Is the Mahali mole-rat (*Cryptomys hottentotus mahali*) a spontaneous or induced ovulator?

D.W. Hart●[1], K. Medger[2], B. van Jaarsveld[1], N.C. Bennett[1,2]

[1] Department of Zoology and Entomology, University of Pretoria, Private Bag X20, Hatfield 0028, South Africa

[2]Mammal Research Institute, Department of Zoology and Entomology, University of Pretoria, Private Bag X20, Hatfield 0028, South Africa

● Corresponding author

D.W. Hart

Department of Zoology and Entomology, University of Pretoria, Pretoria, South Africa

Phone no. +27 84 5744257

Email:hartdaniel7710@gmail.com

Abstract

The Mahali mole-rat (*Cryptomys hottentotus mahali* (Roberts, 1913)) is a social, cooperatively breeding subterranean rodent that breeds aseasonally. Only one female in a colony breeds and the remaining females are reproductively suppressed. When the opportunity arises, these non-reproductive females disperse from the natal colony to escape reproductive suppression and pair up with an unrelated male to start a new colony. This study set out to determine whether female Mahali mole-rats are induced or spontaneous ovulators once separated from the reproductive suppression of the breeding female. Fifteen separated females were subjected to three treatments: housed separately without a male (A), allowed chemical, but not physical contact with a vasectomised male (NPC), and placed in direct contact with a vasectomised male (PC). Urine was collected from all females under each treatment every two days for 40 days. Only females housed in the PC treatment exhibited heightened progesterone concentrations and corpora lutea of ovulation in the ovaries. Furthermore, males possessed epidermal spines on the shaft of the penises which may be used to stimulate the cervix of the female during copulation. These findings suggest that the Mahali mole-rat is an induced ovulator.

Keywords: *Cryptomys,* Mahali mole-rat, induced, ovulation, progesterone, corpora lutea, penile spines.

Introduction

Ovulation and subsequent reproduction in female mammals requires a considerable investment of both energy and resources. Ovulation in mammals may occur by two methods, induced or spontaneous (Milligan 1974, 1975). Spontaneous ovulators exhibit a continuous cycle of follicular development and release of an oocyte during each oestrous cycle even in the absence of male stimuli (Milligan 1974, 1975). Spontaneous ovulation is energetically expensive due to the continuous cycling of hormones and subsequent release of an oocyte, which seldom results in a pregnancy.

Induced ovulators differ from spontaneous ovulators by showing marked changes in hormone concentrations (e.g. oestrogen and progesterone) and ovulation only in the presence of male stimuli. The male stimulus may be visual, olfactory, tactile, auditory, vaginal or perineal (Bibeau et al. 1991; Ramirez and Soufi 1994). Ovulation, usually, occurs in response to a neurogenic stimulus caused by coitus (i.e. penile-vaginal penetration) (Fernandez-Baca et al. 1970; Bibeau et al. 1991; Ramirez and Soufi 1994) and frequently requires repeated copulation events to initiate ovulation. The outer surface of the penis of induced ovulating mammals often possesses protruding structures (spines or ribbing on the shaft of the penis) (Parag et al. 2006; Stoddart and Milligan 2009). During copulation, these structures cause a tactile stimulation of the vaginal wall and cervix, which is essential for initiating ovulation (Jöchle 1975; Viker et al. 1993; Parag et al. 2006). Induced ovulation may be energetically conservative and beneficial for the female as ovulation only occurs when fertilisation is most likely to be successful.

