canada). 1000 ng DNA was sheared using NEBNext dsDNA Fragmentase (New England BioLabs, Whitby, Ontario, Canada), and converted into DNA libraries using a NEBNext Fast DNA Library Prep Set for Ion Torrent (New England Biolabs), and indexed using NEXTflex DNA barcodes for Ion Torrent (Bioo Scientific, Austin, TX, USA). We conducted size selection using an E-Gel electrophoresis system (Thermo Fisher Scientific, Canada) to select approximate 400-bp reads. We re-amplified the libraries (7-10 cycles) in 25 μ l reactions using 11.5 μ l libraries, 12.5 μ l NEB High-Fidelity 2x PCR Master Mix (New England Biolabs, Whitby, Ontario, Canada), and 1 μ l primers for Ion Torrent. PCRs were purified with serapure magnetic beads and DNA was eluted in 20 μ l of nuclease-free water. Biotinylated 120mer MyBaits RNA probes (Mycroarray, Ann Arbor, Michigan, USA) were designed from published HBA (α) and HBD (δ) coding sequences from four mole species (*Condylura cristata*, *Neurotrichus gibbsii*, *Scalopus aquaticus*, *Scapanus orarius*), two shrews (*Episoriculus fumidus*, *Sorex araneus*), and a hedgehog (*Erinaceus europaeus*), plus 30 bp of upstream and downstream intronic flanking sequences when available (see supplemental Table 1 for GenBank Accession numbers); note that the adult expressed β -like globin genes of shrews, moles, and hedgehogs is orthologous to the HBD genes of

other eutherian mammals (Campbell et al., 2012; Gaudry et al., 2014), and thus the protein product is a δ -globin chain (as opposed to a β -globin chain). We followed the manufacturing protocol of Mybait for hybridization using an Eppendorf Mastercycler Nexus Thermal Cycler (Thermo Fisher Scientific, Canada). Post-hybridization PCR amplifications (10-13 cycles according to the efficiency of hybridization) were performed in 25 μ l reactions using 1 μ l resuspended beads, 12.5 μ l NEB High-Fidelity 2x PCR Master Mix, 1 μ l primers for Ion Torrent, and 10.5 μ l nuclease-free water (New England Biolabs, Whitby, Ontario, Canada). PCRs were purified with serapure magnetic beads and DNA was eluted in 20 μ l of nuclease-free water. We determined the concentration using a Qubit Fluorometric Quantitation (Thermo Fisher Scientific, Canada).

The multiplexed libraries were sequenced in one direction using an Ion Torrent Personal Genome Machine with Ion 314v2 and Ion 318v2 chips, and Ion Torrent Proton System with Ion PI chips (Applied Biosystems, Foster City, California, USA). Sequenced reads were binned automatically using the Torrent Suite 4.4 software system according to the 'A' adaptor index sequence and imported into Geneious 8.1 (Biomatters Limited, Auckland, New Zealand). Assemblies were performed against reference exons using five iterations and a maximum mismatch per read of 10%.

To confirm that the obtained *Uropsilus gracilis* HBA and HBD gene sequences are expressed, corresponding mRNA sequences were determined following Signore et al. (2012). Briefly, total RNA was extracted from a ~250 mg spleen sample using the TRIZOL® method, and used to synthesize cDNA using SuperScript™ II Reverse Transcriptase following the manufacture's protocol (Invitrogen). The synthesized cDNA strands were Poly C tagged and used as template strands in RACE (rapid amplification of

cDNA ends) PCRs to amplify the expressed α - and β -like (δ) mRNA transcripts using previously designed α - and δ -globin primers (Signore et al., 2012). PCR conditions, gel extractions, and ligation of amplified products into cloning vectors are described elsewhere (Signore et al., 2012).

