The endocrine control of reproductive suppression in an aseasonally breeding social mole-rat, the Mahali mole-rat (*Cryptomys hottentotus mahali*)

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Highlights

- Reproductive suppression is only evident in females.
- Breeders possess higher levels of testosterone than non-breeders.
- Both prolactin and glucocorticoid are involved in suppression.
- Females are more sensitive to adrenocorticotropic hormone stimulation test than males.
- Link between prolactin and glucocorticoid may be evident.

Abstract

Cooperative behaviour, sociality and reproductive suppression in African mole-rats have been extensively studied. Nevertheless, endocrine correlates of some species of social mole-rats have been neglected, and these species may hold the key to understanding the behavioural and physiological complexity that allows the maintenance of social groups in African mole-rats. In this study, we investigated endocrine correlates implicated in the suppression of reproduction and cooperative behaviours, namely glucocorticoids (a stress-related indicator) through faecal glucocorticoid metabolites (fGCMs), plasma testosterone (an indicator of aggression) and plasma prolactin in the Mahali mole-rat (*Cryptomys hottentotus mahali)* across reproductive classes (breeding females and males, non-breeding females and males) and season (wet and dry). Breeders possessed higher levels of testosterone than non-breeders. In reproductively suppressed non-breeding females, fGCMs were significantly higher than in breeders. Furthermore, an adrenocorticotropic hormone stimulation test (ACTH challenge test) on both male and female non-breeders revealed that female non-breeders show a more significant response to the ACTH challenge than males. At the same time, plasma prolactin levels were equally elevated to similar levels in breeding and non-breeding females. Chronically high levels of prolactin and fGCM are reported to cause reproductive suppression and promote cooperative behaviours in non-breeding animals. Furthermore, there was a negative relationship between plasma prolactin and progesterone in non-breeding females. However, during the wet season, a relaxation of suppression occurs through reduced prolactin which corresponds with elevated levels of plasma progesterone in non-breeding females. Therefore, prolactin is hypothesised to be the primary hormone controlling reproductive

suppression and cooperative behaviours in non-breeding females. This study provides new endocrine findings for the maintenance of social suppression in the genus *Cryptomys*.

Keywords: Mole-rats; Reproductive suppression; Prolactin; Testosterone; Glucocorticoid; Stress

1. Introduction

Through the study of the subterranean family of the Bathyergidae (African mole-rats), many essential findings involving the ultimate and proximate factors that bring about the maintenance of social groups and socially induced infertility in mole-rats specifically and mammals generally have been uncovered. The answer as to how and why (ultimate factors) sociality has evolved in African mole-rats and other mammalian species has been well studied and is relatively well understood (Bennett et al., 1999; Bennett and Faulkes, 2000; Burda et al., 2000; Faulkes and Bennett, 2013; Firman et al., 2020; Jarvis et al., 1994). However, the physiological and/or behavioural mechanisms (proximate factors) that maintain sociality, group cohesion and dominance are less understood (Faulkes and Bennett, 2013).

The social organisation in the various species of African mole-rats ranges from strictly solitary to truly social (colony size range: 2–20) to eusocial (colony size range: 2–300) (Bennett and Faulkes, 2000). Social and eusocial African mole-rat species exhibit cooperative breeding, a reproductive division of labour, cooperative care of young (alloparental care), the overlap of at least two resident litters and often the apparent formation of a hierarchical structure in a colony (Bennett and Faulkes, 2000). Reproduction in these species is often monopolised by a single dominant breeding female (BF) and one or two of the largest dominant breeding males (BMs) within the colony (Bennett and Faulkes, 2000). The subordinate colony members (non-breeding females - NBFs and non-breeding males - NBMs) are reproductively quiescent but are not permanently sterile. Both NBFs and NBMs are able to reproduce but are naturally reproductively suppressed within the confines of their natal colony. These subordinate adults exhibit reproductive hormone concentrations similar to sexually immature juveniles, and anovulatory gonads are often possessed by NBFs (Bennett and Faulkes, 2000). Socially induced infertility is orchestrated through both behavioural, such as incest avoidance, and physiological means, by the dominant breeding individuals (Bennett et al., 1996, Bennett et al., 1999, Bennett et al., 2018; Blecher et al., 2020; Burda, 1995; Lutermann et al., 2013; Medger et al., 2018, Medger et al., 2019). The non-breeding colony members spend a large portion of their time foraging, expanding and defending their burrow system, and caring for the offspring of the BF (alloparental care) (Francioli et al., 2020; Houslay et al., 2020; Lacey and Sherman, 2009; Oosthuizen and Bennett, 2015; Scantlebury et al., 2006; Zöttl et al., 2018).

The two eusocial African mole-rat species, the naked mole-rat (*Heterocephalus glaber*) and the Damaraland mole-rat (*Fukomys damarensis*), have received the greatest attention, and these models have revealed behavioural and physiological control of reproductive inhibition, predominantly through neuroendocrine and endocrine mechanisms (Faulkes and Bennett, 2013). In both species, the reproductively suppressed individuals, NBMs and NBFs in naked mole-rats and NBFs only in Damaraland mole-rats, show a reduced pituitary sensitivity to gonadotropin-releasing hormone (GnRH) compared to their breeding counterparts (Bennett et al., 1993a; Faulkes et al., 1991). This finding implies socially induced impairments to the hypothalamic-pituitary-gonadal axis (HPG axis) in suppressed individuals. As a consequence, this leads to reduced testosterone concentrations detected in NBMs in naked mole-rats and lower or non-detectable oestrogen and progesterone levels recorded in NBFs of both species (Faulkes et al., 1990; Faulkes and Abbott, 1997; Molteno et al., 2004; Molteno and Bennett, 2000; Voigt and Bennett, 2018). However, the two species show dramatic differences in their mechanism of reproductive suppression.

