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Title: A comprehensive profile of reproductive hormones in eusocial Damaraland mole-rats (*Fukomys damarensis*)

Running title: Hormonal profile of Damaraland mole-rats

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Keywords: Cortisol, progesterone, testosterone, prolactin, reproductive suppression, sexual behaviour

Abstract

In species where sociality and group cohesion are primarily determined by the maintenance of a reproductive division of labour and cooperative behaviours, the eusocial Damaraland mole-rat (*Fukomys damarensis)* presents a model which provides behavioural and endocrine distinctions between sex (males and females) and reproductive class (breeders and non-breeders). Although previous studies have demonstrated the endocrine aspects of reproductive suppression and behaviour in Damaraland mole-rats, they have focused on one hormone separately and on different conspecifics and samples across time. Unfortunately, this could introduce extrinsic biases when using these studies to compile complete hormonal profiles for comparisons. This study, therefore, set out to obtain a profile of the reproductive hormones from breeding and non-breeding male and female Damaraland mole-rats at a single point in time, from which circulating plasma prolactin and urinary progesterone, testosterone, and cortisol were measured. As expected, plasma prolactin and urinary cortisol did not differ between the breeders and non-breeders. However, breeders (both male and female) possessed increased urinary testosterone and progesterone concentrations compared to their non-breeding counterparts. These results, in conjunction with the variation in the expression of the respective hormonal receptors within the brains of breeders and non-breeders suggest that elevated testosterone and progesterone in breeders establish a neural dominance phenotype, which ultimately aids in controlling breeding activities. This study has emphasised the need for holistic, comprehensive profiling of reproductive endocrine systems.

Introduction

The reproductive system is a complex amalgamation of interacting hormonal axes modulated by the differential expression of neuroendocrine receptor cells and extrinsic environmental interactions (Brüggemann et al., 2018; Burland et al., 2002; Toufexis et al., 2014; Voigt et al., 2014). The hypothalamic-pituitary-gonadal (HPG) axis is responsible for the production of steroid hormones; testosterone, progesterone, and oestrogen, to name a few, through the activation and stimulatory roles of GnRH (gonadotropin-releasing hormone), LH (luteinising hormone) and FSH (follicle-stimulating hormone) (Brüggemann et al., 2018; Oyola and Handa, 2017; Toufexis et al., 2014). Increases in reproductive steroid hormone concentrations, such as testosterone and progesterone, induce physiological and morphological changes in the animal; however, they also promote several behaviours, such as mating activities and aggression for mate guarding and dominance maintenance (Clarke and Faulkes, 1998; Margulis et al., 1995).

The HPG axis can be disrupted by several other hormones, which in turn can disrupt reproductive activation, success and even behaviour. For example, prolactin is closely linked to the HPG axis; with the anterior pituitary as one of its production sites. Prolactin can naturally suppress steroid hormones, such as progesterone and testosterone, during lactation, often causing infertility at high concentrations (hyperprolactinaemia) (Bennett et al., 2018; Brown et al., 2014; Kauppila et al., 1988; Ziegler, 2000). In addition, prolactin inhibits the release of FSH and LH through a reduction in GnRH release from the hypothalamus and/or reduced sensitivity of the pituitary gland to GnRH (Bennett et al., 2018; Brown et al., 2014; Kauppila et al., 1988; Ziegler, 2000). Glucocorticoids (such as cortisol), released in response to a perceived stressor, produced by the hypothalamic-pituitary-adrenal (HPA) axis, have also been linked to HPG function as a GnRH inhibitor (Toufexis et al., 2014). The HPG and HPA axes are thus interconnected and can be influenced by joint

synergistic and antagonistic feedback mechanisms as an effect of the hypothalamus and anterior pituitary being communal sites for reception and synthesis (Toufexis et al., 2014). The activation of the HPA axis can often lead to the reduction of the HPG axis during periods of acute or chronic stress (Sheng et al., 2021). Previous studies have indicated species-, sex- and reproductive status-specific variations in the endocrine patterns of these reproduction-related hormones (Bens et al., 2018; Carlson et al., 2006; Clarke and Faulkes, 1998; Davies et al., 2016; Gray, 1978; Hart et al., 2022a; Lutermann et al., 2013; Matas et al., 2020; Sapolsky, 1982; Swift-Gallant et al., 2015; Voigt et al., 2021, 2016).

