The pattern of ovulation in the East African Root-Rat (*Tachyoryctes splendens)* from Tanzania: induced or spontaneous ovulator?

Jestina V. Katandukila[1, 2*] & Nigel C. Bennett[1, 3]

[1] *Department of Zoology & Entomology, University of Pretoria, Pretoria 0002, South Africa*

[2] *Department of Zoology, College of Natural and Applied Sciences, University of Dar es Salaam, P.O.Box 35064, Dar es Salaam, Tanzania*

[3] *South African Research Chair for Mammal Behavioural Ecology and Physiology, Department of Zoology & Entomology, University of Pretoria, Pretoria 0002, South Africa*

* **Corresponding author:** Jestina V. Katandukila (email: jestina@zoology.up.ac.za).

Abstract

The East African root rat, *Tachyoryctes splendens* is a solitary dwelling subterranean rodent that exhibits a marked seasonal reproduction linked to the bimodal rainfall pattern of East Africa. The current study set out to determine whether the East African root rat is an induced or spontaneous ovulator. Five wild caught adult females were monitored non-invasively for ovarian cyclicity by measuring urinary progesterone every two days over a period of 120 days. Females were subjected to three different experimental treatments namely, initially singly housed/Control (C), Non-Physical Contact with a male (NPC) and Physical Contact with a vasectomised male (PC), respectively. The concentration of urinary progesterone was higher during PC than during either NPC or C. The act of coitus appears to be necessary for ovulation to occur

in the females, despite the fact that the males were vasectomised and incapable of fertilising the females.

The male root rats were found to possess epidermal spines on the penis which can be used to remove copulatory plugs as well as bring about cervical stimulation during coitus. The spines result in the induction of ovulation as has been proposed for other solitary subterranean rodents. The findings from the female progesterone profiles and the assessment of penile morphology suggest that the female root rat is an induced ovulator stimulated by penile intromission during coitus.

Keywords: *Tachyoryctes splendens*, progesterone, creatinine, ovulation, copulation, penile spines

Introduction

The East African root rat is a solitary subterranean rodent belonging to the family Spalacidae (Jarvis and Sale 1971). The root-rat is endemic to East and central Africa with a patchly distribution and particulary restricted to the highland savannah (Katandukila et al. 2014; Kokiso and Bekele 2008; Jarvis and Sale 1971). It is a seasonal breeder possessing two breeding periods per year. The inter-breeding interval is 166 days with a female producing 2.2 litters per annum (Katandukila et al. 2013a). The breeding periods are tied to bouts of rainfall (long rains and short rains which are characteristic of East Africa). The majority of solitary species studied to date, including solitary subterranean rodents, are reported to exhibit an induced pattern of ovulation which appears to be linked to their life history (Conaway 1971).

The pattern of ovulation in mammals may be one of two patterns, either spontaneous or induced (Milligan, 1980). Spontaneous ovulation does not require vaginal stimulation, with females ovulating at regular periods of the year irrespective of whether copulation takes place (Carlson and Gese 2008; Rasweiler et al. 2011). Spontaneous ovulation is characterised by a continuous cycle of follicular development, where primordial follicles develop into Graafian follicles, and following ovulation a corpus luteum arises. Spontaneous ovulation is under the direct control of a cyclical production of hormones (Milligan 1974). Gonadotrophin releasing hormone plays a significant role in vertebrate reproduction (Bakker and Baum 2000) by bringing about the secretion of the gonadotrophins, follicle stimulating hormone and luteinising hormone, which in turn govern follicular development (Campell and Reece 2005). In induced ovulators a similar process of follicular development to that of spontaneous ovulators arises, but without copulation the female fails to ovulate, despite possessing elevated levels of oestrogen derived from the developing follicles (Knobil 1988). Cervical and vaginal stimulation is crucial to induce the LH surge and subsequent ovulation. Induced ovulators thus do not show the cyclical steroid induced preovulatory GnRH and subsequent LH surges so charactersitic of spontaneous ovulators (Bakker and Baum 2000).