Sequencing reactions were conducted using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the universal sequencing primers M13(F)-40 or M-13(R). A four-capillary Applied Biosystems 3130 Genetic Analyzer was used to sequence the reactions. Sequencher™ (Version 5.0, Gene Codes Corp., Ann Arbor, MI, USA) software was used to align nucleotide sequences and deduce the resulting primary amino acid sequence. These sequences were compared to publically available globin sequences from other members of the order Eulipotyphla (moles, shrews, hedgehogs, and solenodons) to phylogenetically trace amino acid substitutions implicated in altering the DPG sensitivity of European mole Hb. Sequence data collected in this study were deposited in GenBank under accession numbers: XXXXXX - YYYYYY.

3. Results

The isoelectric focusing revealed the presence of a single major Hb component in the blood of both *Uropsilus gracilis* and *Galemys pyrenaicus*, with CO-derivative isoelectric points at 15°C of 8.03 and 7.57, respectively (Fig. S1). Oxygen equilibration curves of the major Hb component of both species in the presence of allosteric effectors were highly concordant (Fig. 1), with both species exhibiting comparable Bohr effects, responses to 0.1 M Cl⁻, and DPG sensitivities (Table 1). By contrast, gracile shrew-like mole Hb exhibited both a slightly higher intrinsic O₂ affinity (P₅₀: 6.7 mm Hg vs. 7.5 mm

Hg at 37°C and pH 7.2, respectively; Table 1) and a higher temperature sensitivity in the presence of 0.1 M Cl⁻ than Hb of the Pyrenean desman ($\Delta H = -26.6 \text{ kJ mol}^{-1} \text{ O}_2$ vs. $-17.36 \text{ kJ mol}^{-1} \text{ O}_2$, respectively). Comparisons to other talpid mole species revealed that all mole species examined to date possess similar sensitivities to allosteric effectors (with the exception of the eastern mole, *Scalopus aquaticus*), although variations in intrinsic O₂ affinity and ΔH were apparent (Table 1).

Our mRNA sequencing results for *Uropsilus* revealed the presence of a single α -like and a single β -like gene. These coding sequences precisely matched those obtained via hybridization capture (data not shown), suggesting they are the only globin loci expressed in the blood of this species. Likewise, our *Galemys* assemblies were also consistent with single adult-expressed α - and β -like loci. Amino acid sequences of the deduced α - and β -type (δ) globin chains of both species are given in Figs. S2 and S3. Amino acid residues forming the DPG binding site are conserved ($\beta 1$ Val, $\beta 2$ His, $\beta 82$ Lys, and $\beta 143$ His) in all species examined, with the exception of a $\beta 143$ His→Ala replacement found within both hedgehog species. The two residues implicated in lowering the DPG sensitivity of European mole Hb ($\beta 4$ Ser and $\beta 5$ Gly) are also conserved among moles (Fig. 2), with exception of the American shrew mole ($\beta 4$ Ser→Thr), and coast and eastern moles ($\beta 5$ Gly→Ala). Notably, $\beta 4$ Ser and $\beta 5$ Gly are also present in the three shrew species for which sequence data is available, although they were substituted by Thr and Ala, respectively, in hedgehogs (Figs. 2 and S2).

4. Discussion

The hypoxic hypercapnic environment of subterranean tunnel systems present inherent challenges to maintaining adequate gas exchange by fossorial mole species, especially during bouts of burrowing. For this reason, the high O₂ affinity of European mole blood (21.4 Torr) it is widely accepted to be beneficial for facilitating O₂ uptake from the ambient air (Quilliam et al., 1971; Jelkmann et al., 1981). Jelkmann et al. (1981) attributed this high blood-O₂ affinity to the presence of two amino acid residues (β 4 Ser and β 5 Gly) that reduce the interaction between DPG and the residues (β 1 Val, β 2 His, β 82 Lys, and β 143 His) involved in the binding of this organophosphate. Accordingly, the DPG sensitivity (measured as: $\text{LogP}_{50}(\text{Cl}^- + \text{DPG}) - \text{LogP}_{50}(\text{Cl}^-)$) of European mole Hb (~ 0.26 ; interpolated from data of Jelkmann et al. (1981)) is lower than that of human HbA (~ 0.40 ; Giardina et al., 1990; Weber, 1992). While these traits were suggested to be an “adaptation of hemoglobin to fossorial life” (Jelkmann et al., 1981), Hbs of the terrestrial gracile shrew-like mole and semi-aquatic Pyrenean desman also have both β 4 Ser and β 5 Gly and exhibit a similarly reduced DPG sensitivity (0.26-0.30; Table 1). These findings unambiguously demonstrate that a low DPG sensitivity predates the radiation of the family Talpidae, and as such the evolution of this trait is not temporally linked to the development of derived fossorial specializations by members of tribes Scalopini and Talpini. Interestingly, β 4 Ser and β 5 Gly are also found in the three species of shrews for which sequence data is available (Fig. 2), while Taiwanese brown-toothed shrew (*Episoriculus fumidus*) Hb—the only other eulipotyphlan species for which comparable data exists—exhibits a comparably depressed DPG sensitivity as moles (0.22; Campbell et al., 2012). Taken together these results strongly suggest that a blunted DPG sensitivity phenotype may in fact be an

