

RESEARCH ARTICLE

O₂ binding and CO₂ sensitivity in haemoglobins of subterranean African mole rats

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ABSTRACT

Inhabiting deep and sealed subterranean burrows, mole rats exhibit a remarkable suite of specializations, including eusociality (living in colonies with single breeding queens), extraordinary longevity, cancer immunity and poikilothermy, and extreme tolerance of hypoxia and hypercapnia. With little information available on adjustments in haemoglobin (Hb) function that may mitigate the impact of exogenous and endogenous constraints on the uptake and internal transport of O₂, we measured haematological characteristics, as well as Hb–O₂ binding affinity and sensitivity to pH (Bohr effect), CO₂, temperature and 2,3-diphosphoglycerate (DPG, the major allosteric modulator of Hb–O₂ affinity in red blood cells) in four social and two solitary species of African mole rats (family Bathyergidae) originating from different biomes and soil types across Central and Southern Africa. We found no consistent patterns in haematocrit (Hct) and blood and red cell DPG and Hb concentrations or in intrinsic Hb–O₂ affinity and its sensitivity to pH and DPG that correlate with burrowing, sociality and soil type. However, the results reveal low specific (pH independent) effects of CO₂ on Hb–O₂ affinity compared with humans that predictably safeguard pulmonary loading under hypoxic and hypercapnic burrow conditions. The O₂ binding characteristics are discussed in relation to available information on the primary structure of Hbs from adult and developmental stages of mammals subjected to hypoxia and hypercapnia and the molecular mechanisms underlying functional variation in rodent Hbs.

KEY WORDS: Bohr effect, Carbon dioxide, Hypoxia, Hypercapnia, Oxygen transport

INTRODUCTION

Among the multiple mammalian lineages that have adopted subterranean habitats, the mole rats – which comprise the families Bathyergidae from Sub-Saharan Africa and Spalacidae from the Middle East – have arguably transitioned the most successfully (Davies et al., 2015; Schuhmacher, 2015). Excavating and inhabiting complex, sealed burrows that may encompass deeper nests, food stores and distinct toilet areas, mole rats exhibit striking behavioural and physiological specializations, including eusociality (living in colonies with a single breeding female), longevity, immunity to cancer and pain insensitivity, low thermoregulatory capacity and marked capacities to detoxify ammonia and express tissue-specific globin proteins including neuroglobin and

cytoglobin (Avivi et al., 2010; Bennett and Faulkes, 2000; Davies and Jarvis, 1986; Fang et al., 2014; Faulkes et al., 1997; Jarvis and Bennett, 1990; Schuhmacher, 2015). Strikingly, mole rats show greater tolerance of hypoxia and hypercapnia than other mammals. The Middle East mole rat, *Spalax ehrenbergi*, survives O₂ tensions below that at the summit of Mount Everest and CO₂ tensions 200-fold higher than in air (Ar et al., 1977; Arieli et al., 1977; Arieli, 1979; Shams et al., 2005), and the African mole rat *Heterocephalus glaber* tolerates 5 h exposure to 80% CO₂ (20% O₂) and circumvents the lethal effects of O₂ deprivation by switching to fructose-fuelled anaerobic metabolism (Park et al., 2017). Although bathyergid and spalacid mole rats face analogous challenges including energy-costly burrowing and food scarcity, the two lineages are only distantly related. Data derived from 38 published studies show that these lineages diverged 67–79 MYA (Adkins et al., 2003; Hedges et al., 2015; Huchon et al., 2007), indicating that physiological adjustments and adaptations observed in both lineages may either have existed in the common ancestors or have arisen via convergent evolution.

Although extreme hypoxia and hypercapnia predictably impose severe constraints on O₂ and CO₂ exchange in the lung and tissue capillaries and thus the internal transport of respiratory gases, their impact may be mitigated by adaptive adjustments at different (systemic, organ, cellular and molecular) levels of biological organization. At the systemic level, burrowing mammals exhibit lower ventilatory responses to increased CO₂ levels than non-burrowing mammals (Boggs, 1995). African mole rats moreover exhibit lower body temperatures and metabolic rates than non-burrowing rodents (Bennett et al., 1993, 1994). Analogously, the Middle East mole rat, *S. ehrenbergi*, displays a greater capacity to survive hypoxia/hypercapnia than rats, coinciding with higher mRNA levels of vascular endothelial growth factor (VEGF) and higher muscle capillary densities that increase the gradients for diffusion of O₂ from the capillaries to the mitochondria (Avivi et al., 1999, 2005).

A permeating evolutionary adjustment characterizing vertebrates subjected to hypoxia is increased blood O₂ binding affinity compared with their relatives living under normoxic conditions (Campbell et al., 2010). Similar to altitude-native mammals (Bartels et al., 1963; Johansen and Weber, 1976; Monge and Leon-Velarde, 1991; Storz and Moriyama, 2008; Storz et al., 2010b; Tufts et al., 2013; Weber, 2007), African and Middle East mole rats display higher blood O₂ affinity than predicted for their body mass – as for armadillo and echidna, which also burrow and exhibit low metabolic rates (Dhindsa et al., 1971). Thus, blood *P*₅₀ (half-saturation O₂ tension) is markedly lower in African mole rats *H. glaber* and *Cryptomys hottentus* than in non-fossorial mouse *Mus musculus* and rat *Rattus norvegicus* (23 and 21 mmHg compared with 33 and 36 mmHg, respectively, at pH 7.4) (Johansen et al., 1976; Van Aardt et al., 2007), and in *S. ehrenbergi* than in mammals of the same body mass (29.5 and 37.9 mmHg, respectively) (Ar, Arieli, Shkolnik, 1977). Commonly, adaptive

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changes in the structure and intrinsic O₂ binding properties of haemoglobin (Hb) (Perutz, 1983; Weber, 2007) are complemented by phenotypical adjustments in haematology and red blood cell (RBC) concentrations of Hb and the cofactors that modulate Hb–O₂ affinity (Tufts et al., 2013). In contrast to the extensive studies on hypoxia-tolerant high-altitude mammals, little is known about the cellular and molecular mechanisms that secure gas transport in mole rats inhabiting hypoxic/hypercapnic burrows. The lack of tangible differences in Hb–O₂ affinity observed in the Lesotho mole rats *Cryptomys hottentotus mahali* and squirrels from different altitudes (Broekman et al., 2006; Revsbech et al., 2013) indicate that burrowing rodents may *a priori* be hypoxia adapted whereby their ability to survive at altitude may not require additional modifications in Hb function.

Mammalian Hbs are tetrameric molecules composed of two α -type and two β -type globin chains that switch between the low-affinity, deoxygenated tense (T) and the high-affinity, oxygenated relaxed (R) states. Increased blood O₂ affinity in species subjected to altitudinal hypoxia commonly results from reductions in intrerythrocytic levels of allosteric effectors [mainly protons, chloride, CO₂ and the organic phosphate 2,3-diphosphoglycerate (DPG)] that reduce affinity by binding at specific sites of deoxyHb, or from gene-based changes (amino acid substitutions) that increase the intrinsic affinity of Hb for O₂ or lower its sensitivity to these effectors (Mairbaurl and Weber, 2012; Perutz and TenEyck, 1972; Storz et al., 2010a; Weber and Fago, 2004). In human Hb, DPG binds at four β -chain amino acid residues (β 1Val, β 2His, β 82Lys and β 143His), chloride ions mainly at one α -chain site (between 131Ser and 1Val) and one β -chain site (between 82Lys and 1Val), protons mainly to histidine residues (primarily β 146His) and α 1Val (the N-terminal residues of the α -chains), and CO₂ binds at the free NH₂ groups of N-terminal (Val) residues of both chains (Berenbrink, 2006; Lukin and Ho, 2004; Perutz, 1983; Weber et al., 2013).

Adaptive increases in Hb's intrinsic O₂ affinity at altitude have commonly been attributed to single amino acid substitutions, as exemplified by Andean llamas, where it results from the loss of one DPG binding site, and Himalayan and Andean geese, where it correlates with single amino acid substitutions that eliminate the same intramolecular, T-state-stabilizing hydrogen bond (Jessen et al., 1991; Weber et al., 1993). Analogously, increased CO₂-carrying capacity of Hb of the burrowing Eastern mole, *Scalopus aquaticus*, is mainly attributed to a single amino acid substitution (Campbell et al., 2010). Recent studies, however, document evolutionary adjustments in Hb function resulting from epistatic interactions between different mutant sites, as in high-altitude-tolerant deer mouse Hb, where specific amino acid replacements may either increase or decrease O₂ affinity depending on mutations

at other sites (Natarajan et al., 2013; Storz et al., 2009). Moreover, tissue O₂ supply under variable O₂ availability may be secured by a 'division of labour' between multiple isoHbs with differentiated O₂ affinities, as observed in the high-altitude yak, which expresses two major adult and two fetal Hbs (Weber et al., 1988).

In order to probe cellular and molecular mechanisms that contribute to securing O₂ delivery to the respiring tissues in African mole rats, we measured haematological characteristics [haematocrit (Hct), Hb multiplicity and Hb and DPG levels] and Hb–O₂ binding properties (intrinsic O₂ affinity and its sensitivity to pH, CO₂ tension, temperature and DPG) in six species of eusocial or solitary mole rats from different soil and biome types in a wide geographical area in Sub-Saharan Africa (Table 1) and relate the results to environmental conditions, modes of life and the available information on the primary structure of Hbs from adult and fetal stages of mole rats and other rodents.