In species whose sociality and group cohesion are primarily determined by their maintenance of a reproductive division of labour, the eusocial Damaraland mole-rat (*Fukomys damarensis)* presents a model which provides behavioural and endocrine distinctions between sex and reproductive class (Bennett, 2009; Cooney and Bennett, 2000; Faulkes et al., 1997; Lovegrove, 1986). The despotic monopolisation of breeding characterises the severe reproductive skew by a single dominant female, the breeding female (BF), and one to three of the most dominant breeding males (BMs) (Bennett, 1990; Jarvis and Bennett, 1993). The remaining non-breeding males (NBMs) and females (NBFs) display cooperative care behaviour and are sexually quiescent due to the suppression of their reproductive system at various stages (Clarke et al., 2001; Molteno and Bennett, 2000).

The downregulation of reproduction in non-breeding Damaraland mole-rats results from the suppression of both behavioural and physiological reproductive characteristics (Bennett et al., 1996, 1993; Jarvis and Bennett, 1993; Kelley et al., 2019; Voigt et al., 2021). As Damaraland mole-rats are obligate outbreeders, inbreeding avoidance is a strong behavioural driver in preventing reproduction in NBFs and NBMs (Burland et al., 2002; Clarke et al., 2001; Kelley et al., 2019; Molteno and Bennett, 2000). Non-breeding males are anatomically and physiologically similar to BMs, yet they fail to reproduce and do not display sexual behaviours. Non-breeding males have similar circulating testosterone and cortisol

concentrations and similar-sized testes and spermatogenesis to BMs (Faulkes et al., 1994; Jarvis and Bennett, 1993; Voigt et al., 2016). In contrast, NBF Damaraland mole-rats are physiologically and anatomically distinct from their reproductively dominant counterparts (Bennett, 2011; Jarvis and Bennett, 1993; Lutermann et al., 2013; Voigt et al., 2021). The BF is reproductively active, exhibiting an ovulatory cyclicity and high progesterone and oestrogen concentrations, while ovulation in NBFs is blocked at the follicle maturation stage, resulting in low progesterone and oestrogen profiles (Bennett, 1994; Bennett et al., 1996, 1994; Clarke et al., 2001; Jarvis and Bennett, 1993; Molteno and Bennett, 2000; Voigt et al., 2021, 2014). These differential profiles have been suggested to stem from lowered pituitary sensitivity to GnRH (Bennett et al., 1993; Voigt et al., 2014; Voigt and Bennett, 2021). As induced ovulators, reproductively inactive females are thus anovulatory and incapable of breeding in the presence of the BF (Kelley et al., 2019; Molteno and Bennett, 2000; Voigt et al., 2021)

In the cooperatively breeding Mahali mole-rats (*Cryptomys hottentotus mahali*), both prolactin and glucocorticoids play a role in reproductive suppression (Hart et al., 2022a). While, only prolactin has been highlighted as a potential driving force behind reproductive suppression in naked mole-rats (*Heterocephalus glaber*) (Bennett et al., 2018; Edwards et al., 2020; Edwards, 2022; Hart et al. 2022b Medger et al., 2019). Yet, in the Damaraland mole-rat, no evidence indicates that prolactin or glucocorticoids drive reproductive suppression (Bennett et al., 2018; Hart et al., 2022b).

While previous studies have demonstrated the endocrine aspects of reproductive suppression in Damaraland mole-rats, they have focused on one hormone separately and on different conspecifics and samples across time. Unfortunately, this could introduce extrinsic biases when using these studies to compile complete hormonal profiles for comparison. Accordingly, now that the endocrine reproductive base has been laid, we can afford to look at the synergistic effects of the reproductive hormones together (Toor et al., 2022). This study,

therefore, set out to obtain a profile of reproductive hormones from breeding and non-breeding male and female Damaraland mole-rats at a single point in time, from which circulating plasma prolactin and urinary progesterone, testosterone, and cortisol correlates can be compiled to form an accumulative reproductive hormone profile. This, therefore, ensures uniformity and eliminates the possible effect of variation in individuals (wild vs captive) (Medger et al., 2018), season (Lutermann et al., 2013), diet (Medger et al., 2018), health (Klein, 2004), or influential dynamic social interactions (Kelley et al., 2019; Medger et al., 2019) within the colony across studies that may alter endocrine correlate values.

This study is also the first to investigate differences in progesterone concentrations between BMs and NBMs and how these relate to BFs and NBFs. This is surprising as an increased expression of progesterone receptors has been found in BMs, compared to NBMs, in most brain regions examined (medial preoptic area, the bed nucleus of the stria terminalis, the ventromedial nucleus of the hypothalamus, the arcuate nucleus and the medial amygdale) (Voigt et al., 2016). This suggests that progesterone might activate sexual behaviour in males (Voigt et al., 2016) as in other mammals (Andersen and Tufik, 2006).

