

Filling in the holes: The reproductive biology of the understudied Mahali mole-rat (*Cryptomys hottentotus mahali*)

D.W. Hart, K. Medger, B. van Jaarsveld, and N.C. Bennett

Abstract: African mole-rats have provided great insight into mammalian evolution of sociality and reproductive strategy. However, some species have not received attention, and these may provide further insights into these evolutionary questions. The cooperatively breeding Mahali mole-rat (*Cryptomys hottentotus mahali* (Roberts, 1913)) is one such species. Body mass, reproductive-tract morphometrics, gonad histology, and plasma reproductive hormone concentrations were studied for breeding and non-breeding males and females over 1 year. This study aimed to discern if this species exhibits a seasonal or aseasonal breeding pattern and whether there is a relaxation of reproductive suppression at any point in the year in non-breeding animals. The pattern of reproductive relaxation during the wetter months is similar to other African mole-rat species. Interestingly, births and pregnant breeding females were recorded throughout the year, thus indicating an aseasonal breeding strategy, despite inhabiting a region that experiences seasonal rainfall. However, there were periods of the year favouring increased reproduction to enable an increased likelihood of offspring survival. This suggests that the Mahali mole-rat may be an opportunistic breeder possibly brought about by the benefits of living in a cooperatively breeding group and potentially moving into more arid environments that were previously unexploited by the genus *Cryptomys* Gray, 1864.

Key words: reproduction, opportunistic breeder, aseasonal, reproductive suppression, Mahali mole-rat, *Cryptomys hottentotus mahali*, cooperative breeding.

Résumé : Les rats-taupes africains ont fourni d'importants renseignements sur l'évolution de la socialité et des stratégies de reproduction chez les mammifères. Certaines espèces qui n'ont pas reçu d'attention pourraient toutefois fournir encore plus d'information sur ces questions touchant à l'évolution. Le rat-taupe de Mahali (*Cryptomys hottentotus mahali* (Roberts, 1913)), dont la reproduction est coopérative, est une de ces espèces. La masse corporelle, la morphométrie du système reproducteur, l'histologie des gonades et les concentrations d'hormones de reproduction dans le plasma ont été étudiées chez des mâles et femelles reproducteurs et non reproducteurs sur une période d'un an. L'étude visait à établir si le motif de reproduction de cette espèce est saisonnier ou non saisonnier et s'il y a relâchement de la suppression de la reproduction à quelque moment de l'année chez les spécimens non reproducteurs. Le motif de relâchement de la suppression de la reproduction durant les mois plus pluvieux est semblable à celui d'autres espèces de rats-taupes africains. Des naissances et des femelles reproductrices gestantes ont notamment été observées tout au long de l'année, ce qui témoigne d'une stratégie de reproduction non saisonnière, malgré la saisonnalité de la pluviosité dans la région étudiée. Certaines périodes de l'année favorisent toutefois une reproduction accrue pour permettre une plus grande probabilité de survie de la progéniture. Cela refléterait la nature opportuniste de la reproduction du rat-taupe de Mahali, découlant possiblement des avantages que confèrent le fait de vivre au sein d'un groupe à reproduction coopérative et, éventuellement, le fait d'investir des milieux plus arides auparavant non exploités par le genre *Cryptomys* Gray, 1864. [Traduit par la Rédaction]

Mots-clés : reproduction, géniteur opportuniste, non saisonnier, suppression de la reproduction, rat-taupe de Mahali, *Cryptomys hottentotus mahali*, reproduction coopérative.

Introduction

The study of the subterranean family, the Bathyergidae (African mole-rats), has allowed for many essential revelations in mammalian evolution, behaviour, and physiology (Bennett and Faulkes 2000; Sherman et al. 2017). Furthermore, this family provides an important study system for the better understanding of how mammals adapt behaviourally, physiologically, and molecularly to their inhabiting environment (Broekman et al. 2006; Eigenbrod et al. 2019; Ivy et al. 2020; Logan et al. 2020; McGowan et al. 2020; Barker et al. 2021).

A focal point of research on African mole-rats includes the broad spectrum of social organisation found in the family Bathyergidae, as well as their range of reproductive strategies. Social organisation in African mole-rats ranges from strictly solitary species through to truly social (colony size range of 2–20 in social species) to eusocial (colony size range of 2–300 in eusocial species), with breeding occurring either seasonally or aseasonally (breeding throughout the year) depending on the species (Bennett and Faulkes 2000). The aridity food-distribution hypothesis, which correlates the sociality of mammals with habitat aridity, is the primary hypothesis used to explain the phenomena

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of differing degrees of sociality in African mole-rats as in other rodents (Jarvis et al. 1994; Bennett et al. 1999; Firman et al. 2020). Breeding in all solitary and some social African mole-rat species is seasonal, with the activation of breeding coinciding with the increase in soil moisture content and the sudden flushes of primary production, which occurs as a consequence of the onset of rainfall (Jarvis 1969; Bennett and Jarvis 1988a; Bennett 1989; Spinks et al. 1997, 1999; Janse Van Rensburg et al. 2002; Herbst et al. 2004; Hart et al. 2006). In contrast, the vast majority of the social and eusocial African mole-rats species breed throughout the year (Bennett and Jarvis 1988b; Bennett and Aguilar 1995; Oosthuizen et al. 2008; Sichilima et al. 2011).

Social and eusocial bathyergids exhibit cooperative breeding and a reproductive division of labour where reproduction is often monopolised by a single breeding female (BF) and one to three of the largest males (breeding males (BMs)) within the colony (Bennett and Faulkes 2000). The remaining colony members (non-breeding females (NBFs) and non-breeding males (NBMs)) are reproductively quiescent, where both NBFs and NBMs can reproduce, but are naturally reproductively suppressed by the dominant breeding individuals (Bennett and Faulkes 2000). This reproductive suppression can be physiological (Bennett et al. 2018; Medger et al. 2019; Blecher et al. 2020) or behavioural (Burda 1995; Bennett et al. 1997; Lutermann et al. 2013), or even a combination of the two (Bennett et al. 1996). The extent of the suppression varies between the species and the sexes (Bennett et al. 1996). Some non-breeding colony members attempt to escape this suppression by dispersing and forming their own colonies (Bennett and Faulkes 2000). 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; Molteno and Bennett 2002a; Scantlebury et al. 2006). If rainfall controls dispersal periods, then reproductive inhibition in non-breeding colony members may be relaxed during these periods because of the greater probability of successful independent reproduction (Molteno and Bennett 2002a). This phenomenon of relaxation of suppression has been found in several social African mole-rat species (Bennett 1989; Spinks et al. 1997, 1999; Janse Van Rensburg et al. 2002).