MATERIALS AND METHODS

Measurements were carried out on two solitary and four social species of African mole rats originating from different geographical localities and soil types (Table 1) that were captured using either the hoe method (Jarvis, 1973) or a modified Hickman live trap (Hickman, 1979). Prior to experiments, the animals were housed for 2 days in glass terraria, containing wood shavings and paper towelling that served as nesting material, at temperatures of 26–28°C, which correspond to those recorded in the foraging burrows (Bennett et al., 1988). The mole rats were killed using an overdose of Halothane anaesthetic (AstraZeneca, Johannesburg, South Africa). Experimental animals were handled in accordance with the Animal Ethics committees of the University of Cape Town and University of Pretoria as well as guidelines of the American Society of Mammalogists (Animal Care and Use Committee, 1998). All animals were captured under permit from the Cape Nature Conservation, Gauteng Nature Conservation and Departments of Nature Conservation in Namibia and Zambia.

Blood was drawn from the right ventricle of the heart using heparinized syringes. DPG concentrations were assayed using Sigma (St Louis, MO, USA) enzymatic test chemicals. Hb concentrations were assessed spectrophotometrically using oxyHb extinction coefficients of $\epsilon_{541}=14.6 \text{ l mmol}^{-1} \text{ cm}^{-1}$ and $\epsilon_{576}=15.8 \text{ l mmol}^{-1} \text{ cm}^{-1}$. RBC DPG and Hb levels were calculated from the respective blood and Hct values.

RBCs were washed twice (centrifuged and resuspended in 0.9% physiological saline) and frozen at –80°C until required. Hb solutions were prepared by mixing the thawed RBCs with 3.3-fold volumes of 0.1 mol l⁻¹ Tris buffer, pH 7.5, removing cell debris by centrifugation, stripping (removal of organic phosphates and small ligand molecules) on a 59×2 cm column of Sephadex G25 gel (GE

Table 1. African mole rat species investigated and their body mass (M_b), habitat and soil type, sociality and origin

Species and taxonomic authority	Mean M_b (g)	Habitat	Soil type	Sociality	Origin
Cape dune mole rat <i>Bathyergus suillus</i> (Schreber 1782)	780	Mesic	Coarse sands	Solitary	Darling, Western Cape (33°22'S 15°25'E).
Cape bleas mole rat <i>Georychus capensis</i> (Pallas 1778)	390	Mesic	Loams and clays	Solitary	Darling, Western Cape (33°22'S 15°25'E).
Common mole rat <i>Cryptomys hottentotus</i> Lesson 1826	67	Mesic/arid	Sands, clays	Social	Stellenbosch, Western Cape (33°56'S 18°51'E).
Giant mole rat <i>Fukomys mechowii</i> (Peters 1881)	272	Mesic	Clays	Social	Chingola, Zambia (the Copperbelt) 12°54'S 27°85'E.
Damaraland mole rat <i>Fukomys damarensis</i> (Ogilby 1838)	100	Arid	Sands	Social	Dordabis, Namibia (22°52'S 17°41'E).
Naked mole rat <i>Heterocephalus glaber</i> Rüppel 1842	33	Arid	Clays	Social	Archers Post and Lerata Water Hole, Northern Kenya (0°38'N 37°40'E).

Healthcare, Uppsala, Sweden) and dialysis against 0.01 mol l⁻¹ Hepes buffer, pH 7.5, containing 5 × 10⁻⁴ mol l⁻¹ EDTA, as previously described (Weber, 1992). Hb solutions were frozen at -80°C in 150 µl aliquots that were thawed individually immediately prior to further analyses. Control experiments showed no difference in P₅₀ values of Hb samples stripped on Sephadex and by adding mixed bed ion exchanger, MB-1. Hb multiplicity was analysed by thin layer isoelectric focusing (TL-IEF) on polyacrylamide gels in the pH range 5–8 (Phast System, Amersham BioSciences, Piscataway, NJ, USA) and staining with Coomassie Blue, as previously described (Broekman et al., 2006).

Preparative isoelectric focusing was carried out at 5°C in 440 ml (LKB, Bromma, Sweden) columns containing ampholines of pH 5–8 (0.55%) and pH 3–10 (0.18%). The pH values of eluted fractions were measured at 25°C. Prior to O₂ binding measurements, ampholines were removed by dialysis against 10 mmol l⁻¹ Hepes buffer pH 7.7 containing 0.5 mmol l⁻¹ EDTA.

O₂ equilibria were measured at 25 and 37°C in the presence of 0.1 mol l⁻¹ chloride and either 0.1 mol l⁻¹ Na-Hepes buffer (at pH values below 8.0–8.2) or 0.1 mol l⁻¹ glycine buffer (at pH above 8.0), using a modified diffusion chamber, coupled to Wösthoff pumps for mixing pure (>99.998%) nitrogen, air, oxygen and CO₂, as earlier described (Weber, 1981, 1992). Hb solutions were 0.30–0.32 mmol l⁻¹ haeme, unless otherwise indicated. Values of P₅₀ (the P_{O₂} at which Hb is 50% saturated) and n₅₀ (Hill's cooperativity coefficient at 50% saturation) were interpolated from linear plots of log[Y/(1-Y)] versus logP_{O₂} (where Y=fractional O₂ saturation) for 5–8 saturation values between 25% and 75%. Using this method, the r² determination coefficients for the fitted curve exceed 0.995 and the standard errors (s.e.m.) are below 3% of the P₅₀ and n₅₀ values (Weber et al., 2014).

The effects of DPG on Hb–O₂ affinity were determined by adding stock, assayed DPG solutions. Chloride was added as KCl and

measured using a Radiometer (Copenhagen, Denmark) CMT10 coulometric titrator. pH was measured using Radiometer PHM 72 Mk 2 equipment on subsamples of oxygenated Hb that were equilibrated to the same CO₂ tensions and temperature as prevailed in the diffusion chamber (Weber et al., 2013). The overall change in enthalpy accompanying oxygenation was estimated from the van't Hoff equation, $\Delta H' = 2.303R \cdot \Delta \log P_{50} / \Delta(1/T_1 - 1/T_2)$, where R is the gas constant (8.314 kJ mol⁻¹ K⁻¹) and T₁ and T₂ are absolute temperatures in degrees Kelvin. The ΔH values quoted exclude the heat of solution of O₂ ($\Delta H_{sol} \approx -12.6$ kJ mol⁻¹).

RESULTS

Haematology

The Hct values and blood and RBC Hb and DPG concentrations measured in the six African mole rat species showed interspecific and intraspecific variation but no clear correlations with body mass, sex, sociality or soil type, and no systematic differences compared with other rodents (Table 2). The measured RBC DPG levels (5.4–8.2 mmol l⁻¹; Table 2) fall within the range reported for 14 species of other rodents (5.0–12.8 mmol l⁻¹, mean 8.2 ± 2.2 mmol l⁻¹) (Scott et al., 1977).

TL-IEF analyses (Fig. 1) indicated similar isoHb multiplicity in *Cryptomys hottentotus hottentotus*, *Fukomys damarensis*, *Fukomys mechowii* and *Bathyergus suillus*, each species expressing two major isoHbs with isoelectric points (pI) ~7.2 and ~7.4, and one to three flanking, minor isoHbs of pI ~7.1 and ~7.6, but revealed intraspecific variation in the relative concentrations of the isoHbs, even amongst conspecific individuals from the same location (Fig. 1). This basic pattern was confirmed by preparative isoelectric focusing of *C. h. hottentotus* Hb (Fig. 2). In contrast to these species, *H. glaber* expresses four isoHbs with lower pI values (6.8–7.2). Significantly, adult and new-born specimens of *H. glaber* showed identical isoHb composition (Fig. 1).

Table 2. Haematological data, including Hct values and blood and RBC Hb (tetramer) and DPG concentrations in African mole rats compared with corresponding values for Middle Eastern mole rats (*Spalax* spp.), other rodents and humans