ancestral trait for Eulipotyphla (i.e. it presumably evolved in an ambulatory forbearer of the group).

Although members of two mole lineages have independently evolved residue substitutions in the N-terminal region of the β -type chains— $\beta 4$ Ser→Thr in the American shrew mole, and $\beta 5$ Gly→Ala in the ancestor of coast and eastern moles (Fig. 2)—neither change appears to markedly alter the sensitivity of the protein for DPG (Table 1). This latter observation contrasts with results obtained from a recombinant human $\beta 5$ Pro→Ala variant that was shown to exhibit a normal DPG effect (Baudin et al., 1996). While these differential results may arise from differences in the genetic background of the protein (Tufts et al., 2015), it is also conceivable that $\beta 4$ and $\beta 5$ are unrelated to variations in DPG sensitivity. For example, the observation that 5 of 6 marmotine rodent species exhibit reduced DPG sensitivities (0.10-0.32) relative to human HbA despite having identical residues to HbA at $\beta 4$ and $\beta 5$ (Revsbech et al., 2013), clearly indicates that replacements at other sites not directly implicated in DPG binding may also strongly affect interactions with this organophosphate. Studies on polar bear Hb, which possesses $\beta 4$ Thr and $\beta 5$ Gly, however, provide evidence in support of a role for $\beta 5$ in altering DPG sensitivity (Pomponi et al., 2004). Specifically, molecular modeling suggests that the $\beta 5$ Pro→Gly replacement in this species not only perturbs the stereochemistry of the DPG binding pocket, but also creates an additional (with respect to human HbA) proton-linked Cl^- binding site between $\beta 82$ Lys and $\beta 143$ His (Pomponi et al., 2004). This prediction is consistent with data revealing the presence of an ‘additional’ Cl^- binding site in both mole and shrew Hbs (Campbell et al. 2010; Campbell et al. 2012; Signore et al. 2012). Hedgehog Hbs, which exhibit $\beta 4$ Thr, $\beta 5$ Ala (Figs. 2 and S3), and a His→Ala replacement at $\beta 143$, offer a natural

model system to test the effects of residue substitutions at these positions on both Cl^- and DPG binding affinity.