Even though extensive work has been conducted on the reproductive biology of African mole-rat species, some subspecies in the genus *Cryptomys* Gray, 1864 have remained neglected. One such subspecies is the social Mahali mole-rat (*Cryptomys hottentotus mahali* (Roberts, 1913)), which is found in the relatively semi-arid bushveld regions of Gauteng, North West Province, and the Northern Cape of South Africa (van Jaarsveld et al. 2019; Fagir et al. 2021; Wallace et al. 2021). Previous studies that have focussed on the reproductive strategies of the various subspecies of *Cryptomys hottentotus* (Lesson, 1826) have shown surprisingly varied results. For example, the common mole-rat (*Cryptomys hottentotus hottentotus* (Lesson, 1826)) (Bennett 1989; Spinks et al. 1997) and highveld mole-rat (*Cryptomys hottentotus pretoriae* (Roberts, 1913)) (Janse Van Rensburg et al. 2002) are found in regions with seasonal climatic variations, including seasonal rainfall, that result in both subspecies exhibiting a strongly seasonal reproductive pattern. In contrast, the Natal mole-rat (*Cryptomys hottentotus natalensis* Roberts, 1913), which also inhabits regions characterised by a seasonal climate but where rainfall can occur at any time of the year, is an aseasonal breeder (Oosthuizen et al. 2008). The diverse reproductive strategies of the *Cryptomys* subspecies allows us to ask an important question: are seasonal climatic variations the root cause of the varying reproductive strategies in African mole-rats and possibly other cooperative breeding and social mammal species? Research on little-studied subspecies, such as the Mahali mole-rat, may allow us to answer this question.

The Mahali mole-rat shares a close genetic relatedness (Faulkes et al. 2004; Broekman et al. 2006) and a close distributional range, as well as similar climatic conditions, with those of the highveld

mole-rat, except that in the former subspecies temperature is, on average, 5 °C warmer and the area has 10% less humidity throughout the year (van Jaarsveld et al. 2019; Fagir et al. 2021; Wallace et al. 2021). The Mahali and highveld mole-rats experience distinct seasonal climates with wet, warm summers and cool, dry winters throughout their distributional range. Since both species occur in close proximity to one another and are phylogenetically closely related, we posited that the Mahali mole-rat should exhibit a similar pattern of reproductive biology and strategies to that of the highveld mole-rat as described in Janse Van Rensburg et al. (2002). Consequently, the specific aims of this study were threefold: (1) to determine if the Mahali mole-rat is a seasonal breeder, (2) to assess whether there is a relaxation of reproductive suppression in non-breeding members of the colony at any point in the year, and (3) to determine if seasonality of breeding (if present) can be linked to seasonal variation in rainfall. We will subsequently compare our findings to current literature in the hope to better understand the root cause of the varying reproductive strategies.

Materials and methods

Animal capture

A total of 31 Mahali mole-rat colonies were captured within the study period of October 2016 and September 2017. Due to the small number of breeding individuals captured each month (≤ 5 queens/month), the 12 months of the year were combined into four southern hemisphere seasons. Nine colonies were captured in summer (December–February), 11 colonies in autumn (March–May), 6 colonies in winter (June–August), and 5 colonies in spring (September–November). A total of 223 animals comprising 109 males and 114 females were captured in total. Mole-rats were captured using Hickman live traps, baited with a small piece of sweet potato (Hickman 1979). The traps were placed at the entrance of excavated burrows. Entire colonies were caught, and a colony was deemed to be entirely trapped out if no trap activity was observed within five consecutive days. The capture of a full colony took no longer than seven consecutive days. Capture sites were on smallholdings and farms in and around the area of Patryshoek, Pretoria (25°39'48.0"S, 28°02'22.5"E), South Africa. On capture, the body mass of each animal was recorded to the nearest 0.01 g (Scout Pro SPU123; Ohaus Corporation, Pine Brook, New Jersey, USA).

Ethics approval

Permission to capture Mahali mole-rats was obtained from all landowners. A collecting permit was obtained from the relevant nature conservation authorities (permit No. CPF6-0127). The Animal Use and Care Committee of the University of Pretoria evaluated and approved the experimental protocol (ethics clearance No. EC044-16).

Environmental conditions

Daily rainfall data were obtained from the South African Weather Service at the weather station in Hartbeespoort (25°44'51.7"S, 27°53'13.9"E), approximately 15 km from the study site, from October 2016 to September 2017. Total monthly rainfall was subsequently calculated (cumulative rainfall) (Table 1).

Determination of reproductive status

All individuals below 40 g with a dark coat colour were classed as juveniles (Bennett and Faulkes 2000). Additionally, individuals captured alone in a tunnel system after a considerable trapping effort of 5 days were considered dispersing individuals. The BMs were distinguishable from NBMs by their large descended inguinal testes and yellow staining around the mouth. Furthermore, the BMs were usually, but not always, the largest male in each colony (Bennett and Faulkes 2000). The BF possessed prominent axillary teats and a perforated vagina, which was absent in the

Table 1. Cumulative rainfall for each month between October 2016 and September 2017.

| | Spring | | Summer | | | Autumn | | | Winter | | | Spring |
|--------------------------|-----------------|------------------|------------------|-----------------|------------------|---------------|---------------|-------------|--------------|--------------|----------------|-------------------|
| | October 2016 | November 2016 | December 2016 | January 2017 | February 2017 | March 2017 | April 2017 | May 2017 | June 2017 | July 2017 | August 2017 | September 2017 |
| Cumulative rainfall (mm) | 35.6 | 85.6 | 118.6 | 211.8 | 173.8 | 12.4 | 64.6 | 20.6 | 0 | 0.2 | 0 | 5.8 |

NBFs or dispersers. During the dissection process, the breeding status of females was confirmed by the presence of foetuses or placental scars on the uterine horns of queens.

Animal housing and dissection procedure

Once captured, the animals were transported back to the mole-rat laboratory at the Department of Zoology and Entomology, University of Pretoria. Entire colonies of animals were housed together in plastic crates (49.5 cm × 28.0 cm). Nesting material, comprising 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. The animals were kept in a climate-controlled room at a constant temperature of 25 ± 1 °C and a photoperiod of 12 h light and 12 h dark.

The capture of animals (entire colonies) was approximately finalised by the middle of each month. However, to ensure that postmortem 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. All animals were then euthanised with an overdose of isoflurane in 1 day. Subsequently, they were weighed to the nearest 0.01 g using a digital scale (Scout Pro SPU123; Ohaus Corporation, Pine Brook, New Jersey, USA). Blood was collected from the animals by exsanguination from the heart and subsequently centrifuged at 3000 r/min for 15 min. The plasma fraction of the blood was separated from blood cells and stored at -70 °C until it was used for hormone analysis. Testes and ovaries, with uterine horns and foetuses, were dissected out of males and females, respectively. All gonads were fixed in Bouin's fixative for approximately 24 h before being rinsed and stored in 70% ethanol.

Histological procedure

The fat and connective tissues were removed from around the gonads, which were weighed to the nearest 0.0001 g using a high precision scale (Ohaus Corp., Pine Brook, New York, USA). The length (mm) and the width (mm) of the fixed gonads were measured to the nearest 0.01 mm using a pair of digital callipers (Sylvac Opto RS 232; Ultra Praezision Messzeuge GmbH, Germany). These dimensions were in turn used to calculate testicular and ovarian volumes (mm³) using the formula for the volume of an ellipsoid (eq. 1, where *a* represents half the maximum length and *b* represents half the maximum width; Woodall and Skinner 1989). The mass and volume for the two testes or ovaries were averaged for each male or female, respectively.

$$(1) \quad V = \frac{4}{3} \pi ab^2$$

The gonads were put through a series of ethanol baths of increasing concentrations for dehydration and were subsequently embedded in wax. Testes and ovaries were cut into 6 µm thick sections with a rotary microtome (820 Spencer; American Optical, Scientific Instrument Division, Buffalo, New York, USA). The sections were mounted in consecutive order on microscope slides with gelatine and finally dried in an oven at 36 °C for 48 h. Dried sections were stained with Ehrlich's haematoxylin and counter-stained with eosin (Bloom and Fawcett 1964).

