

The pattern of ovulation in Ansell's mole-rat, *Fukomys anselli*: phylogenetic or ecological constraints?

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Abstract

The distribution of ovulation patterns and penile ornamentation in mammals is thought to be shaped by sexual selection. Alternatively ovulation pattern have been linked to factors such as phylogeny, social system and ecological constraints but no conclusive pattern has emerged. African mole-rats exhibit a unique range of social organizations and experience diverse ecological condition (i.e. rainfall patterns) with various species exhibiting either induced or spontaneous ovulation in addition to a corresponding variation of penile ornamentation. The members of this family investigated so far do not permit conclusions to be drawn about the importance of phylogenetic versus ecological constraints for the evolution of ovulation pattern since all species of the genus *Cryptomys* studied occur in mesic habitats and exhibit induced ovulation. In contrast the one representative of the genus *Fukomys* is a spontaneous ovulator that occurs in arid habitats. The current study aimed to elucidate the factors creating the observed ovulation pattern by using a species within a genus for which so far only spontaneous ovulation has been recorded but unlike the other species with known ovulation mechanisms of this genus it occurs in a mesic environment. Previously non-reproductive Ansell's mole-rat (*Fukomys anselli*) females were housed individually for a period of six weeks prior to being housed either alone, in chemical or physical contact with a male. Progesterone profiles generated from urine samples collected throughout the study did not differ significantly either before or after the pairing or between the experimental groups suggesting that they ovulate spontaneously. This was supported by the lack of penile ornamentation found in males of this species. The results suggest that phylogenetic rather than ecological constraints determine the ovulation patterns observed in social bathyergids.

Keywords: Bathyergidae, spontaneous ovulation, progesterone, penile ornamentation

Introduction

It has been proposed that penile ornaments and induced ovulation are a result of sexual selection in species that are solitary, have a promiscuous mating system and short periods of interaction between the sexes (Lariviere & Ferguson 2003, Parag et al. 2006). In such systems the potential for pre-copulatory assessment of potential mates and male-male competition may be limited and as a consequence post-copulatory mechanisms such as physical stimulation of the female by penile spines may be a more effective means of intrasexual competition among males. If a certain degree of stimulation of the female vagina and cervix by such structures is necessary for successful ovulation (i.e. induced ovulation) this could be used as criterion for mate choice by females (Eberhard 1985; Parag et al. 2006). Induced ovulation is frequently found in mammals and it has been suggested that this is linked to phylogenetic patterns (Conaway 1971; Milligan 1982; Schiml, Wersinger & Rissmann 2000). Alternatively, ecological factors such as rainfall have been linked to ovulation patterns and such factors and the associated mode of ovulation frequently shows a higher variability at the species than at higher phylogenetic levels (Lariviere & Ferguson 2003). Consequently, there is no general consensus whether phylogenetic constraints or ecological factors play a more prominent role in determining the mode of ovulation and penile morphology (reviewed in Adkins-Regan 2005).

The subterranean family Bathyergidae exhibits a full spectrum of social organization ranging from solitary species through to highly social species (Bennett & Faulkes 2000) and is thought to have evolved in response to dispersal constraints imposed by rainfall patterns in their habitats (Faulkes et al. 1997). Due to their subterranean life-style dispersal is directly linked to the workability of the soil that

increases after rainfalls and foraging activity as well as dispersal is strongly linked to rainfall patterns. The high energetic costs associated (Vleck 1979) with digging constrain burrowing activity including dispersal during dry periods (Bennett & Faulkes 2000). At the one end of a continuum are the strictly solitary genera *Georychus*, *Bathyergus* and *Heliophobius*, which occur in mesic habitats and only pair up for brief periods of time during the breeding season (Bennett & Jarvis 1988a; Sumner et al. 2003; Herbst, Jarvis & Bennett 2004). Male penises in these solitary species *Bathyergus suillus* and *Georychus capensis* are characterised by epidermal spines that aid the stimulation of ovulation in females (Bennett, Faulkes & Molteno 2000; Parag et al. 2006) and in at least two solitary genera females have been shown to be induced ovulators (Bennett et al. 2000; van Sandwyk & Bennett 2005).