The tactile stimuli caused by the outer surface of penis during copulation against the vaginal wall and cervix has been reported to be important for bringing about ovulation in induced ovulators (Jöchle 1975; Viker et al. 1993; Parag et al. 2006). Multiple attempts of mounting and copulation are required to stimulate and bring about ovulation with the subsequent production of a corpus luteum of ovulation (Bennett *et al*. 2000). The solitary Cape dune mole-rat (*Batherygus suillus*; Bennett et al. 2000), social Natal mole-rat (*Cryptomys hotentottus natalensis*; Jackson and Bennett 2005), social

highveld mole-rats (*Cryptomys hotentottus pretoriae*; Malherbe et al. 2005) and solitary Cape mole-rat (*Georychus capensis*; van Sandywk and Bennett 2005) are all confirmed induced ovulators (Parag et al. 2006). Induced ovulation has also been recorded in the solitary *Tuco tuco* (*Ctenomys talarum*; Fanjul and Zenuto 2012) and the solitary blind mole-rat (*Spalax ehrenbergi*; Shanas et al. 1995), which is incidently a phylogentically close relative of the East African root rat, *Tachyoryctes spendens*.

Spontaneous ovulation is the more common pattern of ovulation and has been recorded in a number of small mammals across a broad phylogenetic spectrum including the brown antechinus (*Antechinus stuartii*; Woolley 1966), the Cape ground squirrel (*Xerus inauris*; Bouchie et al. 2006), the Degu (*Octodon degus*: Mahoney et al. 2011) and the southern African spiny mice (*Acomys spinosissimus*; de Bruin et al. 2014). Within subterranean mammals it is reported in the Damaraland mole-rat (*Fukomys damarensis*; Synman *et al.* 2006), the giant Zambian mole-rat (*Fukomys mechowii*; Faulkes et al. 2010), Ansells's mole-rat (*Fukomys anselli*; Bennett et al. 2010) and the naked mole-rat (*Heterocephalus glaber*; Faulkes et al. 1990).

So why would induced ovulation have arisen in mammals? There have been several suggestions posited. Firstly, many small mammals characterisitically possess relatively short life spans and often breed seasonally, consequently they have a narrow window of opportunity to reproduce. Induced ovulation increases the probability of successful breeding following copulation, furthermore, solitary species as well as those species with large home ranges, or low population densities that as a consequence have low mate encounters may benefit from this pattern of ovulation (Lariviere and Ferguson, 2003).

We predicted that male East African root rat will possess epidermal spines on the surface of the glans. We further predicted that the female East African female root

will only exhibit ovarian cyclicity when paired with an unrelated male. Furthermore, we predicted that female East African root rats will only exhibit elevated progesterone concentrations resulting from the production of corpora lutea of ovulation following full contact with the vasectomised male.

Materials and Method

Five male and five female East African root-rats were captured from farmlands in the Arusha region of Tanzania (03˚18 ' 44-45" S, 036˚46' 46-47"E; 1461-1530 metres above sea level) in January 2015. The root-rats were maintained at the Department of Zoology and Wildlife Conservation, University of Dar es Salaam (Republic of Tanzania) for three weeks prior to exportation to the Department of Zoology and Entomology, University of Pretoria, Republic of South Africa. Root-rats were housed singly since the species occurs solitary in the natural habitat (Katandukila et al. 2014). Root rats were fed on chopped carrot and sweet potato and maintained within laboratory at 25^{o}C and a photoperiod of 12L:12D (Katandukila et al., 2013b).

Ethical note

Root-rats were maintained under the guidelines of the American Society of Mammalogists (Animal Care and Use Committee 1998; http://www. mammalogy.org/committees/index/asp) and the research protocol was approved by the Animal Ethics Committee of the University of Pretoria (ethics clearance number ECO62-14), Pretoria, South Africa. Research clearance were granted by the Tanzania Wildlife Research Institute (TAWIRI) and Commission of Science and Technology (COSTECH: 2014-277-NA-2014-204). Export permits were authorized by TAWIRI, the Ministry of Natural Resources & Tourism, Wildlife Division (permit no. 68368) and Ministry of Livestock & Fisheries, Zoosanitary section (permit no. 003986) all in Dar es

Salaam, Tanzania. The import permits (CPB6-2386 and 13/1/1/30/201411000171) were granted by the Gauteng Department of Nature Conservation and the Department of Agriculture, Forestry and Fisheries, Johannesburg, South Africa.

Experimental design

The experiment comprised a sequencial design using the same 5 females for all three protocols and the same males for the non control protocols.