The entire left and right ovaries were cut serially so that the stages of follicular development could be observed with a light

microscope at ×100, ×200, and ×400 magnifications. Follicular stages were identified and classified according to Bloom and Fawcett (1964). Primordial follicles were observed in large numbers on the periphery of the ovary and were counted in every tenth ovarian section. The total number for each of the other follicular stages was counted throughout each ovary. The numbers of secondary and tertiary follicular stages were combined (referred to as secondary follicles).

Testicular sections were investigated for round seminiferous tubules with a light microscope (Diaplan; Ernst LeitzWetzlar GmbH, Germany) and photographed at ×10 magnification with a digital camera (Moticam 1000, 1.3 MP USB 2.0; Motic China Group, Ltd., Xiamen, People's Republic of China). The programme Motic Images Plus 2.0ML (Motic China Group, Ltd., Xiamen, People's Republic of China) was used to measure the diameter of 50 seminiferous tubules (µm) per individual male. The presence or absence of sperm cells was recorded.

Hormonal analysis

Coat-a-count hormone kits (IBL International GmbH, Hamburg, Germany) were used to determine the plasma testosterone, plasma progesterone, and plasma oestradiol concentrations of male and female Mahali mole-rats, respectively. All assays were carried out according to the manufacturer's protocol. The assays were validated by testing for parallelism between serial dilutions of mole-rat plasma (obtained from an individual with high hormone concentrations) and the standard curve (Chard 1978). The aforementioned hormone kits, including cross-reactivity, sensitivity, and protocols, have been recorded in previous studies (see Alagaili et al. 2017; Hart et al. 2019, 2020a, 2020b).

There was no significant difference between the serial dilution curve of plasma testosterone (nmol/L) of a BM and the calibration curve (ANCOVA: $F_{[1,5]} = 0.18$, $p = 0.11$). The intra-assay coefficient of variation was 4.7%. Similarly, no significant difference between the serial dilution curve of plasma progesterone (nmol/L) of a pregnant BF and that of the calibration curve was observed (ANCOVA: $F_{[1,5]} = 2.27$, $p = 0.07$). The intra-assay coefficient of variation for repeated determination of quality control was 7.9%. Furthermore, a serial dilution of plasma oestradiol-17β concentration from a queen with high oestradiol paralleled the reference preparation; thus, the slopes did not differ significantly (ANCOVA: $F_{[1,5]} = 0.66$, $p = 0.09$). The intra-assay coefficient of variation was 16.8%.

Data analysis

IBM SPSS 26 (IBM Corp., Armonk, New York, USA) was used for all statistical analyses and significance was assumed at $p \leq 0.05$. All data are presented as mean ± SE.

Juveniles and dispersers were not considered in the statistical analysis of this study due to their undeveloped reproductive tracts and the low sample size, respectively (Table 2). All reproductive parameters were analysed separately for males and females, whereas males and females were analysed together for body mass.

The normality of the dependent variables (body mass and reproductive parameters) was determined using Shapiro-Wilk's tests. All non-normally distributed dependent variables were log-transformed in an attempt to obtain a normal distribution. Lastly, the homogeneity of all normally distributed dependent variables (including log-transformed data) was confirmed with a Levene's test. Normally distributed dependent variables were

Table 2. Total number of dispersers, juveniles, and pregnant female Mahali mole-rats (*Cryptomys hottentotus mahali*) captured for each season between October 2016 and September 2017.

| | Summer | Autumn | Winter | Spring | Total |
|------------|--------|--------|--------|--------|-------|
| Dispersers | 4 | 2 | 2 | 1 | 9 |
| Juveniles | 10 | 4 | 1 | 6 | 21 |
| Pregnant | 3 | 3 | 3 | 1 | 10 |

Note: A total of 31 colonies were captured within the study period; 9 were captured in summer, 11 in autumn, 6 in winter, and 5 in spring.

analysed using general linear models (GLMs) (Table 3). All non-normal dependent variables were analysed using generalised linear models (GZLMs) fitted with gamma distributions and log-link functions (Table 4). Furthermore, the number of primordial, primary, secondary, Graafian, and atretic follicles, as well as corpora lutea, were not normally distributed; thus, they were analysed using GZLMs fitted with negative binomial distributions with log-link functions (St-Pierre et al. 2018) (Table 5).

Factors for all reproductive parameter models (GLMs and GZLMs) included season (summer, autumn, winter, and spring) and breeding status (BM and NBM or queen and NBF, respectively) and the two-way interactions of season and breeding status (Tables 3, 4, and 5). Breeding status was not included in the model assessing the number of corpora lutea because they were not found in NBFs (Table 6). Body mass recorded at the time of euthanasia and cumulative rainfall were included as covariates (Tables 3, 4, and 5).

Lastly, body mass was not normally distributed; consequently, a generalised linear mixed model (GLMM), with gamma distribution and log-link functions, was used to compare the body masses recorded at capture (across both males and females) of the different breeding states between the four seasons. The analysis included breeding state and season and their two-way interactions as fixed factors and cumulative rainfall as a covariate. Furthermore, the colony from which the individual was captured from was added as a random factor to the body mass model.

All GLMs, GZLMs, and GLMM were followed by least significant difference (LSD) pairwise comparisons. In addition, correlation tests (Pearson if normally distributed or Spearman's rank if not normally distributed) were conducted to investigate any significant effects of the covariates on the dependent variables.

Results

Animal capture

Mahali mole-rat colony sizes ranged from 2 to 20 individuals, with a mean (\pm SE) colony size of 11.3 ± 0.4 individuals. Only female dispersers were captured over the 12-month capture period, with the majority captured during the summer (Table 2). Pregnant queens were captured throughout the year (a total of 10 out of 31), with the highest number of pregnancies observed in summer, winter, and autumn and only one pregnancy in spring (Table 2). Juvenile individuals were captured throughout the year, with the highest number of juveniles captured in summer and spring (Table 2).

