Supplementary material

Title: The relationship between hypoxia exposure and circulating cortisol levels in social and solitary African mole-rats: An initial report

Running title: Hypoxia affects plasma cortisol concentrations

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Animal capture

Mole-rats (except the naked mole-rats) were captured using Hickman live traps, baited with a small piece of sweet potato (Hickman, 1979). The traps were positioned at the entrance of excavated burrows where tunnels were open. Traps were monitored for captures or blocking every 2-3 hours over the course of the day and left overnight, being checked first thing in the morning.

Determination of reproductive status of social mole-rat

In wild caught social mole-rat species, namely the highveld and common mole-rat, all individuals below 40 g with a dark coat colour are considered to be juveniles (Hart et al., 2021). The juveniles were not used in this study. The breeding males were distinguishable from non-breeding males by their large, descended inguinal testes and yellow staining around the mouth. The breeding female possessed prominent auxiliary teats and a perforated vagina, which was absent in the non-breeding females. Breeding animals were not used in the study and the breeding status was confirmed by subsequent dissection.

Animal housing

Naked mole-rats were bred at the University of Ottawa and group housed in interconnected multi-cage systems at 30°C and 21% O_2 in 50% humidity with a 12 L:12D light cycle. Cape dune mole-rat, Cape mole-rat, highveld mole-rat and common mole-rat were wild captured in South Africa and were individually housed at the University of Pretoria at ~26°C and 18.5% O_2 in 50% humidity with a 12 L:12D light cycle. Animals were fed fresh tubers, vegetables, and fruit, and Pronutro cereal supplement (Bokomo Food Products, South Africa) *ad libitum*.

Experimental procedure

All mole-rats were weighed prior to the selected experimental protocol. Mole-rats were individually placed, unrestrained, inside a 1L (naked, Cape, highveld and common mole-rats) or 4.7 L (Cape dune mole-rats) Plexiglass experimental chamber, held at approximately between 28^{o}C (for Cape, highveld and common mole-rats) and 30^{o}C (for naked mole-rats), with a thin layer of bedding on the floor. Control (Normoxia) or treatment (hypoxia) air was supplied to the animal chamber at a flow rate of 600mLmin−1 (small chamber) or 2Lmin−1 (large chamber) for 3 hours. Dried incurrent air and nitrogen were mixed using pre-calibrated rotameters (Matheson Model 7400 Gas Mixer, E700 and E500 flow tubes) and calibrated mass flow meters (Alicat Scientific) to achieve each level of Normoxia or hypoxia.

Plasma cortisol analysis

Cortisol assays used for Cape, highveld, common and Cape dune mole-rats blood samples were performed using a coat-a-count cortisol kit (Coat-a-Count MG12171, IBL International GmBH, Hamburg, Germany). Cross-reactivity with other hormones was 76.0% with prednisolene, 11.4% with 11-deoxycortisol, 2.3% with prednisone, and <1% with aldosterone, corticosterone, cortisone, estriol, estrone, and pregnenolone. Cape, highveld, common and Cape dune mole-rats were assayed in duplicate, and the assay sensitivity (determined at 90% binding) was 6.1ng/ml. The intra-assay coefficient of variation was 5.5% (the samples were run in a single assay). Naked mole-rat cortisol assays were performed using the cortisol coated tube RIA kit (ImmuChem Cortisol CT 0722110, MP Biomedicals LLC, Solon, OH, USA). Cross-reactivity with other hormones was 45.6% with prednisolone, 12.3% with 11-desoxycortisol, 5.5% with corticosterone, 2.7% with prednisone, 2.1% with cortisone, and < 1% with 17α-hydroxyprogesterone, progesterone, dexamethasone, dihydrotestosterone, and testosterone. The minimum detectable dose was 1.7 ng/ml. HG were assayed in singleton, with an intra-assay coefficient of variation of 1.4% and inter-assay coefficient of variation of 4.6%. Serial dilutions of all species plasma were parallel to the standard curve (see table S1).

Table S1: ANCOVA results of the comparison of serial dilution and standard curve to check for parallelism for each species of African mole-rat used in this study.

Species	F	p	n
Cape dune mole-rat	8.31	0.16	4
Cape mole-rat	12.1	0.12	4
highveld mole-rat	17.1	0.25	4
Common mole-rat	6.54	0.37	4
Naked mole-rat	4.11	0.08	5

Asterisks (*) indicates significant difference from normoxia (p ≤ 0.05)

Table S2: The statistical output for the generalised linear model, with gamma distributions and link-identity function, to analyse differences between the species (BS, GC, CHH, CHP, HG) and treatments (normoxia vs hypoxia) and the two-way ineteraction species and treatment (Treatment*Species).

	t	p
Treatment	-0.62	0.54
Species	-2.55	0.01*
Treatment*Species	-2.01	0.05*

Asterisks (*) indicates significant difference from normoxia (p ≤ 0.05)

Table S3: *Post-hoc* comparisons of significant interactions, by Tukey's HSD (honestly significant difference) test, for Table S2 model. BS: Cape dune mole-rat; GC: Cape mole-rat; CHP: highveld mole-rat; CHH: Common mole-rat; HG: Naked mole-rat. Norm: Normoxia treatment; Hypx: Hypoxia treatment.