Species	M _b (g)	Hct (%)	[Hb ₄] _{blood} (mmol l ⁻¹)	[Hb ₄] _{RBC} (mmol l ⁻¹)	[DPG] _{blood} (mmol l ⁻¹)	[DPG] _{RBC} (mmol l ⁻¹)	DPG/Hb ₄	Reference
<i>G. capensis</i> (n=3)		40 ± 6	1.78 ± 0.18	4.58 ± 0.44	3.19 ± 0.13	8.23 ± 1.56	1.79 ± 0.25	This study
<i>F. damarensis</i> (n=7)	112 ± 32	43 ± 4	1.91 ± 0.30	4.41 ± 0.62				This study
<i>C. h. hottentotus</i> (n=2)		52 ± 0	2.00 ± 0.01	3.83 ± 0.02	2.90 ± 0.37	5.53 ± 0.72	1.45 ± 0.19	This study
<i>C. h. hottentotus</i> (n=3)		44 ± 2	2.12 ± 0.27	4.77 ± 0.48	3.07 ± 0.31	7.27 ± 0.93	1.53 ± 0.15	This study
<i>C. h. hottentotus</i> (n=8)	47 ± 17	50 ± 2	2.34 ± 0.25	4.67 ± 0.63				This study
<i>Cryptomys hottentotus pretoria</i> (n=7)	88 ± 12	46 ± 4	1.94 ± 0.23	4.20 ± 0.43				This study
<i>C. h. pretoria</i> within 24 h of capture	115 ± 35	48.4	1.9	3.9 ^a	2.6 ^a	5.4	1.38 ^a	Van Aardt et al., 2007
<i>Cryptomys hottentotus mahali</i> (1600 m)	84 ± 28	49	2.50 ^b	5.10				Broekman et al., 2006
<i>C. h. mahali</i> (3200 m)	55 ± 21	51	2.78 ^b	5.45				
<i>H. glaber</i>	105–165	46	2.11 ^b	4.81 ^a	3.46 ^a	7.3	1.58	Johansen et al., 1976
<i>Spalax ehrenbergi</i> normoxia	196 ± 9	45.6	2.70 ^b	5.92 ^{a,b}	2.14	4.69 ^a	0.87	Ar et al., 1977
<i>S. ehrenbergi</i> hypoxia/hypercapnia	196 ± 9		3.47 ^b		2.15		0.62	Ar et al., 1977
<i>Spalax leucodon</i>	150–200	46.2	1.95 ^b	4.22 ^{a,b}				Turker, 2013
<i>Thomomys bottae</i> (pocket gopher) hypoxia	133 ± 11	55.8	3.08 ^b	5.60 ^{a,b}	–	6.0	1.09	Lechner, 1977
<i>T. bottae</i> sea level	112 ± 6	46.8	2.62 ^b	5.53 ^{a,b}	–	4.7	0.84	Lechner, 1977
<i>Mus musculus</i> (house mouse)		42.2	1.98 ^b	4.70 ^a	3.1 ^a	7.4	1.57	Johansen et al., 1976
<i>Peromyscus maniculatus</i> (deer mouse) 4340 m	–	64	3.33 ^b	5.19 ^{a,b}	5.79 ^{a,b}	8.1 ^{a,b}	1.74	Snyder, 1982
<i>P. maniculatus</i> low altitude	–	45	2.36 ^b	5.24 ^{a,b}	3.49 ^{a,b}	7.75 ^{a,b}	1.48	Snyder, 1982
<i>Rattus norvegicus</i> (rat) normoxia	–		1.98 ^b		1.57		0.88	Ar et al., 1977
<i>R. norvegicus</i> hypoxic–hypercapnic	–		3.55 ^b		2.01		0.65	Ar et al., 1977
<i>Talpa europea</i> (mole)	48–92	44.7	2.60 ^b	5.83 ^{a,b}	2.37 ^a	5.3	0.91	Jelkmann et al., 1981
Humans		44.5	2.33	5.23	1.44	3.21	0.61	Altman and Dittmer, 1971; Spodaryk and Zoladz, 1998

Hct, haematocrit; RBC, red blood cell; Hb, haemoglobin; DPG, 2,3-diphosphoglycerate.

^aCalculated from blood or RBC values and Hct values; ^bmmol l⁻¹ concentrations calculated from other units (g Hb dl⁻¹ or µmol l⁻¹ DPG g⁻¹ Hb) in the cited references.

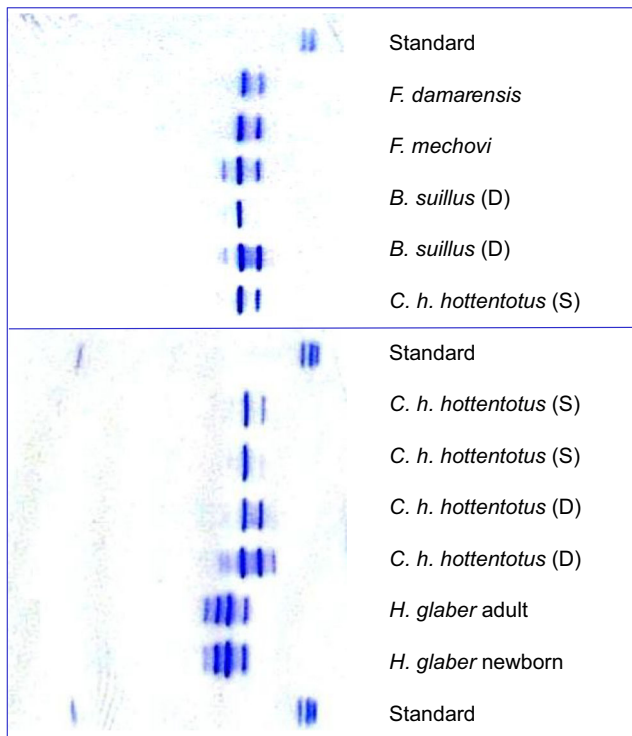


Fig. 1. Thin layer isoelectric focusing (TL-IEF) gels showing iso-haemoglobin (Hb) composition of the mole rat haemolysates. Each lane shows haemolysate from one individual animal. D and S refer to specimens from Darling and Somerset, South African locations that are about 100 km apart. Haemolysates from *Bathyergus suillus* and *Cryptomys hottentotus* were applied at different concentrations in order to distinguish minor isoHb components. Standard proteins with isoelectric points (pI, left to right) of 5.85 and 8.15, 8.45 and 8.65 are also shown. For full species names, see Table 1.

Hb–O₂ binding

The oxygenation properties of stripped haemolysates from the six bathyergid mole rats showed overall uniformity (Table 3, Fig. 3) and no major differences compared with other rodent Hbs under corresponding experimental conditions (Jelkmann et al., 1981; Storz et al., 2010a, 2012; Weber et al., 1994). When measured in the presence of 0.1 mol l⁻¹ chloride (which approximates the condition in mammalian RBCs) and the absence of DPG, the intrinsic O₂ affinity at physiological pH (7.4) and temperature (37°C) was highest (P_{50} =7.4 mmHg) in the social *F. mechowi* living in mesic clays, lowest (P_{50} =9.3 and 10.4 mm Hg, respectively) in solitary *Georychus capensis* from mesic loams and clays and social *H. glaber* found in arid clays, and intermediate (P_{50} =8.1–8.6 mmHg) in social *C. h. hottentotus* and *F. damarensis* and solitary *B. suillus* found in soil types ranging from arid to mesic sands and clays.

The Hbs of the six species displayed pronounced Bohr effects ($\phi = \Delta \log P_{50} / \Delta \text{pH}$ ranging from -0.36 to -0.51 at 37°C) that increased with decreasing temperature (range -0.51 to -0.55 at 25°C; Table 3), consistent with the higher stability of salt bridges that contribute to the Bohr effect. Similarly, all Hbs exhibited highly negative $\Delta H'$ values (i.e. high temperature sensitivity of O₂ affinity) at high pH, where the Bohr effects and the enthalpic contributions from oxygenation-linked proton dissociation approached zero. Thus, at pH 9.5, the intrinsic heats of haeme oxygenation ($\Delta H_{\text{O}_2} = -49$ to -61 kJ mol⁻¹) tally with the calorimetrically determined value (-59 kJ mol⁻¹) for human Hb (Atha and Ackers, 1974; Weber and Campbell, 2011). The numerically lower $\Delta H'$ values at pH 7.4

(-28 to -36 kJ mol⁻¹; Table 3) are consistent with the endothermic, oxygenation-linked dissociation of protons, chloride ions and DPG at this pH (Weber and Campbell, 2011).

The Hbs showed manifest DPG sensitivity, assessed as the decrease in P_{50} induced by saturating DPG:Hb ratios ($\log P_{50, \text{DPG}} - \log P_{50, \text{stripped}} = 0.28 - 0.41$) (Table 3) and marked cooperativity ($n_{50} \approx 2.3$ in the physiological pH range of 7.0–7.4; Fig. 3), indicating an intact quaternary structure under the experimental conditions. Dose–response curves (Fig. 4) for the effects of increasing DPG concentrations on O₂ affinity of *C. h. hottentotus* Hb (Fig. 4) revealed a similar slope to that for human Hb (0.25, consistent with oxygen-linked binding of one DPG molecule per tetrameric Hb).

The major isoHbs of *C. h. hottentotus* showed the same O₂ affinity, cooperativity (P_{50} and n_{50} values), Bohr effect and phosphate sensitivity (Fig. 2). This lack of functional differentiation between component Hbs is consistent with results for other rodents (Condo et al., 1981; Garrick et al., 1975). It indicates that variations in isoHb composition do not contribute to adaptive variation in blood O₂ affinity of mole rats, and provides justification for assessing adaptive traits in Hb function from the haemolysate oxygenation properties.

CO₂ sensitivity

The Hbs of each of the species investigated exhibited distinct specific CO₂ effects (CO₂-induced decreases in O₂ affinity at constant pH) that increased with increasing pH (Fig. 5), as characterizes carbamino (carbamate) formation at uncharged amine groups of N-terminal amino acid residues of the globin chains. As shown in Fig. 5, the CO₂ effects were markedly lower than in human Hb, but similar to those observed in Hb of the bat *Tadarida brasiliensis* that likewise becomes exposed to high CO₂ tensions in densely populated caves (Tuttle, 1994). Assessed as $\Delta \log P_{50}$ [the P_{50} difference between the presence and absence of 6% (42 mmHg) CO₂] at pH 7.2 (which approximates intracellular conditions of mammalian Hbs), the CO₂ effects showed striking interspecific variation (Fig. 5B, Table 3) and were lowest (0.07) in social *C. h. hottentotus* and solitary *G. capensis*, which can occur in mesic clays, intermediate (0.13) in *F. damarensis* from arid sands, and highest (0.15–0.16) in the social species *C. mechowi* and *H. glaber*, which inhabit mesic and arid clays, respectively.

