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RESEARCH ARTICLE

O_2 binding and CO_2 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 O_2, we measured haematological characteristics, as well as Hb–O_2 binding affinity and sensitivity to pH (Bohr effect), CO_2, temperature and 2,3-diphosphoglycerate (DPG, the major allosteric modulator of Hb–O_2 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–O_2 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 CO_2 on Hb–O_2 affinity compared with humans that predictably safeguard pulmonary loading under hypoxic and hypercapnic burrow conditions. The O_2 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 O_2 tensions below that at the summit of Mount Everest and CO_2 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% CO_2 (20% O_2) and circumvents the lethal effects of O_2 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 O_2 and CO_2 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 CO_2 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 O_2 from the capillaries to the mitochondria (Avivi et al., 1999, 2005).

A permeating evolutionary adjustment characterizing vertebrates subjected to hypoxia is increased blood O_2 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 O_2 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 O_2 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 O_2 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–O_2 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–O_2 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 O_2 affinity in species subjected to altitudinal hypoxia commonly results from reductions in intra-erythrocytic levels of allosteric effectors [mainly protons, chloride, CO_2 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 O_2 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 (β1Val, β2His, β82Lys and β143His), 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 β146His) and α1Val (the N-terminal residues of the α-chains), and CO_2 binds at the free NH_2 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 O_2 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 CO_2-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 O_2 affinity depending on mutations at other sites (Natarajan et al., 2013; Storz et al., 2009). Moreover, tissue O_2 supply under variable O_2 availability may be secured by a 'division of labour' between multiple isoHbs with differentiated O_2 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 O_2 delivery to the respiring tissues in African mole rats, we measured haematological characteristics [haematocrit (Hct), Hb multiplicity and Hb and DPG levels] and Hb–O_2 binding properties (intrinsic O_2 affinity and its sensitivity to pH, CO_2 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 ε_{541}=14.6 l mmol^{-1} cm^{-1} and ε_{576}=15.8 l mmol^{-1} 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 *Bathyergus suillus* (Schreber 1782)	780	Mesic	Coarse sands	Solitary	Darling, Western Cape (33°22′S 15°25′E).
Cape blesmol mole rat *Georychus capensis* (Pallas 1778)	390	Mesic	Loams and clays	Solitary	Darling, Western Cape (33°22′S 15°25′E).
Common mole rat *Cryptomys hottentotus* Lesson 1826	67	Mesic/arid	Sands, clays	Social	Stellenbosch, Western Cape (33°56′S 18°51′E).
Giant mole rat *Fukomys mechowi* (Peters 1881)	272	Mesic	Clays	Social	Chingola, Zambia (the Copperbelt) 12°54′S 27°85′E.
Damaraland mole rat *Fukomys damarensis* (Ogilby 1838)	100	Arid	Sands	Social	Dordabis, Namibia (22°52′S 17°41′E).
Naked mole rat *Heterocephalus glaber* Rüppel 1842	33	Arid	Clays	Social	Archers Post and Lerata Water Hole, Northern Kenya (0°38′N 37°40′E).

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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 P_{50} 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 O_2 binding measurements, ampholines were removed by dialysis against 10 mmol l^{-1} Hepes buffer pH 7.7 containing 0.5 mmol l^{-1} EDTA.

O_2 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 CO_2, as earlier described (Weber, 1981, 1992). Hb solutions were 0.30–0.32 mmol l^{-1} haeme, unless otherwise indicated. Values of P_{50} (the P_{O_2} at which Hb is 50% saturated) and n_{50} (Hill's cooperativity coefficient at 50% saturation) were interpolated from linear plots of $\log[Y/(1-Y)]$ versus $\log P_{O_2}$ (where Y=fractional O_2 saturation) for 5–8 saturation values between 25% and 75%. Using this method, the r^2 determination coefficients for the fitted curve exceed 0.995 and the standard errors (s.e.m.) are below 3% of the P_{50} and n_{50} values (Weber et al., 2014).