Given that co-factor sensitivities are strikingly similar among talpid moles, what blood/Hb attributes, if any, are potentially linked to niche specializations in this family? Although we were unable to determine whole blood O_2 affinities for either *U. gracilis* or *G. pyrenaicus*, measurements on whole blood samples from coast, eastern, and star-nosed moles (Campbell et al., 2010) are highly concordant (P_{50} differences <1 mm Hg) with those obtained from purified Hbs measured at 37°C in the presence of 0.1 M Cl^- and saturating DPG (Table 1), indicating that the ‘ Cl^- +DPG’ treatment may be a good proxy for *in vivo* affinities. Our results accordingly suggest that shrew-like moles and desmans exhibit whole blood P_{50} 's (ca. 24 Torr) that are substantially below that of similar sized terrestrial rodents (e.g. mice and rats have P_{50} 's of 52 and 39 Torr, respectively; Hall et al., 1966), though within the range of both fossorial and high-altitude rodents (22 to 30 Torr; Hall et al., 1966). Although comparisons between highland and lowland species pairs failed to find a significant association between Hb- O_2 affinity and elevation among mammals as a whole, this trait was shown to have independently evolved in a number of small mammal species, consistent with theoretical expectations (Storz et al., 2016). Similarly, many semi-aquatic species that rely on lung oxygen stores while diving (e.g. muskrat, sea otter, and platypus) also possess strongly left-shifted O_2 equilibration curves (MacArthur, 1984). As blood O_2 affinities from lowland shrews and hedgehogs range from 34 to 37 Torr (Bartels et al., 1969; Jürgens et al., 1981)—though are estimated to be ~ 25 Torr in the high-elevation Taiwanese brown toothed shrew based on data of Campbell et al. (2012)—these data suggest that a relatively high blood O_2 affinity phenotype is an ancestral trait for the family

Talpidae and may have subsequently facilitated exploitation of derived semi-aquatic and fossorial niches (Fig. 2). A major caveat to this scenario is that the blood O₂ affinity of the high-elevation *Uropsilus* specimen examined in this study is comparable to that of the last common talpid ancestor, which likely lived in the early Eocene (He et al., 2017). It is thus conceivable that a low P₅₀ blood phenotype evolved independently in uropsiline and non-uropsiline talpids. Nonetheless, whole blood data from coast and European moles—which appear to have convergently evolved fossorial lifestyles from a non-fossorial ancestor (Shinohara et al., 2003; He et al., 2017)—reveal the highest O₂ affinities (~20-21 mm Hg; Quilliam et al., 1971; Jelkmann et al., 1981; Campbell et al., 2010; but see Bartels et al., 1969) within the group, consistent with a further specialization to the subterranean environment. Although data from purified Hb solutions are currently lacking for fossorial Eurasian Talpini moles, the elevated blood O₂ affinity of coast moles is a reflection of their higher intrinsic Hb–O₂ affinity (P₅₀'s of 5.2 and 5.4 Torr at 37°C and pH 7.2; Campbell et al. 2010) relative to both semi-aquatic and high-elevation talpid species (6.5 to 7.5 Torr; Table 1). By contrast, P₅₀'s of purified Hb isoforms from the diminutive American shrew mole (9.5-10.3 Torr; Signore et al., 2012) show a strong evolutionary reduction (Fig. 2), consistent with their semi-fossorial lifestyle, very high mass-specific rates of metabolism (Campbell and Hochachka, 2000), and low elevation range (<2,500 m; Nowak, 1999). To summarize, adaptive differences in whole blood affinity within the talpid family predominantly have been driven by changes in the intrinsic O₂ affinity of the protein as opposed to reductions in effector sensitivity.

An exception to this generalization is found in fossorial eastern mole Hb which evolved a Gly→Glu residue exchange at β 136 that abolishes DPG binding and markedly

lowers the intrinsic O₂ affinity of the protein (Campbell et al. 2010). This phenotype was speculated to increase carbamate formation on the N-termini of the β -type chains, thus increasing blood CO₂ carrying capacity during burst tunneling activities. While CO₂ has little influence on the O₂ affinity of European mole blood (Jelkmann et al., 1981), which presumably safeguards pulmonary O₂ loading under hypoxic and hypercapnic burrow conditions (Weber et al., 2017), the effects of this metabolic by-product on other mole Hbs are currently unknown. Acetylation of the α - and β -type chain N-terminal residues, as occurs when N-terminal Val is replaced by Ser, is one factor known to impede CO₂ binding (Weber et al., 2017). While it is plausible that evolutionary reductions in CO₂ sensitivity underlie both fossorial and semi-aquatic adaptations in this group, the N-termini of both the α - and β -type globin chains are perfectly conserved among eulipotyphlans (Val in all cases; Figs. S2 and S3), suggesting that this trait may be ancestral and unrelated to habitat shifts in this order.