Methods

Ethical note

The University of Pretoria animal ethics committee approved the experimental procedures of this study (NAS017-2021 and NAS022/2021).

Study species

Forty-nine Damaraland mole-rats (10 BFs, 8 BMs, 16 NBFs, 15 NBMs) from ten captive colonies housed at the University of Pretoria were used. All individuals used in this experiment were considered adults (Bennett and Faulkes, 2000). Breeding

females were identified by the presence of prominent axillary and inguinal teats, well-developed external genitalia with a perforate vagina, and/or pregnancy-related changes in girth/body size, as well as a history of producing young. Only one BF was identified as being heavily gravid, while only one BF was suckling young (one pup). At the time of the sampling, all NBFs had never mated, bore young or lactated and likely never ovulated before. Breeding males were identified based on observations of copulation with the BFs, a dark stain around the periphery of the mouth, and bulging testes which project from abdominal pockets. Long-term observational records were used to confirm the identity of the BMs and NBFs and the age of non-breeding individuals. All animals have been part of long-term (>20 years) monitoring and breeding projects at the University of Pretoria. Therefore, age data (accurate to 1 day) was available for non-breeding Damaraland mole-rats, while less accurate age data was available for the breeding Damaraland mole-rat colony members and, therefore, not included in this study.

Animal housing

Damaraland mole-rats were housed in their natal colonies in large plastic crates (1 m × 0.5 m × 0.5 m), with wood shavings and paper towelling provided as nesting material. Housing room temperatures ranged between 26.5 and 27.5 °C, with relative humidity around 50–60%. Animal rooms were maintained on a 12L:12D photoperiod. Photoperiod has not been shown to affect Damaraland mole-rat behaviour (Oosthuizen et al., 2003). Animals were fed *ad libitum* on a variety of chopped vegetables and drank no free water (Hart et al., 2022c).

Urine collection

Urine was collected from all Damaraland mole-rats over two days (approximately 25 animals a day). Each animal was kept in a cylindrical plastic cage with a wire-mesh

base on top of the urine collection tray. The wire-mesh base prevented the contamination of the urine sample with faecal matter. Apple and sweet potato were provided. Urine was collected between the hours of 0900 and 1300, and as soon as urine had been voided (usually within 15 minutes), the animal was placed back into its natal colony. Urine was collected with a single-use plastic pipette and immediately frozen at −40 °C.

Blood sampling

Blood samples were collected three days after urine collection between 09h00 and 13h00. Bleeding occurred after urine collection to avoid the stress of handling and bleeding affecting urinary hormone levels (namely cortisol). Furthermore, a three-day break between urine collection, which is minimally invasive, and bleeding was selected to ensure any stress experienced by urine collection did not affect circulating hormone levels, namely plasma prolactin. The mole-rats were handheld, and venous blood samples were collected from the hind foot after sterilisation of the bleed site. Approximately 0.3 – 0.5ml of blood was collected into heparinised micro-haematocrit tubes, depending on the body mass of the animal. The blood was centrifuged at 1500 g, and the resulting plasma decanted and stored at – 80 °C until further analysis. Only 1% of the total body mass of the individual blood was collected, as permitted by the University of Pretoria Animal Ethics Committee.

Hormone analysis

Plasma prolactin

Plasma prolactin concentrations were quantified using an Elabscience Guinea pig Prolactin ELISA kit (Elabscience Biotechnology Inc., Wuhan, China), as described by Bennett et al. (2018). The sensitivity of the assay was 0.09 ng/mL plasma, and

intra-assay precision and repeatability are <10%, according to the manufacturer's guidelines.

Urinary steroid hormones

Urine samples were analysed for testosterone, progesterone and cortisol concentrations using coat-a-count kits (Diagnostic Products Corporation, Los Angeles, California, USA). All assays were conducted according to the manufacturer's protocol. Assays were validated by testing for parallelism between serial dilutions of mole-rat urine (obtained from an individual with high hormone concentrations) and the standard curve (Chard, 1988). The aforementioned hormone kits, including cross-reactivity, sensitivity, and protocols, have been described in previous studies (Hart et al., 2021, 2020; Medger et al., 2018).