Experimental animals were placed under three consecutive experimental treatments namely, Control (C), non-physical contact (NPC) and physical contact (PC). Each experimental treatment was executed over 40 consecutive days. It is hypothesised that if the females are going to cycle they will do during this period of time.

Control (C). Five females were housed singly in cages (34cm x 34cm x 20cm) with no males present in the same room, thus preventing any chemical interaction between the males and females. The treatment was used as a measure for baseline progesterone concentrations.

Non Physical Contact (NPC). Five females were housed in individual cages (48cm x 28.5cm x 48cm) next to five males individual males . A wire mesh separated the males from the females and thus prevented any physical contact. There was, however, visual, olfactory and auditory contact between the sexes. The males and females remained in these conditions for the duration of the experiment. The males were present for the entire forty day period.

Physical Contact (PC). Five females were each housed with vasectomised males in cages (48cm x 28.5cm x 48cm). This allowed for full physical contact as well as chemical contact of the females with the males and consequently the opportunity for copulations to occur. Males were only allowed with the females for 5 hours per day and were under supervision from the authors to ensure that no aggressive interaction

resulted. Males were introduced to females every second day for for a period of five hours for the duration of the full 40 day period irrespective of how many bouts of copulation took place over the period.

Urine collection

Urine was collected from female *T. splendens* (n = 5) on every second day from the onset of experimental phases and labelled accordingly. Each female was kept in a cylindrical plastic cage with a wire mesh base settled on top of urine collection tray; the wire mesh base prevent faecal material to enter the urine. Urine was collected between 09h00 and 13h00, as soon as urine had been voided the female was placed back into her container. Urine was collected from March to July 2015; urine was kept in a freezer at −40°C immediately following collection.

Male vasectomy

A qualified veterinarian vasectomized five males (mass 352.20 ± 24.99 g) three months prior to being placed in PC. Vasectomy was performed to remove the vas deferens and epididymis from each testis to ensure that during the PC experimental phase no pregnancy resulted. During vasectomy, the males were mask-induced anaesthetized using 5% isofluorane gas and subsequently maintained using 2-2.5% isofluorane gas until the end of procedure. The analgesic Meloxicam (0.5mg/kg) was administered after vasectomy to assist with pain post-surgery.Vasectomy was performed three months prior to experimentation to ensure the epididymus was clear of spematozoa.

Assessment of penis morphology

Penises were dissected out from frozen material of additional adult males collected on a prior occasion, these organs were placed in 10% formalin on thawing. This fixing procedure was followed by a series of treatments with a 0.15M phosphate

buffer. Specimens were subjected to a series of dehydration steps using ethanol of increasing concentration (50–100%). Critical point drying (CPD) was reached (CPD from liquid CO_2) and the material was further dehydrated using the BIORAD 3000 critical point drier (Watford, UK). At this point, the material was effectively coated with dehydrated cells that carry heavy metals (osmium and phosphate fixation) to which minute particles of gold can adhere. A Polaron E5200C (Watford, UK) sputter coater was used to sputter a few nanometres of gold, coating the fixed dehydrated material, which was mounted on a carbon tape on a lead stage. The gold-plated material was then viewed with a scanning electron microscope – J SM-840 (JEOL, Tokyo, Japan), and subsequently images were produced.

Determination of Creatinine

Creatinine is a breakdown product from tissue proteins, usually formed by muscle tissues in mammals (Schmidt-Nielsen 1997), it is excreted at a relatively constant rate. Before progesterone assay, all urine samples were analysed for creatinine concentration (Bonney et al. 1982) using a modified Jaffe reaction (Folin 1914). Creatinine standards were made up fresh on a daily basis and aliqouted into epindorf tubes and kept frozen until use. Standards were made from a stock of creatinine powder and dissolved in distilled water. The procedure was as follows; 6 µl of standard or sample was added to the wells of a microtitre plate in duplicate, 2 wells were left empty as duplicate blanks. To each of the wells a 180 µl of fresh picrate reagent was added. Picrate reagent consisted of saturated picric acid solution, alkaline triton and distilled deionized water (1:1:10). The alkaline triton was composed of 4.2 ml triton X-100, 12.5ml 1 N NaOH and 66.0ml distilled deionized water; the picric reagent was continuous stirred to make homogenous solution. The standards were prepared from 3mg/ml of Creatinine (600mg (0.6 g) of creatinine (Sigma) in 200 ml of DIDW) and