A second possible adaptive difference among mole Hbs pertains to temperature sensitivity, with both the Pyrenean desman and scalopin moles showing evolutionary reductions in ΔH (Table 1). In the latter case, this trait is associated with a heightened Bohr effect that may both promote O₂ offloading at the tissues while minimizing impairment of O₂ loading at the lungs during exercise-induced hyperthermia (Signore et al., 2012). By contrast, the reduced temperature sensitivity of *G. pyrenaicus* Hb appears to arise from a reduction in the intrinsic ΔH of the protein since effector sensitivities are unaltered. As this small (35-80 g) species exploits swift moving streams in the mountain ranges of the Pyrenees and northern Iberian peninsula (Palmeirim and Hoffman, 1983), it may be expected to show regional reductions in body temperature and hence benefit from a

numerically reduced oxygenation enthalpy to minimally impair O₂ offloading to cool tissues (Weber and Campbell, 2011). Additional studies are required to determine if these traits are common to Russian desmans (*Desmana moschatus*) and other fully fossorial species.

5. Conclusion

The results of our comparative study reveal that possession of Hb with a low DPG sensitivity is ancestral for the talpid family, and likely even predates the evolution of this group, and hence did not evolve as a specific adaptation to fossorial life. Our findings instead suggest that adaptive variations in whole blood affinity associated with habitat shifts among members of this clade predominantly result from amino acid substitutions that alter the inherent O₂-affinity of the protein. Unfortunately, sequence variation within both the HBA and HBD loci (Figs. S2 and S3) currently preclude pinpointing the specific replacements underlying these differences. Finally, reductions in the thermal sensitivity of O₂ binding and unloading evolved at least twice in the talpid lineage, though it is presently unclear if this trait is universally found among fossorial and semi-aquatic mole species.

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Table 1. Intrinsic oxygen affinity, expressed as half-saturation oxygen tension, P_{50} (mm Hg) of the major hemoglobin component of six talpid species, and their sensitivity to allosteric effectors and temperature. Allosteric sensitivities were calculated at 37°C and pH 7.2 using linear equations derived from Figure 1, while the temperature sensitivity is expressed as the enthalpy of oxygenation (ΔH ; kJ mol⁻¹ O₂) over the temperature range 25–37°C.

	Shrew-like mole	Desman	Shrew mole ¹	Star-nosed mole ¹	Coast mole ²	Eastern mole ²
P_{50} 'stripped'	6.69	7.46	9.54	6.45	5.16	14.77
P_{50} 0.1 M Cl ⁻	11.94	13.19	18.64	12.94	10.80	25.96
P_{50} 0.1 M Cl ⁻ + DPG	23.99	23.97	30.53	23.22	18.33	27.28
Cl ⁻ effect ³	0.25	0.25	0.29	0.30	0.33	0.25
DPG effect ⁴	0.30	0.26	0.21	0.25	0.23	0.02
Bohr effect ⁵						
'stripped'	-0.13	-0.19	-0.18	-0.13	-0.44	-0.41
0.1 M Cl ⁻	-0.26	-0.35	-0.38	-0.41	-0.59	-0.52
0.1 M Cl ⁻ + DPG	-0.75	-0.70	-0.63	-0.68	-0.78	-0.73
ΔH ⁶	-26.7	-17.4	-25.3	-24.2	-7.6	-10.3

¹ data from Signore et al. (2012)

² data from Campbell et al. (2010)

³ $\text{Log}P_{50} (0.1 \text{ M Cl}^-) - \text{Log}P_{50} (\text{stripped})$

⁴ $\text{Log}P_{50} (\text{DPG} + 0.1 \text{ M Cl}^-) - \text{Log}P_{50} (0.1 \text{ M Cl}^-)$

⁵ $\Delta \text{Log}P_{50} / \Delta \log \text{pH}$; over the pH range 6.8–7.2

⁶ in 0.1 M Cl⁻

Figure 1. Values of P_{50} and Hill's cooperativity coefficient at half oxygen saturation (P_{50} and n_{50}) for the major hemoglobin component of (A) the gracile shrew-like mole, *Uropsilus gracilis* and (B) Pyrenean desman, *Galemys pyrenaicus* at 25 and 37°C, and its pH dependence in the absence and presence of 0.1 M Cl^- and 2,3-DPG (DPG/Hb₄ ratios ~30). Insets: diagram of isoelectric focusing column illustrating the relative abundance and pI of the major hemoglobin component.