The effects of DPG on Hb–O_2 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 CO_2 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 T_1 and T_2 are absolute temperatures in degrees Kelvin. The ΔH values quoted exclude the heat of solution of O_2 ($\Delta H_{sol}\approx-12.6$ 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 *Cryptomus hottentotus hottentotus*, *Fukomys damarensis*, *Fukomys mechowi* 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
G. capensis (*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
F. damarensis (*n*=7)	112±32	43±4	1.91±0.30	4.41±0.62				This study
C. h. hottentotus (*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
C. h. hottentotus (*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
C. h. hottentotus (*n*=8)	47±17	50±2	2.34±0.25	4.67±0.63				This study
Cryptomys hottentotus pretoria (*n*=7)	88±12	46±4	1.94±0.23	4.20±0.43				This study
C. h. pretoria 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
Cryptomys hottentotus mahali (1600 m)	84±28	49	2.50[b]	5.10				Broekman et al., 2006
C. h. mahali (3200 m)	55±21	51	2.78[b]	5.45				
H. glaber	105-165	46	2.11[b]	4.81[a]	3.46[a]	7.3	1.58	Johansen et al., 1976
Spalax ehrenbergi normoxia	196±9	45.6	2.70[b]	5.92[a,b]	2.14	4.69[a]	0.87	Ar et al., 1977
S. ehrenbergi hypoxia/hypercapnia	196±9		3.47[b]		2.15		0.62	Ar et al., 1977
Spalax leucodon	150–200	46.2	1.95[b]	4.22[a,b]				Turker, 2013
Thomomys bottae (pocket gopher) hypoxia	133±11	55.8	3.08[b]	5.60[a,b]	–	6.0	1.09	Lechner, 1977
T. bottae sea level	112±6	46.8	2.62[b]	5.53[a,b]	–	4.7	0.84	Lechner, 1977
Mus musculus (house mouse)		42.2	1.98[b]	4.70[a]	3.1[a]	7.4	1.57	Johansen et al., 1976
Peromyscus maniculatus (deer mouse) 4340 m	–	64	3.33[b]	5.19[a,b]	5.79[a,b]	8.1[a,b]	1.74	Snyder, 1982
P. maniculatus low altitude	–	45	2.36[b]	5.24[a,b]	3.49[a,b]	7.75[a,b]	1.48	Snyder, 1982
Rattus norvegicus (rat) normoxia	–		1.98[b]		1.57		0.88	Ar et al., 1977
R. norvegicus hypoxic–hypercapnic	–		3.55[b]		2.01		0.65	Ar et al., 1977
Talpa europea (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.
[a]Calculated from blood or RBC values and Hct values; [b]mmol l^{-1} concentrations calculated from other units (g Hb dl^{-1} or µmol l^{-1} DPG g^{-1} Hb) in the cited references.

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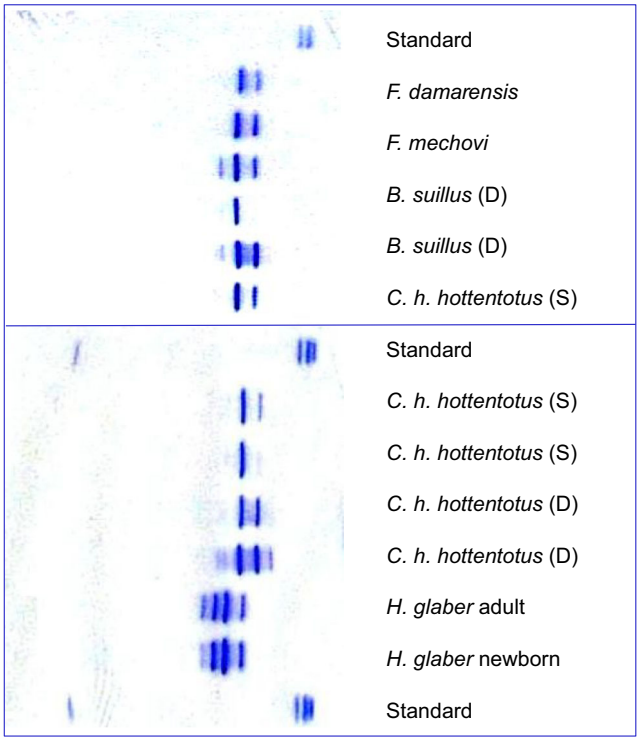