In the spontaneously ovulating naked mole-rat, social suppression in subordinates has been suggested to be the result of interaction with the dominant BF who shoves non-breeders and exerts aggressive interaction towards them (as a result of increased testosterone) (Faulkes and Abbott, 2009; Clarke and Faulkes, 1997, Clarke and Faulkes, 2001). As a consequence, increased glucocorticoid levels in the subordinates (a proxy for an increase in both acute and chronic stress) may result (Bennett and Faulkes, 2000; Clarke and Faulkes, 1997; Faulkes and Abbott, 1997). Chronically high levels of glucocorticoids, such as cortisol, have been associated with decreased reproductive fertility and even reproductive suppression (Toufexis et al., 2014). Furthermore, in cooperatively breeding species, increased glucocorticoid concentrations have been linked to cooperative behaviour such as offspring care (alloparental care) in subordinate group members (Carlson et al., 2006a). Recently, breeding and non-breeding naked mole-rats were observed to possess similar levels of glucocorticoids, thus challenging the notion that glucocorticoids and chronic stress play a role in reproductive suppression and alloparental care in naked mole-rats (Edwards et al., 2020; Medger et al., 2019). A more plausible hypothesis for the control of physiological suppression of reproduction in naked mole-rats has come from the peptide hormone prolactin (Bennett et al., 2018). In mammals, prolactin regulates several processes, including the inhibition of the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), the control of milk production and the activation of parental care (Brown et al., 2014). Prolactin is also well-known for suppressing reproduction during lactation, and elevated levels (hyperprolactinemia) can disrupt reproductive function in both sexes (Kauppila et al., 1988; Ziegler, 2000). In naked mole-rat NBFs and NBMs, prolactin concentrations are as high or even higher than those recorded in BFs, even during lactation (Bennett et al., 2018). In contrast, hyperprolactinemia is absent in Damaraland mole-rats regardless of breeding status or sex; therefore, it is unlikely that prolactin plays a pivotal role in the reproductive suppression or alloparental care in Damaraland mole-rats (Bennett et al., 2018). In this species, NBFs show anovulation and reduced progesterone concentrations, while NBMs are reproductively similar to BMs, but lack the opportunity to breed due to an absence of unrelated NBFs while in the confines of their natal colonies (Faulkes et al., 1994; Jarvis and Bennett, 1993; Bennett et al., 1994). However, unlike their naked mole-rat counterparts, induced ovulation appears in Damaraland mole-rats (Voigt et al., 2021), and there is a strong aversion to inbreeding in this species (Burland et al., 2002; Cooney and Bennett, 2000). It has been hypothesised that a behavioural mode of suppression through the avoidance of breeding with related individuals in a colony (incest avoidance) or, if unrelated individuals are present in the colony, their reproductive attempts may be interrupted by a breeder of the same sex (Burland et al., 2004; Clarke et al., 2001), however, physiological suppression is still evident in NBFs (Bennett et al., 1996; Bennett et al., 1993b; Rickard and Bennett, 1997). As in naked mole-rats, breeding and non-breeding Damaraland mole-rats possess similar levels of glucocorticoids regardless of their sex, implying that chronic stress may not play a role in their reproductive suppression or alloparental care (Medger et al., 2018).

In contrast to the comparatively well-studied naked and Damaraland mole-rats, little is known regarding the mechanisms of reproductive suppression operating in the social genus *Cryptomys*. Thus, to further understand the potential mechanisms of reproductive suppression and the underlying endocrine control in African mole-rats and other cooperatively breeding mammal species, the genus *Cryptomys* provides an ideal model to unravel the underlying physiological mechanisms for socially induced infertility.

The basic reproductive biology of many subspecies of *Cryptomys*, including the Mahali mole-rat (*Cryptomys hottentotus mahali*), has been described (Janse van Rensburg et al., 2002; Oosthuizen et al., 2008; Spinks et al., 1997, Spinks et al., 1999; Hart et al., 2020; Hart et al., 2021). Hart et al. (2021) observed reproductive suppression in female subordinates but not male subordinates. Furthermore, year-round breeding and offspring production was observed in the Mahali mole-rat;

however, there were periods of the year (dry season) favouring increased reproduction to enable an increased likelihood of offspring survival (more offspring born in the wet season) (Hart et al., 2021). Similar to other African mole-rat species, the Mahali mole-rat showed a pattern of reproductive suppression relaxation during the wet season (Hart et al., 2021). However, the mechanisms of reproductive suppression and its potential endocrine control are unknown for most subspecies of *Cryptomys*. To date, it is believed that behavioural interactions (through aggression as a result of increased testosterone in breeders) and/or inbreeding avoidance are the primary strategies underlying the suppression of reproduction in the non-breeding colony members in the genus *Cryptomys* (Lutermann et al., 2013; Oosthuizen et al., 2008).

In this study, we investigate this assumption for the Mahali mole-rat by comparing several endocrine correlates implicated in the suppression of reproduction and alloparental care between the reproductive classes (BF, BM, NBF and NBM) and season (wet and dry). The hormone classes investigated include glucocorticoids (a stress-related indicator) through faecal glucocorticoid metabolites (fGCM), plasma testosterone (an indicator of aggression) and plasma prolactin. As a prerequisite, we examine the suitability of enzyme immunoassays for detecting changes in fGCM concentrations of Mahali mole-rats by performing an adrenocorticotropic hormone stimulation test (ACTH challenge test) on both male and female non-breeders.

Glucocorticoids were measured in faeces rather than in plasma, since animals need to be captured and restrained first for plasma sample collection and the induced stress response appears in plasma within minutes (Medger et al., 2018). The measurement of glucocorticoid metabolites in faeces alleviates this problem. Additionally, faecal sampling allows repeated monitoring of hormones over short intervals (similar to the methods used in ACTH challenges), which is especially advantageous in smaller mammals in which repeated blood sampling is impossible (Medger et al., 2018).

2. Materials and methods

2.1. Animal capture

A total of 224 Mahali mole-rats, comprising 109 males and 115 females, were captured within the study period between October 2016 and September 2017; with mole-rat colonies being captured monthly for an entire calendar year (Hart et al., 2021). A total of 118 individuals were captured within the wet season (December – May), and 106 individuals were captured within the dry season (June – November) (see Table 1 for full capture breakdown). 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 h over the course of the day and left overnight, being checked first thing in the morning. Entire colonies were caught out, with a colony being deemed to be completely trapped if no trap activity was observed five consecutive days after capture of the last animal (Hart et al., 2020). Capture sites were at smallholdings or farms in and around the area of Patryshoek, Pretoria (25°40' S, 28°2' E), South Africa. Permission to capture the mole-rats was obtained from all landowners, and a collecting permit was obtained from the relevant nature conservation authorities (Permit number: CPF6-0127). On capture, the sex and body mass (to the nearest 0.1 g) was recorded for each animal (Scout Pro SPU123, Ohaus Corporation, Pine Brook, New Jersey, U.S.A). The Animal Use and Care Committee of the University of Pretoria evaluated and approved the experimental protocol (ethics clearance number EC044-16 and NAS 128/2020) and DAFF section 20 approval (SDAH-Epi-20070806200).

Table 1. The total number of Mahali mole-rats (*C. h. mahali*) captured between October 2016 and September 2017. Animals captured were split between reproductive class (BF: Breeding female, BM: Breeding male, NBF: Non-breeding female, NBM: non-breeding male) and season (Wet: December – May, Dry: June – November).

Season	Reproductive class				
	BF	**BM**	**NBF**	**NBM**	**Total**
Wet	10	16	57	35	118
Dry	10	14	38	44	106
Total	20	30	95	79	224

2.2. Animal housing and dissection procedure

The captured animals were transported to the mole-rat laboratory at the University of Pretoria, Department of Zoology and Entomology. Complete colonies of mole-rats were housed together in plastic crates (49.5 × 28.0 cm). Nesting material, comprising of wood shavings and paper towelling were supplied to each colony. The mole-rats were fed daily on sweet potatoes and apples. All water requirements of the animals were satisfied with the provided food (Bennett and Jarvis, 1995). The animals were maintained in a climate-controlled room at a constant temperature of 25 ± 1 °C and a light cycle of 12 h light and 12 h dark.