Female reproductive parameter

Throughout the sampling period, BFs (ovarian mass 0.019 ± 0.002 mg and ovarian volume 16.9 ± 2.1 mm³) possessed both heavier and larger ovaries compared with NBFs (ovarian mass 0.007 ± 0.0005 mg and ovarian volume 7.0 ± 0.65 mm³) (Tables 3 and 4; Figs. 1a and 1b). Furthermore, the BFs (plasma oestrogen 102.7 ± 11.7 pg/mL and plasma progesterone 4.0 ± 0.33 ng/mL) exhibited significantly higher concentrations of both plasma oestrogen and plasma progesterone compared with NBFs (plasma oestrogen 61.9 ± 5.84 pg/mL and plasma progesterone 1.2 ± 0.23 ng/mL) (Tables 3 and 4; Figs. 1c and 1d). More so, corpora lutea

Table 3. The statistical outputs, through the use of general linear models, of the effects of season, breeding status, their two-way interactions, rainfall, and body mass on the normally distributed continuous reproductive parameter data measured for male ($n = 109$) and female ($n = 114$) Mahali mole-rats (*Cryptomys hottentotus mahali*).

| Dependent variable | Normality | Model distribution | Season | | | Breeding status | | | Season × Breeding status | | | Rainfall | | | Body mass | | |
|------------------------------|--------------------------|--------------------|--------|----|-------|-----------------|----|--------|--------------------------|----|-------|----------|----|------|-----------|----|--------|
| | | | F | df | p | F | df | p | F | df | p | F | df | p | F | df | p |
| Ovarian volume | Normal (log-transformed) | Normal | 0.74 | 3 | 0.53 | 25.3 | 1 | 0.001* | 0.10 | 7 | 0.96 | 0.08 | 1 | 0.78 | 0.09 | 1 | 0.77 |
| Plasma oestrogen | Normal (log-transformed) | Normal | 3.86 | 3 | 0.01* | 18.3 | 1 | 0.001* | 0.63 | 7 | 0.60 | 0.18 | 1 | 0.68 | 0.16 | 1 | 0.69 |
| Seminiferous tubule diameter | Normal | Normal | 1.20 | 3 | 0.28 | 1.50 | 1 | 0.22 | 3.67 | 7 | 0.02* | 0.63 | 1 | 0.43 | 15.3 | 1 | 0.001* |

Note: An asterisk indicates significance ($p \leq 0.05$).

Table 4. The statistical outputs, through the use of general linearized models, of the effects of season, breeding status, their two-way interactions, rainfall, and body mass on the not normally distributed continuous reproductive parameter data measured for male ($n = 109$) and female ($n = 114$) Mahali mole-rats (*Cryptomys hottentotus mahali*).

| Dependent variable | Normality | Model distribution | Season | | | Breeding status | | | Season × Breeding status | | | Rainfall | | | Body mass | | |
|-----------------------------------|------------|--|----------|----|----------|-----------------|----|----------|-----------------------------|----|----------|----------|----|----------|-----------|----|----------|
| | | | χ^2 | df | <i>p</i> | χ^2 | df | <i>p</i> | χ^2 | df | <i>p</i> | χ^2 | df | <i>p</i> | χ^2 | df | <i>p</i> |
| Ovarian mass | Non-normal | Gamma distributions and log-link functions | 9.36 | 3 | 0.03* | 78.6 | 1 | 0.001* | 2.22 | 7 | 0.53 | 1.85 | 1 | 0.17 | 0.008 | 1 | 0.93 |
| Plasma progesterone concentration | Non-normal | Gamma distributions and log-link functions | 8.46 | 3 | 0.04* | 10.6 | 1 | 0.001* | 33.7 | 7 | 0.001* | 0.09 | 1 | 0.76 | 1.15 | 1 | 0.28 |
| Testicular mass | Non-normal | Gamma distributions and log-link functions | 0.46 | 3 | 0.93 | 0.01 | 1 | 0.91 | 11.1 | 7 | 0.14 | 0.00 | 1 | 0.99 | 59.1 | 1 | 0.001* |
| Testicular volume | Non-normal | Gamma distributions and log-link functions | 0.72 | 3 | 0.88 | 1.59 | 1 | 0.21 | 2.13 | 7 | 0.55 | 0.38 | 1 | 0.54 | 35.5 | 1 | 0.001* |
| Plasma testosterone concentration | Non-normal | Gamma distributions and log-link functions | 4.33 | 3 | 0.23 | 10.8 | 1 | 0.001* | 21.1 | 7 | 0.02* | 0.004 | 1 | 0.95 | 0.98 | 1 | 0.32 |

Note: An asterisk indicates significance ($p \leq 0.05$).

Table 5. The statistical outputs, through the use of general linearized models, of the effects of season, breeding status, their two-way interactions, rainfall, and body mass on the count reproductive parameters data measured for male ($n = 109$) and female ($n = 114$) Mahali mole-rats (*Cryptomys hottentotus mahali*).

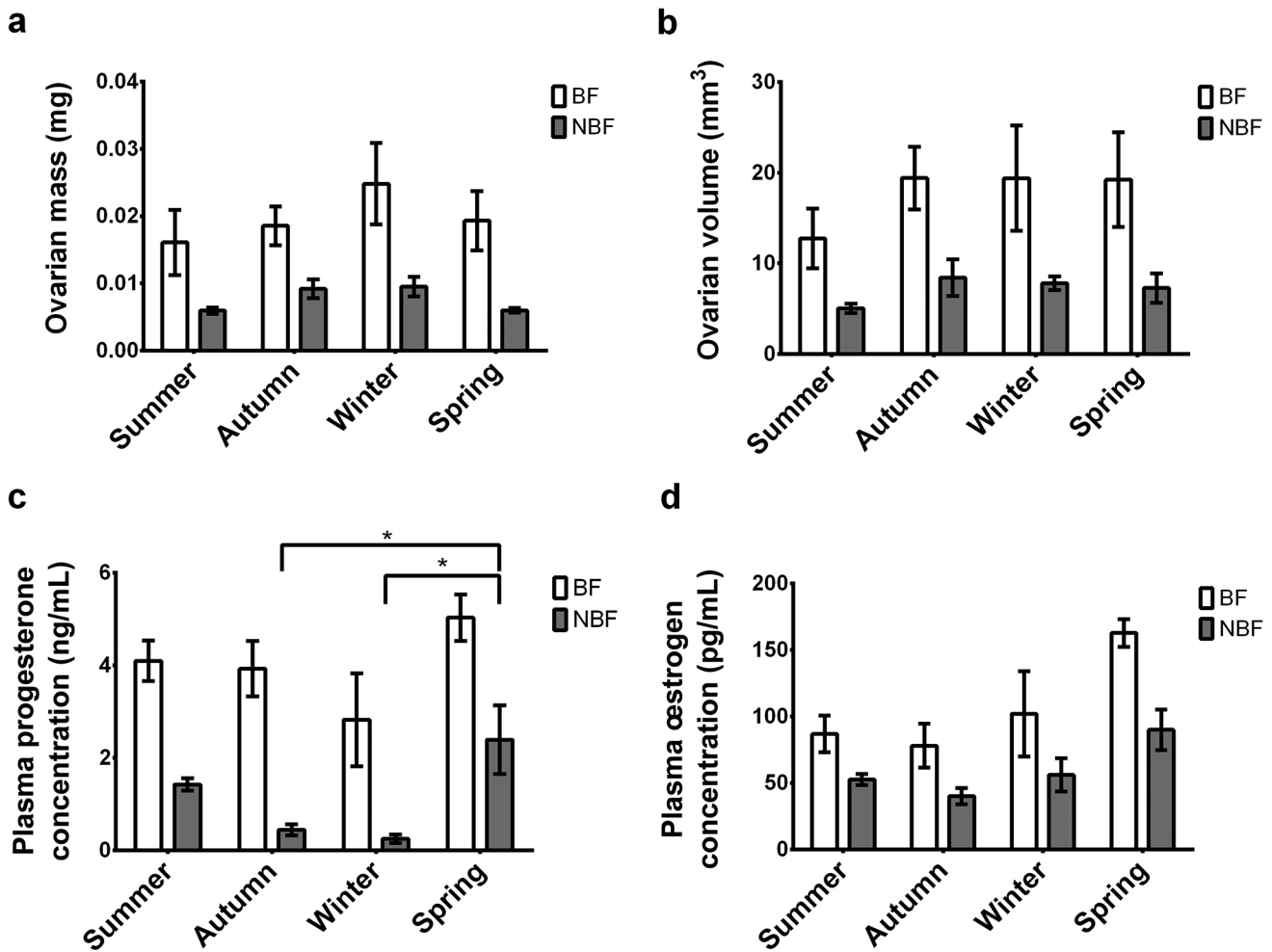
| Dependent variable | Model distribution | Season | | | Breeding status | | | Season × Breeding status | | | Rainfall | | | Body mass | | |
|-----------------------------|---|----------|----|----------|-----------------|----|----------|-----------------------------|----|----------|----------|----|----------|-----------|----|----------|
| | | χ^2 | df | <i>p</i> | χ^2 | df | <i>p</i> | χ^2 | df | <i>p</i> | χ^2 | df | <i>p</i> | χ^2 | df | <i>p</i> |
| No. of primordial follicles | Negative binomial distributions with log-link functions | 0.45 | 3 | 0.93 | 6.43 | 1 | 0.01* | 7.14 | 7 | 0.41 | 0.01 | 1 | 0.94 | 0.11 | 1 | 0.74 |
| No. of primary follicles | Negative binomial distributions with log-link functions | 0.7 | 3 | 0.99 | 0.01 | 1 | 0.98 | 0.47 | 7 | 0.95 | 0.01 | 1 | 0.99 | 0.002 | 1 | 0.97 |
| No. of secondary follicles | Negative binomial distributions with log-link functions | 2.26 | 3 | 0.46 | 9.03 | 1 | 0.03* | 0.79 | 7 | 0.85 | 0.24 | 1 | 0.63 | 0.78 | 1 | 0.38 |
| No. of Graafian follicles | Negative binomial distributions with log-link functions | 0.22 | 3 | 0.97 | 0.03 | 1 | 0.86 | 1.10 | 7 | 0.78 | 0.004 | 1 | 0.95 | 0.001 | 1 | 0.99 |
| No. of atretic follicles | Negative binomial distributions with log-link functions | 0.19 | 3 | 0.98 | 2.55 | 1 | 0.11 | 4.13 | 7 | 0.76 | 0.03 | 1 | 0.88 | 0.20 | 1 | 0.66 |
| No. of corpora lutea | Negative binomial distributions with log-link functions | 0.15 | 3 | 0.99 | — | — | — | — | — | — | 0.46 | 1 | 0.50 | 0.18 | 1 | 0.67 |