Due to the constraints of living underground and burrowing, seasonally breeding, solitary subterranean rodents, such as some species of African mole-rats (Bathyergidae), have only a brief window of opportunity to come into contact with mates and acquire enough food for successful offspring rearing (Bennett and Faulkes 2000). These periods typically occur during the wet season (breeding season) when the mole-rats can efficiently excavate the soft soil and food is more abundant (Bennett and Faulkes 2000). Spontaneous ovulation would be energetically wasteful under such conditions when males are not present for most of the year. The solitary and seasonally breeding Cape dune mole-rat (*Bathyergus suillus* (Schreber 1782)) and the Cape mole-rat (*Georychus capensis* (Pallas 1778)), for example, are induced ovulators (Malherbe et al. 2004; Jackson and Bennett 2005; Van Sandwyk and Bennett 2005; Parag et al. 2006). More generally, induced ovulation occurs more frequently in solitary and seasonally breeding rodents, whereas social and aseasonally breeding rodents are more likely to be spontaneous ovulators (Zarrow and Clark 1968; Kauffman and Rissman 2006). Spontaneous ovulators are found in the eusocial and aseasonally breeding African mole-rat species, such as the naked mole-rat (*Heterocephalus glaber* Rüppell 1842), the giant mole-rat (*Fukomys mechowii* (Peters 1881)), Ansell's mole-rat (*Fukomys anselli* (Burda, Zima, Scharff, Macholán and Kawalika 1999)) and the Damaraland mole-rat (*Fukomys damarensis* (Ogilby 1838)(Bennett and Jarvis 1988; Faulkes et al. 1990, 2010; Jarvis and Bennett 1990; Molteno and Bennett 2000; Snyman et al. 2006; Bennett et al. 2010).

All four sub-species of *Cryptomys hottentotus* are social and cooperatively breeding subterranean African mole-rats, and all subspecies studied so far are induced ovulators despite differences in their seasonal breeding strategies. The Highveld (*C. h. pretoriae* (Roberts 1913)) (Malherbe et al. 2004) and the common mole-rat (*C. h. hottentotus* (Lesson

1826)) (Bennett 1989; Spinks et al. 1997, 1999) breed seasonally and the Natal mole-rat (*C. h. natalensis* Roberts 1913) (Jackson and Bennett 2005) breeds throughout the year. Until recently, no studies had been conducted on the biology of the Mahali mole-rat (*C. h. mahali* (Roberts, 1913)) and in particular their reproductive biology and breeding strategy are largely unknown (Hart 2019).

Mahali mole-rats are nocturnal (van Jaarsveld et al. 2019) and subterranean social rodents distributed in the relatively arid bushveld regions of Gauteng, North West Province and the Northern Cape of South Africa. The Mahali mole-rat is phylogenetically closely related to *C. h. pretoriae* (Faulkes et al. 2004; Broekman et al. 2006) and shares similar climatic seasonal conditions. Reproduction is monopolised by a single female (queen) within the colony. As in other social African mole-rats, non-reproductive females (NRFs) Mahali mole-rats exhibit socially induced infertility (Bennett et al. 1994; Hart 2019). Reproduction appears to occur throughout the year in the Mahali mole-rats (Hart 2019).

In African mole-rats, which are typically found in habitats with distinct wet and dry seasons, like those experienced by the Mahali mole-rat, dispersal from their natal colony is most common during the part of the year when precipitation occurs. During the rainy season, the soil is soft enough to permit easy burrowing (Jarvis et al. 1994) allowing for dispersal and finding of the typical food resources of mole-rats, geophytes (Jarvis and Bennett 1990).

For the present study, NRF Mahali mole-rats were removed from their natal colony (i.e. removal from the physical presence of the queen and other related non-reproductive animals) to simulate dispersal and induce reproductive activation. These females were either

housed singly or paired with non-related vasectomised males to pose a simple but fundamental question: is the social aseasonally breeding Mahali mole-rat a spontaneous or induced ovulator? We predict that ovulation of the female Mahali mole-rat is induced as is the pattern in other subspecies of *Cryptomys hottentotus* and furthermore that females would only show elevated progesterone levels and corpora lutea of ovulation after physical contact with a male. Further support for this prediction may be found in the penile spines occurring on the shaft of the penis of male Mahali mole-rats. Since the Mahali mole-rat is an agricultural pest information pertaining to the biology and particularly the reproductive biology could be crucial for their future control.