The capture of animals (entire colonies) was finalised by the middle of each month (a total of 31 colonies were captured during the entire study, mean (±SE) colony size of 11.3 ± 0.4 individuals), and all animals were euthanised with an overdose of isoflurane in one day. However, to ensure that post-mortem examination was as accurate as possible, functionally complete colonies were maintained together for approximately 1 week after all individuals in a system had been trapped. Blood was obtained from the mole-rats by exsanguination from the heart and centrifuged at 1500*g* for 15 min. The separated plasma was stored at −70 °C until hormone analysis.

2.3. Determination of reproductive status

All individuals below 40 g with a dark coat colour were considered to be juveniles (Hart et al., 2021). Additionally, individuals captured alone in a tunnel system after a considerable trapping effort of 5 days were assumed to be dispersing individuals. The juveniles and dispersing individuals were not considered further for this study. The BMs were distinguishable from NBMs by their large, descended inguinal testes and yellow staining around the mouth. The BFs possessed prominent auxiliary teats and a perforated vagina, which was absent in the NBFs or dispersers. During the dissection process, the breeding status of a female was confirmed by the presence of foetuses or placental scars on the uterine horns of queens.

2.4. ACTH challenge and faecal collection

Three additional NBMs and three NBFs were captured during May 2018 and maintained in captivity for six months. These captive mole-rats were housed separately throughout the six month period. The mole-rats were subsequently moved to collection chambers with a wire-mesh bottom and a collection dish underneath for faecal collection. The wire-mesh bottom prevented any contamination of the faecal samples by urine. Collection chambers were checked hourly or bi-daily for faeces, depending on the protocol. Throughout the experiment, mole-rats were fed pieces of sweet potato and apple daily. No additional water was provided as the animals received all the water from their food (Bennett and Jarvis, 1995). Animals were placed in the collection chambers for ten days before the administration of ACTH to allow the mole-rats to acclimatise to their new

surroundings. The faecal collection started seven days prior to the ACTH challenge to obtain baseline values, and the collection continued for five days post-ACTH challenge. Chambers were checked for faeces every hour for the first 24 h post-ACTH injection and twice a day for the rest of the experimental days before and after treatment. Synthetic ACTH (Synacthen ® depot, Novartis, South Africa (Pty) Ltd) was dissolved in a sterile isotonic saline solution, and individuals were injected intramuscularly (0.3 ml) at 10:00 a.m. with a dose of 10 IU (100 μg) per 100 g body mass (Medger et al., 2018). The handling time during injection was <1 min. Sampling commenced one hour after injection (11:00 a.m.). Faeces were collected in Eppendorf tubes using tweezers. Tweezers were cleaned thoroughly with 70% ethanol between sampling events. All samples were frozen immediately after collection and stored at −20 °C until further preparation. The Animal Use and Care Committee of the University of Pretoria evaluated and approved the experimental protocol (ethics clearance number EC031–18).

2.5. Faecal collection of wild animals

The above collection protocol was similarly used for faecal collection from wild-caught individuals. Within the first 4 h, only the first faecal sample dropped by the wild-caught individuals was collected, frozen and subsequently analysed for the fCGM. In general, animals usually defecate within 2 h of capture.

2.6. Faecal hormone metabolite extraction and analysis

Faecal samples were lyophilised, pulverised and sieved through a wire-mesh strainer (Medger et al., 2018). Between 0.050 and 0.055 g of faecal powder was weighed out per sample and extracted using 1.5 ml of 80% ethanol. The suspension was shaken for 15 min on a multi-vortex and subsequently centrifuged for 10 min at 1500*g* (Medger et al., 2018). The supernatant was transferred into a clean Eppendorf tube and stored at −20 °C until analysis.

Faecal steroid extracts ($n = 72$) from all six ACTH treated animals were measured for immunoreactive fGCM concentrations using five different enzyme immune-assays (EIAs): (i) Cortisol; (ii) 11-oxoaetiocholanolone I (detecting 11,17 dioxoandrostanes); (iii) 11-oxoaetiocholanolone II (detecting fGCMs with a 5β- 3α-ol-11-one structure), (iv) Corticosterone, and (v) 5α-pregnane-3β,11β,21-triol-20-one (detecting fGCMs with a 5α-3β- 11β-diol structure). Detailed assay characteristics, including full descriptions of the assay components and cross-reactivities, have been provided for the 11-oxoaetiocholanolone I, cortisol and corticosterone EIAs by Palme (1997), 11-oxoaetiocholanolone II EIA by Möstl and Palme (2002) and for the 5α-pregnane-3β,11β,21-triol-20-one EIA by Touma et al. (2003). The sensitivities of the EIAs used are 1.8 ng/g faecal dry weight Corticosterone, 0.6 ng/g faecal dry weight for Cortisol, 11-oxoaetiocholanolone I, and 11-oxoaetiocholanolone II, and 2.4 ng/g faecal dry weight for 5α-pregnane-3β,11β,21-triol-20-one, respectively. The coefficients of variance for intra-assay variance were 5.67% and 6.90% for the Cortisol, 5.61% and 7.03% for the Corticosterone, 5.65% and 6.11% for the 11-oxoaetiocholanolone II, 2.21% and 2.15% for the 11-oxoaetiocholanolone I, and 4.27% and 4.34% for the 5α-pregnane-3β,11β,21-triol-20-one EIA, respectively. The coefficients of variance for inter-assay variance were 10.19% and 14.70% for the Cortisol, 12.83% and 13.58% for the Corticosterone, 10.07% and 13.48% for the 11-oxoaetiocholanolone II, 11.69% and 12.36% for the 11-oxoaetiocholanolone I, and 5.30% and 8.09% for the 5α-pregnane-3β,11β,21-triol-20-one EIA, respectively.

The Cortisol EIA discriminated best between pre- and peak ACTH-injection fGCM levels (see 3.2 Results) and was solely used for quantifying fGCMs in the sample extracts of the wild animals. Faecal steroid concentrations are presented as μg/g faecal dry weight (DW). EIAs were performed

at the Endocrine Research Laboratory, University of Pretoria, as described by Ganswindt et al., 2012, Ganswindt et al., 2014.