DISCUSSION

Is the tolerance of African mole rats to extreme hypoxia and hypercapnia attributable to distinctive haematological properties (Hct, cellular Hb and DPG values, etc.) or to O₂ binding characteristics (intrinsic O₂ affinity and sensitivity to allosteric effectors)? Do mole rat Hbs share molecular mechanisms that safeguard tissue O₂ supply in other hypoxia- and hypercapnia-tolerant mammals? Considering the intensively documented links between amino acid substitutions and Hb function (Perutz, 1983; Mairbaurl and Weber, 2012), we address these questions by comparing blood and Hb properties of African mole rats with those of animals subjected to hypoxia and hypercapnia at high altitude or during gestational development (Weber, 1995).

Haematology

The absence of distinguishing differences in Hct and blood and RBC Hb and DPG concentrations among the mole rat species investigated, and compared with other rodent species (Table 2) indicates that variations in haematological parameters do not contribute materially to the capacity of mole rats to colonize subterranean habitats. That housing the animals above ground for 2 days before blood sampling is

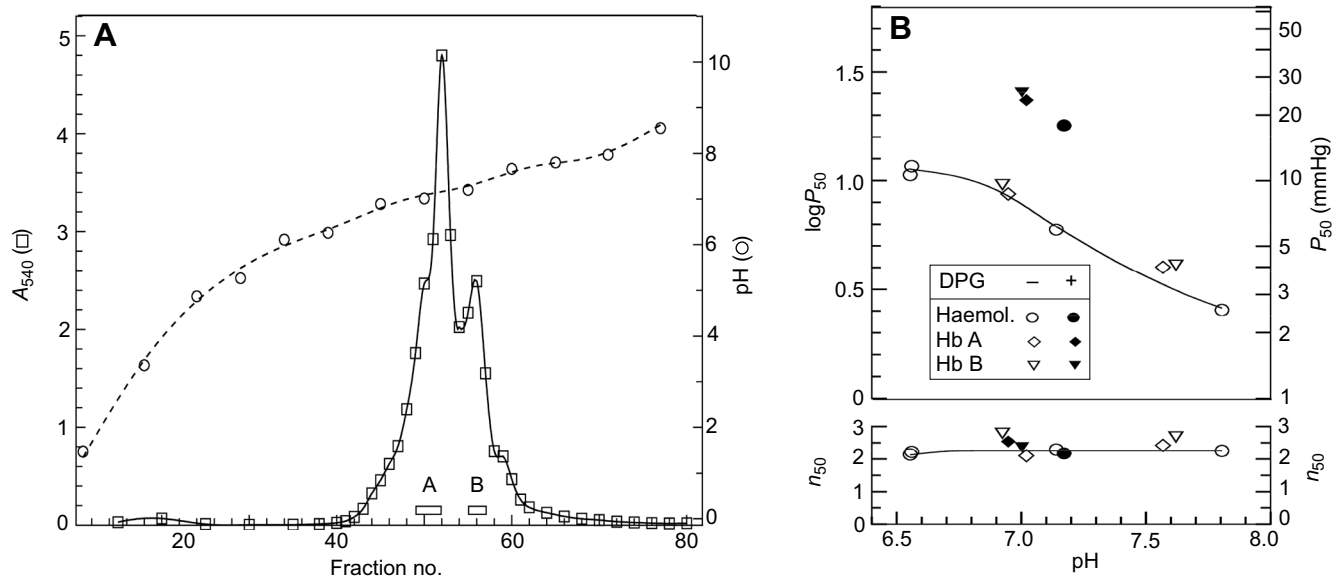


Fig. 2. IsoHb differentiation in *C. h. hottentotus*. (A) Preparative isoelectric focusing of the haemolysate showing two major isoHbs with $pI \sim 7.2$ and ~ 7.4 , where horizontal bars A and B show fractions pooled for O_2 equilibrium measurements. (B) Hb- O_2 affinity and cooperativity of O_2 binding [indexed as P_{50} (half-saturation O_2 tension, mmHg) and n_{50} (Hill's cooperativity coefficient at 50% saturation) values, respectively] of Hb fractions A (diamonds) and B (triangles) and the composite haemolysate (circles), measured in 0.1 mol l^{-1} KCl at 25°C , and the absence (open symbols) and presence (filled symbols) of saturating levels of 2,3-diphosphoglycerate (DPG; DPG:Hb ratio >50).

unlikely to have influenced the measurements significantly is indicated by the observation that translocation of high-altitude rodents to sea level does not reduce Hct values within 6 months (Morrison et al., 1963b). This inference is consistent with the lack of significant differences in Hct and blood Hb concentrations between high- and low-altitude Peruvian rodent species (Morrison et al., 1963a), although Hct may vary with ambient differences in humidity and the gas permeability of the soil in genetically distinct populations of *S. ehrenbergi* (Arieli et al., 1986).

The manifest isoHb multiplicity in African mole rats (four major components in *H. glaber* and two in the other species; Fig. 1) contrasts with the spalacid mole rat *S. ehrenbergi*, which expresses a single Hb component (Kleinschmidt et al., 1984). This difference aligns with species differences in allozyme pattern that argue for placing *H. glaber* and the other African mole rats in distinct subfamilies (Heterocephalinae and Batherginae, respectively; Janacek et al., 2017). Implicit in their different isoelectric points, multiple isoHb components extend the pH range for the capacity of Hbs to buffer protons and free ions (Weber, 1990).

Hb- O_2 binding and its molecular underpinnings

Although the intrinsic O_2 affinity of African mole rat Hbs is high ($P_{50}=7.4\text{--}10.5$ mmHg; Table 3) compared with that of the burrowing European mole, *E. europea* ($P_{50}=14.2$ mmHg; Jelkmann et al., 1981), the values do not differ markedly from those measured under similar conditions in other rodents ($P_{50}=7.4\text{--}8.8$ mmHg in house mouse, *Mus musculus*, and high-altitude-tolerant deer mouse, *Peromyscus maniculatus*; Storz et al., 2012). In contrast with the mole, where the high blood- O_2 affinity ($P_{50}=21.4$ mmHg) is attributed to low RBC DPG concentrations and a weak Hb-DPG interaction (Jelkmann et al., 1981), the DPG levels (Table 2) as well as the DPG sensitivity of Hb- O_2 affinity in mole rats (Fig. 4) correspond well with those in other rodents and humans.

The pronounced DPG sensitivity of O_2 affinity in the mole rat Hbs (Fig. 3, Table 3) is consistent with conservation of the amino acid residues directly implicated in DPG binding ($\beta 1\text{Val}$, $\beta 2\text{His}$, $\beta 82\text{Lys}$ and $\beta 143\text{His}$) in the hitherto-sequenced mole rat Hbs (*S. ehrenbergi*, *H. glaber* and *F. damarensis*) (Fang et al., 2014; Kim et al., 2011; Kleinschmidt et al., 1985) (Table S1) and contrasts with

Table 3. O_2 affinity (indexed as P_{50} values) of purified (stripped) mole rat Hbs and their sensitivity to pH (ϕ), temperature, DPG and CO_2 at the indicated pH values

	37°C					25°C					25–37°C		37°C	
	P_{50} (mmHg)		ϕ			P_{50} (mmHg)		ϕ			$\Delta H'$ (kJ mol $^{-1}$)	DPG sensitivity	CO_2 sensitivity	
pH	7.0	7.4	8.0	9.5	7–8	7.0	7.4	8.0	9.5	7–8	7.4	9.5	7.2	7.2
<i>C. h. hottentotus</i>	13.36	8.35	4.45	2.54	-0.48	7.15	4.13	2.13	0.88	-0.53	-32.41	-55.12	0.35	0.07
<i>F. damarensis</i>	11.76	8.09	4.78	2.71	-0.39	6.27	3.81	1.91	0.94	-0.52	-35.60	-55.12	0.28	0.13
<i>F. mechowii</i>	10.98	7.39	4.11	2.45	-0.43	5.89	3.66	1.86	0.93	-0.50	-32.41	-49.15	0.38	0.16
<i>B. suillus</i>	13.23	8.56	5.71	2.32	-0.36	6.74	4.32	2.01	0.88	-0.52	-31.22	-49.15	0.41	
<i>G. capensis</i>	15.95	9.32	4.92	2.85	-0.51	8.08	4.84	2.32	0.90	-0.55	-27.83	-61.10	0.38	0.07
<i>H. glaber</i>	15.78	10.45	5.95	3.53	-0.42	8.47	4.97	2.52	1.15	-0.51	-35.00	-59.11		0.15

Sensitivity to pH was measured as the Bohr factor $\phi = \Delta \log P_{50} / \Delta \text{pH}$. Temperature sensitivity is expressed as apparent heats of oxygenation, $\Delta H'$, at pH 7.4 and 9.5; note, these values exclude the heat of solution of O_2 ($\Delta H_{\text{sol}} = -12.6 \text{ kJ mol}^{-1}$). The effect of DPG is given by the increase in P_{50} induced by saturating DPG concentrations ($\log P_{50, \text{DPG}} - \log P_{50, \text{stripped}}$). Sensitivity to CO_2 was measured as the increase in P_{50} induced by 6% CO_2 ($\log P_{50, \text{CO}_2} - \log P_{50, \text{stripped}}$).