Note: An asterisk indicates significance ($p \leq 0.05$).

Table 6. Numbers of different follicle types (mean \pm SE) for breeding female (BF) and non-breeding female (NBF) Mahali mole-rats (*Cryptomys hottentotus mahali*) for four seasons.

| | Summer | | Autumn | | Winter | | Spring | |
|-------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| | BF | NBF | BF | NBF | BF | NBF | BF | NBF |
| Primordial | 119.0 \pm 26.6 | 317.4 \pm 24.2 | 102.7 \pm 19.4 | 337.4 \pm 38.7 | 100.0 \pm 25.0 | 236.7 \pm 15.7 | 138.5 \pm 22.8 | 279.3 \pm 17.4 |
| Primary | 54.0 \pm 11.5 | 55.4 \pm 5.3 | 39.7 \pm 3.2 | 55.2 \pm 4.1 | 46.8 \pm 5.6 | 42.3 \pm 6.8 | 46.3 \pm 2.7 | 39.8 \pm 4.7 |
| Secondary | 3.3 \pm 0.5 | 17.6 \pm 4.8 | 3.8 \pm 0.5 | 11.3 \pm 2.1 | 5.0 \pm 0.4 | 27.6 \pm 2.4 | 5.8 \pm 0.9 | 24.8 \pm 3.7 |
| Graafian follicle | 4.8 \pm 1.0 | 3.1 \pm 0.5 | 2.7 \pm 0.3 | 3.0 \pm 0.5 | 3.0 \pm 0.6 | 3.8 \pm 0.4 | 2.8 \pm 0.5 | 3.8 \pm 0.4 |
| Atretic follicle | 54.5 \pm 11.4 | 23.8 \pm 3.5 | 39.7 \pm 3.2 | 31.0 \pm 3.3 | 46.8 \pm 5.6 | 21.6 \pm 3.4 | 48.8 \pm 1.8 | 27.5 \pm 4.6 |

Fig. 1. (a) Ovarian mass (mg), (b) ovarian volume (mm³), (c) plasma progesterone concentration (ng/mL), and (d) plasma oestrogen concentration (pg/mL) of breeding female (BF) and non-breeding female (NBF) Mahali mole-rats (*Cryptomys hottentotus mahali*) compared over four seasons (summer, autumn, winter, and spring). Data are shown as mean \pm SE. An asterisk indicates significance ($p \leq 0.05$).