Fig. 1. Thin layer isoelectric focusing (TL-IEF) gels showing isohaemoglobin (Hb) composition of the mole rat haemolysates. Each lane shows haemolysate from one individual animal. D and S refer to specimens from Darling and Somerset, South African locations that are about 100 km apart. Haemolysates from *Bathyergus suillus* and *Cryptomus 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–O_2 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 O_2 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 ($\phi=\Delta\log P_{50}/\Delta$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 O_2 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 (ΔH_{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 ($\log P_{50,DPG}-\log P_{50,stripped}$=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 O_2 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 O_2 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 O_2 affinity of mole rats, and provides justification for assessing adaptive traits in Hb function from the haemolysate oxygenation properties.

CO_2 sensitivity

The Hbs of each of the species investigated exhibited distinct specific CO_2 effects (CO_2-induced decreases in O_2 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 CO_2 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 CO_2 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) CO_2] at pH 7.2 (which approximates intracellular conditions of mammalian Hbs), the CO_2 effects showed striking interspecific variation (Fig. 5B, Table 3) and were lowest (0.07) in social *C. h. hottentus* 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 O_2 binding characteristics (intrinsic O_2 affinity and sensitivity to allosteric effectors)? Do mole rat Hbs share molecular mechanisms that safeguard tissue O_2 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

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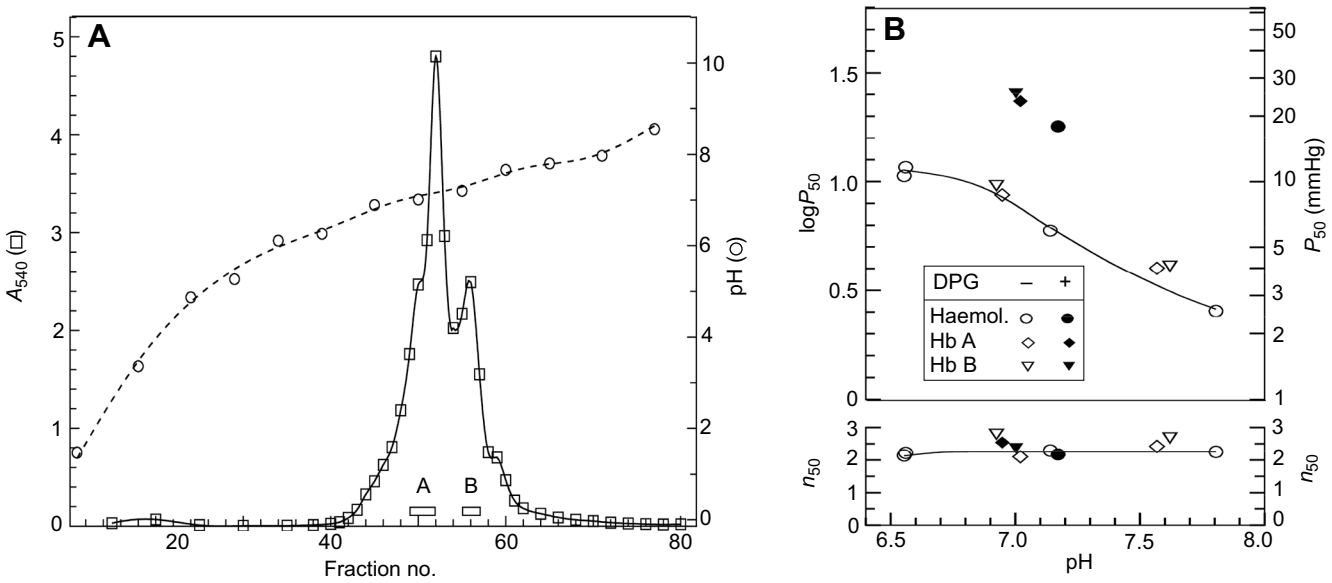

