



## 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 O<sub>2</sub> affinity of European mole (*Talpa europaea*) blood is postulated to largely arise from the presence of two β-globin chain residues (β4 Ser and β5 Gly) that weaken 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 O<sub>2</sub> 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 O<sub>2</sub> affinity and co-factor sensitivity of the major Hb component of the gracile shrew-like mole (*Uropsilus gracilis*) and the Pyrenean desman (*Galemys pyrenaicus*). Our results unequivocally demonstrate that the presence of β4 Ser and β5 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 O<sub>2</sub> affinity among members of this family predominantly arose from amino acid substitutions that increase or decrease the intrinsic O<sub>2</sub> 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<sub>50's</sub>) of shrew and hedgehog blood (~37 Torr), for example, are 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<sub>2</sub> affinity are widely accepted to have arisen via selection pressures to optimize the delicate balance between the uptake of O<sub>2</sub> at the pulmonary alveoli and its unloading at the tissues (Tenney, 1995; Weber 1995; Storz 2016). Accordingly, the relatively high blood P<sub>50's</sub> typically observed in small terrestrial species (e.g. shrews, mice, and

voles) are considered to favour a rapid delivery of O<sub>2</sub> necessary to meet their high mass-specific cellular demands (Schmidt-Nielsen and Larimer 1958; Hall 1966; Jürgens et al. 1981). Conversely, the relatively low blood P<sub>50's</sub> of subterranean mammals (e.g. moles, ground squirrels, and prairie dogs) are presumed to facilitate maximal oxygenation of Hb at low environmental O<sub>2</sub> pressures associated with underground habitats (Hall 1966; Campbell et al. 2010; Revsbech et al. 2013). Although variations in blood O<sub>2</sub> affinity may arise from modifications in allosteric effector (e.g. H<sup>+</sup>, Cl<sup>-</sup>, 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<sub>2</sub> affinity of the protein and/or the strength of its interaction with erythrocytic effector molecules (Weber 1995).

The high O<sub>2</sub> affinity of European mole (*Talpa europaea*) blood in particular has been attributed to a weak interaction of its Hb with 2,3-

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diphosphoglycerate (DPG) due to two amino acid substitutions relative to human HbA ( $\beta 4$  Thr  $\rightarrow$  Ser and  $\beta 5$  Pro  $\rightarrow$  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 when this low DPG sensitivity Hb phenotype arose, or if it evolved as a specific adaptation for fossorial life, as no data are currently available for talpid mole species that do not have an evolutionary history of subterranean habitation. These are represented by the ‘basal’ shrew-like moles (subfamily Uropsilinae) and the semi-aquatic Eurasian desmans. The small (~10 g) ambulatory shrew-like moles are the earliest offshoot of the talpid family lineage, exhibit no specializations for burrowing (Shinohara et al. 2003; He et al. 2017) 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 thus 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. In contrast, the semi-aquatic Eurasian desmans (tribe Desmanini) represent a phylogenetically and morphologically more derived talpid lineage (He et al. 2017). As their forelimbs remain unspecialized for digging, however, both extant desman species employ their large webbed hind feet, long laterally flattened tails, and prehensile snouts 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; Weber 1995; Storz 2016), it is thus conceivable that evolution of a 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–O<sub>2</sub> equilibrium curves (including their responses to temperature and the red cell ligands H<sup>+</sup>, DPG, and Cl<sup>−</sup>) were determined for the major Hb component of the gracile shrew-like mole (*Uropsilus gracilis*) and the Pyrenean desman (*Galemys pyrenaicus*). The  $\alpha$ - and  $\beta$ -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 obtained from a female *Uropsilus gracilis nivatus* 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  $-80^{\circ}\text{C}$ . The spleen was excised and placed in RNAlater (Ambion), then stored at  $-20^{\circ}\text{C}$ . 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

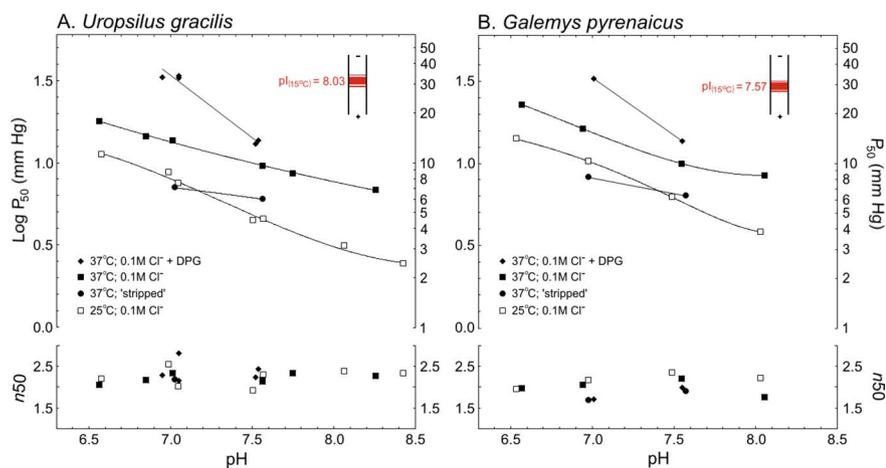
spleen sample from the female specimen (Rio Paiva) preserved in 95% ethanol was used for DNA extraction and sequencing.