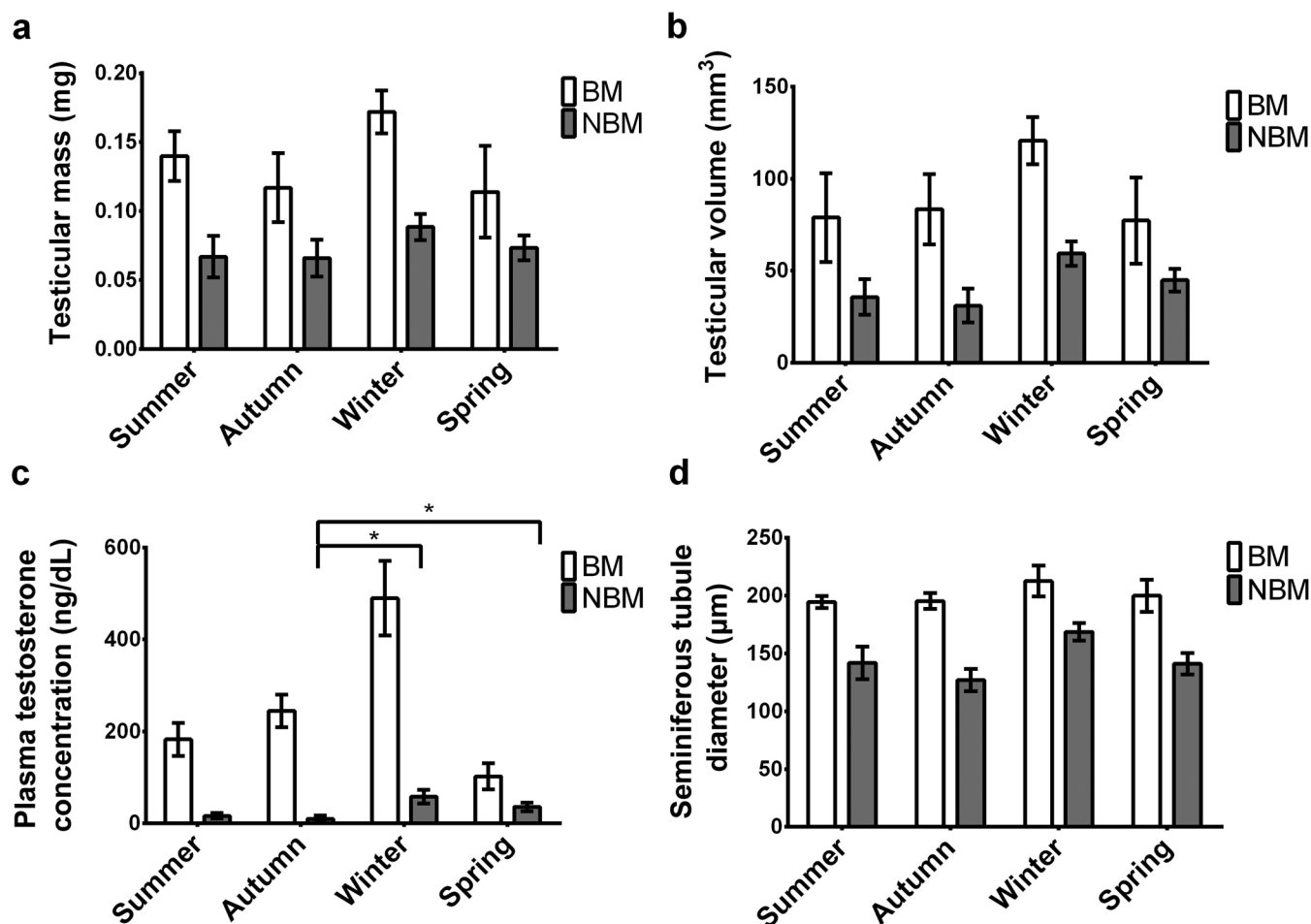


were not present in the NBFs; however, all BF had between 1 and 3 corpora lutea present in their ovaries throughout the year (these being either corpora lutea of ovulation or corpora lutea of pregnancy). In contrast, NBFs possessed more primordial and secondary follicles than BF (LSD: $p < 0.001$; Tables 5 and 6). There was no difference in the number of primary, Graafian, and atretic follicles between BF and NBF (Tables 5 and 6).

There was a seasonal effect on ovarian mass and plasma progesterone concentration and plasma oestrogen concentration (Tables 3 and 4; Figs. 1a, 1c, and 1d). Females (BFs and NBFs) captured in summer (0.008 ± 0.001 mg) had lighter ovaries than those captured in autumn (0.012 ± 0.002 mg) and winter ($0.013 \pm$

0.002 mg) (LSD: $p \leq 0.007$; Fig. 1a). Furthermore, females captured in winter possessed heavier ovaries than females captured in spring (0.008 ± 0.001 mg) (LSD: $p = 0.02$; Fig. 1a). Ovarian mass was similar between summer and spring, winter and autumn, and autumn and spring, respectively (LSD: $p \geq 0.13$, for all; Fig. 1a). Females captured during winter (plasma oestrogen 52.8 ± 7.8 pg/mL and plasma progesterone 0.77 ± 0.31 ng/mL) and autumn (plasma oestrogen 60.6 ± 5.2 pg/mL and plasma progesterone 1.4 ± 0.40 ng/mL) had lower concentrations of both plasma oestrogen and progesterone compared with females captured in spring (plasma oestrogen 102.7 ± 13.9 pg/mL and plasma progesterone 2.9 ± 0.65 ng/mL) (LSD: $p \leq 0.02$; Figs. 1c and 1d). Plasma oestrogen concentrations were

Fig. 2. (a) Testicular mass (mg), (b) testicular volume (mm^3), (c) plasma testosterone concentration (ng/dL), and (d) seminiferous tubule diameter (μm) of breeding male (BM) and non-breeding male (NBM) Mahali mole-rats (*Cryptomys hottentotus mahali*) compared over four seasons (summer, autumn, winter, and spring). Data are shown as mean \pm SE. An asterisk indicates significance ($p \leq 0.05$).



similar between spring and summer (plasma oestrogen 65.3 ± 12.2 pg/mL and plasma progesterone 2.0 ± 0.26 ng/mL), winter and autumn, winter and summer, and autumn and winter, respectively (LSD: $p \geq 0.17$). Furthermore, females from spring and summer, winter and autumn, winter and summer, autumn and spring, and autumn and winter had similar concentrations of plasma progesterone (LSD: $p \geq 0.07$; Figs. 1c and 1d). The season did not affect female Mahali mole-rat ovarian volume and numbers of primordial, primary, secondary, Graafian, and atretic follicles, and corpora lutea (Fig. 1b; Tables 3, 5, and 6).

The interaction between breeding status and season only affected plasma progesterone concentrations (Tables 3 and 4; Fig. 1c). The plasma progesterone concentrations of BMs remained constant throughout the sampling period (LSD: $p > 0.05$; Fig. 1c); however, plasma progesterone concentrations of NBMs were higher during spring than during autumn and winter (LSD: $p \leq 0.001$; Fig. 1c). NBMs captured in autumn, summer, and winter had similar plasma progesterone concentrations (LSD: $p > 0.05$; Fig. 1c). The interaction between breeding status and season was not significant for the remaining female reproductive parameters (Tables 4 and 5; Figs. 1a–1d).

Both body mass and rainfall did not affect any female reproductive parameters (Tables 4, 5, and 6).

Male reproductive parameters

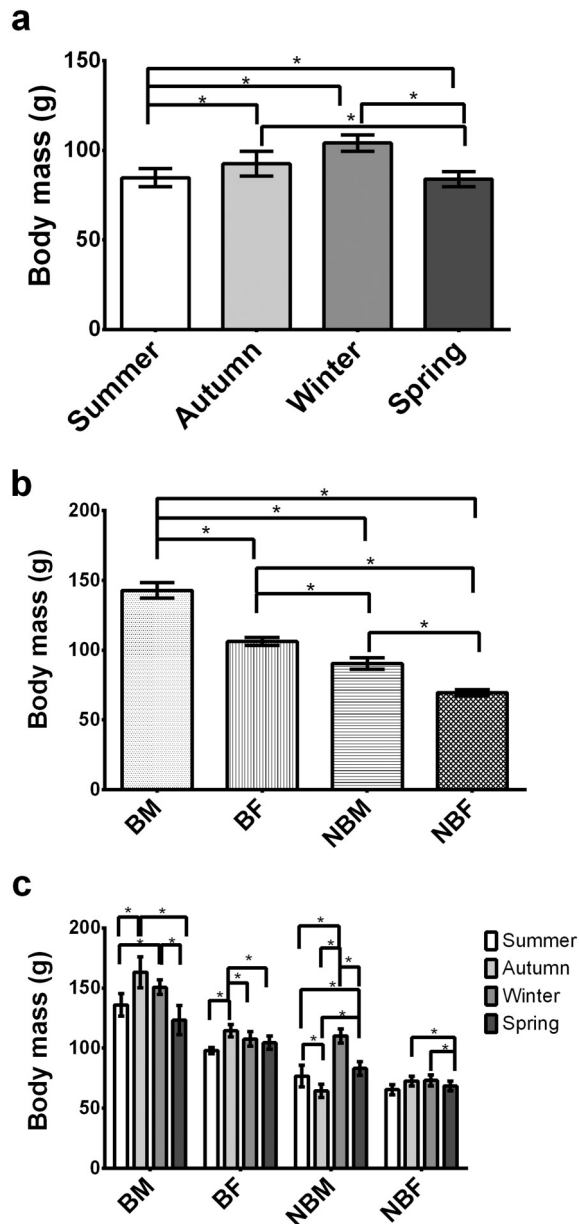
Few significant differences between BM and NBM Mahali mole-rat reproductive parameters were observed within this study.