Fig. 2. IsoHb differentiation in *C. h. hottentotus*. (A) Preparative isoelectric focusing of the haemolysate showing two major isoHbs with pI ~7.2 and ~7.4, where horizontal bars A and B show fractions pooled for O_2 equilibrium measurements. (B) Hb–O_2 affinity and cooperativity of O_2 binding [indexed 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}=7.4–10.5 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}=7.4–8.8 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 mm Hg) 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 (β1Val, β2His, β82Lys and β143His) 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	ΔH' (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
C. h. hottentotus	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
F. damarensis	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
F. mechowi	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
B. suillus	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	
G. capensis	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
H. glaber	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 $\phi=\Delta\log P_{50}/\Delta pH$. 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 ($\Delta H_{sol}=-12.6$ kJ mol^{-1}). The effect of DPG is given by the increase in P_{50} induced by saturating DPG concentrations ($\log P_{50,DPG}-\log P_{50,stripped}$). Sensitivity to CO_2 was measured as the increase in P_{50} induced by 6% CO_2 ($\log P_{50,CO_2}-\log P_{50,stripped}$).

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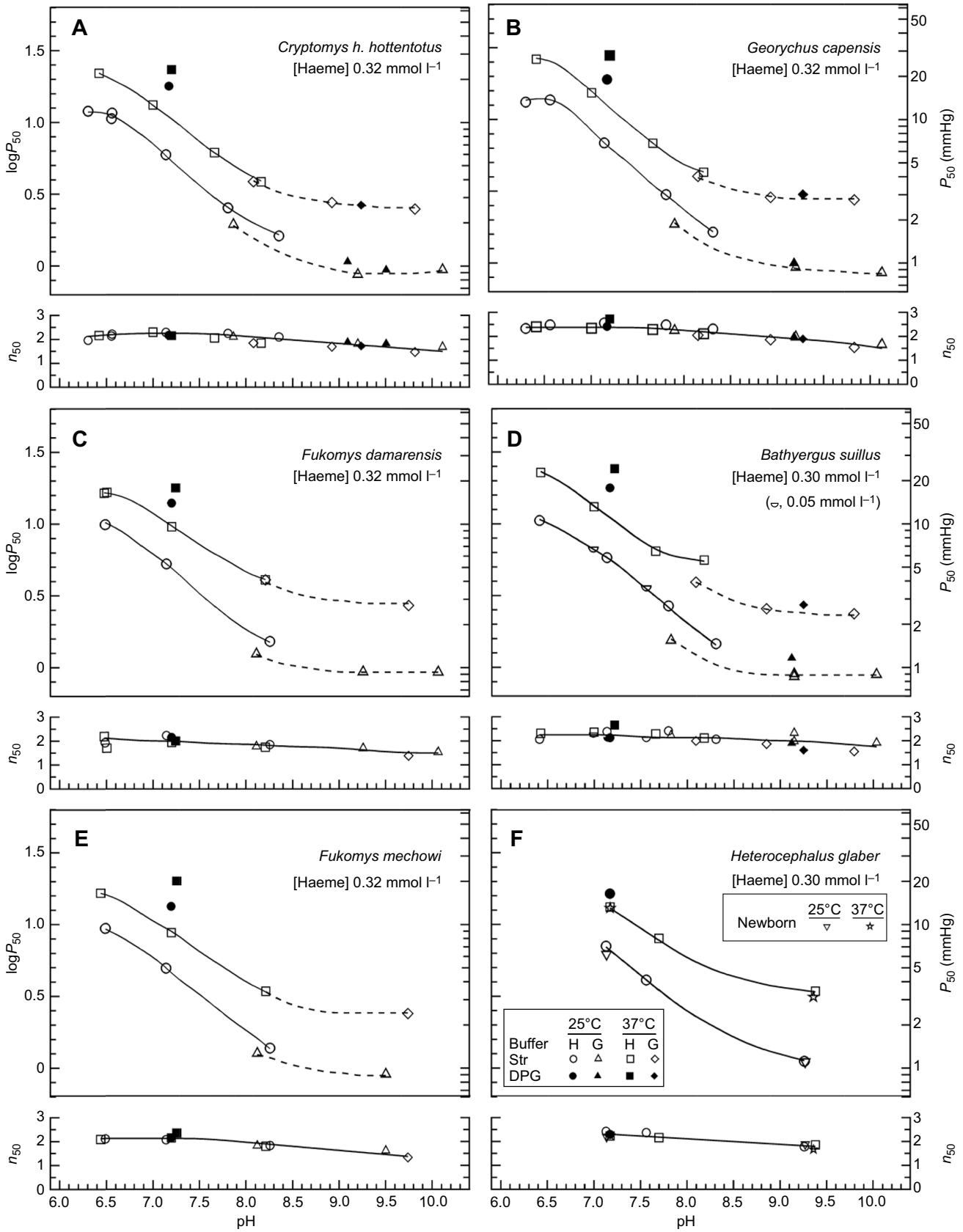