In contrast to the solitary bathyergids, the social mole-rat genera *Cryptomys*, *Fukomys* and *Heterocephalus* live in groups where reproduction is confined to a single female per colony and breeding may occur throughout the year (Jarvis 1981, Bennett & Jarvis 1988b; Bennett 1989; Jarvis & Bennett 1993; Burda & Kwalika 1993; van Rensburg et al. 2002). The genus *Cryptomys* has representatives which occur in habitats with moderate to high seasonal rainfall (Bennett & Faulkes 2000) that facilitates breeding dispersal and the extent of promiscuity can be low to moderate (Bishop et al. 2004). As in solitary species with predictable seasonal rainfall females in this genus typically exhibit induced ovulation (Malherbe, Bennett & Schoeman 2004; Jackson & Bennett 2005). The males characteristically possess penile ornamentation that is thought to stimulate the female vaginal and cervical tract to trigger ovulation (Parag et al. 2006).

In contrast, two species of the other social genera, the Damaraland mole-rat, *Fukomys damarensis*, and the naked mole-rat, *Heterocephalus glaber*, experience strong ecological constraints that provide individuals with limited opportunities for breeding dispersal (Jarvis et al. 1994) and the degree of promiscuity is comparatively low (Burland et al. 2002; Burland et al. 2004). In the habitats occupied by these mole-rats rainfall is unpredictable and sporadic, and several months or even years may pass before the soil properties are suitable for extended foraging or dispersal activity (Bennett & Faulkes 2000). Males of both species lack the elaborate ornamentation of the penis found in the other Bathyergidae (Parag et al. 2006) and females of both species exhibit spontaneous ovulation (Faulkes, Abbott & Jarvis 1990; Snyman, Jackson & Bennett 2006). This mode of ovulation may have arisen or persisted in these two species because colony turnover is less frequent compared to the species in mesic habitats. At the same time selection may not have acted strongly on the male morphological structures that induce ovulation.

Among the social bathyergid species studied so far ovulation patterns coincide with both the phylogeny of the species and the rainfall pattern in their habitat (Table 1) that correlate with the degree of mating dispersal and promiscuity (Bishop et al. 2004; Burland et al. 2004). As a result, it remains unclear which of these factors is the key determinant of the patterns observed. A recent molecular phylogeny places Ansell's mole-rat (*Fukomys anelli*) in close relationship with the Damaraland mole-rat (Faulkes et al. 2004; Ingram, Burda & Honeycutt 2004). A study by Willingstorfer, Burda & Winckler (1998) suggests that Ansell's mole-rat non-reproductive females are unovulatory in their natal colony. However, since this is the case for non-breeding

females in this genus as well as in *Cryptomys* species this allows no conclusions regarding the mode of ovulation.

Ansell's mole-rat appears to breed throughout the year (Burda 1989), however, in contrast to Damaraland mole-rats they occur in areas with moderate to high rainfall and consequently dispersal opportunities and hence extra-colony mating opportunities may arise frequently (Bennett & Faulkes 2000). Thus, they experience ecological constraints similar to members of the Bathyergidae for which induced ovulation has been reported while they are phylogenetically closer to mole-rat species that exhibit spontaneous ovulation. This renders them an ideal model species to evaluate the importance of phylogenetic versus ecological constraints on ovulation patterns in bathyergids.

Assuming that the mode of ovulation is determined by phylogenetic constraints we would predict that Ansell's mole-rat is a spontaneous ovulator as is the case of its sister representative and males should lack elaborate penile ornamentation. In contrast, if ecological constraints (i.e. rainfall patterns) are the selective force shaping ovulation patterns in bathyergids we would expect induced ovulation and penile ornamentation in the study species.

Materials and methods

Experimental animals were captured about 25km west of Lusaka, Zambia, from February to April 2007. The trapping methods consisted of modified Hickman live traps which were placed at an opening to the burrow system, or by trapping an animal in the burrow system by blocking the retreat of the mole-rat in the tunnel by use of a hoe. Seven males and eleven non-reproductive females, discerned by their non-perforate vagina, were

collected. All males used in the study had a mass above 80g and females had a mass exceeding 60g to ensure sexual maturity. The males used in this study originated from different colonies than the females. After capture, the animals were transported to the Republic of South Africa (Permit no. 13/1/30/2/98-127) to the University of Pretoria (Permit no. CPB6 000036) where the experiments took place. The protocol was approved by the Animal Use and Care Committee of the University of Pretoria (AUCC060719-020). Animals were housed in a temperature-controlled room maintained at between 26 and 28°C with a relative humidity of between 50 and 60% and a lighting regime of 12L:12D. Wood shavings were used as nesting material. Mole-rats were fed sweet potatoes, chopped gem squash and apple daily, no free water was provided since they obtain all of their water requirements from the food resource to maintain a positive water balance.