The BMs (testicular mass 0.14 ± 0.01 mg and testicular volume 89.6 ± 10.9 mm^3) exhibited similar testicular mass and volume to that of the NBMs (testicular mass 0.07 ± 0.005 mg and testicular volume 46.3 ± 4.11 mm^3) (Table 4; Figs. 2a and 2b). Furthermore, sperm cells were observed in the seminiferous tubules of both BMs and NBMs. BMs (202.9 ± 51.3 μm) had similar seminiferous tubule diameters to NBMs (148.6 ± 5.70 μm) (Table 3; Fig. 2d). However, BMs (252.8 ± 34.7 ng/dL) exhibited higher plasma testosterone concentrations than NBMs (35.6 ± 6.82 ng/dL) (Table 4; Fig. 2c).

On initial observation, increased levels of all male reproductive parameters, in both BMs and NBMs, were observed during the dry months of winter (Figs. 2a–2d); however, there was no statistically significant effect of season on any of the male reproductive parameter (Fig. 2a–2d; Table 4).

The interaction between season and breeding status did not affect testicular mass and volume (Table 4; Figs. 2a and 2b). Contrastingly, the interaction between season and breeding status was significant for plasma testosterone concentration and seminiferous tubule diameter of male Mahali mole-rats (Table 4; Figs. 2c and 2d). Plasma testosterone concentrations of NBMs captured in autumn were significantly lower than those of NBMs captured in winter and spring (LSD: $p \leq 0.028$; Fig. 2c). All other post hoc comparisons were not significant (LSD: $p \geq 0.63$). The post hoc test revealed no biological meaningful, significant statistical comparisons for the seminiferous tubule diameter measurements.

Fig. 3. Comparisons of body masses (g) of Mahali mole-rats (*Cryptomys hottentotus mahali*) over (a) four seasons (summer, autumn, winter, and spring), (b) breeding status (BM, breeding male; BF, breeding female; NBM, non-breeding male; NBF, non-breeding female), and (c) the interaction between season and breeding status. Data are shown as mean \pm SE. An asterisk indicates significance ($p \leq 0.05$).



Cumulative rainfall did not affect any of the male reproductive parameters (Tables 3 and 4). However, body mass had a positive relationship with testicular mass and volume and the diameter of the seminiferous tubules (Tables 3 and 4), with heavier males possessing greater testicular masses and volumes and larger seminiferous tubules ($r \geq 0.73$, $p \leq 0.001$, for all).

Body mass

Mahali mole-rat body mass was significantly influenced by the season (GLMM: $F_{[3,223]} = 13.9$, $p \leq 0.001$; Fig. 3a). Animals captured in summer were significantly lighter than the animals captured in the other three seasons (LSD: $p \leq 0.001$; Fig. 3a). Furthermore, animals captured in spring were lighter than the animals

captured in winter and autumn (LSD: $p \leq 0.02$; Fig. 3a). Animals captured in winter were significantly heavier than those captured in autumn (LSD: $p = 0.004$; Fig. 3a).

The breeding state of the animal significantly affected their body mass (GLMM: $F_{[3,223]} = 143.6$, $p \leq 0.001$; Fig. 3b). BMs were the heaviest mole-rats, followed by BFs and then NBMs, with NBFs being the lightest individuals in a colony besides for juveniles, which were not considered in the analysis (LSD: $p \leq 0.001$; Fig. 3b).

The interaction between breeding status and season was significant (GLMM: $F_{[9,223]} = 17.3$, $p \leq 0.001$). BMs were significantly heavier in winter and autumn than in spring and summer (LSD: $p \leq 0.01$; Fig. 3c). There was no difference in BM body mass between individuals measured in the spring compared with those measured in the summer, or between individuals measured in the winter compared with those measured in the autumn (LSD: $p \geq 0.31$; Fig. 3c). Breeding females were heavier in autumn than in summer, winter, and spring (LSD: $p \leq 0.05$; Fig. 3c). There was no difference in the body mass of BFs between spring, summer, and winter (LSD: $p \geq 0.08$; Fig. 3c).

The heaviest NBMs were captured in winter, followed by those captured in spring and summer, and NBMs captured during autumn were the lightest (LSD: $p \leq 0.05$; Fig. 3c). NBFs captured in summer were significantly lighter than NBFs captured during the other three seasons (LSD: $p \leq 0.002$; Fig. 3c). Additionally, NBFs captured in spring were significantly lighter than those captured in winter and autumn (LSD: $p \leq 0.03$; Fig. 3c). There was no difference in body mass of NBFs between winter and autumn (LSD: $p \geq 0.26$; Fig. 3c).

Cumulative rainfall played a significant role in the body mass of Mahali mole-rats (GZLM (cumulative rainfall): $F_{[1,223]} = 18.4$, $p = 0.01$). Mahali mole-rats were heavier during periods with less rainfall (Spearman's rank correlation: $r = -0.22$, $p = 0.002$).

Discussion

The current study successfully describes the reproductive biology of a relatively understudied African mole-rat species, the Mahali mole-rat, in which reproduction was hypothesised to be controlled by rainfall pattern. We subsequently compared our findings with other African mole-rat species in the hope to better understand the plethora of reproductive strategies seen in this unique rodent family.

As seen in the highveld mole-rat (Janse Van Rensburg et al. 2002) and all other social and eusocial mole-rat species (Bennett 1989; Spinks et al. 1999; Bennett and Faulkes 2000; Oosthuizen et al. 2008; Faulkes and Abbott 2009), socially induced infertility was observed in NBF Mahali mole-rats. Mahali mole-rat BFs exhibited greater ovarian mass and volume, as well as increased concentrations of both plasma progesterone and oestrogen, compared with NBFs. Furthermore, corpora lutea of pregnancy and ovulation were found only in the ovaries of BFs throughout the year but not in the NBFs. This suggests regular periods of ovarian cyclicity and the production of corpora lutea following ovulation and, if fertilised, subsequent pregnancy in the BFs. The NBFs are anovulatory while in the confines of the colony, whereas the BFs exhibit the ability to ovulate throughout the year. The absence of corpora lutea in the NBFs may be the result of a lack of opportunity for coitus. It is speculated that in the induced ovulating social mole-rat subspecies of *C. hottentotus* (Malherbe et al. 2004; Jackson and Bennett 2005; Hart et al. 2020b), the females are behaviourally suppressed by methods such as interruption of mating or lack of an opportunity to mate with an unrelated opposite-sex conspecific (Oosthuizen et al. 2008; N.C. Bennett, unpublished data). Not surprisingly, the Mahali mole-rat BFs were heavier than the NBFs, which lends support to the hypothesis that the larger BF could more easily behaviourally suppress the smaller NBFs preventing them from copulating as proposed for other *Cryptomys* subspecies (N.C. Bennett, unpublished data).