Methods and material

Animal capture and housing

A total of 15 NRF and seven mature male Mahali mole-rats (*Cryptomys hottentotus mahali*) were collected in Pretoria, Patryshoek (25° 40 ′ S, 28° 2′ E), South Africa. The animals were caught using Hickman live traps baited with sweet potatoes. Once captured, the animals were moved to the laboratory at the Department of Zoology and Entomology, University of Pretoria, South Africa.

In the laboratory, the animals were placed in a climate-controlled room set at a temperature of 25 °*C* and a constant humidity of 50% throughout the experiment. The photoperiod was 12*L*:12*D*. All females were initially kept in isolation from possible chemical and behavioural stimulation by males for five weeks (habituation period modelling dispersal). A period of five weeks was chosen for all experiments since oestrous cycles in bathyergid mole-rats have been

estimated to be around one month (Faulkes et al. 1990). The experimental protocol was evaluated by the Animal Use and Care Committee of the University of Pretoria (ethics clearance number EC044-16).

Male vasectomy

A qualified veterinarian vasectomised seven males three months before their participation in the experiment to ensure that the epididymis was clear of spermatozoa. During the vasectomy, mask-induced anaesthesia was performed using 5% isofluorane gas and subsequently maintained using 2%–2.5% isoflurane gas until the end of the procedure. The analgesic Meloxicam (0.5 mg/kg) was administered after vasectomy as a post-surgery analgesic.

Experimental design

After the five-week habituation period, the experimental animals were placed under three experimental treatments: alone (*A*), non-physical contact with a male (NPC), and physical contact with a male (PC). Each experimental treatment was executed over 40 consecutive days. Three out of the 15 females experienced all three treatments. These three females were first kept alone and then placed into non-physical contact with a male. In the end, they were in direct physical contact with a male, after which they were euthanised. All euthanasia was done using an overdose of isoflurane. The remaining 12 animals experienced only one out of three experimental treatments (*A*: $n = 7$; NPC: $n = 4$; PC: $n = 1$).

Alone (A)

Ten females (three used throughout the three trials and seven animals used solely for this experiment) were housed singly in cages (34 cm × 34 cm × 20 cm) with no males present in the same room; thus, preventing any chemical interaction between males and females. The treatment was used as a measure for baseline progesterone concentrations. Seven of the females used for A were euthanised at the end of this treatment.

Non-physical contact (NPC)

Seven females were housed in individual cages (three used throughout the three trials and four animals used solely for this experiment)(48 cm × 28.5 cm × 48 cm) were placed next to a vasectomised male. A wire mesh separated the males from the females to prevent any physical contact but allowed visual, olfactory, and auditory contact between the sexes. The males and females remained in these conditions for the duration of the 40-day treatment period, after which four of the females used for NPC were euthanised.

Physical contact (PC)

Four females, comprised of the three females that had experienced the previous two treatments (*A*, PC) and a single female used only for PC, were each housed with a vasectomised male in large holding cages (48 cm × 28.5 cm × 48 cm). This treatment allowed for full physical contact of the females with the males and consequently, the opportunity for copulations to occur. Males were supervised for the first eight hours to ensure that there were no aggressive interactions. Mounting and subsequent copulation occurred during the first five days. The males were present for the entire 40-day period. All females were euthanised at the end of the treatment period.

Urine collection

Urine was collected from all-female Mahali mole-rats every second day from the onset of each treatment, resulting in 20 samples of urine collected for each animal per treatment. Each female was kept in a cylindrical plastic cage with a wire-mesh base on top of the urine collection tray. The wire-mesh base prevented the contamination of the urine sample with faecal matter. Urine was collected between 09*h*00 and 13*h*00, and as soon as urine had been voided, the female was placed back into her container. Urine was collected with a single-use plastic pipette and frozen at -40°*C*.

Urine progesterone determination

Urine samples were analysed for progesterone using a coat-a-count kit for progestogen determination (Diagnostic Products Corporation, Los Angeles). The antiserum is highly specific for progesterone, with cross-reactivity to all naturally occurring steroids of < 0.5%, except 17α-dihydroprogesterone (3.4%), 11-eoxycorticosterone (2.4%), 5β-pregnan-3, 20-dione (3.2%) and 5α-pregnan-3, 20-dione (9%). Standard concentrations ranged from 0.3 to 127.2 nmol.l^{-1}. Steroids were neither purified nor separated by chromatography. A serial dilution of a high progesterone sample paralleled the standard curve (ANCOVA: $F = 2.5$, $p > 0.05$). The intra- and inter-assay coefficient of variations were 7.8 % and 12.0 %, respectively.

