Supplementary material

Short Communication: Elevated Testosterone Correlates with Enhanced Innate Immune Function In A Cooperatively Breeding Rodent

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Animal Housing

All animals were fed *ad libitum* and housed in large polyurethane crates (measuring 1 m × 0.5 m × 0.5 m), equipped with wood shavings and paper towelling for nesting. Animals were maintained in climate-controlled rooms with humidity maintained between 40–60% and ambient temperatures set between 24–26°C (Grenfell et al., 2024). Breeding and non-breeding males were classified based on a combination of anatomical, morphological, behavioural, and historical colony records.

Sample Collection

Urine samples were collected by placing each of the 26 male Damaraland mole-rats individually into cylindrical plastic chambers with elevated mesh floors above a collection tray (Wallace et al., 2023). Animals were provided apple and sweet potato during this period. Urine was typically excreted within 15 minutes and was immediately pipetted into sterile 1.5 mL Eppendorf tubes and stored at –80°C until further hormone analysis. All urine collections occurred between 09:00 and 12:00 to avoid confounding effects of circadian hormone fluctuations. Animals were promptly returned to their colonies following sampling.

Three days after urine collection, whole blood samples were obtained from each individual. The dorsal tarsal vein was accessed after cleaning the hindfoot with

Biotaine (0.5% chlorhexidine gluconate, 70% ethanol; Braun Medical Pty Ltd). A sterile 23-gauge needle was used to lance the vein, and blood was collected into heparinised microhaematocrit capillary tubes or sterile 1.5 mL Eppendorf tubes. Volumes ranged from 400–600 μL depending on individual size. Of this, approximately 300–400 μL was allocated to haematological analyses, while 100 μL was used for microbicidal killing assays (MKA). No more than 1% of total body weight was drawn in accordance with ethical guidelines approved by the University of Pretoria Animal Ethics Committee. Where sample volumes were limited, priority was given to MKA, and diluted blood samples (1:3 with phosphate-buffered saline) were used for haematological testing. All blood samples used in MKA were kept at 4°C and used within two hours of collection.

Microbicidal Killing Assay Procedure

Given the small volumes of blood collected, the MKA protocol followed modifications of established methods from Millet et al., (2007) and DeRogatis et al., (2020). Preliminary assessments indicated that mole-rat blood carried a high microbial load (Wallace et al., 2023), necessitating the use of antibiotic-supplemented media for microbial control. As a result, *Escherichia coli* was selected as the challenge microbe rather than *Candida albicans* or *Staphylococcus aureus*, which are incompatible with the modified media conditions.

Tetracycline was determined to be the most effective antibiotic for controlling background microbial growth. A working solution of tetracycline at 0.075 g per 5 mL of nuclease-free water was used, and SURE competent tetracycline-resistant E. coli (Agilent, #200238) was chosen for the assays. Bacteria were re-streaked weekly on tetracycline-infused tryptic soy agar to maintain resistance and viability. Each experimental day, a fresh working solution of 10^4 colony-forming units per millilitre (CFU/mL) was prepared and serially diluted for assay use.

Whole blood was diluted 1:4 in a medium containing L-glutamine and CO_2-independent media (Sigma-Aldrich No. G3126 and Gibco-Invitrogen No. 18045, respectively), both supplemented to 4 mM. For each assay, 15 μL of diluted blood was mixed with 7.5 μL of the bacterial working solution and vortexed. Samples were incubated at 41°C for 30 minutes in a water bath, then plated onto tetracycline-infused tryptic soy agar and incubated at 37°C for 24 hours. Colonies were manually counted to determine CFU. All samples were run in duplicate.

Positive controls included 7.5 μL of bacterial solution plated without blood. Two negative controls per individual were also included: (i) blood alone and (ii) blood mixed with L-glutamine and CO_2-independent media. All pipetting and plating were conducted in sterile laminar flow hoods under aseptic conditions.

Haematological Analyses

Automated complete blood counts and manual leukocyte differentials were performed by the Clinical Pathology Unit at the Faculty of Veterinary Science, University of Pretoria. An ADVIA 2120i automated haematology analyser (Siemens) was used.