2.7. Plasma testosterone, progesterone, and prolaction analyses

Coat-a-count hormone kits (IBL International GmBH, Hamburg, Germany) were used to determine plasma testosterone and progesterone concentrations as described by Hart et al. (2021). All assays were carried out according to the manufacturer's protocol. Serial dilutions of respective plasma samples gave displacement curves that were parallel to the respective standard curves for both testosterone and progesterone. (Testosterone: $F_{[1,5]} = 0.18$; $p = 0.11$; Progesterone: $F_{[1,5]} = 2.27$; $p = 0.07$ (Chard, 1978)). The sensitivity of the testosterone assay was 0.015 ng/dl plasma, and the intra-assay coefficient of variation was 4.7%. The sensitivity of the progesterone assay was 1.48 nmol/L or 0.47 ng/ml, and the intra-assay coefficient of variation was 7.9%.

Plasma prolactin was quantified using an Elabscience Guinea pig PRL (Prolactin) ELISA kit (Elabscience Biotechnology Inc., Wuhan, China) as described by Bennett et al. (2018). The sensitivity of the assay was 0.1 ng/ml plasma and intra-assay precision and repeatability are <10%, according to the manufacturers guidelines. Serial dilutions of plasma samples gave displacement curves that were parallel to the respective standard curve with relative variation of the slope of the trend lines <2%. See supplementary material for the standard curve (Fig. S1).

2.8. Data analyses

All statistical analyses were performed in R 3.5.2, and statistical significance was assumed at $p \leq 0.05$. All data are presented as mean ± standard error (SE).

Mean individual baseline values for fGCM were calculated from the samples collected seven days prior to ACTH administration. Baseline values were used to calculate the percentage change of fGCM concentration during the ACTH challenge. Average individual fGCM concentrations were calculated every 12 h from 0 to 24 h post-treatment and for 24 h intervals for the rest of the post ACTH challenge period. Faecal GCM concentrations of captive individuals were compared between the sexes and the 13 experimental times before and after the ACTH challenge using generalised mixed models (GLMM) with the individual as a random factor. Sex and sampling time and the interaction of sex and sampling time were included as independent factors in the GLMM.

The normality of dependent variables (body mass and hormone concentrations) was determined using Shapiro-Wilk tests (S—W). Non-normally distributed dependent variables were transformed (either log or square-root) to obtain normal distributions if possible. The homogeneity of normally distributed data was confirmed using Levene's test. Normally distributed dependent variables were analysed using General linear models (GLMs). All non-normal dependent variables were analysed using Generalised linear models (GLZMs) fitted with gamma distributions and link-identity functions using the *lme4* package. *Post-hoc* comparisons were made using Tukey's honestly significant difference (HSD) tests.

Models contained hormone concentrations (plasma prolactin, plasma testosterone, or fGCM) or body mass as response variables and reproductive class (BF, NBF, BM and NBM) and season (Dry and Wet) as predictors, with two-way interactions included. Model selection was conducted for each model using the *dredge* function of the *Mumin* package (Barton and Barton, 2015). Model suitability was assessed using Akaike information criterion values corrected for a small sample size (AICc). Models with ΔAICc <2 were considered equally parsimonious, the coefficients of which were subsequently averaged to construct a final model. Conditional average values were reported

for final models with more than one competing model. The final models selected are presented in the supplementary material.

A total of 180 individuals were assessed for fGCMs (BF: n = 17, NBF: n = 68, BM: n = 29, NBM: n = 66). The fGCM data were log-transformed to obtain a normal distribution. Two separate models were used to analyse plasma testosterone data due to the extremely high values of BMs. The first model retained BMs (213 individuals) (BF: n = 20, NBF: n = 89, BM: n = 30, NBM: n = 74), while the second model possessed only BF, NBF and NBM data with the exclusion of BM data (197 individuals; NBF: n = 89, BM: n = 30, NBM: n = 74. Plasma testosterone data for both models could not be transformed into a normal distribution. A total of 90 individuals were assessed for plasma prolactin (BF: n = 13, NBF: n = 52, BM: n = 10, NBM: n = 15). Plasma prolactin data were log-transformed to obtain a normal distribution. Due to insufficient data, seasonal comparisons of plasma prolactin were made for female individuals only in a separate model (65 females; BF: n = 13, NBF: n = 52). Additionally, 90 females (BF: 20, NBF: 70) were assessed for variation of plasma progesterone concentrations between reproductive class and season (non-normally distributed data). Body mass data of 212 individuals were analysed (BF: n = 20; NBF: n = 88; BM: n = 28; NBM: n = 76), and a normal distribution was obtained after a square-root transformation. See supplementary material for further details.

To investigate if prolactin was linked to reproductive suppression in NBFs, Spearman's rank-order correlations were conducted between plasma prolactin and plasma progesterone. Likewise, to investigate if stress is linked to reproductive suppression in NBFs, Spearman's rank-order correlations were conducted between fGCM and plasma progesterone. Furthermore, the correlation between plasma testosterone and plasma progesterone was investigated in NBFs. Further correlations were conducted between body mass and four variables, respectively, namely fGCM, plasma prolactin, plasma testosterone and plasma progesterone.

To investigate if prolactin or stress (fGCM) were linked to reproductive suppression, Spearman's rank-order correlations were conducted for NBMs between plasma prolactin and plasma testosterone, as well as between fGCMs and plasma testosterone, respectively. Furthermore, Spearman rank correlations were conducted between body mass and three variables respectively, namely plasma prolactin, plasma testosterone and fGCM, respectively.

3. Results

3.1. Body mass

The body mass of the Mahali mole-rat was explained by season and reproductive class (Table S1). Body mass differed significantly between reproductive status throughout the year (F = 44.3, df = 3, $p < 0.001$, Fig. 1a) and was significantly affected by season (F = 10.65, df = 1, $p \leq 0.002$, Fig. 1b). Breeding males were significantly heavier than BFs ($p \leq 0.008$), NBFs ($p < 0.001$) and NBMs ($p < 0.001$) respectively, throughout the year (Fig. 1a). BFs were significantly heavier than both NBMs ($p \leq 0.003$) and NBFs ($p < 0.001$), while NBMs were significantly heavier than NBFs throughout the year ($p \leq 0.004$). Furthermore, individuals captured in the dry season were significantly heavier than those captured in the wet season (F = 13.39, df = 1, $p < 0.001$, Fig. 1b).

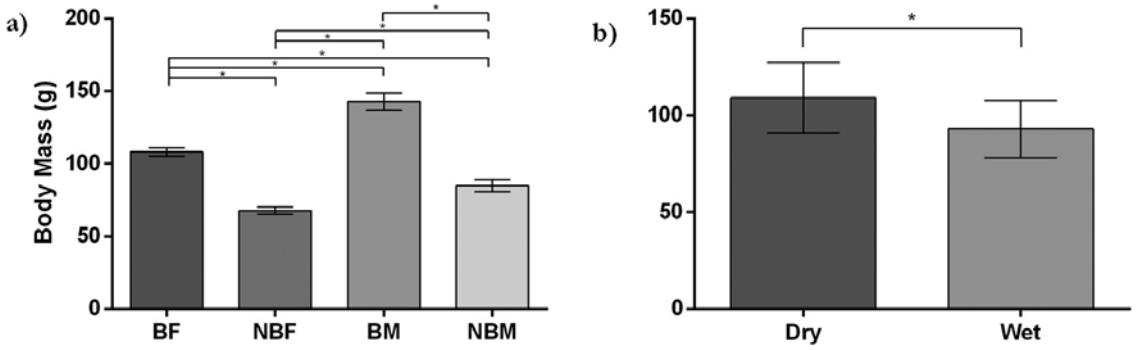

Fig. 1. Bar graphs showing differences in body mass (g) of Mahali mole-rats (*C. h. mahali)* by a) reproductive class (BF: Breeding female; BM: Breeding male; NBF: Non-breeding female; NBM: non-breeding male) and b) season (Wet: December – May; Dry: June – November). * indicates significant difference ($p < 0.05$).