Post-hoc comparison	**p**
BS - CHH	<0.001*
BS - CHP	<0.001*
BS - GC	0.10
BS - HG	<0.001*

CHH - CHP	0.97
CHH - GC	0.16
CHH - HG	0.87
CHP - GC	0.39
CHP - HG	1.00
GC - HG	0.35
Norm GC - Norm HG	0.82
Norm GC - Hypx HG	1.00
Norm CHP - Norm HG	0.97
Norm CHP - Norm GC	0.47
Norm CHP - Hypx HG	0.06
Norm CHP - Hypx GC	0.03*
Norm CHH - Norm HG	0.98
Norm CHH - Norm GC	0.47
Norm CHH - Norm CHP	1.00
Norm CHH - Hypx HG	0.04*
Norm CHH - Hypx GC	0.02*
Norm CHH - Hypx CHP	0.01*
Norm BS - Norm HG	<0.001*
Norm BS - Norm GC	0.61
Norm BS - Norm CHP	<0.001*
Norm BS - Norm CHH	<0.001*
Norm BS - Hypx HG	0.33
Norm BS - Hypx GC	0.97
Norm BS - Hypx CHP	0.94
Norm BS - Hypx CHH	0.51
Hypx HG - Norm HG	0.05*
Hypx GC - Norm HG	0.04*
Hypx GC - Norm GC	0.99
Hypx GC - Hypx HG	0.99
Hypx CHP - Norm HG	0.01*
Hypx CHP - Norm GC	0.99
Hypx CHP - Norm CHP	0.02*
Hypx CHP - Hypx HG	0.99
Hypx CHP - Hypx GC	1.00
Hypx CHH - Norm HG	0.67
Hypx CHH - Norm GC	1.00
Hypx CHH - Norm CHP	0.34
Hypx CHH - Norm CHH	0.33
Hypx CHH - Hypx HG	1.00
Hypx CHH - Hypx GC	0.99
Hypx CHH - Hypx CHP	0.99
Hypx BS - Norm HG	<0.001*
Hypx BS - Norm GC	0.43

Hypx BS - Norm CHP	<0.001*
Hypx BS - Norm CHH	<0.001*
Hypx BS - Norm BS	1.00
Hypx BS - Hypx HG	0.26
Hypx BS - Hypx GC	0.86
Hypx BS - Hypx CHP	0.78
Hypx BS - Hypx CHH	0.36

Asterisks (*) indicates significant difference from normoxia ($p \leq 0.05$)