There was no significant difference between the serial dilution curve of urinary testosterone of a BM and the calibration curve (ANCOVA: $F_{[1,5]} = 1.1$, $p = 0.53$). The intra- and inter-assay coefficient of variation was 4.7% and 6.0%, respectively. Furthermore, a serial dilution of urinary cortisol concentration from an individual with high cortisol paralleled the reference preparation; thus, the slopes did not differ significantly (ANCOVA: $F_{[1,5]} = 15.2$, $p = 0.22$). The intra- and inter-assay coefficient of variation was 5.5% and 11.0%, respectively. Similarly, no significant difference was observed between a BF with a high urinary progesterone serial dilution curve and the calibration curve (ANCOVA: $F_{[1,5]} = 2.9$, $p = 0.39$). The intra- and inter-assay variation coefficient for repeated quality control determination was 7.9% and 12.3%, respectively.

Creatinine determination

Urine hormone concentration varied due to variable fluid intake; thus, the concentrations of steroid hormones (testosterone, progesterone, and cortisol) had

to be corrected. The correction was accomplished by analysing each urine sample for creatinine concentration, as creatinine is excreted at a relatively constant rate. The creatinine concentration of each urine sample was determined using a modified Jaffe reaction (Folin, 1914). Final standardised results are presented as steroid hormone [testosterone (ng), progesterone (ng) and cortisol (μg)] per milligram of creatinine (mg creatinine).

Data analysis

Statistical analyses were performed on R 2022.02.0, Microsoft Excel (Version 2205) and Graphpad Prism 8.4.3. Statistical significance was denoted by $p < 0.05$, and data is presented as mean ± standard error (SEM).

Normality was tested visually using the QQ plot, and Levene's test on model residuals. Data that were not normally distributed were log-transformed. General linear models (GLM's) were used to analyse normally distributed dependant variables, while generalised linear models (GLZM's) with gamma distributions and link-identity functions from the *lme4* package were used to analyse non-normal dependant variables. Urinary testosterone, cortisol and progesterone concentrations data had to be log-transformed into a normal distribution, while the plasma prolactin data failed to become normally distributed even after log transformation.

All models possessed colony size and body mass as covariates and breeding caste (BF, BM, NBF, NBM) as the primary predictor variable. *Post-hoc* comparisons of significant interactions, namely breeding caste, were obtained by Fisher's least significant difference (LSD) tests. Due to insufficient data on the breeding individuals, age analyses were only conducted for non-breeders (NBFs and NBMs). Linear regressions were conducted between urinary testosterone, cortisol, progesterone and plasma prolactin and age, respectively, separately for NBMs and NBFs. Lastly, Pearson correlations were conducted between log-transformed

urinary testosterone and cortisol and progesterone concentrations, respectively. At the same time, Spearman-rank correlations were performed between plasma prolactin and urinary testosterone, cortisol, and progesterone concentrations, respectively.

Results

Testosterone

Both breeding caste and body mass significantly affected urinary testosterone concentrations in the Damaraland mole-rat (Table 1, Figures 1a and 2). While, colony size did not significantly affect urinary testosterone concentrations (Table 1). There was a significant positive relationship between urinary testosterone and body mass, whereby heavier animals possessed higher urinary testosterone concentrations (Figure 2a, Table 1). Furthermore, BMs possessed significantly higher urinary testosterone levels in comparison to BFs, NBFs and NBMs (Figure 1a). Both NBMs and BFs possessed significantly higher urinary testosterone concentrations than NBFs (Figure 1a). No difference was noted between BF's and NBM's urinary testosterone levels (p=0.62; Figure 1a).

Table 1. The statistical outputs from the models investigating the effects of body mass, colony size and breeding caste (BF: Breeding female; BM: Breeding male; NBF: Non-breeding female; NBM: Non-breeding male) on the urinary testosterone (T; ng/mg creatinine), cortisol (C; µg/mg creatine), progesterone (P; ng/mg creatinine) and plasma prolactin (PRL; ng/ml) of Damaraland mole-rats (*Fukomys damarenesis*).

	T			C			PRL			P		
	F	*p*	ηp^2	F	*p*	ηp^2	χ^2	*p*	ηp^2	F	*p*	ηp^2
Colony Size	0.67	0.80	0.01	1.41	0.24	0.02	0.19	0.48	0.01	0.44	0.51	0.03
Body Mass	2.10	0.04*	0.09	5.32	0.03*	0.12	0.35	0.55	0.01	2.57	0.12	0.05
Breeding caste	11.7	0.0001*	0.41	1.00	0.41	0.05	0.66	0.88	0.02	14.0	0.0001*	0.49

Significant relationships (p-value < 0.05) are indicated with '*'. ηp^2 represent partial eta-squared (effect size).