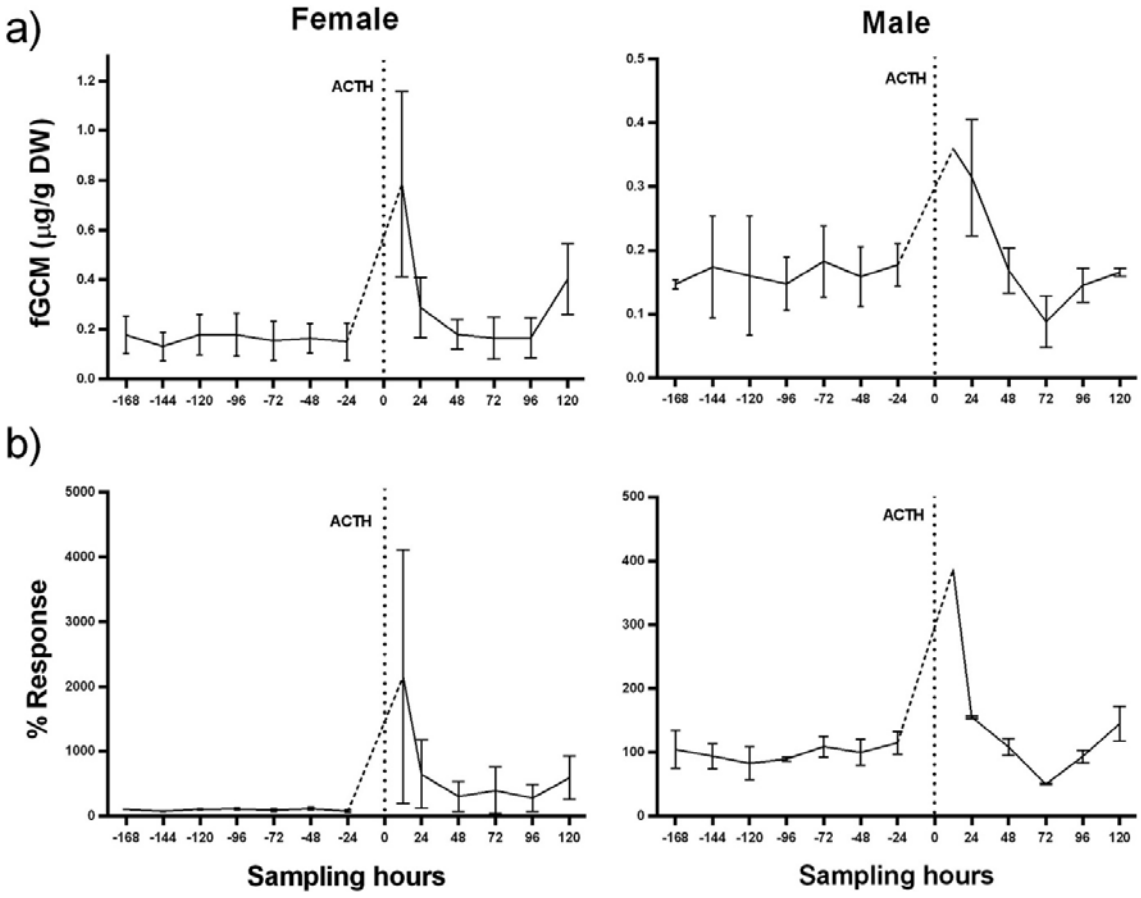

Fig. 2. Concentrations (µg/g DW) of a) faecal glucocorticoid metabolites (fGCM) and b) % response to baseline values of male and female Mahali mole-rats (*C. h. mahali)*. fGCM and % response values (mean ± standard error) are shown for 168 h prior to and 120 h after a challenge with synthetic ACTH. Note: only one faecal sample was collected for the male cohort at the sampling hour 24.

3.2. ACTH challenge

Faecal GCM concentrations were significantly elevated after the injection of ACTH ($F = 4.54$, $df = 12$, $p \leq 0.001$). At 12 h post-ACTH administration, fGCM concentrations were 200 ± 113.0% above baseline ($p \leq 0.03$) and remained significantly elevated until 24 h post-treatment ($p \leq 0.05$). Faecal GCM concentrations returned to baseline levels approximately 48 h after treatment. Overall mean fGCM concentrations differed significantly between females (0.22 ± 0.03 μg/g DW) and males (0.17 ± 0.02 μg/g DW) ($F = 0.64$, $df = 1$, $p \leq 0.001$, Fig. 2a and b).

The interaction between sex and time was not significant (GLMM: $F = 0.82$, $df = 12$, $p = 0.63$), and as such, the response of fGCM concentrations in the mole-rats to an ACTH challenge appeared to be similar for both females and males (Fig. 2a and b). However, the response of males 12 h (387.0%) and 24 h (155.6 ± 2.0%) post-ACTH challenge was considerably less than that of the response arising in females 12 h (2155.0 ± 1957.1%) and 24 h (649.9 ± 525.8%) post-ACTH challenge (Fig. 2a and b). This difference, however, is not significant ($F = 0.82$, $df = 12$, $p = 0.63$).

3.3. fGCMs

The variation in fGCM concentrations between individuals was different depending on their reproductive status (Table S2). Reproductive status had a significant effect on the fGCM concentrations ($F = 3.2$, $df = 3$, $p \leq 0.03$; Fig. 3) with NBFs showing significantly higher levels of fGCMs than BFs ($p \leq 0.05$) throughout the year. Breeding males showed similar fGCM levels to NBMs ($p = 0.66$) and BFs ($p = 0.89$), respectively (Fig. 3). Furthermore, NBFs also showed similar concentrations of fGCMs to BMs ($p = 0.12$) and NBMs ($p = 0.51$), respectively (Fig. 3).