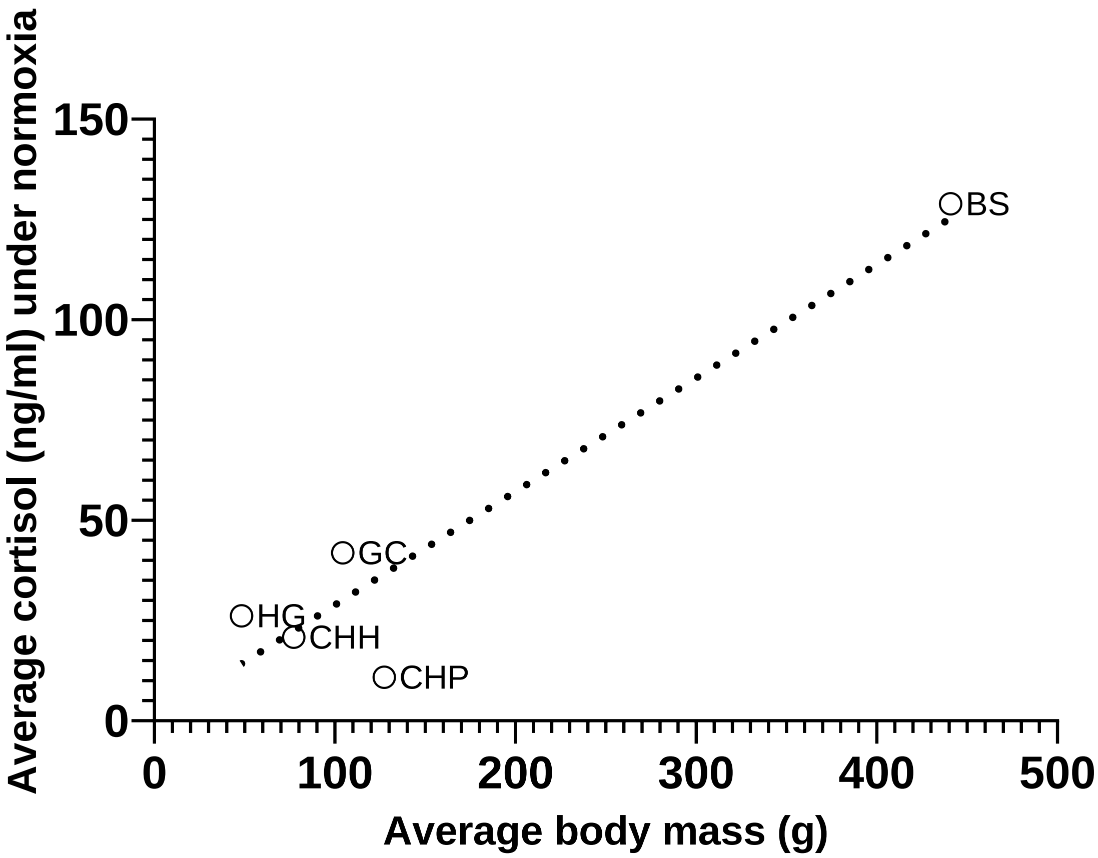

Figure S1: The relationship between the average body mass and plasma cortisol (ng/ml) level under normoxia of five African mole-rat species. BS: Cape Dune mole-rats; GC: Cape mole-rats; CHH: common mole-rats: CHP: highveld mole-rats; HG: naked mole-rats.

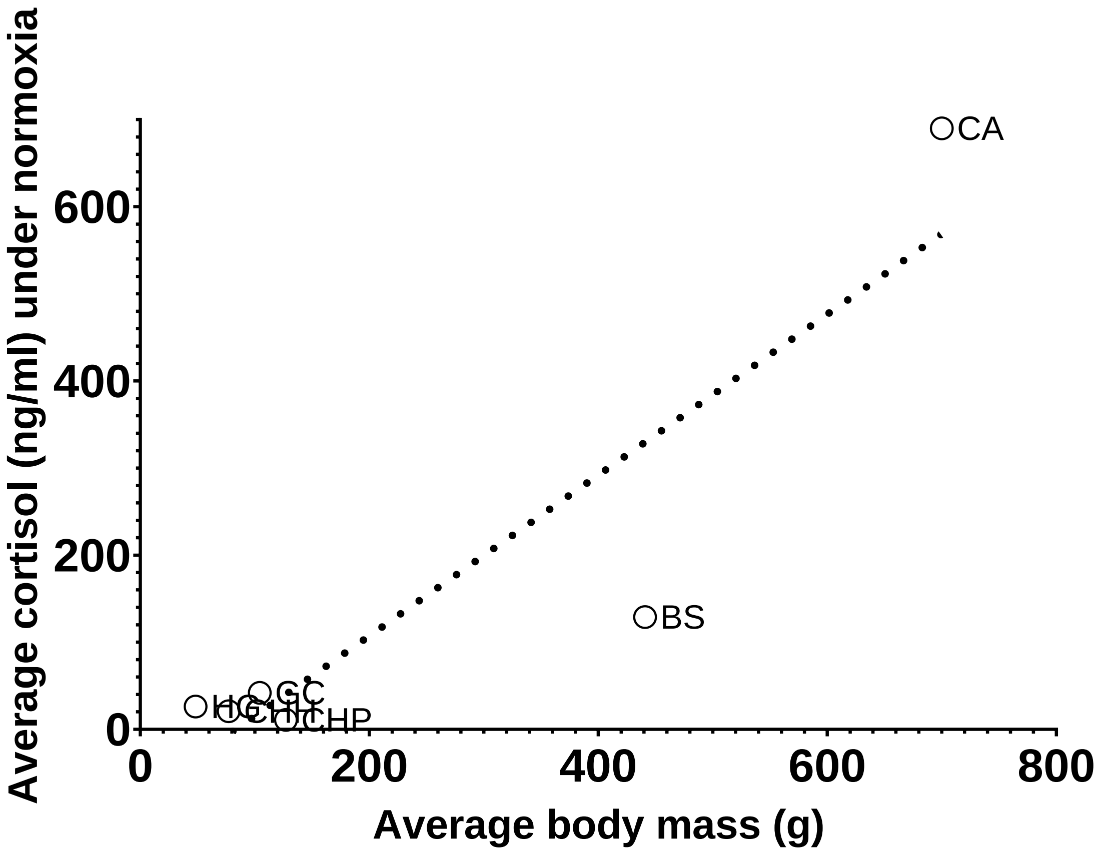

Figure S: The relationship between the average body mass and plasma cortisol (ng/ml) level under normoxia of five African mole-rat species (BS: Cape Dune mole-rats; GC: Cape mole-rats; CHH: common mole-rats: CHP: highveld mole-rats; HG: naked mole-rats) and guinea pigs (CA).

References

Hart. D.W.. Medger. K.. van Jaarsveld. B.. Bennett. N.C.. 2021. Filling in the holes: The reproductive biology of the understudied Mahali mole-rat (*Cryptomys hottentotus mahali*). Can. J. Zool. 99(9). 801-811.

Hickman. G.C.. 1979. A Live-Trap and Trapping Technique for Fossorial Mammals. South African J. Zool. 9–12. https://doi.org/10.1080/02541858.1979.11447641