Creatinine determination

Urine concentration varied due to variable fluid intake; thus, progesterone concentrations had to be corrected. The correction was accomplished by analysing each urine sample for creatinine concentration, as creatinine is excreted at a relatively constant rate. The creatinine concentration of each urine sample was determined using a modified Jaffe reaction (Folin

1914). Final standardised results are presented as ng progesterone/mg creatinine (corrected progesterone).

Ovarian histology

On euthanasia, immediately after the last urine sample had been taken, all females were weighed to the nearest 0.01 *g* using a digital scale (Scout Pro SPU123, Ohaus Corporation, Pine Brook, New Jersey, U.S.A.). Ovaries were removed and placed in Bouin's solution for 24 hours after which they were transferred into 70% ethanol. The fat and connective tissue were removed, and ovaries were weighed to the nearest 0.0001 *g* using a high precision scale (Ohaus Corp. Pine Brook, N.Y., U.S.A.). The length (mm) and the width (mm) of the fixed ovaries 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 ovarian volume (mm^3) using the formula for the volume of an ellipsoid: $V = 4/3 \times \pi \times a \times b^2$ where a represents half the maximum length and b half the maximum width (Woodall and Skinner 1989). Ovarian mass was corrected for body mass (corrected ovarian mass). The corrected ovarian mass and ovarian volume were averaged for the two ovaries per female. Standard histological techniques using serial ethanol dehydration and paraffin wax embedding were carried out on the ovaries (Drury and Wallington 1980). Ovarian sections, for both ovaries for each female, were cut 6 μm thick and stained with Ehrlich's haematoxylin and eosin. The sections were examined serially for stages of follicular growth with a light microscope at x100, x200 and x400 magnifications. Follicular stages were identified, according to Bloom and Fawcett (1964). The presence or absence of each follicular stage was recorded.

Penis electron microscopy

Penises were dissected out from frozen material of additional adult males collected on a prior occasion, fixed in a 2.5% glutaraldehyde/formaldehyde solution and then processed for scanning electron microscopy following standard procedures. After fixation, the samples were rinsed in 1.5 *M* phosphate buffer, dehydrated through a graded ethanol series and finally dried in hexamethyldisilizane. The samples were then mounted onto aluminium stubs, sputter-coated with carbon (Polaron E5200C Carbon Coater, Quorum Technologies, Watford, UK) and examined with a field emission scanning electron microscope (Zeiss Ultra PLUS FEG SEM) operated at 5 kV.

Data analysis

Corrected ovarian mass and log-transformed ovarian volume were normally distributed (Shapiro–Wilk's test: $p \geq 0.26$, for both), whereas progesterone concentration was not normally distributed even after log-transformation (Shapiro–Wilk's test: $p \leq 0.003$). One way ANOVAs were used to compare the corrected ovarian mass and log-transformed ovarian volume between the three treatments. A generalised linear mixed model (GLMM) fitted with a gamma distribution with log link function was conducted to compare the mean urinary progesterone concentrations between the three treatments. Sampling day was included as a repeated measure. A second GLMM was used to compare progesterone concentration between treatments for the three individuals that experienced all three treatments. Individual was included as a random factor for all GLMMs. Post-hoc comparisons were made following all models using least-significant difference (LSD) pairwise comparisons.

IBM SPSS 25 (IBM Corp., 2017) was used for all statistical analyses and significance was assumed at $p \leq 0.05$. All data are given as mean ± standard error (SE).