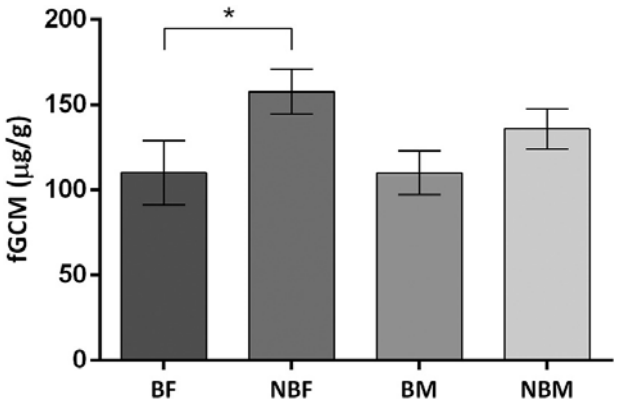

Fig. 3. Bar graph showing differences in faecal glucocorticoid metabolite (fGCM) (μg/g DW) concentrations of Mahali mole-rats (*C. h. mahali)* by reproductive status (BF: Breeding female; BM: Breeding male; NBF: Non-breeding female; NBM: non-breeding male). Data shown as mean ± SEM. * indicates significant difference ($p < 0.05$).

3.4. Plasma testosterone

The variation in plasma testosterone concentration of BM data was explained by reproductive status and season, respectively (Table S3). Reproductive status significantly affected plasma testosterone concentrations ($z = 3.00$, $p < 0.003$, Fig. 4a), while season did not have a significant effect throughout the sampling period ($z = 1.01$, $p \geq 0.31$). Breeding males had significantly higher plasma testosterone concentrations compared to BFs, NBFs and NBMs ($p < 0.001$, for all, Fig. 4a). Similar concentrations of plasma testosterone concentrations were found in BFs, NBFs and NBMs ($p > 0.05$, for all, Fig. 4a).

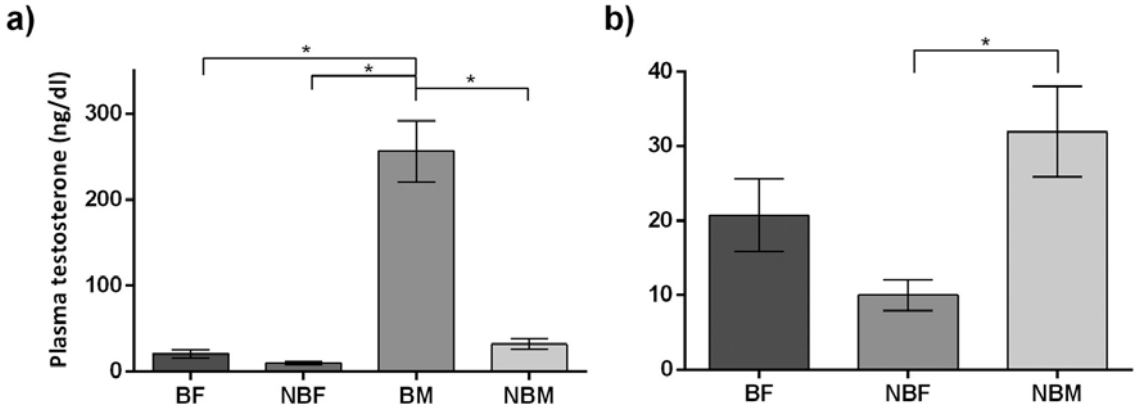

Fig. 4. Bar graph showing differences in plasma testosterone concentrations (ng/dl) of Mahali mole-rats (*C. h. mahali)* by a) reproductive class (BF: Breeding female; BM: Breeding male; NBF: Non-breeding female; NBM: non-breeding male) and b) reproductive status, excluding BMs. * indicates significant difference ($p < 0.05$).

Once excluding BMs, the variation in plasma testosterone concentrations between NBMs, BFs and NBFs was explained by both reproductive status and season (Table S4). Plasma testosterone concentrations were significantly affected by reproductive status ($z = 3.178$, $p < 0.002$, Fig. 4b), but not by season ($z = 1.018$, $p \geq 0.3$). NBMs showed significantly higher levels of plasma testosterone compared to NBFs ($p < 0.001$) and similar levels to BFs ($p = 0.45$, Fig. 4b). Additionally, BFs and NBFs possessed similar plasma testosterone concentrations throughout the sampling period (HSD: $p = 0.47$).

3.5. Plasma progesterone

The variation in female plasma progesterone concentrations was explained by reproductive status and season (Table S4). Both season ($t = 4.57$, $p < 0.001$) and reproductive status ($t = -3.07$, $p < 0.003$) had a significant effect on plasma progesterone concentrations (Fig. 6). Breeding female plasma progesterone concentrations were significantly higher than that of NBFs ($p < 0.001$, Fig. 5a) throughout the sampling period. Further analysis revealed that plasma progesterone concentrations were found to be significantly higher during the wet season compared with the dry season ($p < 0.001$, Fig. 5b).

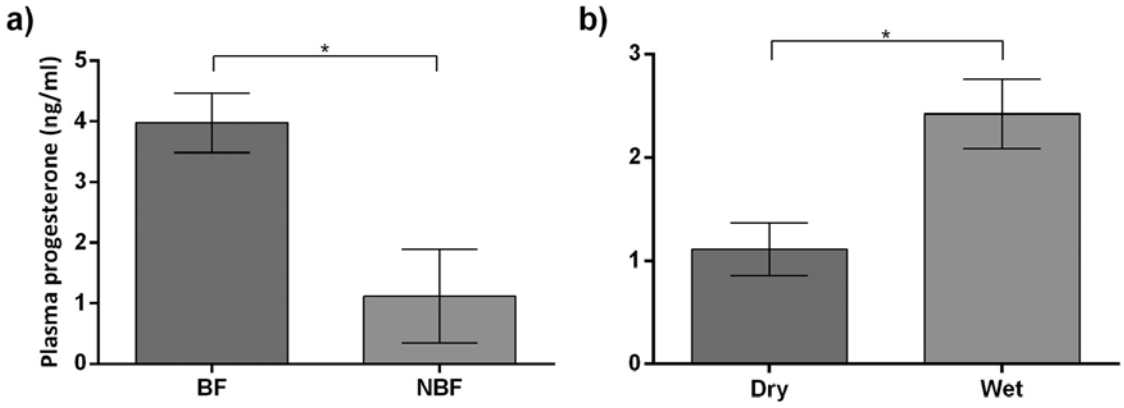

Fig. 5. Bar graphs showing differences in plasma progesterone (ng/ml) concentrations of Mahali mole-rat (*C. h. mahali)* females by a) reproductive status (BF: Breeding female; NBF: Non-breeding female) and b) season (Wet: December – May; Dry: June – November). Data shown as mean ± SEM. * indicates significant difference ($p < 0.05$).