Progesterone

Both colony size and body mass did not significantly affect urinary progesterone concentrations in the Damaraland mole-rat (Table 1). While, breeding caste did significantly affect urinary cortisol concentration (Table 1, Figure 1b). Breeding females possessed significantly higher urinary progesterone levels in comparison to BMs, NBFs and NBMs (Figure 1b). On the other hand, NBFs possessed similar urinary progesterone concentrations to BMs ($p = 0.43$) and NBMS ($p = 0.052$) (Figure 1b). While, a significant difference was noted between BM's and NBM's urinary progesterone levels (Figure 1b), with BMs possessing higher urinary progesterone levels than NBMs. Only one BF was identified as being gravid, while only one BF was suckling young (one pup). The gravid BF had urinary progesterone of 276.26ng/mg creatinine, while, the BF that was nursing a pup had high urinary progesterone of 106.76ng/mg creatinine. These values, however, fell within the range of urinary progesterone of the remaining BFs (25-334ng/mg).

Cortisol

There were no significant effects of breeding caste or colony size on the urinary cortisol concentrations (μg/mg creatinine) of Damaraland mole-rats (Table 1 and 2). However, there was a significant negative relationship between urinary cortisol concentrations and body mass in Damaraland mole-rats (Figure 2b, Table 1). Heavier individuals were observed to have higher urinary cortisol concentrations.

Prolactin

Neither breeding caste, colony size nor body mass significantly affected plasma prolactin concentrations in Damaraland mole-rats (Table 1 and 2). The gravid BF had plasma prolactin of 7.93 pg/ml, while, the BF that was nursing a pup had high plasma prolactin of 10.53 pg/ml. These values, however, fell within or just outside plasma prolactin (2-8 pg/ml) range of the remaining BFs.

Table 2: The urinary cortisol (µg/mg creatine) and plasma prolactin (PRL; ng/ml) (mean±SE) of the different breeding caste (BF: Breeding female; BM: Breeding male; NBF: Non-breeding female; NBM: Non-breeding male) of 49 Damaraland mole-rats (*Fukomys damarenesis*).

	BF	BM	NBF	NBM
n	10	8	16	15
Urinary cortisol	50.4±14.7	22.2±3.32	83.2±29.2	92.9±24.4
Plasma prolactin	4.91±0.87	4.14±1.61	3.77±1.14	3.53±1.06

Effect of age in non-breeders

A significant inverse relationship between age and urinary testosterone concentrations was present in NBMs, but not present in NBFs (Table 3). This implies decreased urinary testosterone concentrations in NBMs as they age. No further significant interactions exist between urinary cortisol, progesterone, plasma prolactin, and age in either NBMs or NBFs (Table 3).

Table 3. Linear equations and regression statistical outputs for the relationships between the age of a non-breeding male (NBM) Damaraland mole-rats and non-breeding female (NBF) Damaraland mole-rats with body mass and the endocrinal correlates; urinary

testosterone (T; ng/mg creatinine), cortisol (C; µg/mg creatinine), progesterone (P; ng/mg creatinine) and plasma prolactin (PRL; ng/ml).

Significant relationships (p-value ≤ 0.05) are indicated with '*'.

	NBM				NBF			
	Slope	y-intercept	F	p	Slope	y-intercept	F	p
T	-0.74	1526.0	5.45	0.04*	0.04	-18.76	0.73	0.41
C	-0.00	96.59	0.00	0.96	-0.04	144.0	0.39	0.54
P	-0.001	15.6	0.02	0.25	0.91	-6.16	2.82	0.12
PRL	0.00	-0.95	0.74	0.40	0.00	1.51	0.35	0.56
Body Mass	-0.01	171.3	0.11	0.74	0.01	119.7	0.50	0.49

Correlations between hormones

No correlations were found between plasma prolactin and urinary testosterone, cortisol, and progesterone ($r \leq 0.11$, $p \geq 0.25$). Similarly, no significant correlation was observed between urinary testosterone and progesterone ($r = 0.27$, $p = 0.06$). In contrast, significant correlations were observed between urinary cortisol and urinary progesterone ($r = 0.42$, $p = 0.02$) and urinary cortisol and urinary testosterone concentrations ($r = 0.31$, $p \leq 0.03$).