prepared on different concentration including 0.0, 0.05, 0.1, 0.5, 1.0, 1.5 and 2mg/ml. The microplate was then left in the dark at room temperature for 2 hours to allow development of colour. Absorbance of the plate was measured using a microplate reader at an optical density of 492 nm using (Multiscan Ascent V1.24, Amersham) . Standard curves (R^2 > 0.99) were used to determine all sample values. Urinary progesterone concentrations were expressed as ng/mg creatinine (ng/mg Cr or ng mg^{-1}Cr); expressing urinary hormone concentrations relative to creatinine standardizes measurements over differing concentrations of urine.

Radioimmunoassay

The urine from experimental females was assayed for progesterone using Coat-A-Count kit following the manufacturer's specifications. The kit was validated for use in mole-rats as previously described in Bennett et al. (1994) and the total hormonal concentrations were determined using a gamma counter. Extracted urine samples from two pools were compared with unextracted urine fom the two pools. There was no significant difference in progesterone concentration between the extracted and non-extracted pools and as a consequence we used the neat urine to measure native progesterone. The use of non-extracted urine to investigate patterns of ovulation has been succesfully used in other roednet moles (Malherbe et al. 2004; Jackson and Bennett 2005; van Sandwyk and Bennett 2005; Snyman et al. 2006; Faulkes et al. 2010). We spiked the standard curve with 6 samples at a dilution of 1:16 from a pool of urine of low concentration to these 50µL of progesterone in increasing concentrations (0.3, 1.6, 6.4, 31.8, 63.6 and 127.2 nmols/l) whucg were subsequently assayed in dupicate. The resulting curve was perfectly parallel to the standard curve.

Progesterone: A volume of 50µL of neat unextracted urine was dispensed in duplicate into polypropylene tubes following the Coat-A-Count progesterone kit (IBL

International GMBH Germany.) procedures. The progesterone antiserum is highly specific for progesterone with a low cross-reactivity (< 1%) to other naturally occurring steroids except in 5α-Pregnan-3, 20-dione (3.46%), 20-β-Dihydroxyprogesterone (3.27%) and 17- α- Hydroxyprogesterone (1.50%). The urine sample with a high concentration of progesterone was double-diluted using the assay buffer as a matrix 1:1 to 1:16 then assayed. The slopes of serial double dilution and standard curve were compared to check for parallelism (Analysis of covariance (ANCOVA): ($F_{1,5}$) = 0.101; n = 6; P > 0.05) following a log-logit transformation of the data (Chard 1987). The intra-assay coefficient of variation for the urine pool was 3% whereas the inter-assay coefficient was 6.1% and sensitivity of the assay defined as twice the buffered blank was 1.35 nmol/L.

Data analysis

Data were subjected to R algorithms (R Core team 2015). The data were tested for normality using ShapiroWilk Normality test (W = 0.7623 p < 0.05, n = 296). A comparison of urinary progesterone between experimental phases/treatments and between females based on Friedm an statistical test as our data fitted non-parametric parameters for related samples (Faulkes et al. 2010). The Wilcoxon Signed-rank statistical test was a post hoc. Other measurements wer e expressed as mean ± standard deviation (SD). Graphics were generated by Grammer of Gr aphics plotting2 (ggplot2) package. The level of significance for all statistical tests was α < 0.05.

Results

Cyclicity of urinary progesterone

During the Control (C) experimental phase the concentration of urinary progesterone was 0.80± 0.34 ng/mg Cr whereas during Non-physical contact (NPC) and the Physical contact (PC) phases the concentrations were 0.71± 0.23 ng/mg Cr and 1.29± 0.75 ng/mg Cr, respectively (Table 1).

Concentrations of urinary progesterone varied significantly between experimental phases ($F(2,0.05) = 30.94$, p-value = 0.000, n = 100). The comparisons of concentration of urinary progesterone between experimental phases showed significantly higher concentrations during the PC phase than during C and NPC phases (C & NPC: $W(1,0.05) = 0.64$, p = 1.000, N = 100; C & PC: $W(1,0.05) = 4.46$, p=0.0001, N = 100; NPC & PC: $W(1,0.05) = 5.10$, p-value = 0.0001, N = 100: (Table 1).