### 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  $-80^{\circ}\text{C}$  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 Hb<sub>4</sub> 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 (~3  $\mu\text{l}$ ) aliquots via absorbance changes at 436 nm at both 25 and 37  $^{\circ}\text{C}$  using the thin-film technique (Weber 1992; Campbell et al. 2010). For each run, P<sub>50</sub> and n<sub>50</sub> (Hill's cooperativity coefficients at 50% saturation) values were interpolated from linear plots of Log (percent oxyhemoglobin/percent deoxyhemoglobin) vs. Log PO<sub>2</sub> using a minimum of four oxygen equilibration steps between 30 and 70% saturation. Bohr coefficients were calculated from P<sub>50</sub> measurements obtained within the pH range 7.0–7.5. The enthalpy of oxygenation ( $\Delta\text{H}$ , kJ mol<sup>−1</sup> O<sub>2</sub>) in the presence of 0.1 M Cl<sup>−</sup>, corrected for the solubilization heat of O<sub>2</sub> ( $-12.55$  kJ mol<sup>−1</sup>), 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  $\alpha$ - and  $\beta$ -like ( $\delta$ ) 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 DNA fragments ~400 bp in size. We re-amplified the libraries (7–10 cycles) in 25  $\mu\text{l}$  reactions using 11.5  $\mu\text{l}$  libraries, 12.5  $\mu\text{l}$  NEB High-Fidelity 2  $\times$  PCR Master Mix (New England Biolabs, Whitby, Ontario, Canada), and 1  $\mu\text{l}$  primers for Ion Torrent. PCRs were purified with Serapure magnetic beads and DNA was eluted in 20  $\mu\text{l}$  of nuclease-free water. Biotinylated 120mer MyBaits RNA probes (Mycroarray, Ann Arbor, Michigan, USA) were designed from published HBA ( $\alpha$ ) and HBD ( $\delta$ ) 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 S1 for GenBank Accession numbers); note that the adult expressed  $\beta$ -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  $\delta$ -globin chain (as opposed to a  $\beta$ -globin chain). We followed the manufacturer's (MyBaits) protocol for DNA 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  $\mu\text{l}$  reactions using 1  $\mu\text{l}$  resuspended beads, 12.5  $\mu\text{l}$  NEB High-Fidelity 2  $\times$  PCR Master Mix, 1  $\mu\text{l}$  primers for Ion Torrent, and 10.5  $\mu\text{l}$  nuclease-free water (New England Biolabs, Whitby, Ontario, Canada). PCRs were purified with Serapure



**Fig. 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/Hb<sub>4</sub> ratios ~30). Insets: diagram of isoelectric focusing column illustrating the relative abundance and pI of the major hemoglobin component.

magnetic beads and DNA was eluted in 20  $\mu$ l of nuclease-free water. We determined DNA concentrations using an Invitrogen Qubit 4 fluorometer (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 9.15 (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 manufacturer'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  $\alpha$ - and  $\beta$ -like ( $\delta$ ) mRNA transcripts using previously designed  $\alpha$ - and  $\delta$ -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: MG699126 – MG699129.

### 3. Results

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 of 8.03 and 7.57, respectively, measured at 15 °C (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  $O_2$  affinity ( $P_{50}$ : 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 ( $\Delta H = -26.6$  kJ mol<sup>-1</sup>  $O_2$  vs.  $-17.36$  kJ mol<sup>-1</sup>  $O_2$ , respectively). Comparisons to other talpid mole species revealed that all mole species examined to date possess similar sensitivities to these allosteric effectors (with the exception of the eastern mole, *Scalopus aquaticus*), although variations in intrinsic  $O_2$  affinity and  $\Delta H$  were apparent (Table 1).