Fig. 3. Hb–O_2 binding properties of African mole rats. P_{50} (mmHg) and n_{50} values and the pH dependence of stripped Hbs measured at 37°C (squares and diamonds) and 25°C (triangles, circles and semicircles) in 0.1 mol l^{-1} KCl, and in either 0.1 mol l^{-1} Hepes buffer (H; circles and squares) or 0.1 mol l^{-1} glycine 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.

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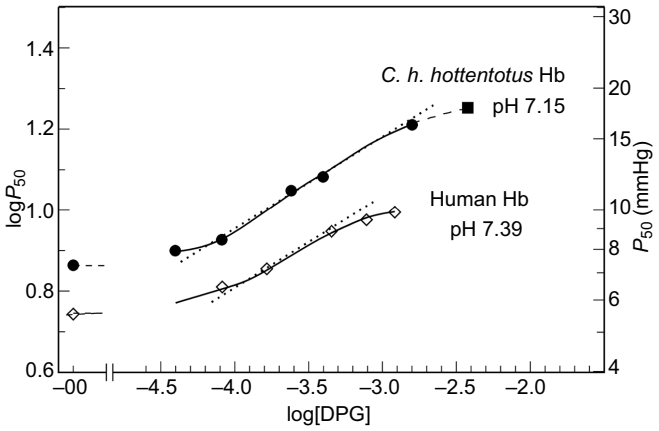

Fig. 4. Dose–response curves for the effects of DPG on O_2 affinity of *C. h. hottentotus* and human Hbs. P_{50} (mmHg) values are plotted against log[DPG] (mol l^{-1}) concentration. Dotted lines show a gradient of 0.25, congruent with the displacement of one log[DPG] molecule per four O_2 molecules. 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 (β2His→Asn). This indicates an unabridged capacity for DPG-mediated, adaptive variation in blood-O_2 affinity in adult mole rats. In contrast, the γ143His→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. β4Thr→Ser and β5Pro→Gly (Jelkmann et al., 1981). However, Eastern and Coast mole (*S. aquaticus and Scalopus orarius*) Hbs that show the β4Thr→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 β5 (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 α1Val and α131Ser/Thr and between β1Val and β82Lys) in most rodents, including *S. ehrenbergi* and *F. damarensis* (Table S1), *H. glaber* has α1Ser, which is prone to acetylation (Driessen et al., 1985), and non-polar α131Ala, 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 (α50, 57, 60, 64, 71, 113, 115 and 116) and four β-chain positions (β62, 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 (β5, 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