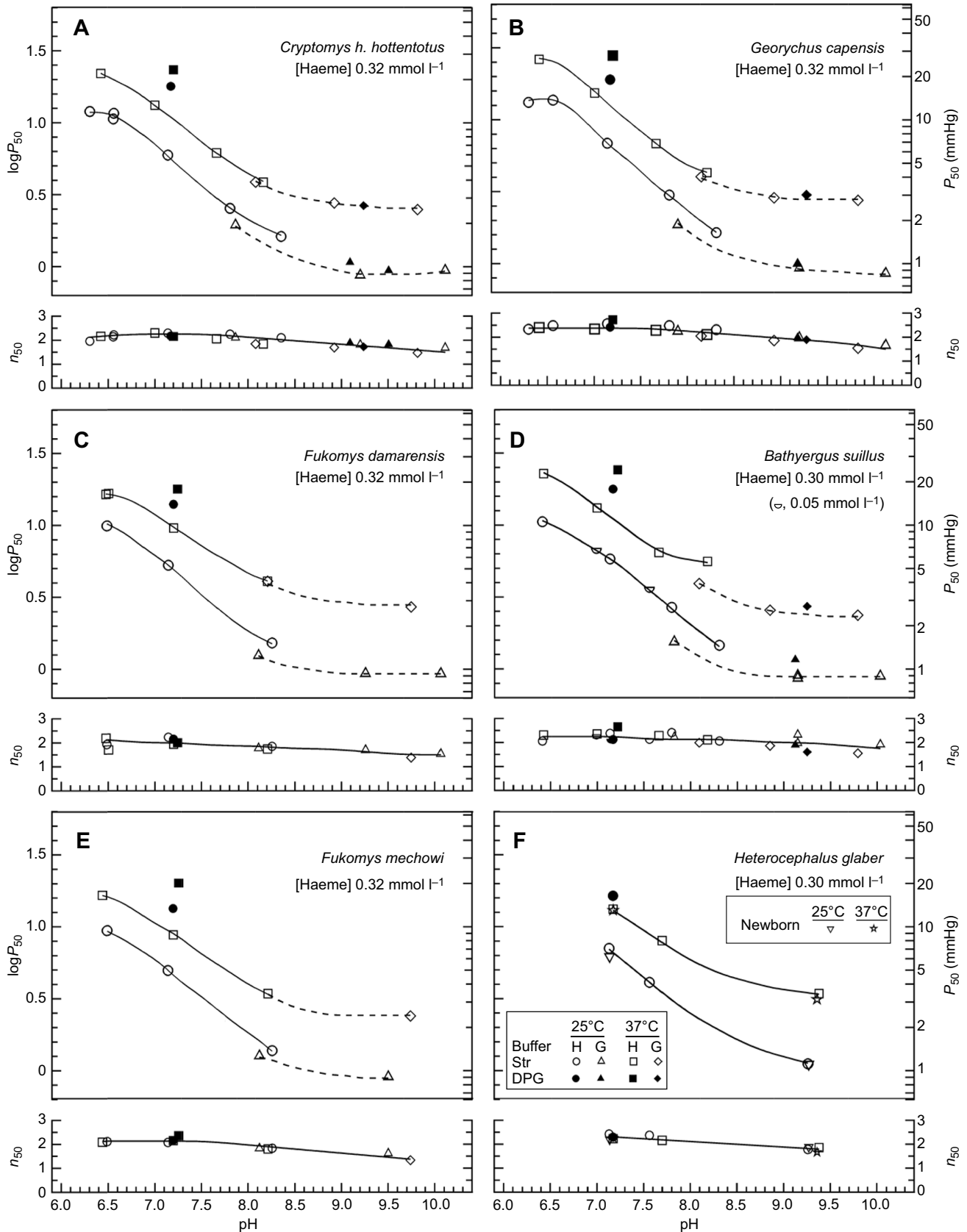


Fig. 3. Hb–O₂ binding properties of African mole rats. P_{50} (mmHg) and n_{50} values and the pH dependence of stripped Hbs measured at 37°C (squares and diamonds) and 25°C (triangles, circles and semicircles) in 0.1 mol l⁻¹ KCl, and in either 0.1 mol l⁻¹ Hesperes buffer (H; circles and squares) or 0.1 mol l⁻¹ glycyls buffer (G; triangles and diamonds), in the absence (open symbols) and presence (filled symbols) of saturating DPG levels. Haeme concentration, 0.30–0.32 mmol l⁻¹ as indicated, except semicircles in D (0.05 mmol l⁻¹). Str, values for stripped (purified) Hbs. Other details as in Fig. 2.

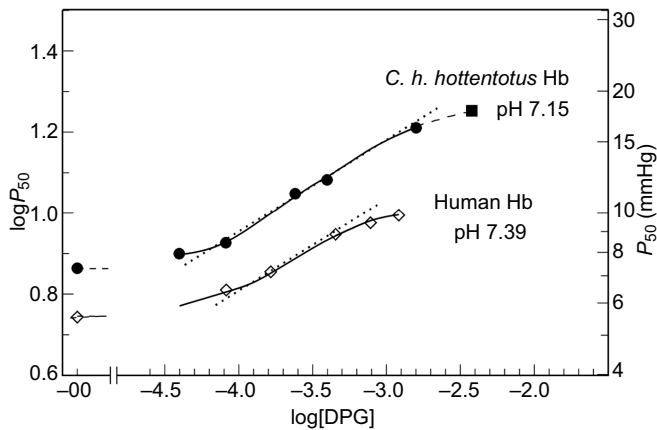


Fig. 4. Dose–response curves for the effects of DPG on O_2 affinity of *C. h. hottentotus* and human Hbs. P_{50} (mmHg) values are plotted against $\log[DPG]$ (mol l^{-1}) concentration. Dotted lines show a gradient of 0.25, congruent with the displacement of one $\log[DPG]$ molecule per four O_2 molecules. Temperature, 25°C .

the reduced DPG effects in Hbs of high-altitude-tolerant mammals like llama, alpaca and elephant, which lack one positively charged DPG binding residue ($\beta 2\text{His} \rightarrow \text{Asn}$). This indicates an unabridged capacity for DPG-mediated, adaptive variation in blood- O_2 affinity in adult mole rats. In contrast, the $\gamma 143\text{His} \rightarrow \text{Tyr}$ exchange in fetal Hb of *H. glaber* (Table S1) is predicted to increase fetal blood O_2 affinity via decreased DPG sensitivity, thus favouring maternal to fetal O_2 transfer in the placenta. In the mole (*T. europea*) Hb, the reduced DPG sensitivity has been attributed to the exchange of residues close to the N-terminal binding sites, viz. $\beta 4\text{Thr} \rightarrow \text{Ser}$ and $\beta 5\text{Pro} \rightarrow \text{Gly}$ (Jelkmann et al., 1981). However, Eastern and Coast mole (*S. aquaticus* and *Scalopus orarius*) Hbs that show the $\beta 4\text{Thr} \rightarrow \text{Ser}$ exchange retain high DPG sensitivity (Campbell et al., 2010), indicating that this substitution does not exert a determining effect. Assessment of the effects of this exchange, and of substitutions at adjacent $\beta 5$ (to negatively charged Asp in *S.*

ehrenbergi, mouse and deer mouse, and neutral Asn in *H. glaber*; Table S1) awaits solution of the molecular structures.

In contrast to conserved chloride binding sites (between $\alpha 1\text{Val}$ and $\alpha 131\text{Ser/Thr}$ and between $\beta 1\text{Val}$ and $\beta 82\text{Lys}$) in most rodents, including *S. ehrenbergi* and *F. damarensis* (Table S1), *H. glaber* has $\alpha 1\text{Ser}$, which is prone to acetylation (Driessen et al., 1985), and non-polar $\alpha 131\text{Ala}$, which predictably eliminates α -chain chloride binding (Weber et al., 2002) and increases Hb- O_2 affinity. The sequenced adult mole rat Hbs moreover lack the ‘additional’ chloride site formed by three cationic residues at β -chain positions 8, 76 and 77, which reduces the temperature sensitivity of Hb- O_2 binding in some mammals (De Rosa et al., 2004; Fronticelli et al., 1995; Signore et al., 2012), although this site is present in *S. ehrenbergi* Hb and fetal *H. glaber* Hb (Table S1).

In addition to adaptations in Hb function attributed to single amino acid exchanges at key sites (Perutz, 1983), functional differentiation may result from multiple substitutions with individually small effects and their context dependence (epistasis), whereby the oxygenation effects of an exchange at one site depend on the allelic state of other, structurally distant sites (Natarajan et al., 2013; Tufts et al., 2015). As recently demonstrated (Kumar et al., 2017), the effect of individual substitutions on Hb- O_2 affinity may be masked by mutational effects of opposite sign that control quaternary structural stability. In deer mouse, *P. maniculatus*, which inhabits a wide range of altitudes, genetically determined differences in Hb- O_2 affinity correlate with amino acid polymorphisms at eight α -chain positions ($\alpha 50$, 57, 60, 64, 71, 113, 115 and 116) and four β -chain positions ($\beta 62$, 72, 128 and 135) (Natarajan et al., 2013) (Table S1). Analogously, the amino acid residues in Hb of high-altitude pika (rodent) *Ochotona princeps* differ from those in its low-altitude sister species *Ochotona collaris* at five β -chain positions ($\beta 5$, 58, 62, 123 and 126; Tufts et al., 2015) (Table S1B). Of note, the primary structures available for Hbs of adult mole rats and their developmental stages – which commonly have higher affinities than adult Hb – show several of the substitutions found in the high-altitude deer mouse and pika Hbs, notably at α -chain positions 50, 113 and 115, and β -chain positions 5, 123, 126, 128 and 135 (Table S1), suggesting possible convergence in the