3.6. Plasma prolactin

Plasma prolactin concentrations differed significantly between reproductive statuses throughout the sampling period ($F = 5.412$, $df = 3$, $p \leq 0.002$, Fig. 6a). Breeding females and NBFs showed comparable concentrations of plasma prolactin ($p = 0.98$, Fig. 7a). Likewise, BMs and NBMs also showed similar concentrations of plasma prolactin ($p = 0.89$, Fig. 6a), while NBFs showed significantly higher plasma prolactin concentrations compared to BMs and NBMs, respectively ($p \leq 0.05$ for both, Fig. 6a). Furthermore, BFs had significantly higher plasma prolactin concentrations than BMs (HSD: $p \leq 0.03$) but similar levels to NBMs (HSD: $p = 0.089$, Fig. 6a).

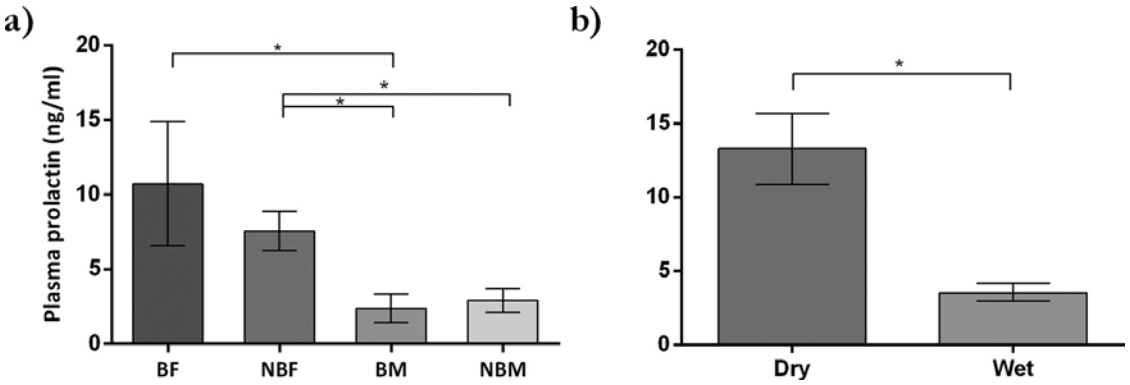

Fig. 6. Bar graphs showing differences in plasma prolactin (ng/ml) concentrations of Mahali mole-rats (*C. h. mahali)* by a) reproductive status (BF: Breeding female; BM: Breeding male; NBF: Non-breeding female; NBM: non-breeding male) and b) season for female individuals. Data shown as mean ± SEM. * indicates significant difference ($p < 0.05$).

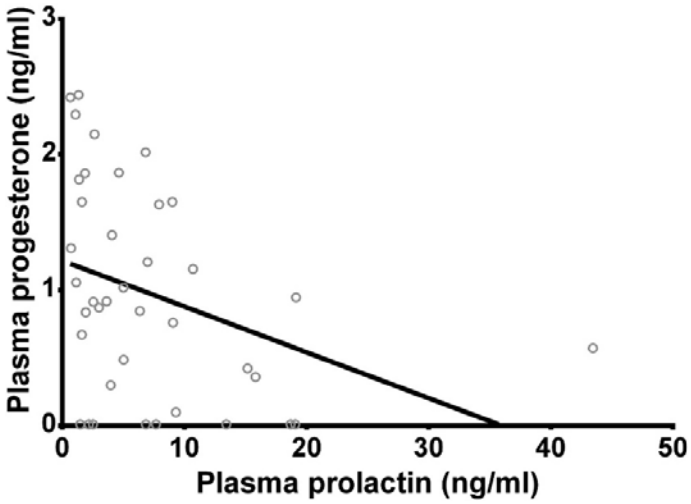

Fig. 7. The negative relationship between plasma prolactin (ng/ml) and progesterone (ng/ml) in non-breeding female mole-rats (*C. h. mahali).*

On removing males, season alone explained the variation in plasma prolactin concentrations between breeding and non-breeding females (Table S4). Plasma prolactin levels of both breeding and non-breeding females were observed to be significantly higher in the dry season than in the wet season ($F = 32.8$, $df = 1$, $p < 0.001$, Fig. 6b).

3.7. Correlational links between hormones and body mass in non-breeders

Non-breeding females with the highest concentration of plasma prolactin also had the lowest concentrations of plasma progesterone ($r = -0.44$, $p = 0.06$, Fig. 7). Contrastingly, plasma testosterone and fGCM concentrations did not affect plasma progesterone concentrations in NBFs ($r \leq -0.07$, $p \geq 0.57$). Body mass did not affect fGCM, or plasma prolactin, testosterone, or progesterone concentrations ($r \leq 0.05$, $p \geq 0.63$).

Neither plasma prolactin ($r \leq 0.13$, $p \geq 0.62$) nor fGCM concentrations ($r \leq -0.12$, $p \geq 0.34$) were significantly correlated with plasma testosterone concentrations. Non-breeding males with the highest body mass had the highest testosterone concentrations ($r = 0.39$, $p < 0.001$). Contrastingly, there were no significant correlations between body mass and prolactin ($r \leq -0.42$, $p \geq 0.12$), nor between body mass and fGCM concentrations ($r \leq -0.13$, $p \geq 0.27$).

4. Discussion

This study suggests that a combination of behavioural and physiological reproductive suppression modes are operational in the Mahali mole-rat. Similar to the Natal (*C. h. natalensis*), Damaraland and naked mole-rats, increased circulating testosterone levels were observed in breeding Mahali mole-rats of both sexes compared to their non-breeding counterparts (Faulkes et al., 1991; Lutermann et al., 2013; Medger et al., 2019; Swift-Gallant et al., 2015). Therefore, the increased testosterone, coupled with increased body mass, would result in the physical dominance of breeders over their non-breeding counterparts. As a result, breeders may interrupt breeding attempts between unrelated colony members, or unrelated NBMs, and the BF. Furthermore, non-breeders may also be the object of aggression that may lead to chronic stress and thus chronic exposure to increased levels of glucocorticoids.

Unlike the scenario presented in Damaraland and naked mole-rats, a clear relationship is visible between the hypothalamic-pituitary-adrenal (HPA) axis and the HPG axis, and consequently reproductive hormones, such as testosterone and progesterone, in the Mahali mole-rat. This study provides evidence that reproductive suppression and possibly alloparental care may arise from increased fGCM concentrations (glucocorticoids), possibly by chronic stress. Non-breeding females possessed significantly higher fGCMs than BFs throughout the year. It is possible that this may arise as a result of them being targeted by increased aggression directed towards them by BFs. Chronically high glucocorticoid levels have been linked to reduced reproductive abilities (Toufexis et al., 2014) and increased cooperative behaviours such as offspring care (Carlson et al., 2006a) in cooperatively breeding meerkats. Consequently, one of the possible mechanisms inhibiting NBF breeding (reproductive suppression) within the colony may be chronically high levels of glucocorticoids, but further research is required to substantiate this statement. In contrast, NBMs, which are not reproductively suppressed within the confines of the colony (Hart et al., 2021), exhibited similar levels of fGCMs to BMs throughout the year. Unrelated NBMs cannot attempt breeding with NBFs, as these NBFs are reproductively suppressed within the colony and, therefore, it is likely that BMs interrupt breeding attempts between unrelated NBMs or the BF (Bennett and Faulkes, 2000). Breeding interruptions would be a short confrontation (acute stress) and therefore would not result in chronic stress, or prolonged levels of elevated glucocorticoids, resulting in no reproductive suppression. Likewise, incest avoidance by the NBMs would also not induce increased levels of fGCMs.