Our mRNA sequencing results for *Uropsilus* revealed the presence of a single  $\alpha$ -like and a single  $\beta$ -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  $\alpha$ - and  $\beta$ -like loci. Amino acid sequences of the deduced  $\alpha$ - and  $\beta$ -type ( $\delta$ ) globin chains of both species are given in Figs. S2 and S3. Amino acid residues forming the DPG binding site ( $\beta$ 1 Val,  $\beta$ 2 His,  $\beta$ 82 Lys, and  $\beta$ 143 His) are conserved in all species examined, with the exception of a  $\beta$ 143 His  $\rightarrow$  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  $\rightarrow$  Thr), and coast and eastern moles ( $\beta$ 5 Gly  $\rightarrow$  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  $O_2$  affinity of European mole blood (21.4 Torr) is widely accepted to be beneficial for facilitating  $O_2$  uptake from the ambient air (Quilliam et al. 1971; Jelkmann et al. 1981). Jelkmann et al. (1981) attributed this high blood- $O_2$  affinity to the presence of two amino acid residues ( $\beta$ 4 Ser and  $\beta$ 5 Gly) that reduce the interaction between DPG and the residues ( $\beta$ 1 Val,  $\beta$ 2 His,  $\beta$ 82 Lys, and  $\beta$ 143 His) involved in the binding of this organophosphate. Accordingly, the DPG sensitivity (measured as:  $\text{Log}P_{50}(Cl^- + \text{DPG}) - \text{Log}P_{50}(Cl^-)$ ) 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  $\beta$ 4 Ser and  $\beta$ 5 Gly and exhibit a similarly reduced DPG sensitivity

**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 Fig. 1, while the temperature sensitivity is expressed as the enthalpy of oxygenation ( $\Delta H$ ; kJ mol<sup>-1</sup> O<sub>2</sub>) over the temperature range 25–37 °C.

|                                      | Shrew-like mole | Desman | Shrew mole <sup>a</sup> | Star-nosed mole <sup>a</sup> | Coast mole <sup>b</sup> | Eastern mole <sup>b</sup> |
|--------------------------------------|-----------------|--------|-------------------------|------------------------------|-------------------------|---------------------------|
| $P_{50}$ 'stripped'                  | 6.69            | 7.46   | 9.54                    | 6.45                         | 5.16                    | 14.77                     |
| $P_{50}$ 0.1 M Cl <sup>-</sup>       | 11.94           | 13.19  | 18.64                   | 12.94                        | 10.80                   | 25.96                     |
| $P_{50}$ 0.1 M Cl <sup>-</sup> + DPG | 23.99           | 23.97  | 30.53                   | 23.22                        | 18.33                   | 27.28                     |
| Cl <sup>-</sup> effect <sup>c</sup>  | 0.25            | 0.25   | 0.29                    | 0.30                         | 0.33                    | 0.25                      |
| DPG effect <sup>d</sup>              | 0.30            | 0.26   | 0.21                    | 0.25                         | 0.23                    | 0.02                      |
| Bohr effect <sup>e</sup>             |                 |        |                         |                              |                         |                           |
| 'stripped'                           | -0.13           | -0.19  | -0.18                   | -0.13                        | -0.44                   | -0.41                     |
| 0.1 M Cl <sup>-</sup>                | -0.26           | -0.35  | -0.38                   | -0.41                        | -0.59                   | -0.52                     |
| 0.1 M Cl <sup>-</sup> + DPG          | -0.75           | -0.70  | -0.63                   | -0.68                        | -0.78                   | -0.73                     |
| $\Delta H^f$                         | -26.7           | -17.4  | -25.3                   | -24.2                        | -7.6                    | -10.3                     |

<sup>a</sup> data from Signore et al. (2012).

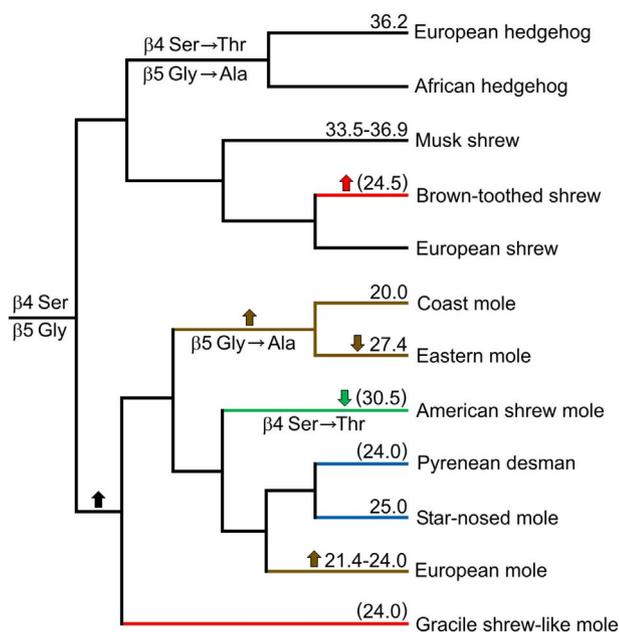
<sup>b</sup> data from Campbell et al. (2010).