Figure 2. Blood oxygen affinity (in mmHg) of moles, shrews, and hedgehogs. Values obtained from whole blood are corrected to pH 7.4, while those estimated from purified hemoglobin samples measured at 37°C and pH 7.2 are given in parentheses. The authors preferred interpretation of evolutionary increases (up arrows) and decreases (down arrow) in blood oxygen affinity are shown. Mapping residue replacements at positions implicated in altering the DPG sensitivity of European mole blood ($\beta 4$ and $\beta 5$) onto this phylogeny suggests that $\beta 4$ Ser and $\beta 5$ Gly, and hence evolution of a low DPG sensitive hemoglobin phenotype, predates the radiation of the family Talpidae. Coloured lines denote ecological niches (black: lowland terrestrial; red: highland terrestrial; brown: fossorial; green: semi-fossorial; blue: semi-aquatic). Data from Bartels et al. (1969), Jürgens et al. (1979), Jelkmann et al. (1981), Campbell et al. (2010), Campbell et al. (2012), Signore et al. (2012), and this study. Phylogenetic tree of the Order Eulipotyphla derived from Sato et al. (2016) and He et al. (2017).

Table S1. GenBank accession numbers of globin protein/gene sequences employed in this study. Asterisks denote sequences used to design hybridization probes.

Species	α -type loci	β -type loci
<i>Talpa europaea</i>	' α ': P01951.1	' β ': P02061.1
<i>Uropsilus gracilis</i>	This study	This study
<i>Galemys pyrenaicus</i>	This study	This study
<i>Condylura cristata</i>	HBA1: JN208868.1* HBA2: NM001281855.1* HBA2: JN208869.1*	HBD: AJFV01042238.1* HBD: AJFV01042236.1*
<i>Neurotrichus gibbsii</i>	HBA: JN208871.1*	HBD1: JN208873.1* HBD2: JN208872.1*
<i>Scapanus orarius</i>	HBA: HM060231.1* HBA: HM060230.1*	HBD: HM060232.1*
<i>Scalopus aquaticus</i>	HBA1: HM060237.1*	HBD: HM060239.1*
<i>Sorex araneus</i>	HBA1&2: ALT02039077.1*	HBD: AALT02155696.1*
<i>Episoriculus fumidus</i>	HBA: JQ582443.1*	HBD1: JQ582442.1 HBD2: JQ582445.1*
<i>Suncus murinus</i>	' α ': P01950.1	' β ': P02060.1
<i>Erinaceus europaeus</i>	HBA1&2: MDU01126209.1*	HBD1&3: AMDU01169339.1*
<i>Atelerix albiventris</i>	HBA1&2: AC186458.2	HBD1&3: AC171157.2

Figure S1. Preparative isoelectric focusing of (A) gracile shrew-like mole (*Uropsilus gracilis*), and (B) Pyrenean desman (*Galemys pyrenaicus*) hemolysates. Closed circles denote absorption at 540 nm while open triangles represent the pH values of eluted fractions at 15°C. Horizontal red bar denotes fractions pooled and dialyzed for O₂ equilibrium measurements (see Figure 1).

A.

B.

Figure S2. Amino acid alignment of the α -globin chains for 12 eulipotyphlan species. Residues are shown only at positions where they differ from those of the European mole.