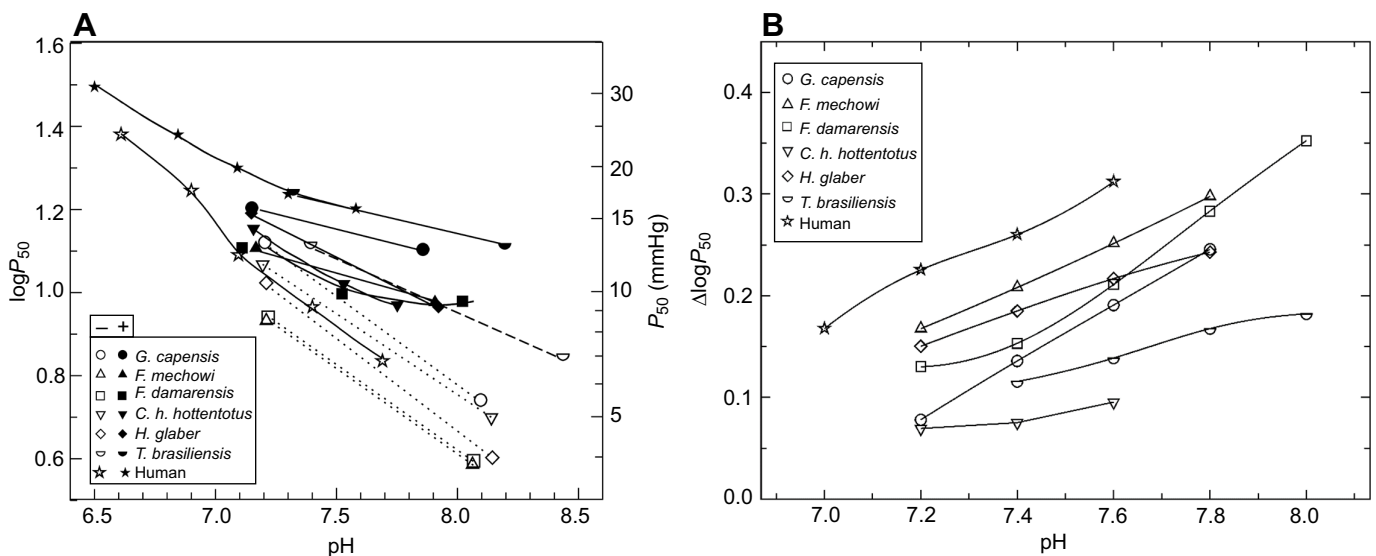


Fig. 5. CO_2 sensitivity of O_2 binding. (A) O_2 affinity (expressed as P_{50} values, mmHg) of mole rat, bat (*Tadarida brasiliensis*) and human Hbs measured at 37°C in the presence of 0.1 mol l^{-1} KCl and in the absence (open symbols) and presence (filled symbols) of 6% CO_2 ($P_{\text{CO}_2}=42 \text{ mmHg}$). (B) pH dependence of the CO_2 -induced decreases in O_2 affinity (increases in $\log P_{50}$) interpolated from A. Data for human and bat (*T. brasiliensis*) Hbs are from Dahms et al. (1972) and Kleinschmidt et al. (1987), respectively. Other details as in Fig. 2.

molecular mechanisms underpinning high Hb–O₂ affinity. In this regard, future studies on the functional properties of fetal and embryonic mole rat Hbs (Table S1) promise clarification.

CO₂ effects

A striking result of this study is the consistently low specific CO₂ effects observed in mole rat Hbs compared with human Hb, which was lowest in social *C. h. hottentotus* found in mesic and arid sands and clays (Fig. 4, Table 1). Although the specific effect of CO₂ on Hb–O₂ affinity augments O₂ unloading in metabolizing tissues, it may also hamper pulmonary O₂ loading under hypercapnic conditions. Thus, irrespective of the magnitude of the Bohr effect (which is decreased in the presence of CO₂ as a result of the pH dependence of carbamate formation), low specific CO₂ effects on Hb oxygenation would safeguard pulmonary Hb–O₂ loading under hypoxic and hypercapnic conditions that predictably are acute in crowded burrows of social species inhabiting compact soils. This interpretation is consistent with the low specific CO₂ effect on Hb of the bat *T. brasiliensis* (Fig. 5), which faces high CO₂ tensions in densely populated caves (Kleinschmidt et al., 1987; Tuttle, 1994). In this species, the passively retained CO₂ neutralizes excess alkali resulting from concomitant increases in ammonia levels (Studier and Fresquez, 1969). However, other factors such as body mass and phylogeny may be the cause of the lower CO₂ sensitivity observed in the African mole rats and bats compared with humans. The present results thus call for further studies on the possible correlations between the magnitude of specific CO₂ effects in mammals, and endogenous and exogenous factors, including phylogeny, body size and environmental conditions (including hypoxia and hypercapnia).

The exact molecular mechanisms underlying the low CO₂ effects in the mole rats is unknown. As CO₂ binds at the N-terminal residues of α and β (type) chains, a primary cause could be acetylation of these residues, as occurs when N-terminal Val is replaced by Ser (Ashiuchi et al., 2005). As shown in Table S1, the adult α chains and embryonic (α -type) ζ chains of *H. glaber* have N-terminal Ser residues, which are susceptible to acetylation (Driessen et al., 1985), in turn reducing Cl[−] and CO₂ binding and thus increasing O₂ affinity. Acetylated Ser or Ala residues at N-terminal α -chains may moreover increase O₂ affinity by decreasing the strength of the tetramer–dimer interface (Ashiuchi et al., 2005).

The high O₂ affinity, high DPG sensitivity and low CO₂ sensitivity Hbs of mole rats contrast sharply with the Hb from the strictly fossorial Eastern mole (*S. aquaticus*), whose low O₂ affinity and DPG insensitivity are attributed to a charge-changing (δ 136Gly→Glu) substitution in the β -type chains (Table S1). This replacement increases the blood's CO₂-carrying capacity by obstructing DPG binding at the shared N-terminal amino acid residues (Campbell et al., 2010). Analogously, low CO₂ sensitivity of mole rat Hbs may also result from the strong interaction with anionic allosteric effectors, in particular DPG, but possibly also lactic acid, which exerts a similar effect (Nielsen and Weber, 2007).

In conclusion, the results of this study provide evidence that variations in the specific effects of CO₂ on Hb affinity may constitute a key component of the spectrum of adjustments that secure pulmonary uptake of O₂ and its flux to the working musculature in burrowing animals exposed to hypoxia/hypercapnia. As the intrinsic O₂ affinities of African mole rat Hbs are similar to those in other rodents, the higher blood O₂ affinities observed in mole rats compared with values in other, similar-size mammals appear to result from differences in the molecular interactions with allosteric effectors, including CO₂, DPG and chloride, lactate and protons. The observations that African mole rat Hbs share components of the

critical sets of amino acid exchanges encountered in Hbs of high-altitude rodents (deer mouse and pika) indicate possible convergence in mechanisms regulating Hb–O₂ affinity in response to ambient conditions.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: R.E.W., J.U.M.J., N.C.B.; Methodology: R.E.W., J.U.M.J., A.F., N.C.B.; Validation: R.E.W., A.F., N.C.B.; Formal analysis: R.E.W., J.U.M.J., A.F., N.C.B.; Investigation: R.E.W., J.U.M.J., N.C.B.; Writing - original draft: R.E.W., J.U.M.J., N.C.B.; Writing - review & editing: R.E.W., A.F.; Project administration: R.E.W., N.C.B.; Funding acquisition: A.F., R.E.W.

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Supplementary information

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References

- Adkins, R. M., Walton, A. H. and Honeycutt, R. L. (2003). Higher-level systematics of rodents and divergence time estimates based on two congruent nuclear genes. *Mol. Phylogenet. Evol.* **26**, 409–420.
- Altman, P. L. and Dittmer, D. S. (1971). *Biological Handbooks: Respiration and Circulation*, pp. 1–930. Bethesda: Federation of American Societies for Experimental Biology.
- Animal Care and Use Committee (1998). Guidelines for the capture, handling and care of mammals as approved by the American Society of Mammalogists. *J. Mammal.* **79**, 1416–1431.
- Ar, A., Arieli, R. and Shkolnik, A. (1977). Blood-gas properties and function in the fossorial mole rat under normal and hypoxic-hypercapnic atmospheric conditions. *Respir. Physiol.* **30**, 201–219.
- Arieli, R. (1979). The atmospheric environment of the fossorial mole rat (*Spalax ehrenbergi*): Effects of season, soil texture, rain, temperature and activity. *Comp. Biochem. Physiol. Part A Physiol.* **63**, 569–575.
- Arieli, R., Ar, A. and Shkolnik, A. (1977). Metabolic responses of a fossorial rodent (*Spalax ehrenbergi*) to simulated burrow conditions. *Physiol. Zool.* **50**, 61–75.
- Arieli, R., Heth, G., Nevo, E. and Hoch, D. (1986). Hematocrit and hemoglobin concentration in four chromosomal species and some isolated populations of actively speciating subterranean mole rats in Israel. *Experientia* **42**, 441–443.
- Ashiuchi, M., Yagami, T., Willey, R. J., Padovan, J. C., Chait, B. T., Popowicz, A., Manning, L. R. and Manning, J. M. (2005). N-terminal acetylation and protonation of individual hemoglobin subunits: position-dependent effects on tetramer strength and cooperativity. *Protein Sci.* **14**, 1458–1471.
- Atha, D. H. and Ackers, G. K. (1974). Calorimetric determination of the heat of oxygenation of human hemoglobin as a function of pH and the extent of reaction. *Biochemistry* **13**, 2376–2382.
- Avivi, A., Resnick, M. B., Nevo, E., Joel, A. and Levy, A. P. (1999). Adaptive hypoxic tolerance in the subterranean mole rat *Spalax ehrenbergi*: the role of vascular endothelial growth factor. *FEBS Lett.* **452**, 133–140.
- Avivi, A., Shams, I., Joel, A., Lache, O., Levy, A. P. and Nevo, E. (2005). Increased blood vessel density provides the mole rat physiological tolerance to its hypoxic subterranean habitat. *FASEB J.* **19**, 1314–1316.
- Avivi, A., Gerlach, F., Joel, A., Reuss, S., Burmester, T., Nevo, E. and Hankeln, T. (2010). Neuroglobin, cytoglobin, and myoglobin contribute to hypoxia adaptation of the subterranean mole rat *Spalax*. *Proc. Natl. Acad. Sci. USA* **107**, 21570–21575.
- Bartels, H., Hilpert, P., Barbey, K., Betke, K., Riegel, K., Lang, E. M. and Metcalfe, J. (1963). Respiratory functions of blood of the yak, llama, camel, Dybowski deer, and African elephant. *Am. J. Physiol.* **205**, 331–336.
- Bennett, N. C. and Faulkes, C. G. (2000). *African Mole-Rats: Ecology and Eusociality*, pp. 1–273. Cambridge: Cambridge University Press.
- Bennett, N. C., Jarvis, J. U. M. and Davies, K. C. (1988). Daily and seasonal temperatures in the burrows of African rodent moles. *S. Afr. J. Zool.* **23**, 189–195.
- Bennett, N. C., Jarvis, J. U. M. and Cotterill, F. P. D. (1993). Poikilothermic traits and thermoregulation in the Afrotropical social subterranean Mashona mole-rat (*Cryptomys hottentotus darlingi*) (Rodentia, Bathyergidae). *J. Zool.* **231**, 179–186.