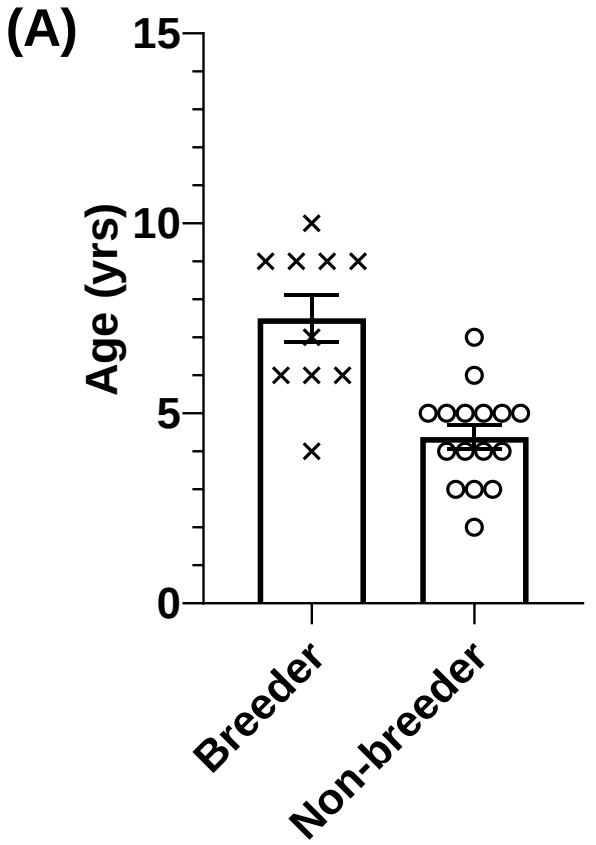
Endocrinological Analyses

Urinary Hormone Quantification

Testosterone and cortisol concentrations in urine were measured using Coat-A-Count radioimmunoassay kits (Diagnostic Products Corporation, Los Angeles, California, USA), following the manufacturer's instructions. Validation was performed by testing for parallelism between serial dilutions of mole-rat urine and the standard curve. Serial dilution of urine from a breeding male did not differ significantly from the standard curve (ANCOVA: $F_{[1,5]} = 1.1$, $p = 0.53$), and intra-assay variation for testosterone was 4.7%. For cortisol, serial dilutions also paralleled the reference standard (ANCOVA: $F_{[1,5]} = 15.2$, $p = 0.22$), with an intra-assay variation of 5.5%.

Creatinine Correction

Urinary steroid hormone concentrations were corrected for urine dilution by quantifying creatinine concentration using a modified Jaffe reaction (Folin, 1914). Hormone concentrations are expressed as testosterone (ng) and cortisol (μg) per milligram of creatinine (mg creatinine).


(A)
15
10
5
0
Age (yrs)
Breeder
Non-breeder

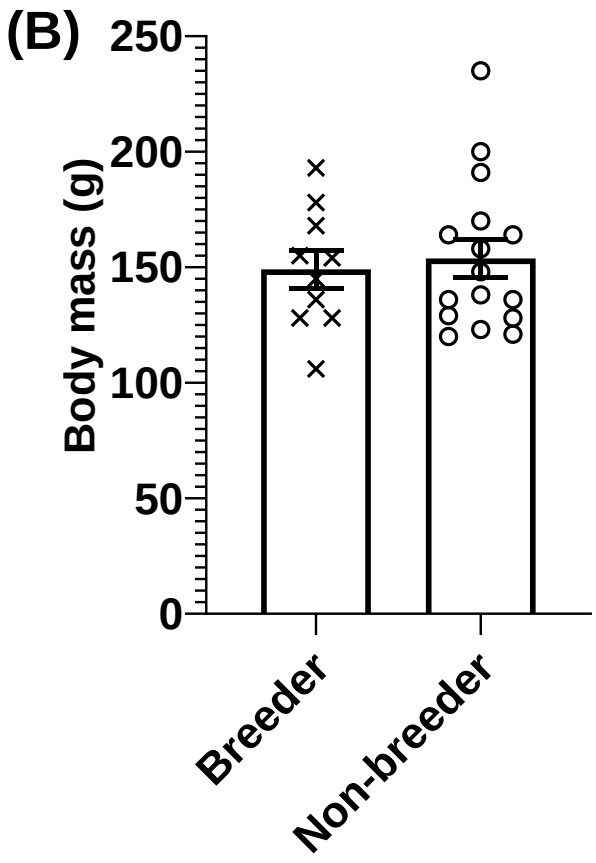
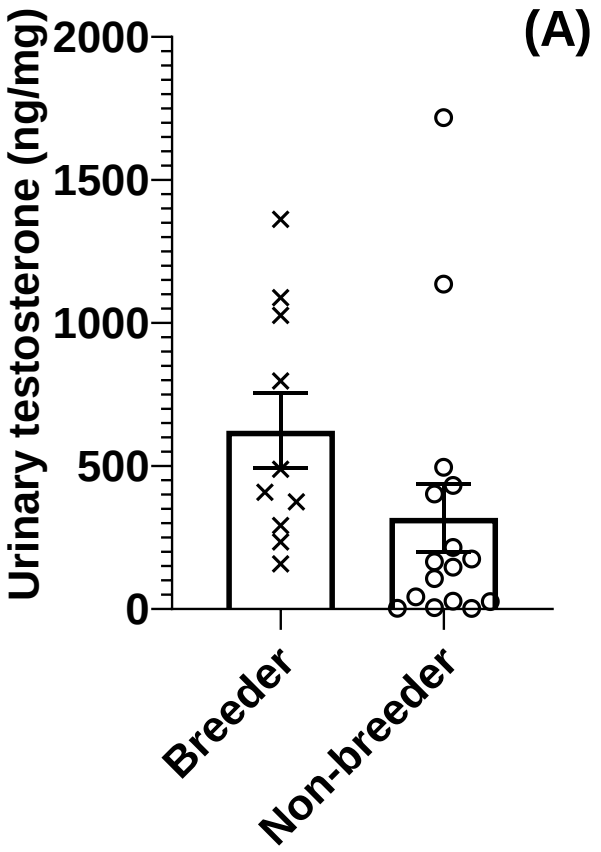

(B)
250
200
150
100
50
0
Body mass (g)
Breeder
Non-breeder

Figure S1. Age (years) and body mass (g) of the 26 male Damaraland mole-rats used in this study, comprising 10 breeders and 16 non-breeders.