Table 1. Summary of urinary progesterone hormone concentrations (ng/mg Cr, where Cr is creatinine; mean ± SD) from experimental female East African root rats (*Tachyoryctes splendens*) ($n = 5$) within experimental phases.

	Progesterone concentration (ng/mg Cr)			
Variable	Overall	C	NPC	PC
F1	0.96±0.59	0.72±0.33	0.72±0.19	1.45±0.76
F2	0.91±0.48	0.83±0.36	0.64±0.15	1.24±0.61
F3	0.96±0.55	0.73±0.38	0.88±0.31	1.38±0.75
F4	0.91±0.51	0.88±0.35	0.66±0.21	1.18±0.70
F5	0.90±0.61	0.81±0.32	0.67±0.17	1.22±0.92
C	0.80±0.34*a*	—	—	—
NPC	0.71±0.23*a*	—	—	—
PC	1.29±0.75*b*	—	—	—

Note: Variation in progesterone concentrations between the five female East African root rats (F1–F5) for each experimental phase was not significantly different from each other ($P > 0.05$). Overall variation in progesterone concentrations in East African root rats between experimental phases was significantly different ($P < 0.001$) as indicated by the different lowercased italic letters (for more details see Results). C, control; NPC, nonphysical contact; PC, physical contact.

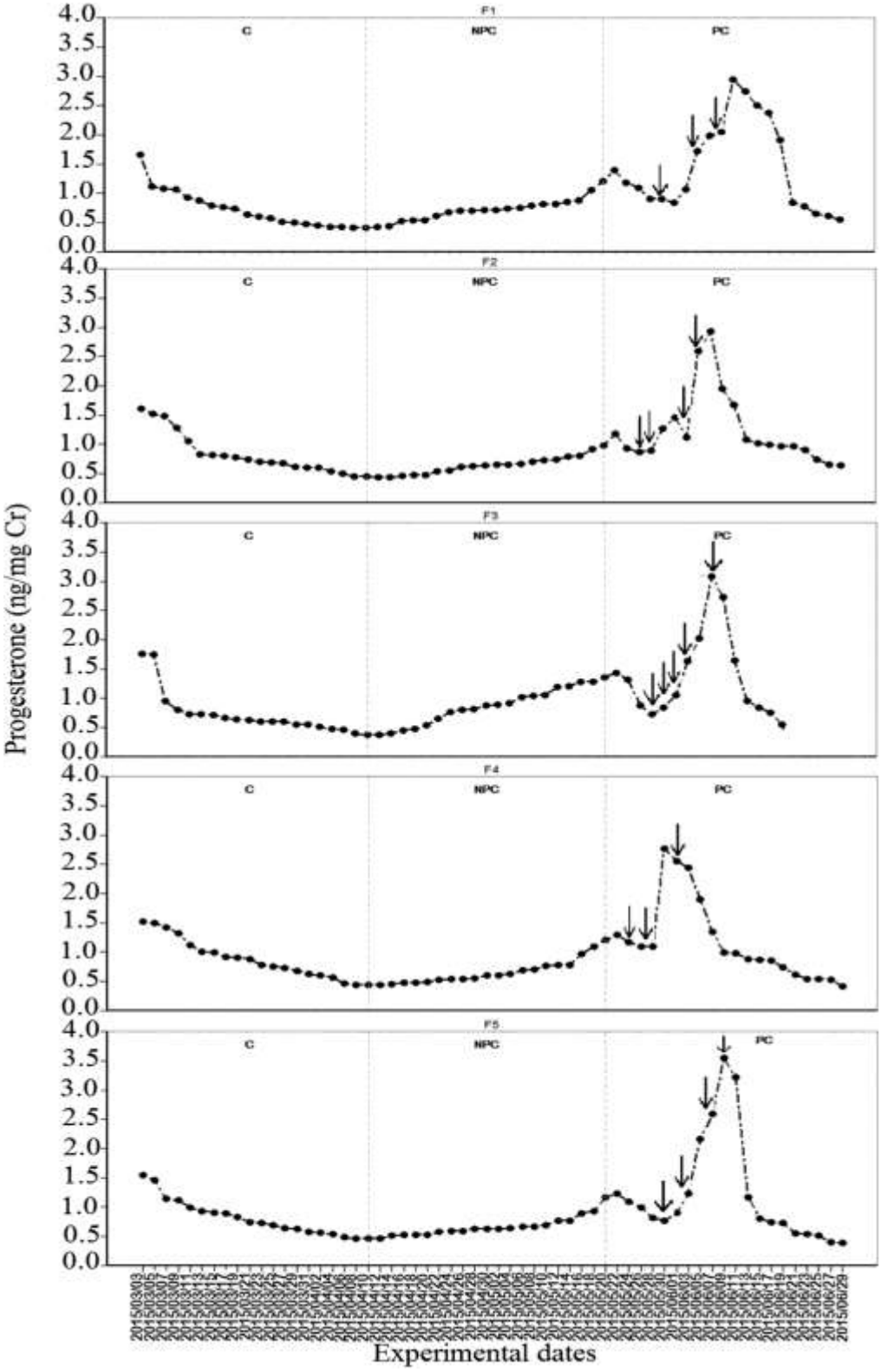

Figure 1: Trend of concentration of urinary progesterone hormone in five females with respective dates (Year/Month/day) of urine collection during three experimental phases; C= Control; NPC = Non-Physical Contact; PC = Physical Contact: F = female; arrows show days when copulation arose.