- Bennett, N. C., Aguilar, G. H., Jarvis, J. U. M. and Faulkes, C. G. (1994). Thermoregulation in three species of Afrotropical subterranean mole-rats (Rodentia: Bathyergidae) from Zambia and Angola and scaling within the genus *Cryptomys*. *Oecologia* **97**, 222–227.
- Berenbrink, M. (2006). Evolution of vertebrate haemoglobins: Histidine side chains, specific buffer value and Bohr effect. *Respir. Physiol. Neurobiol.* **154**, 165–184.
- Boggs, D. F. (1995). Hypoxic ventilatory control and hemoglobin oxygen affinity. In *Hypoxia and the Brain* (ed. J. R. Sutton, C. S. Houston and G. Coates), pp. 69–86. Burlington, Vermont, USA: Queen City Printers, Inc.
- Broekman, M. S., Bennett, N. C., Jackson, C. R. and Weber, R. E. (2006). Does altitudinal difference modulate the respiratory properties in subterranean rodents' (*Cryptomys hottentotus mahali*) blood? *Physiol. Behav.* **88**, 77–81.
- Campbell, K. L., Storz, J. F., Signore, A. V., Moriyama, H., Catania, K. C., Payson, A. P., Bonaventura, J., Stetefeld, J. and Weber, R. E. (2010). Molecular basis of a novel adaptation to hypoxic-hypercapnia in a strictly fossorial mole. *BMC Evol. Biol.* **10**, 214.
- Condo, S. G., Giardina, B., Barra, D., Gill, S. J. and Brunori, M. (1981). Purification and functional properties of the hemoglobin components from the rat (*Wistar*). *Eur. J. Biochem.* **116**, 243–247.
- Dahms, T., Horvath, S. M., Luzzana, M., Rossi-Bernardi, L., Roughton, F. J. W. and Stella, G. (1972). The regulation of oxygen affinity of human haemoglobin. *J. Physiol.* **223**, 29P–31P.
- Davies, K. C. and Jarvis, J. U. M. (1986). The burrow systems and burrowing dynamics of the mole-rats *Bathyergus suillus* and *Cryptomys hottentotus* in the fynbos of the Southwestern Cape, South-Africa. *J. Zool., Lond.* **209**, 125–147.
- Davies, K. T., Bennett, N. C., Tsagkozeorga, G., Rossiter, S. J. and Faulkes, C. G. (2015). Family wide molecular adaptations to underground life in African mole-rats revealed by phylogenomic analysis. *Mol. Biol. Evol.* **32**, 3089–3107.
- 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.
- Dhindsa, D. S., Hoversland, A. S. and Metcalfe, J. (1971). Comparative studies of the respiratory functions of mammalian blood. VII. Armadillo (*Dasypus novemcinctus*). *Respir. Physiol.* **13**, 198–208.
- Driessen, H. P. C., de Jong, W. W., Tesser, G. I. and Bloemendal, H. (1985). The mechanism of N-terminal acetylation of proteins. *CRC Crit. Rev. Biochem.* **18**, 281–325.
- Fang, X., Seim, I., Huang, Z., Gerashchenko, M. V., Xiong, Z., Turanov, A. A., Zhu, Y., Lobanov, A. V., Fan, D., Yim, S. H. et al. (2014). Adaptations to a subterranean environment and longevity revealed by the analysis of mole rat genomes. *Cell Reports* **8**, 1354–1364.
- Faulkes, C. G., Bennett, N. C., Bruford, M. W., O'Brien, H. P., Aguilar, G. H., Jarvis, J. U. M. (1997). Ecological constraints drive social evolution in the African mole-rats. *Proc. R. Soc. Lond. B. Biol. Sci.* **264**, 1619–1627.
- Fronticelli, C., Sanna, M. T., Perez-Alvarado, G. C., Karavitis, M., Lu, A. L. 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.
- Garrick, L. M., Sharma, V. S., McDonald, M. J. and Ranney, H. M. (1975). Rat hemoglobin heterogeneity. Two structurally distinct chains and functional behaviour of selected components. *Biochem. J.* **149**, 245–258.
- Hedges, S. B., Marin, J., Suleski, M., Paymer, M. and Kumar, S. (2015). Tree of life reveals clock-like speciation and diversification. *Mol. Biol. Evol.* **32**, 835–845.
- Hickman, G. C. (1979). Live-trap and trapping technique for fossorial mammals. *S. Afr. J. Zool.* **14**, 9–12.
- Huchon, D., Chevret, P., Jordan, U., Kilpatrick, C. W., Ranwez, V., Jenkins, P. D., Brosius, J. and Schmitz, J. (2007). Multiple molecular evidences for a living mammalian fossil. *Proc. Natl. Acad. Sci. USA* **104**, 7495–7499.
- Janacek, L. L., Honeycutt, R. L., Rautenbach, I. L., Erasmus, B. H., Reigi, S. and Schlitter, D. H. (2017). Allozyme variation and systematics of African mole-rats (Rodentia: Bathyergidae). *Biochem. Syst. Ecol.* **2**, 401–416.
- Jarvis, J. U. M. (1973). Structure of a population of mole-rats, *Tachyoryctes splendens* (Rodentia-Rhizomyidae). *J. Zool.* **171**, 1–14.
- Jarvis, J. U. M. and Bennett, N. C. (1990). The evolutionary history, population biology and social structure of African mole-rats: family Bathyergidae. *Prog. Clin. Biol. Res.* **335**, 97–128.
- Jelkmann, W., Oberthür, W., Kleinschmidt, T., Braunitzer, G. and Oberthür, W. (1981). Adaptation of hemoglobin function to subterranean life in the mole, *Talpa europaea*. *Respir. Physiol.* **46**, 7–16.
- Jessen, T.-H., Weber, R. E., Fermi, G., Tame, J. and Braunitzer, G. (1991). Adaptation of bird hemoglobins to high altitudes: Demonstration of molecular mechanism by protein engineering. *Proc. Natl. Acad. Sci. USA* **88**, 6519–6522.
- Johansen, K. and Weber, R. E. (1976). On the adaptability of haemoglobin function to environmental conditions. In: *Perspectives in Experimental Biology, Vol. 1, Zoology* (ed. P. Spencer Davies), pp. 219–234. Oxford & New York: Pergamon Press.
- Johansen, K., Lykkeboe, G., Weber, R. E., Maloij, G. M. O. and Maloij, G. M. (1976). Blood respiratory properties in the naked mole rat *Heterocephalus glaber*, a mammal of low body temperature. *Respir. Physiol.* **28**, 303–314.
- Kim, E. B., Fang, X., Fushan, A. A., Huang, Z., Lobanov, A. V., Han, L., Marino, S. M., Sun, X., Turanov, A. A., Yang, P. et al. (2011). Genome sequencing reveals insights into physiology and longevity of the naked mole rat. *Nature* **479**, 223–227.
- Kleinschmidt, T., Nevo, E. and Braunitzer, G. (1984). The primary structure of the hemoglobin of the mole rat (*Spalax ehrenbergi*, rodentia, chromosome species 60). *Hoppe Seylers. Z. Physiol. Chem.* **365**, 531–538.
- Kleinschmidt, T., Nevo, E., Goodman, M. and Braunitzer, G. (1985). Mole rat hemoglobin: primary structure and evolutionary aspects in a second karyotype of *Spalax ehrenbergi*, Rodentia, (2n=52). *Biol. Chem. Hoppe Seyler* **366**, 679–685.
- Kleinschmidt, T., Rucknagel, P. K., Weber, R. E., Koop, B. F. and Braunitzer, G. (1987). Primary structure and functional properties of the hemoglobin from the free-tailed bat *Tadarida brasiliensis* (Chiroptera). Small effect of carbon dioxide on oxygen affinity. *Biol. Chem. Hoppe-Seyler* **368**, 681–690.
- Kumar, A., Natarajan, C., Moriyama, H., Witt, C. C., Weber, R. E., Fago, A. and Storz, J. F. (2017). Stability-mediated epistasis restricts accessible mutational pathways in the functional evolution of avian hemoglobin. *Mol. Biol. Evol.* **34**, 1240–1251.
- Lechner, A. J. (1977). Metabolic performance during hypoxia in native and acclimated pocket gophers. *J. Appl. Physiol. Respir. Environ. Exerc. Physiol.* **43**, 965–970.
- Lukin, J. A. and Ho, C. (2004). The structure–function relationship of hemoglobin in solution at atomic resolution. *Chem. Rev.* **104**, 1219–1230.
- Mairbaurl, H. and Weber, R. E. (2012). Oxygen transport by hemoglobin. *Compr. Physiol.* **2**, 1463–1489.
- Monge, C. and Leon-Velarde, F. (1991). Physiological adaptation to high altitude: oxygen transport in mammals and birds. *Physiol. Rev.* **71**, 1135–1172.
- Morrison, P. R., Kerst, K. and Rosenmann, M. (1963a). Hematocrit and hemoglobin levels in some Chilean rodents from high and low altitude. *Int. J. Biometeorol.* **7**, 45–50.
- Morrison, P. R., Kerst, K., Reynafarje, C. and Ramos, J. (1963b). Hematocrit and hemoglobin levels in some peruvian rodents from high and low altitude. *Int. J. Biometeorol.* **7**, 51–58.
- Natarajan, C., Inoguchi, N., Weber, R. E., Fago, A., Moriyama, H. and Storz, J. F. (2013). Epistasis among adaptive mutations in Deer mouse hemoglobin. *Science* **340**, 1324–1327.
- Nielsen, M. S. and Weber, R. E. (2007). Antagonistic interaction between oxygenation-linked lactate and CO₂ binding to human hemoglobin. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **146**, 429–434.
- Park, T. J., Reznick, J., Peterson, B. L., Blass, G., Omerbašić, D., Bennett, N. C., Kuich, P. H. J. L., Zasada, C., Browe, B. M., Hamann, W. et al. (2017). Fructose-driven glycolysis supports anoxia resistance in the naked mole-rat. *Science* **356**, 307–311.
- Perutz, M. F. (1983). Species adaptation in a protein molecule. *Mol. Biol. Evol.* **1**, 1–28.
- Perutz, M. F. and TenEyck, L. F. (1972). Stereochemistry of cooperative effects in hemoglobin. *Cold Spring Harb. Symp. Quant. Biol.* **36**, 295–310.
- Revsbech, I. G., Tufts, D. M., Projecto-Garcia, J., Moriyama, H., Weber, R. E., Storz, J. F. and Fago, A. (2013). Hemoglobin function and allosteric regulation in semi-fossorial rodents (family Sciuridae) with different altitudinal ranges. *J. Exp. Biol.* **216**, 4264–4271.
- Schuhmacher, L.-N. (2015). The naked mole-rat as an animal model in biomedical research: current perspectives. *Open Access Anim. Physiol.* **7**, 137–148.
- Scott, A. F., Bunn, H. F. and Brush, A. H. (1977). The phylogenetic distribution of red cell 2,3 diphosphoglycerate and its interaction with mammalian hemoglobins. *J. Exp. Zool.* **201**, 269–288.
- Shams, I., Avivi, A. and Nevo, E. (2005). Oxygen and carbon dioxide fluctuations in burrows of subterranean blind mole rats indicate tolerance to hypoxic-hypercapnic stresses. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **142**, 376–382.
- Signore, A. V., Stetefeld, J., Weber, R. E. and Campbell, K. L. (2012). Origin and mechanism of thermal insensitivity in mole hemoglobins: a test of the 'additional' chloride binding site hypothesis. *J. Exp. Biol.* **215**, 518–525.
- Snyder, L. R. G. (1982). 2,3-diphosphoglycerate in high- and low-altitude populations of the deer mouse. *Respir. Physiol.* **48**, 107–123.
- Spodaryk, K. and Zoladz, J. A. (1998). The 2,3-DPG levels of human red blood cells during an incremental exercise test: Relationship to the blood acid-base balance. *Physiol. Res.* **47**, 17–22.
- Storz, J. F. and Moriyama, H. (2008). Mechanisms of hemoglobin adaptation to high altitude hypoxia. *High Alt. Med. Biol.* **9**, 148–157.
- Storz, J. F., Runck, A. M., Sabatino, S. J., Kelly, J. K., Ferrand, N., Moriyama, H., Weber, R. E. and Fago, A. (2009). Evolutionary and functional insights into the mechanism underlying high-altitude adaptation of deer mouse hemoglobin. *Proc. Natl. Acad. Sci. USA* **106**, 14450–14455.
- Storz, J. F., Runck, A. M., Moriyama, H., Weber, R. E. and Fago, A. (2010a). Genetic differences in hemoglobin function between highland and lowland deer mice. *J. Exp. Biol.* **213**, 2565–2574.
- Storz, J. F., Scott, G. R. and Cheviron, Z. A. (2010b). Phenotypic plasticity and genetic adaptation to high-altitude hypoxia in vertebrates. *J. Exp. Biol.* **213**, 4125–4136.