Discussion

This study set out to obtain a profile of the reproductive hormones from breeding and non-breeding male and female Damaraland mole-rats at a single point in time, from which circulating plasma prolactin and urinary progesterone, testosterone, and cortisol. As expected, plasma prolactin and urinary cortisol did not differ between the breeders and non-breeders of both sexes. However, breeders of both sexes possessed increased urinary testosterone and progesterone concentrations compared to their non-breeding counterparts. These results, in conjunction with the variation in the expression of the respective hormonal receptors within the brains suggest that

362 elevated testosterone and progesterone in breeders establish a neural dominance
363 phenotype, which ultimately aids in controlling breeding activities.

364 Breeding female Damaraland mole-rats possessed the highest urinary
365 progesterone concentrations. As BFs have access to unrelated males (the BM), they
366 ovulate and fall pregnant regularly, having three to four litters throughout the year
367 (Bennett and Faulkes, 2000). Through this and the increased pituitary gland
368 sensitivity to GnRH (Bennett et al., 1993), along with an increased expression pattern
369 of oestrogen receptor α and aromatase (androgen-converting enzyme), the
370 circulating progesterone in BFs is greater than in NBFs and males (Voigt et al., 2014).
371 Interestingly, BMs possess similar urinary progesterone concentration to NBFs,
372 possibly due to two factors: reduced circulating progesterone levels in NBFs (a fact
373 that is highlighted as NBMs and NBFs have similar urinary progesterone levels) and
374 increased circulating progesterone levels in BMs. Non-breeding males have the
375 lowest urinary progesterone concentration measured, implying that BMs inherently
376 possess higher circulating progesterone levels. Variation between breeding and non-
377 breeding Damaraland mole-rat males has been found in the differential
378 neuroendocrine expression of androgen-, progesterone- and Rfrp-receptors (AR,
379 PGR, and RFRP-3, respectively), which directly regulate GnRH activity in the
380 hypothalamus (Matas et al., 2020; Swift-Gallant et al., 2015; Voigt et al., 2016, 2014;
381 Voigt and Bennett, 2021). Breeding males have a greater expression of AR and PGR
382 than NBMs, both of which, when stimulated by their respective hormones, activate
383 GnRH neurons to release excess gonadotropins, therefore enabling heightened
384 reproductive behaviour (Voigt et al., 2016). Conversely, NBMs have lower AR and
385 PGR distributions, but elevated RFRP-3, which acts as an inhibitory function on
386 GnRH neurons and subsequent reproductive phenotypes (Voigt et al., 2016; Voigt
387 and Bennett, 2021). The expression of these receptor cells thus alludes to and
388 permits the endocrine variations that facilitate each individual's reproductive state.
389 Progesterone is a steroid required for reproduction, whereby previous studies have
390 implicated the synergistic activity of progesterone and testosterone via PGR and AR

activity on GnRH stimulation and, thus, reproductive ability and behaviour (Voigt et al., 2016). Further implications have been made regarding the role of progesterone in reproductive success and spermatozoa morphology, that while breeding and non-breeding male Damaraland mole-rats have comparable spermatozoa production (Jarvis and Bennett, 1993), the spermatozoa of NBMs have more dysmorphologies in terms of double heads, multiple and/or shorted tails and thus a possible additive cause of failure in breeding (N.C. Bennett, personal observation and communication). Additively, NBMs have been reported to be oligospermic and even azoospermic compared to their breeding counterparts (Maswanganye et al., 1999).

The highest urinary testosterone concentrations were seen in the BMs, a somewhat expected trend in mole-rat species (Hart et al., 2022a; Hart et al., 2021), but unexpected in Damaraland mole-rats. Other studies investigating the relationship between testosterone concentrations in Damaraland mole-rat males, did not report BMs having significantly higher testosterone concentrations than NBMs (Bennett, 1988; Medger et al., 2018). Likewise, the majority of previous studies on testosterone in Damaraland mole-rats females indicated no significant differences between reproductive states (Bennett, 1994; Bennett et al., 1994; Clarke et al., 2001; Medger et al., 2018), which contrasts with the present study that found BFs possessing higher urinary testosterone concentrations than NBFs and similar urinary testosterone concentrations to NBMs. Breeding males not only maintain increased testosterone concentrations (this study), but also increased expression of AR in their brains compared to NBMs; in conjunction with increased progesterone concentrations and expression of PGR, this would establish a neural dominance phenotype, which ultimately aids in controlling breeding activities (Voigt et al., 2014). Even though the BFs urinary testosterone concentrations were lower than BMs in this study, the increased expression of AR in BFs compared to NBFs can exploit the lower circulating testosterone concentrations in order to establish this neural dominance phenotype in the BFs (Voigt et al., 2014). Upon observing increased concentrations of testosterone in BFs compared to NBFs in Damaraland

and Natal mole-rats (*C. h. natalensis*), a pattern also seen in the other mole-rat species (Clarke and Faulkes, 1998; Hart et al., 2022a; Spinks et al., 1999), Lutermann et al. (2013) linked elevated testosterone concentrations to the ability of an individual to attain and defend the breeding monopoly. This re-enforces the hypothesis that female intra-sexual competition exerts selective pressures on testosterone-mediated traits thought to enhance reproductive success (Clutton-Brock, 2007; Lutermann et al., 2013; Medger et al., 2019).