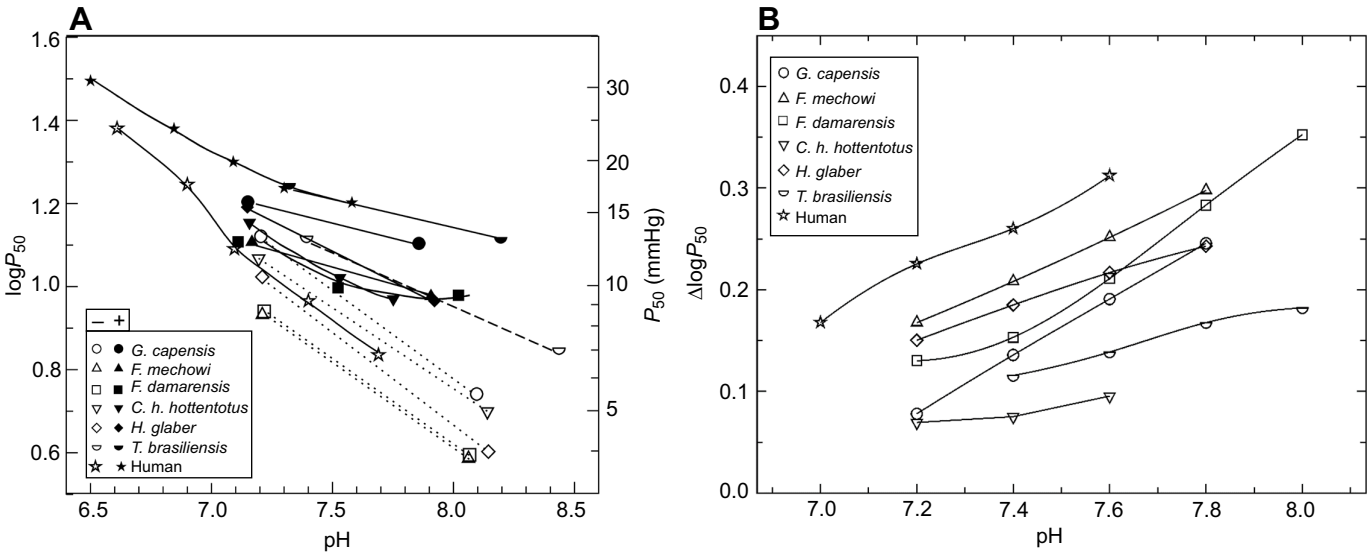

Fig. 5. CO_2 sensitivity of O_2 binding. (A) O_2 affinity (expressed as P_{50} values, mmHg) of mole rat, bat (*Tadarida brasiliensis*) and human Hbs measured at 37°C in the presence of 0.1 mol l^{-1} KCl and in the absence (open symbols) and presence (filled symbols) of 6% CO_2 (P_{CO_2}=42 mmHg). (B) pH dependence of the CO_2-induced decreases in O_2 affinity (increases in logP_{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–O_2 affinity. In this regard, future studies on the functional properties of fetal and embryonic mole rat Hbs (Table S1) promise clarification.

CO_2 effects

A striking result of this study is the consistently low specific CO_2 effects observed in mole rat Hbs compared with human Hb, which was lowest in social *C. h. hottentus* found in mesic and arid sands and clays (Fig. 4, Table 1). Although the specific effect of CO_2 on Hb–O_2 affinity augments O_2 unloading in metabolizing tissues, it may also hamper pulmonary O_2 loading under hypercapnic conditions. Thus, irrespective of the magnitude of the Bohr effect (which is decreased in the presence of CO_2 as a result of the pH dependence of carbamate formation), low specific CO_2 effects on Hb oxygenation would safeguard pulmonary Hb–O_2 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 CO_2 effect on Hb of the bat *T. brasiliensis* (Fig. 5), which faces high CO_2 tensions in densely populated caves (Kleinschmidt et al., 1987; Tuttle, 1994). In this species, the passively retained CO_2 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 CO_2 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 CO_2 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 CO_2 effects in the mole rats is unknown. As CO_2 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 CO_2 binding and thus increasing O_2 affinity. Acetylated Ser or Ala residues at N-terminal α-chains may moreover increase O_2 affinity by decreasing the strength of the tetramer–dimer interface (Ashiuchi et al., 2005).

The high O_2 affinity, high DPG sensitivity and low CO_2 sensitivity Hbs of mole rats contrast sharply with the Hb from the strictly fossorial Eastern mole (*S. aquaticus*), whose low O_2 affinity and DPG insensitivity are attributed to a charge-changing (δ136Gly→Glu) substitution in the β-type chains (Table S1). This replacement increases the blood's CO_2-carrying capacity by obstructing DPG binding at the shared N-terminal amino acid residues (Campbell et al., 2010). Analogously, low CO_2 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 CO_2 on Hb affinity may constitute a key component of the spectrum of adjustments that secure pulmonary uptake of O_2 and its flux to the working musculature in burrowing animals exposed to hypoxia/hypercapnia. As the intrinsic O_2 affinities of African mole rat Hbs are similar to those in other rodents, the higher blood O_2 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 CO_2, 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–O_2 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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