Results

Urinary progesterone

For the three females that experienced all three treatments, urinary progesterone concentrations were significantly different between the three treatments (GLMM: $F_{[2]} = 25.2$, $p \leq 0.001$, Fig. 1). Urinary progesterone was considerably increased under physical contact with a male (PC) (19.4 ± 4.2 ng/mg creatinine) in comparison to when they were maintained on their own (A) (3.7 ± 0.4 ng/mg creatinine) and during non-physical contact with a male (NPC) (4.2 ± 0.4 ng/mg creatinine) (LSD: $p \leq 0.03$, Fig. 1). NPC urinary progesterone concentrations and A urinary progesterone concentrations were similar (LSD: $p = 0.93$, Fig. 1) for the three females.

The females, which were exposed to a specific treatment only, showed similar results. There was a significant difference in urinary progesterone concentrations between the three treatments (GLMM: $F_{[2]} = 28.0$, $p \leq 0.001$). The mean concentration of urinary progesterone for the females that were housed with a vasectomised male (PC) (15.3 ± 3.2 ng/mg creatinine, $n = 4$) were significantly higher than the mean concentration of urinary progesterone of both the females housed alone (*A*) (4.7 ± 0.4 ng/mg creatinine, $n = 10$) and in non-physical contact with a vasectomised male (NPC) (2.6 ± 0.2 ng/mg creatinine, $n = 7$) (LSD: $p \leq 0.001$, Fig. 2). The females under A and NPC treatments had similar mean concentrations of urinary progesterone (LSD: $p = 0.26$, Fig. 2).

Ovarian histology

Corrected ovarian mass and ovarian volumes were indistinguishable between the three treatments ($F_{[2, 12]} \leq 1.34$, $p \geq 0.3$). All ovaries contained primordial, primary, secondary and Graafian follicles. All females also showed evidence of follicular regression with the presence of atretic follicles. Corpora lutea of ovulation were only found in females exposed to the PC treatment indicating that ovulation had taken place in only those females.

Penile morphology

Numerous spines were observed on the outer surface of the shaft of the penises ($n = 3$). These sharp structures were unevenly distributed toward the base of the shaft of the penis (Fig. 3). The protrusions on the penis of the Mahali mole-rat are spine-like and sharp at the apex. The tip of the glans penis was smooth (Fig. 3).

Discussion

Histological evidence and sequential monitoring of urinary progesterone have revealed that the Mahali mole-rat is an induced ovulator. High progesterone concentrations and the presence of corpora lutea of ovulation were only observed in the females exposed to the PC treatment; these are indicative of successful ovulation following copulation. Merely the physical presence of a male without copulation was not able to stimulate a female to ovulate as indicated by low progesterone concentrations and the absence of corpora lutea of ovulation in the NPC treatment. The presence of the sharp spine-like protrusions on the shaft

of the penis of the male adds support to the notion that female Mahali mole-rats need vaginal stimulation to induce ovulation.

Coitus and thus, penile-vaginal penetration in mammals has long been reported to cause a neuroendocrine reflex in females, which results in ovulation and a subsequent surge in progesterone (Dewsbury 1972; McClintock 1983). The penile morphology of male Mahali mole-rats reinforces the concept of this sub-species of *Cryptomys hottentotus* being an induced ovulator. The penile morphology is similar to that observed in the Natal mole-rat, which possesses ornamentation in the form of small protrusions that are rounded at the apex (Parag et al. 2006). The protrusions on the shaft of the penis of the Mahali mole-rat are more spinose and sharp at the apex, similar to those found in male Cape dune mole-rats (Parag et al. 2006). The smooth glans and the small protrusions located at the base of the erect Mahali mole-rat penis indicate that full and deep penetration would be needed to allow for the necessary stimulation of the vaginal wall and cervix. In two solitary bathyergid mole-rats, the Cape dune and the Cape mole-rat, males have numerous epidermal spines on the penis, while the eusocial spontaneous ovulating mole-rat species *Fukomys damarensis* and *Heterocephalus glaber* have ridges on the penis, but lack any elaborate ornamentation in the form of spines and protrusions (Parag et al. 2006).