On closer examination of the HPA axis of the Mahali mole-rat, sex differences are evident in the adrenal sensitivity to ACTH. Female Mahali mole-rats show increased sensitivity to administered ACTH when compared to males resulting in females producing higher fGCMs for the same ACTH

dose related stimulation. The dominant route of excretion (urine or faeces) of glucocorticoids may vary between the sexes (Teskey-Gerstl et al., 2000); however, it can be hypothesised that the female HPA axis has become more sensitive to stressors to allow for efficient cooperative breeding to occur in this species. In humans (*Homo sapiens*), females produce more ACTH from their pituitary gland than males in response to the same stressor, but have similar adrenal responses to ACTH, resulting in similar glucocorticoid levels (Gallucci, 1991). While in rats (*Rattus norvegicus*), as with the Mahali mole-rat, females typically have a more robust neuroendocrine response to stress, as evidenced by their increased glucocorticoid and ACTH response compared to males (Heck and Handa, 2019). In contrast to the Mahali mole-rat, however, female rats have been shown to have a delayed return to baseline ACTH and glucocorticoid levels after stress (Heck and Handa, 2019). Male Mahali mole-rats show a delayed return in glucocorticoid levels when compared to females, indicating sex differences in the negative feedback regulation of the HPA axis, which is in contrast to rats (Heck and Handa, 2019).

As with fGCMs, plasma prolactin appears to play a critical role in reproductive suppression and cooperative care in the Mahali mole-rat. Similar to naked mole-rats (Bennett et al., 2018), NBFs possessed comparable concentrations of plasma prolactin to BF Mahali mole-rats. However, the plasma prolactin titres of both NBFs and BFs were not at the level of hyperprolactinemia (greater than 20 ng/ml), or the levels of prolactin recorded for the naked mole-rat by Bennett et al. (2018). Plasma prolactin levels for the Mahali mole-rats were, however, higher than that of naked mole-rats reported in Medger et al. (2019) and Damaraland mole-rats in Bennett et al. (2018). Elevated prolactin is well known to suppress fertility and mediate parental and alloparental care in cooperatively breeding mammals and birds (Carlson et al., 2006b; Schoech et al., 1996). One would expect that NBFs, which do not breed or lactate, would have low or even undetectable levels of prolactin; thus the unexpected high plasma prolactin levels in NBFs may inhibit GnRH release, and as a consequence the downstream levels of LH, FSH and subsequent production of oestrogen and progesterone, leading to the well-characterized anovulation in NBF Mahali mole-rats (Hart et al., 2021). This conclusion is supported by NBFs possessing lower plasma progesterone concentrations than BFs in addition to the lack of corpora lutea of ovulation (Hart et al., 2021). Furthermore, NBFs that possess higher plasma prolactin levels also possessed lower plasma progesterone levels, suggesting that prolactin acts as an inhibitory factor of progesterone release in the Mahali mole-rat (this study). In addition, we can speculate that increased plasma prolactin, as with the increased glucocorticoids of NBFs, may also play a part in the mechanisms eliciting cooperative behaviour (Bennett et al., 2018). Both BMs and NBMs possessed lower plasma prolactin titres than BFs and NBFs; this is somewhat expected as both NBMs and BMs can breed within the colony, and it is unlikely that they perform many alloparental care tasks (Hart et al., 2021).

Unlike fGCMs, plasma prolactin in female Mahali mole-rats showed a variation between seasons, as females captured in the dry season possessed higher plasma prolactin concentrations than those captured in the wet season. The possible reason for this could be two-fold: firstly, a greater number of pregnant BFs were captured in the dry season ($n = 6$) compared to the wet season ($n = 4$) (see Hart et al., 2021). Pregnancy has been observed to increase circulating levels of prolactin (Grattan, 2001). Secondly, there is an apparent relaxation of suppression of reproduction during the wet season in NBF Mahali mole-rats, as illustrated by increases in progesterone levels of females in the wet season, even though there are fewer pregnant BFs (this study; Hart et al., 2021). Hart et al. (2021) reported indications of relaxation of suppression in NBF Mahali mole-rats supported by increased plasma progesterone concentrations and more frequent events of female dispersal during the months of the wet season, whereas increased suppression in NBFs was observed during the months of the dry season. Dispersal from the natal colony occurs under favourable environmental conditions when the soil characteristics are optimal for excavation and digging, such as periods of

good rainfall (Jarvis et al., 1994; Kotze et al., 2008). This phenomenon has been recorded in several social and eusocial mole-rats, including the common mole-rat (*C.h hottentotus*) (Spinks et al., 1997, Spinks et al., 1999), highveld mole-rat (*C. h. pretoriae*) (Janse van Rensburg et al., 2002), Damaraland mole-rat (Molteno and Bennett, 2002) and the naked mole-rat (Westlin et al., 1994).

The relaxation of suppression in NBFs seen during the wet season may have an effect on fGCM concentrations, as BFs may increase aggression towards NBFs to discourage dispersal (Kutsukake and Clutton-Brock, 2006). This may, in turn, result in similar fGCM levels between the seasons in NBF Mahali mole-rats. Consequently, prolactin may be the primary controlling factor in reproductive suppression in the Mahali mole-rat; as adrenocortical cell hypertrophy (Silva et al., 2004) and increased adrenal sensitivity to ACTH stimulation has been observed to result in increased glucocorticoid levels in individuals exhibiting increased levels of prolactin (Jaroenporn et al., 2007).

5. Conclusion

This study provides an overview of the controlling endocrine mechanisms that possibly mediate both reproductive suppression and alloparental care in the cooperative breeding, social Mahali mole-rat. The study presents a novel understanding of the proximate factors involved in the maintenance of sociality in African mole-rats and possibly other social mammalian species. Furthermore, the results show possible interactions between prolactin, the HPA axis and the HPG axis and highlight the dearth of knowledge regarding these interactions. A better understanding of how natural reproductive suppression occurs may allow for greater strides in hormone therapies and contraceptives. Furthermore, the health benefits of being naturally suppressed have been recently highlighted in African mole-rats, with the causes of the benefits being linked to endocrine mechanisms controlling reproductive suppression (Jacobs et al., 2021a, Jacobs et al., 2021). Further research on the understudied species, such as those in the genus *Cryptomys,* will be required to further our knowledge on how social groups are kept together in mammals as well as the potential benefits of reproductive suppression and social living.

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