Comparison of variation in the concentration of urinary progesterone between females was not statistically significant ($F_{(4,0.05)}$ = 2.35, p= 0.67, n = 60) (Table 1).

The highest levels of urinary progesterone were recorded when females were housed with a male during physical contact and the progesterone profiles show definitive follicular and luteal phases. It was calcualted from the urinary progesterone concentrations that a mean follicular phase of 9.2±1.03 days and a mean luteal phase of 15.2±3.03 days could be estimated from the profiles (Figure 1).

Penile morphology

Numerous spines were observed on the outer surface of penises (n = 5). These sharp structures were unevenly distributed (Figure 2). At higher magnification the protruding spines overlapped the skin-like layers (Figure 2). The shape of spines on the outer surface of penis of *T. spendens* showed much similarity to those of *Georychus capensis*, but were disimilar to those recorded for *Bathyergus suillus.* However, the shape of a distal part of penis of *T. splendenes* showed similarity to the later species (Figure 3).

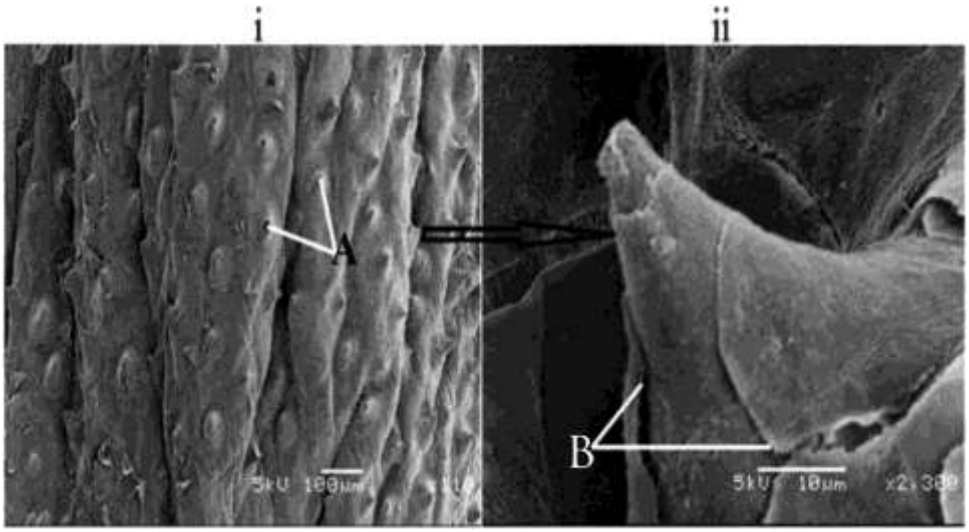

Figure 2: Surface morphology of the penis of male *Tachyoryctes splendens* seen with Scanning Electronic Microscope (SEM); i = outer surface of the penis, ii = magnified protruding structure on the penile surface: A = protruding structures like spines, B = overlaying skin layers on the protruding structure of the penis.