- Storz, J. F., Weber, R. E. and Fago, A.** (2012). Oxygenation properties and oxidation rates of mouse hemoglobins that differ in reactive cysteine content. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **161**, 265-270.
- Studier, E. M. and Fresquez, A. A.** (1969). Carbon dioxide retention: a mechanism of ammonia tolerance in mammals. *Ecology* **50**, 492-494.
- Tufts, D. M., Revsbech, I. G., Chevion, Z. A., Weber, R. E., Fago, A. and Storz, J. F.** (2013). Phenotypic plasticity in blood-oxygen transport in highland and lowland deer mice. *J. Exp. Biol.* **216**, 1167-1173.
- Tufts, D. M., Natarajan, C., Revsbech, I. G., Projecto-Garcia, J., Hoffmann, F. G., Weber, R. E., Fago, A., Moriyama, H. and Storz, J. F.** (2015). Epistasis constrains mutational pathways of hemoglobin adaptation in high-altitude pikas. *Mol. Biol. Evol.* **32**, 287-298.
- Turker, H.** (2013). Potential effects of ultraviolet-C radiation on the mole-rats (*Spalax leucodon*) hematological values. *Amer. J. Molec. Biol.* **3**, 235-240.
- Tuttle, M. D.** (1994). The lives of mexican free-tailed bats. *BATS Magazine* **12**. http://www.batcon.org/resources/media-education/bats-magazine/bat_article/656.
- Van Aardt, W. J., Bronner, G. and Buffenstein, R.** (2007). Hemoglobin-oxygen-affinity and acid-base properties of blood from the fossorial mole-rat, *Cryptomys hottentotus pretoriae*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **147**, 50-56.
- Weber, R. E.** (1981). Cationic control of O₂ affinity in lugworm erythrocytes. *Nature* **292**, 386-387.
- Weber, R. E.** (1990). Functional significance and structural basis of multiple hemoglobins with special reference to ectothermic vertebrates. In *Animal Nutrition and Transport Processes. 2. Transport, Respiration and Excretion: Comparative and Environmental Aspects*. Mol. Comp. Physiol., Vol. 6 (ed. J.-P. Truchot and B. Lahlou), pp. 58-75. Basel: S. Karger.
- 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.** (1995). Hemoglobin adaptations to hypoxia and altitude - The phylogenetic perspective. In *Hypoxia and the Brain (Proceedings of the 9th International Hypoxia Symposium, Lake Louise, Canada)* (ed. J. R. Sutton, C. S. Houston and G. Coates), pp. 31-44. Burlington, Vermont, USA: Queen City Printers.
- Weber, R. E.** (2007). High-altitude adaptations in vertebrate hemoglobins. *Respir. 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.
- Weber, R. E. and Fago, A.** (2004). Functional adaptation and its molecular basis in vertebrate hemoglobins, neuroglobins and cytoglobins. *Respir. Physiol. Neurobiol.* **144**, 141-159.
- Weber, R. E., Lalthantluanga, R. and Braunitzer, G.** (1988). Functional characterization of fetal and adult yak hemoglobins: an oxygen binding cascade and its molecular basis. *Arch. Biochem. Biophys.* **263**, 199-203.
- Weber, R. E., Jessen, T.-H., Malte, H. and Tame, J.** (1993). Mutant hemoglobins (a¹¹⁹-Ala and b⁵⁵-Ser): Functions related to high-altitude respiration in geese. *J. Appl. Physiol.* **75**, 2646-2655.
- Weber, R. E., Böning, D., Fago, A., Schmidt, W. and Correa, R.** (1994). Hemoglobins from *Plasmodium*-infected rat erythrocytes: functional and molecular characteristics. *Blood* **84**, 638-642.
- Weber, R. E., Ostojic, H., Fago, A., Dewilde, S., Van Hauwaert, M.-L., Moens, L. and Monge, C.** (2002). Novel mechanism for high-altitude adaptation in hemoglobin of the Andean frog *Telmatobius peruvianus*. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **283**, R1052-R1060.
- Weber, R. E., Fago, A., Malte, H., Storz, J. F. and Gorr, T. A.** (2013). Lack of conventional oxygen-linked proton and anion binding sites does not impair allosteric regulation of oxygen binding in dwarf caiman hemoglobin. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **305**, R300-R312.
- Weber, R. E., Fago, A. and Campbell, K. L.** (2014). Enthalpic partitioning of the reduced temperature sensitivity of O₂ binding in bovine hemoglobin. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **176**, 20-25.