The increase in the testosterone concentration of breeders (both male and females) may suggest increased aggression or dominance displayed by these males toward their non-breeding colony members in breeding monopoly maintenance, which has been suggested to cause an increase in glucocorticoids, namely cortisol (Hart et al., 2022a). However, to date, including in this study, no significant difference in glucocorticoid concentrations between breeding and non-breeding Damaraland mole-rats (from stable colonies) has been observed (Medger et al., 2018), thus indicating no physiological stress in non-breeders (Hart et al., 2022b for review). While, the role of prolactin in reproduction suppression in African mole-rats is still unclear as each species appears to have its own unique pattern (Bennett et al., 2018; Hart et al., 2022a). However, this study confirms the findings of Bennett et al. (2018), which found almost undetectable prolactin concentrations in Damaraland mole-rats, suggesting it plays no role in reproductive suppression in this species.

This study has aided in elucidating inconsistencies in prior reproductive endocrinological work and has highlighted a gap in the knowledge base, largely the role of progesterone in male reproductive systems. This study also reinforces the current hypothesises suggesting that neither cortisol nor prolactin is the key driving mechanism of reproductive suppression in Damaraland mole-rats (Hart et al., 2022b). Furthermore, this study has emphasised the need for holistic, comprehensive profiling of reproductive endocrine systems.

Acknowledgements

The project was funded by the SARChI chair for Mammal Behavioural Ecology and Physiology, South Africa, # 64796, for the DST-NRF awarded to NCB. We would also like to thank Dr Phoebe Edwards and the anonymous reviewer for their helpful comments and suggestions while reviewing this article.

Author Contributions

N.C.B., D.W.H. and K.M.E.W. designed the study. K.M.E.W., N.C.B. and D.W.H. collected the data. N.C.B., D.W.H., K.M.E.W. and N.H. conducted hormonal analysis. K.M.E.W. compiled the data. D.W.H and K.M.E.W. analysed the data. All authors contributed to the writing of the first draft of the manuscript. N.C.B., D.W.H. and K.M.E.W. revised the manuscript after review.