Atretic follicles were present in all females in the *A* and NPC treatments indicating that, despite active folliculogenesis, ovulation did not occur possibly because of a lack of coitus. Moreover, male chemical cues (NPC treatment) had no significant effect on the induction of ovulation in female Mahali mole-rat, which is in drastic contrast to other *Cryptomys hottentotus* sub-species (Malherbe et al. 2004; Jackson and Bennett 2005).

Mahali mole-rats placed in isolation (*A* treatment) and chemical contact (NPC treatment) showed similar follicular development to the female in the PC treatment; all females possessed Graafian follicles, but only the PC possessed corpora lutea of ovulation. In both the Natal (Jackson and Bennett 2005) and Highveld mole-rats (Malherbe et al. 2004), higher follicular development was found during the NPC treatment (male chemical stimuli was present) than during the A treatment indicating the positive effect of male chemical stimuli on the ovulation of these two sub-species. Similarly, semiochemicals emitted from the males resulted in ovulation in the Cape mole-rat (Van Sandwyk and Bennett 2005), mouse (Marsden and Bronson 1964) and grey short-tailed opossum (*Monodelphis domestica* (Wagner 1842)) (Fadem 1989). In wild-caught Mahali mole-rats, both NRFs within colonies and dispersing females exhibited follicular development up until the Graafian follicle stage; however, no corpora lutea of ovulation were observed in these females (Hart 2019). The year-round follicular activity in the ovaries of NRF Mahali mole-rats implies that they can reproduce with individuals from neighbouring unrelated colonies after dispersal from their natal colonies throughout the year. Pairing usually results in the formation of new colonies from unrelated and genetically distinct conspecifics (Bennett and Faulkes 2000). These dispersing females would actively seek mating opportunities. Dispersing females exhibit spontaneous growth and development of primary follicles into mature follicles but lack the final stimulus of coitus to cause ovulation. Pairing with a suitable mate and subsequent mating is likely the final trigger for ovulation and subsequent pregnancy.

The onset of rainfall softens the soil and increases food abundance which in turn reduces the energetic costs of searching for a mate in the solitary species, or dispersal in social species

(Faulkes and Bennett 2013). Seasonal reproduction and true induced ovulation are common in the solitary species of mole-rats which inhabit regions with predictable and seasonal rainfall (Faulkes and Bennett 2013). The social Highveld (Janse van Rensburg et al. 2002; Malherbe et al. 2004) and common mole-rats (Bennett 1989; Spinks et al. 1997, 1999) exhibit the same selected traits of seasonal reproduction and induced ovulation, whereas the Mahali and Natal mole-rats (Oosthuizen et al. 2008) exhibit induced ovulation, but aseasonal reproduction. Aseasonal reproduction is shared with the eusocial genera *Fukomys* (Snyman et al. 2006; Bennett et al. 2010; Faulkes et al. 2010) and *Heterocephalus* (Faulkes et al. 1990; Jarvis and Bennett 1990); however, these species are spontaneous ovulators. Phylogenetic studies have revealed a positive correlation between seasonality in breeding and induced ovulation (Faulkes et al. 2010). A reconstruction of the evolution of African mole-rats has revealed that induced ovulation was likely the ancestral state and that this trait has been convergently lost in the eusocial genera *Fukomys* and *Heterocephalus* (Faulkes et al. 2010). The Mahali and Natal mole-rat have maintained the trait of induced ovulation, although they are aseasonal breeders.