Surgery

Three males were vasectomised three months prior to being placed in physical contact with one of three females. Males were anaesthetized using iso-fluorane gas induction and maintenance with a mask. Both the vas deferens and epididymis were removed from each of the testis. All vasectomies were completed three months prior to pairing up to ensure clearance of sperm from the vas deferens. Vasectomy was performed to ensure that physical contact alone and not hormonal changes resulting from fertilization were recorded.

Experimental design

Females were placed individually in plastic containers (1m x 0.5m x 0.5m) for an initial period of six weeks prior to being divided into three groups for an additional period of six weeks. The first group (4 animals) was left in isolation as control animals. The second group (4 animals) was placed in non-physical contact with gonadally intact males. Holding cages were provided with a wire mesh (2mm grid) separation preventing any physical contact but allowing semio-chemical communication. The females in the third group (3 animals) were placed in a holding chamber with vasectomised males to permit physical interaction. In all these cases mating took place within a couple of hours of pairing.

Sample collection

Over the 88 days of the experiment, females were placed in urine collection chambers every second day between 08h00 and 14h00 during which urine samples were collected. The chambers were cylindrical and had a smooth mesh floor to allow urine to fall through to a collecting dish. This set up prevented faecal contamination of the urine. The mole-rats were checked hourly throughout the day and urine samples were collected by sterile pipette being stored in plastic eppendorf tubes with the animal number and date. The plastic tubes were stored at -40°C. On completion of collection the mole-rat was returned to the holding cage.

Progesterone determination.

Progesterone is an important indicator hormone because it rises with increasing follicular development and subsequent production of the corpora lutea of ovulation (Espey & Lipner 1994). The cyclical pattern of progesterone secretion in spontaneous ovulators provides the rationale behind the use of progesterone concentrations for detecting ovulation (Bauman 1981) and longitudinal studies have shown that the assessment of progesterone profiles is a suitable non-invasive method for the detection of ovulation (Brown 2000). This protocol has previously been successfully employed to investigate patterns of ovulation in the African mole-rats (Faulkes et al. 1990; Clarke, Miethe & Bennett 2001) and to differentiate patterns of induced ovulation (Malherbe et al. 2004; Jackson & Bennett 2005) from spontaneous ovulation (Snyman et al. 2006). The progesterone concentrations were measured using a coat-a-count kit (Diagnostic Products Corporation, Los Angeles, USA). The antiserum is highly specific for progesterone with a cross reactivity to all naturally occurring steroids <0.5%, with the exception of 17 α hydroprogesterone (3.4%), 11-eoxycorticosterone (2.4%), 5 β -pregnan-3, 20-dione (3.2%) and 5 α -pregnan-3, 20 dione (9.0%). The concentrations of the standards ranged from 0.3 to 127.2 nmol/l. The assay has previously been validated for use in mole-rats by testing for parallelism in the slope of the curve produced using serial dilutions (over the range of 1:1 to 1:64) of mole-rat urine obtained from a pregnant female against that of a standard curve (Jackson & Bennett 2005). The minimum detection limit of the assay was 0.36 nmol/l and intra- and inter-assay coefficient of variation was 5.1% (n=6) and 9.3% (n=6) respectively.

Creatinine determination

Prior to hormone assay all urine samples were analysed for their creatinine content (Booney, Wood & Kleinman 1982). Creatinine concentration was determined by using a modified version of the Jaffe reaction (Folin 1914). The process involves adding 10µl of standard or sample to the well of a microplate, in duplicate, and leaving two wells empty as duplicate control blank. A further 200µl of picrate reagent is added to the wells, including the blanks. Fresh alkaline picrate was mixed and comprised a saturated picric acid solution, alkaline titron and deionised water (1:1:10). The alkaline triton is composed of 4.2ml triton x-100, 12.5 ml 1N NaOH and 66.0 ml distilled deionised water. After adding the alkaline picrate the microplate was placed in the dark for a period of 1.5 h, at room temperature to allow colour development to occur. A standard curve ($R^2 > 0.99$) was used to determine all sample values.

Electron microscopy

Penises were dissected out from frozen material of adult males and placed in 10% formalin on thawing. This fixing procedure was followed by a series of treatments with a 0.075M phosphate buffer, whereas post-fixation was achieved using a treatment of 1% osmium tetroxide. Specimens were subjected to a series of dehydration steps using ethanol of increasing concentration (30-100%). Critical point drying (CPD) was reached (CPD from liquid CO₂) and the material was further dehydrated using the BIORAD 3000 critical point dryer (Watford, UK). At this point, the material is effectively coated with dehydrated cells that carry heavy metals (osmium and phosphate fixative) to which minute particles of gold can adhere. A Polaron E5200C (Watford, UK) sputter coater was

used to sputter a few nanometers 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 subsequent images were produced.