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Figure legends

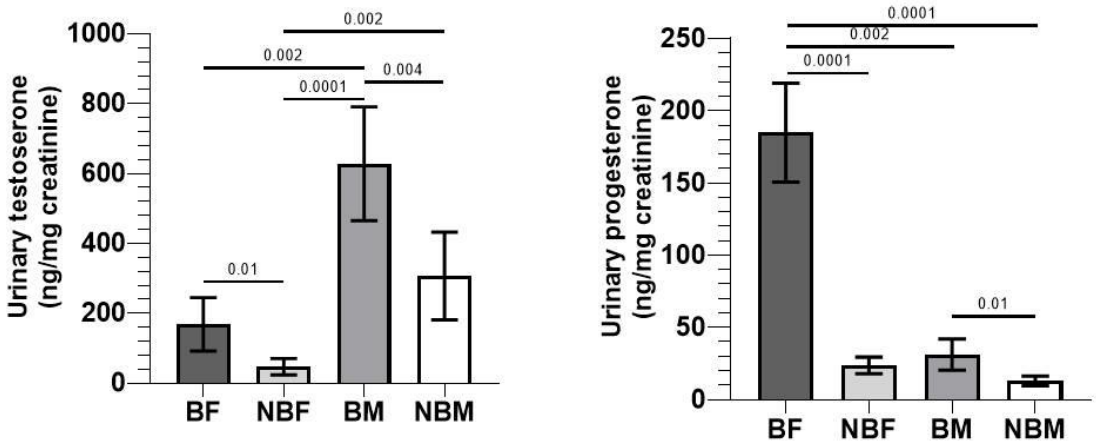
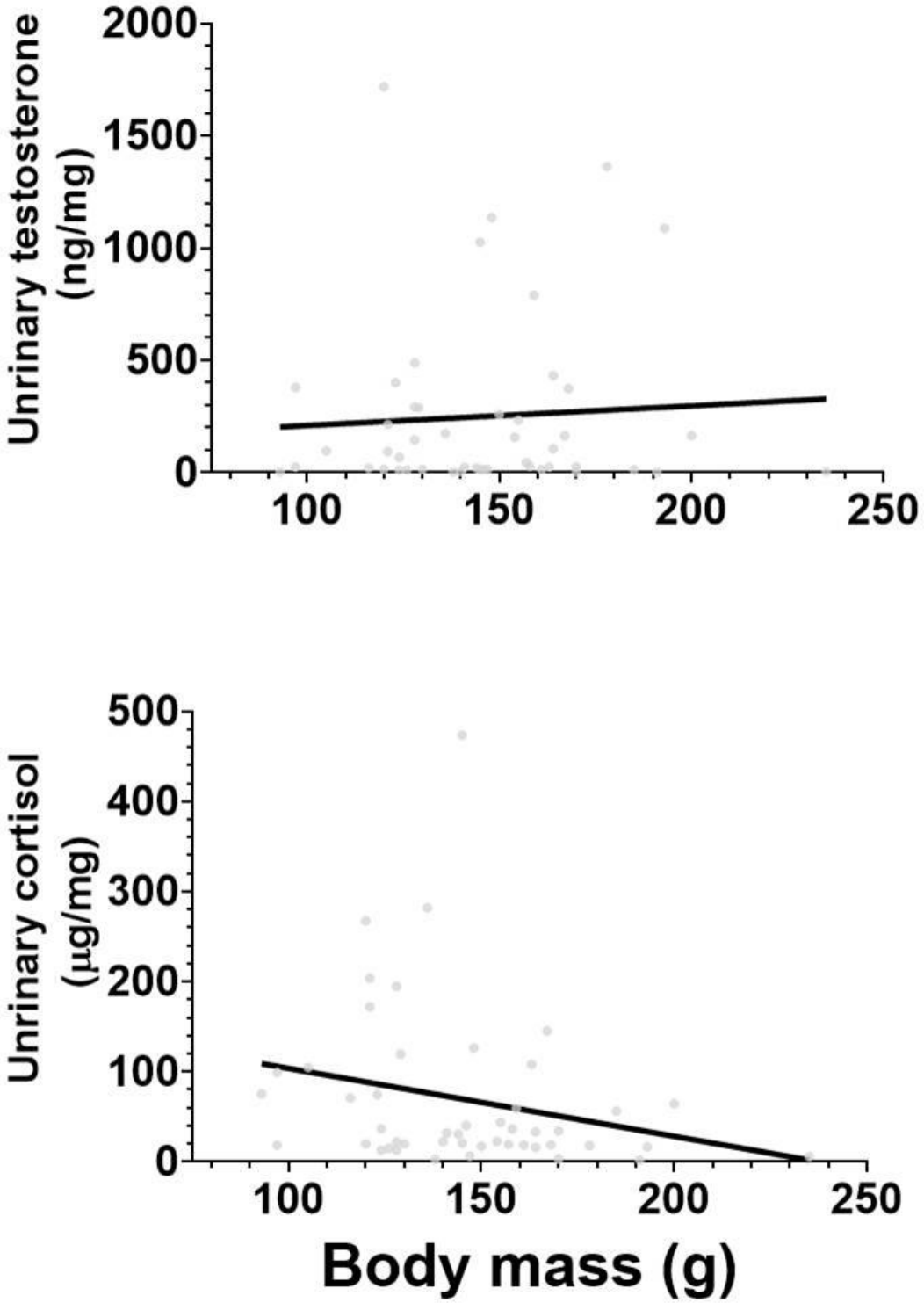


Figure 1. Bar graphs displaying breeding caste (BF: Breeding female; BM: Breeding male; NBF: Non-breeding female; NBM: Non-breeding male) differences in urinary (a) testosterone (ng/mg creatinine) and (b) progesterone (ng/mg creatinine) concentrations in Damaraland mole-rats (*Fukomys damarensis*). Data presented as mean ± SEM. Results for significant post-hoc Fisher's least significant difference (LSD) tests between each breeding caste is presented in the figure. Statistical significance was assumed at $p < 0.05$.



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672 **Figure 2.** The relationship between urinary (a) testosterone (ng/mg creatinine) and
673 (b) cortisol (µg/mg creatinine) concentrations and body mass (g) in Damaraland
674 mole-rats (*Fukomys damarensis*).

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