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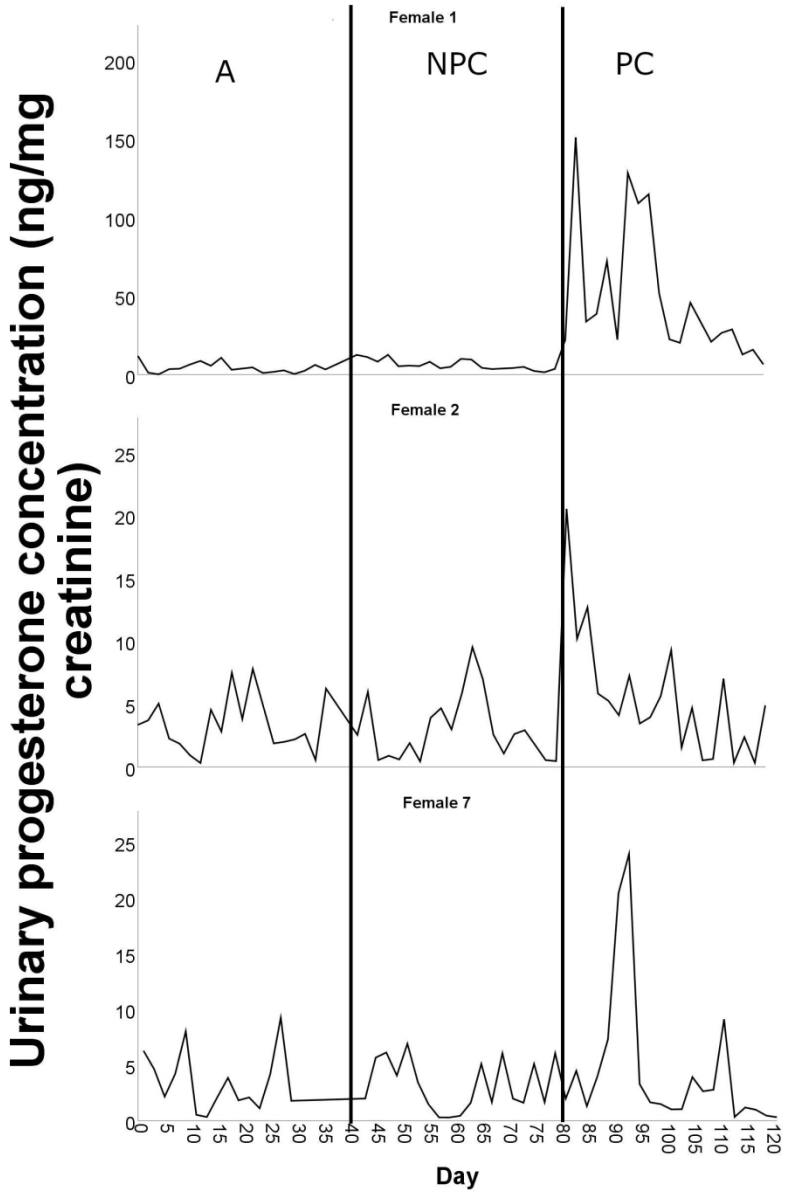
Figure legends

Fig. 1: Urinary progesterone concentrations (ng/mg creatinine) in three female Mahali mole-rats, subjected to all three treatments: *A*: Alone; NPC: non-physical contact with a male; PC: physical contact with a male. Progesterone values were measured every second day over a total of 40 days per treatment.

Fig. 2: Mean ± SE urinary progesterone concentrations (ng/mg creatinine) for female Mahali mole-rats which were housed either alone (*A*, *n* = 10); in non-physical contact with a male (NPC, *n* = 7), or in physical contact with a vasectomized male (PC, *n* = 4). Each treatment lasted for 40 days with urine collections every second day.

Fig. 3: Surface morphology of the penis of male Mahali mole-rats (*Cryptomys hottentotus mahali*) seen with a scanning electronic microscope (SEM): (i) outer surface of the penis and (ii) magnified protruding structure on the penile surface. Arrows indicate protruding structures like spines.