Statistical analysis

Despite the intense effort put into urine collection sample volumes obtained from a study animal were not always sufficient to conduct the assay for a particular day. Data was thus averaged for the periods before and after experimental manipulation before further analyses. Data were log-transformed to satisfy the criteria of a normal distribution and subjected to a repeated measurement analysis of variance (ANOVA) to account for repeated measurements of an individual (SPSS 17). The significance level for all tests was set at $p < 0.05$.

Results

The number of samples collected that amounted to sufficient urine for hormone analyses was 132 for controls (range: 25-39), 142 for animals in chemical contact (range: 25-40) and 116 for females in physical contact with vasectomised males (range: 31-45). The progesterone values varied greatly throughout the experimental period and between experimental treatments. Baseline fluctuations of progesterone between about 0 and 5 ng progesterone/mg creatinine contrasted with distinct progesterone peaks observed across groups during the first experimental period in all experimental groups (Fig. 1). These peaks were less pronounced in the control group possibly due to the lack of sufficient

amounts of urine samples. Although some synchrony of peaks of progesterone of different females could be observed during the experimental period large individual variation precluded the identification of a clear cycling pattern. Despite copulations occurring within hours of pairing we did not observe increases of progesterone levels in females in response to physical contact with males that would indicate that ovulation had occurred. Such increases in response to stimulation would have been clearly visible as the progesterone levels measured during this period were at baseline levels (Fig. 1c)

Progesterone levels averaged at 5.6 ± 12.2 ng progesterone/mg creatinine for control, 20.6 ± 62.0 ng progesterone/mg creatinine for females with chemical stimulation and 18.2 ± 62.0 ng progesterone/mg creatinine for females in physical contact with a male during the first part of the experimental period. During the second part of the experiment progesterone levels were 9.5 ± 29.0 ng progesterone/mg creatinine for control females, 18.4 ± 9.6 ng progesterone/mg creatinine for females in chemical contact and 9.4 ± 21.0 ng progesterone/mg creatinine for females in physical contact (Fig. 2). The repeated measurement ANOVA found no significant effect of the trial period on the progesterone levels measured ($F_{1,8}=0.106$, $p=0.753$). Similarly, there were no significant differences between the different experimental groups ($F_{2,8}=3.228$, $p=0.094$) and we found no significant effect of the interaction between trial period and experimental group ($F_{2,8}=1.646$, $p=0.252$). The highest absolute progesterone levels were recorded for females in the chemical stimulus experiment trial in both the control and experimental part (Fig. 1b). Similarly, progesterone peaks were observed during the control period of females that were subsequently placed in physical contact with males (Fig. 1c) suggesting

that neither a chemical nor a physical stimulus by males is necessary to induce the progesterone cycle in non-breeding Ansell's mole-rat.

Electron microscopy of penises of Ansell's mole-rat revealed them to possess a number of slightly raised longitudinal ridges. At the same time, it is characterised by a distinct lack of obvious spines or rounded protrusions (Fig. 3).

Discussion

Induced ovulation is common in solitary and non-gregarious rodents that synchronise their reproduction with seasonal variation in environmental factors such as rainfall. It is assumed to be an adaptive trait in solitary species that rely on chance or transient encounters for periods of mating and copulation (Zarrow & Clark 1968). The seasonality of rainfall and the degree of promiscuity have also been identified as key determinants for induced ovulation among carnivore species (Laviere & Ferguson 2003).

The rainfall patterns are directly linked with breeding dispersal and extra-colony mating in social mole-rats (Spinks, Jarvis & Bennett 2000; Bishop et al. 2004; Burland 2004) and the mesic habitat of Ansell's mole-rats suggests a high potential for promiscuity in this species. The unusual recording of more than one breeding female in a closely related species, the Giant mole-rat (*Fukomys mechowii*) (Sichilima, Faulkes & Bennett 2008), that occurs in the same habitat, corroborates this hypothesis. If ecological constraints govern ovulation pattern it could thus be expected that Ansell's mole-rats exhibit induced ovulation. However, this was not the case and increases in progesterone were neither linked to copulations observed nor was the presence of a male conspecific necessary to generate significant peaks in urinary progesterone metabolites. This suggests

that Ansell's mole-rat is a spontaneous ovulator like its sister species, the Damaraland mole-rat. Baseline levels of progesterone recorded during the current study were in a similar range as those reported for Damaraland mole-rats (Clarke et al. 2001, Snyman et al. 2006), In contrast to Damaraland mole-rats peaks in progesterone concentrations were already observed during the first days of urine collection while this occurred after 35 days in the sister species (Clarke et al. 2001, Snyman et al. 2006). However, this may be related to the fact that a minimum of three weeks of isolation had elapsed before urine collection was commenced in the current study.