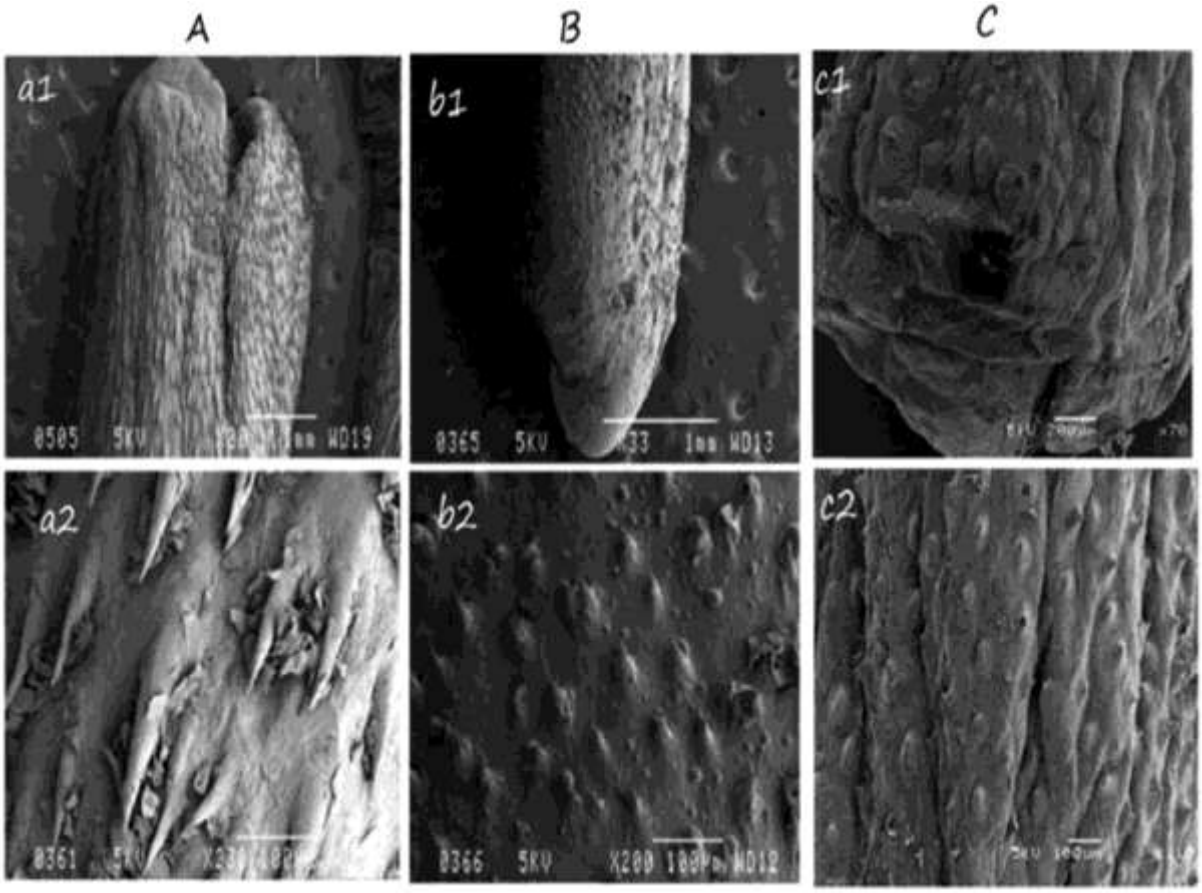

Figure 3: Morphology of outer surface on penile structure of solitary African subteranean rodents; A = *Bathyergus suillus*; B = *Georychus capensis*; C = *Tachyoryctes splendens*; a1, b1 and c1 = penile shape of each species respectively; a2, b2 and c2 = magnified surface showing protruding structure (i.e. spine-like) (source for A and B: Parag et al. 2006).

Discussion

Sequential monitoring of urinary progesterone has shown that in the physical presence of a male, female root rats undergo ovulation following copulation by the male, suggesting the East African root rat is an induced ovulator.