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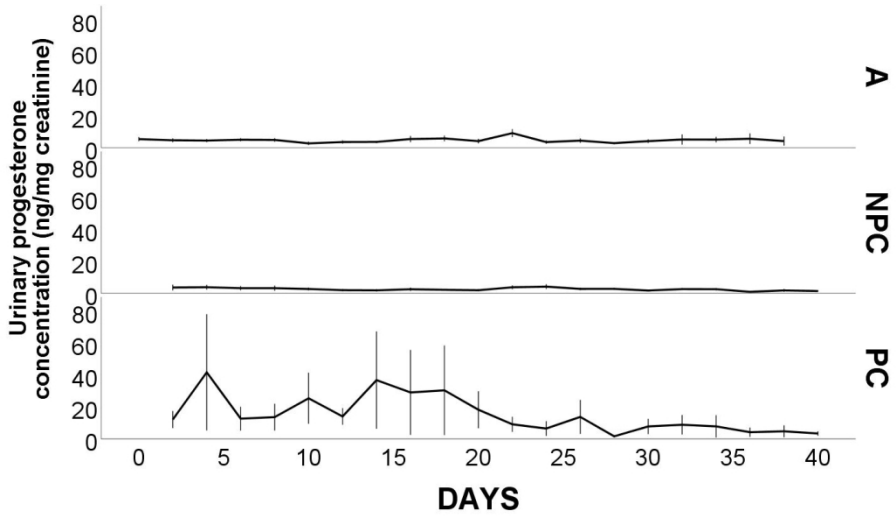

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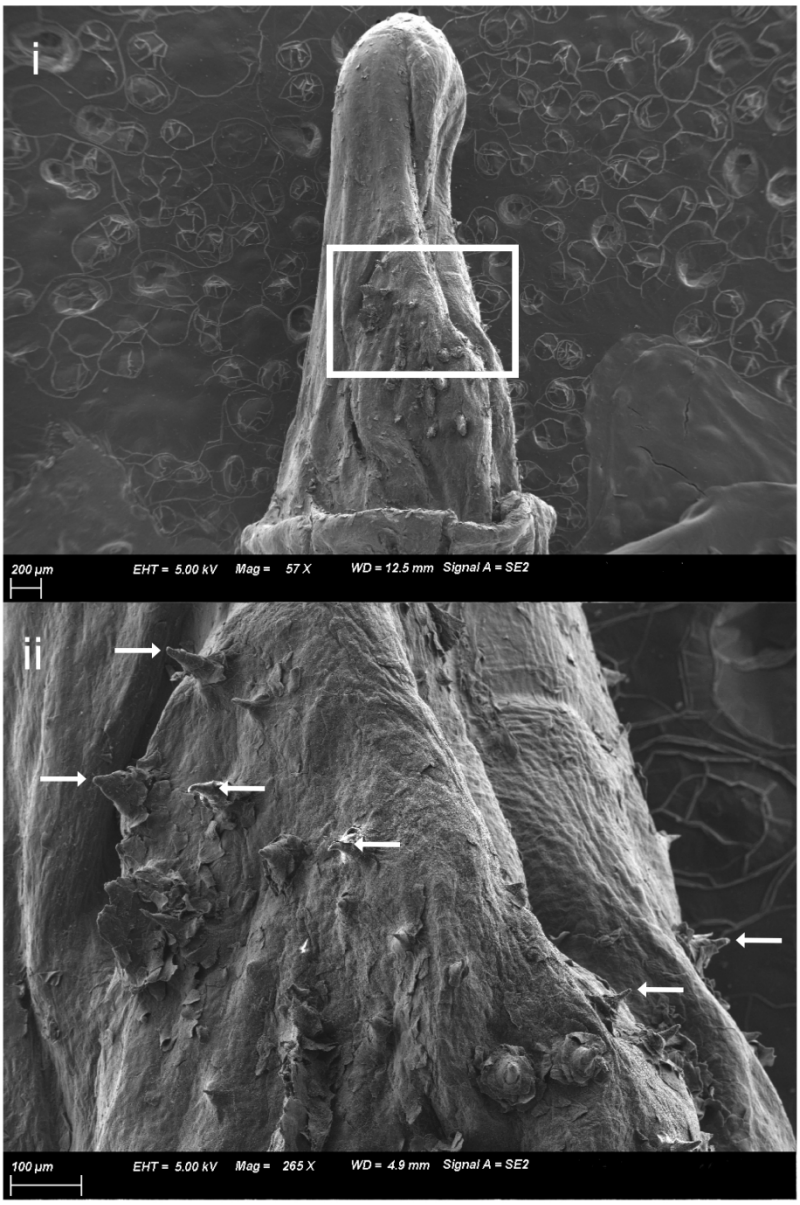

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