	Residue 1	10	20	30	40	50
						
<i>T. europaea</i> 'α'	VLSGTDKSNIKAAWDKVG	AHAGEYGA	EALERTFT	SFPTTK	TYFP	PHFDLSH
<i>U. gracilis</i> HBA	...PA..T.....	P..D.....	A.....	P		
<i>G. pyrenaicus</i> HBA	...A...T.....	S...D.....	M..			
<i>C. cristata</i> HBA1	...A.....	F.G.I.D.....	AA.....			
<i>C. cristata</i> HBA2	...A.....	F.G.I.D.....	AA.....			
<i>N. gibbsii</i> HBA	...AA..T.....	S...D.....	A.....	NP		
<i>S. orarius</i> HBA	..YD.....	Q...T.....	G....M.E.....	K.		
<i>S. aquaticus</i> HBA1	..YD.....	Q...T.....	G....M.DC.....	KP		
<i>S. aquaticus</i> HBA2	..YD.....	Q...T.....	G....M.DC.....	M.P		
<i>S. araneus</i> HBA	...AN..A.....	I..N..T.....	A.....	NP		
<i>E. fumidus</i> HBA	...AN..A.....	I..N..T.....	A.....	NP		
<i>S. murinus</i> 'α'	...AN..A.V.....	GQ..AN.....	A.....	Y...P		
<i>E. europaeus</i> HBA1&2	...A...A.V.TF.G.L.G.G....	G...D.M.QAH.....	NP			
<i>A. albiventris</i> HBA1	...AA..A.V..F.G.L.G....	G...D.M.QAH.....	P			
<i>A. albiventris</i> HBA2	...AA..A.V..F.G.L.G....	G...D.M.QAH.....	P			
	51	60	70	80	90	100
						
<i>T. europaea</i> 'α'	GSAQVKAHGKKVADALTNA	VGHLDLPG	AMSALSDL	HAHKLR	VD	VPVNFKL
<i>U. gracilis</i> HBA		R...S.....				
<i>G. pyrenaicus</i> HBA	I.....	S.....				
<i>C. cristata</i> HBA1	G.....	N.....	L.....			
<i>C. cristata</i> HBA2	I.G.....	N.....	L.....			
<i>N. gibbsii</i> HBA	G.....	A.....				
<i>S. orarius</i> HBA	N.....	A.....	LNT.....			
<i>S. aquaticus</i> HBA1	S...E...K...D.....	LNV.....				
<i>S. aquaticus</i> HBA2	S...E...K...D.....	LNV.....				
<i>S. araneus</i> HBA	G.....	K...S.....	L.....			
<i>E. fumidus</i> HBA	G.....	K...S.....	L.....			
<i>S. murinus</i> 'α'		K...SM.....	L.....			
<i>E. europaeus</i> HBA1&2	G.....	T..NN...V...L.....				
<i>A. albiventris</i> HBA1	S.....	S..SS...V...L.....				
<i>A. albiventris</i> HBA2	S.....	S..SS...V...L.....				
	101	110	120	130	140	
						
<i>T. europaea</i> 'α'	LSHCLLVTLACHHPNDFTP	AVHASLDK	FLATVST	VLTSKYR		
<i>U. gracilis</i> HBA		A.....				
<i>G. pyrenaicus</i> HBA		S.....				
<i>C. cristata</i> HBA1		S.IS.....				
<i>C. cristata</i> HBA2		S.IS.....				
<i>N. gibbsii</i> HBA	...S.....	A.....				
<i>S. orarius</i> HBA		S...A.....M.....				
<i>S. aquaticus</i> HBA1		S.LSA.....M.....	F.....			
<i>S. aquaticus</i> HBA2		S.LSA.....	F.....			
<i>S. araneus</i> HBA		S...AE.....M.S.....				
<i>E. fumidus</i> HBA		S...AE.....M.S.....				
<i>S. murinus</i> 'α'		A...A.....S.....				
<i>E. europaeus</i> HBA1&2		L...A.....A.....				
<i>A. albiventris</i> HBA1		L...A.....A.....				
<i>A. albiventris</i> HBA2		L...A.....A.....				