<sup>c</sup>  $\text{Log}P_{50}$  (0.1 M Cl<sup>-</sup>) -  $\text{Log}P_{50}$  (stripped).

<sup>d</sup>  $\text{Log}P_{50}$  (DPG + 0.1 M Cl<sup>-</sup>) -  $\text{Log}P_{50}$  (0.1 M Cl<sup>-</sup>).

<sup>e</sup>  $\Delta\text{Log}P_{50}/\Delta\text{log pH}$ ; over the pH range 6.8–7.2.

<sup>f</sup> in 0.1 M Cl<sup>-</sup>.



**Fig. 2.** Blood oxygen affinity (in mm Hg) 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 arrows) 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 indicates 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). Blood oxygen affinity data from Bartels et al. (1969), Jürgens et al. (1981), 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). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(0.26–0.30; Table 1). These findings unambiguously demonstrate that a low DPG sensitivity Hb phenotype 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 the tribes Scalopini and Talpini. Interestingly,  $\beta 4$  Ser and  $\beta 5$  Gly are also found in the three species of shrews for which sequence data is

available (Fig. 2), with Taiwanese brown-toothed shrew (*Episorculus fumidus*) Hb—the only other eulipotyphlan species for which comparable data exists—exhibiting a comparably depressed DPG sensitivity as moles (0.22; Campbell et al. 2012). Taken together these results indicate that a blunted DPG sensitivity phenotype may in fact be an ancestral trait for Eulipotyphla (i.e. it presumably evolved in an ambulatory forebearer of the group).

Although members of two mole lineages have independently evolved residue substitutions in the N-terminal region of the  $\beta$ -type chains— $\beta 4$  Ser  $\rightarrow$  Thr in the American shrew mole, and  $\beta 5$  Gly  $\rightarrow$  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  $\rightarrow$  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  $\rightarrow$  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<sup>-</sup> 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<sup>-</sup> 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 (Fig. 2 and S3), and a His  $\rightarrow$  Ala replacement at  $\beta 143$ , offer a natural model system to test the effects of residue substitutions at these positions on both Cl<sup>-</sup> 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<sub>2</sub> 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<sup>-</sup> and saturating DPG (Table 1), indicating that the 'Cl<sup>-</sup> + DPG' treatment may be a good proxy for in vivo O<sub>2</sub> affinities. Our results accordingly suggest that shrew-like moles and desmans exhibit whole blood P<sub>50s</sub> (ca. 24 Torr) that are substantially below that of similar sized terrestrial rodents (e.g. mice and rats have P<sub>50s</sub> of 52 and 39 Torr, respectively; Hall, 1966), though within the range of both fossorial and high-altitude rodents (22 to 30 Torr; Hall, 1966). Although comparisons between highland and lowland species pairs failed to find a significant association between Hb-O<sub>2</sub> 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, 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<sub>2</sub> equilibration curves (MacArthur 1984). As blood O<sub>2</sub> 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<sub>2</sub> affinity phenotype is an ancestral trait for the family Talpidae and may have subsequently facilitated exploitation of derived high-altitude, semi-aquatic, and fossorial niches (Fig. 2). A major caveat to this scenario is that the blood O<sub>2</sub> 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 P<sub>50</sub> blood phenotype evolved independently in uropsiline and non-uropsiline talpid lineages. 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 O<sub>2</sub> 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 O<sub>2</sub> affinity of coast moles is a reflection of their higher intrinsic Hb-O<sub>2</sub> affinity (P<sub>50s</sub> 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, P<sub>50s</sub> 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 (< 2500 m; Nowak 1999). To summarize, adaptive differences in whole blood affinity within the talpid family predominantly have been driven by changes in the intrinsic O<sub>2</sub> 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 β136 that abolishes DPG binding and markedly lowers the intrinsic O<sub>2</sub> 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 CO<sub>2</sub> carrying capacity during burst tunneling activities. While CO<sub>2</sub> has little influence on the O<sub>2</sub> affinity of European mole blood (Jelkmann et al. 1981), which presumably safeguards pulmonary O<sub>2</sub> 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 CO<sub>2</sub> binding (Weber et al. 2017). While it is plausible that evolutionary reductions in CO<sub>2</sub> 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 O<sub>2</sub> offloading at the tissues while minimizing impairment of O<sub>2</sub> 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 Hoffmann 1983), it may be expected to show regional reductions in body temperature and hence benefit from a numerically reduced oxygenation enthalpy to minimally impair O<sub>2</sub> 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 O<sub>2</sub>-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 O<sub>2</sub> 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.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cbpb.2018.01.001>.

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