There were, however, indications of relaxation of suppression in NBFs that were supported by increased plasma progesterone concentrations and more frequent events of female dispersal during the wetter months of summer, whereas increased suppression in NBFs was observed during the dry months of autumn and winter. Similarly, relaxation of reproductive suppression and increased dispersal events have been observed in the seasonally breeding social common and highveld mole-rat NBFs in the genus *Cryptomys* (Spinks et al. 1997, 1999; Janse Van Rensburg et al. 2002) and in the aseasonal, eusocial naked mole-rats (*Heterocephalus glaber* Rüppell, 1842) (Westlin et al. 1994) and Damaraland mole-rats (*Spicomys damarensis* (Ogilby, 1838)) (Molteno and Bennett 2002b).

Surprisingly and against our initial predictions, pregnant BFs and juvenile Mahali mole-rats were captured at all times of the year, which implies that reproduction occurs throughout the year. Furthermore, over the calendar year of sampling, there was a lack of seasonality in follicular development and an increase in the size of the ovaries in BF Mahali mole-rats. However, there was an observable skew in the period of the year where reproduction occurred. The highest number of juvenile Mahali mole-rats was captured in the wetter months of spring and summer. The mean gestation period for *C. hottentotus* is around 66 days (Bennett 1989); thus, if breeding occurs towards the end of the dry season (winter in the case for Mahali mole-rats), then the offspring would be born during the wet season and stand a greater chance of survival. Thus, even though the Mahali mole-rats have the capability to breed and produce offspring throughout the year, particular periods are favoured to allow for the increased likelihood of survival of their offspring. Interestingly, increased reproductive suppression occurs during the period of increased breeding activity (winter). Year-round follicular development has been observed in the BFs and NBFs of the seasonally breeding common and highveld mole-rats (Spinks et al. 1999; Janse Van Rensburg et al. 2002). This suggests that the reproductive axis in the genus *Cryptomys* is not ultimately switched off throughout the year, giving the BF in colonies the capability to breed throughout the year; however, in highveld and common mole-rats, breeding only occurs during the wet season (Spinks et al. 1999; Janse Van Rensburg et al. 2002). Contrastingly, the females of the seasonally breeding solitary mole-rat species show complete regression of follicular development in the non-breeding season (dry season) (Jarvis 1969; Bennett and Jarvis 1988a; Herbst et al. 2004; Hart et al. 2006), whereas both Damaraland mole-rat NBFs and BFs show year-round follicular development; however, NBFs do not ovulate (Molteno and Bennett 2002a, 2002b).

Similarly to the highveld mole-rat (Janse Van Rensburg et al. 2002) and all other social and eusocial mole-rat species (Bennett 1989; Spinks et al. 1997; Bennett and Faulkes 2000; Oosthuizen et al. 2008; Faulkes and Abbott 2009), excluding the eusocial naked mole-rats, both the BMs and NBMs exhibited sperm production and a lack of regression in testicular mass and volume throughout the year. This indicates that the Mahali mole-rat NBMs are not reproductively suppressed while in the confines of the colony. Therefore, the maintenance of reproductive activity in both BM and NBM Mahali mole-rats is hypothesised to be for reproductive readiness if opportunities arise to disperse (Bennett and Faulkes 2000). The BMs, however, possessed testosterone concentrations and body masses that were significantly higher than those of the NBMs. These higher testosterone concentrations may result from the larger BMs copulating frequently with the BF because NBMs are behaviourally inhibited from copulating with the BF or the other NBFs in the confines of the colony owing to incest avoidance.

Although no significant differences in testicular mass and volume, seminiferous tubule diameter, or plasma testosterone concentration were found over the four seasons, reproductive parameters of both BMs and NBMs were highest during the dry winter months. This may possibly indicate that there are increased

frequencies of breeding, particularly winter, which is also supported by the larger number of offspring found during the subsequent spring and summer (see above). This could result in enhanced inhibitory mechanisms directed towards the NBMs and NBFs, such as aggressive sparring.

As a likely result of the aseasonal reproduction found by our study, rainfall did not affect reproduction in Mahali mole-rats, which is in stark contrast to the common (Bennett 1989), highveld (Janse van Rensburg et al. 2002), and solitary (Jarvis 1969; Bennett and Jarvis 1988a; Hart et al. 2006) mole-rat species. However, seasonality, driven by rainfall, did significantly affect the body mass of the Mahali mole-rat. The heavier animals were caught in winter and autumn, whereas the lighter animals were caught in spring and summer. The reason for this may be due to a combination of factors. Firstly, increases in reproductive hormones, such as testosterone, progesterone, and oestrogen, during winter may increase body mass (Saltzman and Ziegler 2014; Alagaili et al. 2017; Hart et al. 2020a). Secondly, the geophytes and tubers that mole-rats feed on are storage organs for plants, and during the drier seasons, these storage organs are large and contain more nutrients to be able to maintain the plants through the dry period, thus providing the mole-rats with a higher quality diet (Spinks et al. 2000). Thirdly, when rainfall and temperatures are low, the ground is hard and compacted, thus decreasing the amount of digging activity the colony can perform (Bennett and Faulkes 2000). This possible decrease in work by each individual and feeding on higher quality food may increase body mass during the drier months.

The current study adds to the wealth of knowledge of African mole-rat reproductive biology. We find an interesting pattern of reproductive strategies among the four subspecies of the genus *Cryptomys*. Strict seasonal reproduction in social mole-rat species has only been observed in the common mole-rat and the highveld mole-rat, whereas aseasonal reproduction was observed in the Natal mole-rat. The Mahali mole-rat has a breeding strategy more akin to the Natal mole-rat in that the subspecies breeds throughout the year. Rainfall occurs over more months in the habitat of the Natal mole-rat than in the bushveld habitat of the Mahali mole-rat. Some common ancestor of the Mahali and highveld mole-rats may have moved into the drier bushveld, and as a consequence over evolutionary time, evolved into the Mahali mole-rat and adopted an opportunistic breeding strategy, whereas those that moved into the less arid habitats evolved into the highveld mole-rat and adopted a seasonal breeding strategy. The opportunistic breeding strategy seen in the Mahali mole-rat, and possibly the Natal mole-rat, may be brought about by the benefits of social living and cooperative breeding (both Mahali and Natal mole-rats form large colonies) and having access to a constant supply of food in the form of rootstocks of bushes and rhizomes of grasses year-round. Increased colony sizes of the eusocial species and some social species (like the Mahali and Natal mole-rats) may minimise the effects of yearly variation in environmental conditions, such as digging capabilities, foraging abilities, and food abundance, leading to those species possessing strict aseasonal breeding strategies. African mole-rats thus provide a unique model to unravel the causes and consequences of cooperative breeding and, more importantly, into the understanding of mammalian reproductive strategies in both solitary- and colonial-living species.

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