Figure S3. Amino acid alignment of the δ -globin chains for 12 eulipotyphlan species. Residues are shown only at positions where they differ from those of the European mole. Residues implicated in DPG binding (β 1 Val, β 2 His, β 82 Lys and β 143 His) are underlined. β 4 Ser and β 5 Gly (bold) are present in the European mole, gracile shrew-like mole, star-nosed mole, Asian house shrew, Taiwanese brown-toothed shrew, and Eurasian shrew. Question marks denote missing data. The HBD-T2 loci from both hedgehog species are slightly truncated and found on the complimentary strand relative to other β family genes (Gaudry et al., 2014), and are not included here.

Species	Residue #	1	10	20	30	40	50
<i>T. europaea</i> ' β '		VHL	SGEEKGLVTGMW	GKVN	VDEVGGEALGRLL	VVYPWTQRFF	DSFGDLSS
<i>U. gracilus</i> HBD			M	L	A	A	N
<i>G. pyrenaicus</i> HBD			AA	D	??????????		
<i>C. cristata</i> HBD1			S				
<i>C. cristata</i> HBD2			S				
<i>N. gibbsii</i> HBD1		T	D	S	L	D	I
<i>N. gibbsii</i> HBD2		T	D		L		A
<i>S. orarius</i> HBD		A		L		D	A
<i>S. aquaticus</i> HBD		M	A	I	S	D	I
<i>S. araneus</i> HBD			V	L			
<i>E. fumidus</i> HBD1&2		M		SAI	AL	D	
<i>S. murinus</i> ' β 1'			AC	L	E	A	
<i>S. murinus</i> ' β 1'			AC	L	E	A	
<i>E. europaeus</i> HBD1&3		TA	A	L	K	E	F
<i>A. albiventris</i> HBD1&3		TA	A	L	K	E	F
	51	60	70	80	90	100	
<i>T. europaea</i> ' β '		ASAIMGNAKVK	AHGKKVANSIT	DGVKNL	DNL	KGTYAKLSEL	HCDKLHVDP
<i>U. gracilus</i>		G		G	I	S	
<i>G. pyrenaicus</i>		P	P	H	D	I	??????????
<i>C. cristata</i> HBD1				H	I		
<i>C. cristata</i> HBD2				H	N		
<i>N. gibbsii</i> HBD1				S			
<i>N. gibbsii</i> HBD2		P	M	S			
<i>S. orarius</i> HBD		G	P	S	I		
<i>S. aquaticus</i> HBD		PA	P	H	S	I	
<i>S. araneus</i> HBD			P	LQ	LG	IN	F
<i>E. fumidus</i> HBD1&2		V	P	LM	MA	N	M
<i>S. murinus</i> ' β 1'		V	P	LH	LGE	A	F
<i>S. murinus</i> ' β 1'		V	P	LH	LGE	A	F
<i>E. europaeus</i> HBD1&3		D	V	P	A	LQ	MG
<i>A. albiventris</i> HBD1&3		D	V	P	A	LQ	MG
	101	110	120	130	140	146	
<i>T. europaea</i> ' β '		ENFRLLGNVL	VLCVLARN	LGKEFT	PQAQAA	FQKVVL	LGVATALAHKYH
<i>U. gracilus</i> HBD					M	N	
<i>G. pyrenaicus</i> HBD			M				
<i>C. cristata</i> HBD1							
<i>C. cristata</i> HBD2							
<i>N. gibbsii</i> HBD1							
<i>N. gibbsii</i> HBD2							

<i>S. orarius</i> HBD	M..T.....H.....M.....
<i>S. aquaticus</i> HBD	H.RT....M..EV.A.....
<i>S. araneus</i> HBD	HF.....SV.....M.A...A.....
<i>E. fumidus</i> HBD1&2	V...HF.....TV.....L.A.....
<i>S. murinus</i> 'β1'	V...HF.....PV.....A...N.....
<i>S. murinus</i> 'β2'	V...SKF.....MT.....A...N.....
<i>E. europaeus</i> HBD1&3	HF..D...A.....A...N...A...
<i>A. albiventris</i> HBD1&3	HF..D...A.....V...N...A...