(A)
Urinary testosterone (ng/mg)
2000
1500
1000
500
0
Breeder
Non-breeder

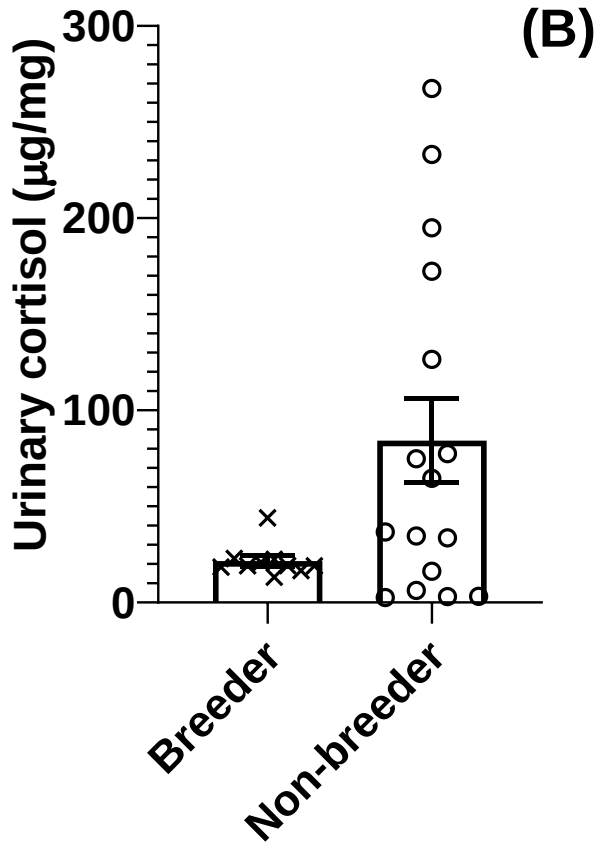

(B)
Urinary cortisol (µg/mg)
300
200
100
0
Breeder
Non-breeder

Figure S2. (A) Urinary testosterone (ng/mg) and (B) cortisol (µg/mg) of the 26 male Damaraland mole-rats used in this study, comprising 10 breeders and 16 non-breeders.

Table S1: Summary of beta regression model results for effects of urinary steroid hormones (testosterone and cortisol), body mass, age, breeding status and breeding status and urinary steroid hormones interaction on proportional immune cell counts

Variable	Z-value	P-value
Neutrophil		
Breeding status	-0.48	0.63
Urinary testosterone	0.39	0.70
Body mass	-2.43	0.02*
Age	-2.13	0.03*
Breeding status × Testosterone	-1.03	0.31
Breeding status	-0.21	0.84
Urinary cortisol	0.39	0.70
Body mass	-2.03	0.04*
Age	-1.62	0.11
Breeding status × Cortisol	-0.42	0.67
Lymphocytes		
Breeding status	0.44	0.66
Urinary testosterone	-0.39	0.70
Body mass	2.52	0.01*
Age	2.19	0.03*
Breeding status × Testosterone	1.08	0.28
Breeding status	0.25	0.80
Urinary cortisol	-0.36	0.72
Body mass	2.05	0.04*
Age	1.67	0.10
Breeding status × Cortisol	0.38	0.71
Monocytes		
Breeding status	-0.41	0.68
Urinary testosterone	-0.32	0.75
Body mass	-0.54	0.59
Age	-1.15	0.25
Breeding status × Testosterone	-0.47	0.64

Breeding status	-0.42	0.67
Urinary cortisol	0.44	0.66
Body mass	-0.08	0.94
Age	-1.28	0.20
Breeding status × Cortisol	-0.30	0.76
Eosinophils		
Breeding status	-0.01	0.99
Urinary testosterone	-0.03	0.98
Body mass	0.01	0.99
Age	-0.18	0.86
Breeding status × Testosterone	0.25	0.81
Breeding status	0.21	0.83
Urinary cortisol	-0.01	0.99
Body mass	-0.27	0.78
Age	-0.13	0.90
Breeding status × Cortisol	-0.04	0.97
Basophils		
Breeding status	-0.02	0.98
Urinary testosterone	-0.37	0.72
Body mass	-0.43	0.67
Age	-0.15	0.88
Breeding status × Testosterone	0.26	0.80
Breeding status	-0.65	0.52
Urinary cortisol	-0.26	0.80
Body mass	-0.22	0.83
Age	-0.75	0.45
Breeding status × Cortisol	0.41	0.68

*: Indicates statistical significance ($p < 0.05$)

References

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