The highest levels of urinary progesterone were recorded when females were housed with a male during physical contact and the progesterone profiles show definitive follicular and luteal phases. Experimental animals were observed copulating approximately 5 days after the pairing of females with males; the first four days were preceded by agonistic behaviour and the general avoidance of one another. Copulation

has been reported to induce ovulation following penile intromission in a number of mammals (Dewsbury 1972, 1988).

The elevation of urinary progesterone concentrations had a mean length of 15.2±3.03 days and prior to the proposed luteal phase, a follicular phase of 9.2±1.03 days is estimated. The available data suggests that the length of the ovarian cycle is approximately 24 days. The luteal phase is shorter than that of the estimated 26 days for the spontaneous ovulator *F. mechowii* (Faulkes et al. 2010) and 27.5 days estimated for the *H. glaber* (Faulkes *et al*. 1990). Because the females used in this study had baseline concentrations at the start of the experiment, it is assumed that these females were acyclic at the start of the series of experiments. Breeding usually takes place during the long rains (March, April and May) as well as the short rains in late September. October and November. The fact that females housed on their own and also housed in non-physical contact failed to exhibit ovarian cyclicity in April and May (the period usually when they breed), but exhibited distinct cyclicity in June and July (a time when they do not breed) tends to suggest that the cycles we observed in these females were not as a result of an endogenous circannual event. We purposely paired the animals in the non-breeding period in order to negate these types of effects.

The finding that ovulation is induced in the East African root rat is drawn from the progesterone profiles and is consistent with the observed penile morphology obtained using scanning electron microscopy, which is similar to that observed in two solitary bathyergid mole-rats, namely *Bathyergus suillus* and *Georychus capensis* in that the glans of the penis is covered in epidermal; spines. Conversely, SEM examination of the penises of *H. glaber*, *F. mechowii* and *F. damarensis* confirm the progesterone profiles that reveal a spontaneous pattern of ovulation. The SEM profiles reveal that the

glans is smooth and lacks protrusions or spines which would be necessary for coital stimulation in induced ovulators (Parag et al. 2006).

Induced ovulators require the mechanical stimulation of the cervix and vaginal walls to trigger the onset of ovulation (Zarrow and Clarke 1968). As a consequence the anatomy and morphology of the penis is adapted to meet this necessary requirement (Harcourt & Gardiner, 1994). Penile morphology of the South American *tuco tuco* of the genus *Ctenomys* is similarly characterised by spinous protrusions (Altuna & Lessa 1985). Indeed, in the *tuco tucos*, the presence of spines may also facilitate lock during mating and the removal of copulatory plugs, thus facilitating sperm competition (Ramm, Parker and Stockley 2005). Induced ovulation has been found in both solitary and cooperatively breeding species (van Sandwyk and Bennett, 2005, Malherbe et al. 2004 and Jackson and Bennett, 2005). A characterstic feature of these taxa is that they are either seasonally breeding or ar depemdent on short lived mating encounters. In contrast, the two eusocial species of mole-rat, the Damaraland mole-rat and the naked mole-rat are both spontaneous ovulators and are non seasonal, breeding throughout the year and have regular mating encounters (Faulkes et al. 1990; Snyman et al. 2006). These two species reside in habitats where rainfall is sporadic and unpredictable, as a consequence dispersal is infrequent with the result that tunrover of breeders is reduced and new colony formation not so frequent (Jarvis et al. 1994).

Tachyoryctes splendens is a strictly solitary species that comes together to breed during periods of rainfall when the soil is more friable for digging and when fresh above ground vegetation is abundant to feed the young. Individuals advertise their sex and presence by tapping their head on the burrow ceiling (Hrouzkova et al. 2013), indeed this sesimic communication may be advantageous for breaking down the strict xenophobia that arises in this species. When pairing up the root rats, the first few days were

characterised by agonistic behaviours and vocalisation, it is possible that the lack of seismic communication did not enable the break down in xenophobia (Katandukila pers. obs.). As in African mole-rats, seasonal breeding is accompanied by regular and predictable periods of dispersal, but where encounters for mating are brief and opportunistic these appear to be the drivers that have shaped an induced pattern of ovulation in the East African root rat (Bennett and Jarvis 1988). This finding is consistent with comparative studies in other mammals (e.g. carnivores, Lariviere & Ferguson 2003) where induced ovulation has arisen to overcome the risks of unsuccessful mating that arises in seasonal environments.

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