The apparent lack of penile protrusions or spines is in accordance with findings from the taxonomic distribution of such ornaments. In contrast, among social bathyergids only members of the mesic-dwelling genus *Cryptomys* have been found to exhibit penile ornaments (Parag et al. 2006). This indicates that phylogenetic constraints may play a more prominent role in determining ovulation patterns and penile structures in bathyergids than ecological factors. Further support for this hypothesis comes from the striking differences in ecological parameters between the *Fukomys* species and precipitation is three times higher in the habitat of Ansell's mole-rat compared to that of the Damaraland mole-rat (Faulkes et al. 1997).

Although phylogenetic rather than ecological constraints seem to have determined the mode of ovulation in social mole-rats there is evidence that ecological factors have played a regulatory role at other stages of the hypothalamic-pituitary axis in the genus *Fukomys*. In the Damaraland mole-rat non-reproductive female colony members are physiologically suppressed at the level of the pituitary and exhibit reduced concentrations of circulating LH in the blood (Bennett et al. 1993). Studies suggest that this is a result of

the combined effects of inhibition by the breeding female and incest avoidance by the non-breeding female (Bennett, Faulkes & Molteno 1996; Cooney & Bennett 2000). In contrast, Burda (1995) has shown that in Ansell's mole-rat reproductive inhibition appears to be due to an avoidance of inbreeding. This is in accordance with rainfall patterns rather than phylogenetic relationships as members of the genus *Cryptomys* lack a physiological suppression (Faulkes & Bennett 2001) but experience similarly elevated precipitation patterns compared to Damaraland mole-rats (Faulkes et al. 1997).

Damaraland mole-rats occur in regions which are arid and where rainfall is sporadic and unpredictable and there are few chances for dispersal and mating opportunities for non-reproductive females are limited (Jarvis et al. 1994). From the perspective of a non-reproductive female it would thus be energetically costly to invest resources in the production of gametes that are not likely to be fertilized. However, the reproductive axis will be switched on when an unfamiliar male is made available (Bennett, Faulkes & Molteno 1996; Cooney & Bennett 2000) and the extended periods of co-habitation necessary until successful reproduction would allow sufficient time for mate assessment. In the more mesic habitats explored by the *Cryptomys* species and Ansell's mole-rats such limitations have been lifted from non-reproductive females and accordingly a purely behavioural mechanism has evolved (Faulkes & Bennett 2001).

The emerging pattern suggests that while the mode of reproductive suppression in social mole-rat species has evolved in response to ecological parameters such as rainfall, ovulation methods have been more inert towards ecological factors and are determined by phylogenetic relationships. The former allows for more flexibility under varying environmental conditions similar to the opportunistic potential reported from a number of

solitary rodents that will extend their breeding season when environmental conditions are favourable (Heideman & Bronson 1994). At the same time, the evolution or loss of morphological characteristics associated with induced ovulation requires a high investment and once evolved is not easily reversible. This may account for the observed association of ovulation method and phylogenetic patterns in bathyergids. If this hypothesis is warranted it illustrates how competing demands can exert selective forces at different stages of the hypothalamic-pituitary axis to shape the reproductive pattern found among mammals. The family Bathyergidae is characterised by a great diversity of ecological factors, social and mating systems and reproductive patterns reflected in a multitude of mammal species. Thus, the results of the current study are not only shedding further light on factors shaping the reproductive biology of African mole-rats but may be useful in identifying the factors determining the ovulation mechanisms and reproductive physiology in other mammals as well.

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Figure legends

Figure 1 Progesterone profiles for non-reproductive females removed from their natal colony and housed for 6 weeks initially on their own and subsequently for a further six weeks either a) alone, b) in non-physical but chemical contact or c) paired with a vasectomised male. Bar indicates point at which females were separated into respective trials.

Figure 2 Urinary progesterone concentrations (means $\pm$ standard deviation) in female Ansell's mole-rats in the three experimental groups before and after experimental manipulation. Experimental groups: controls, with no contact; chemical, only olfactory contact with a male; physical contact, paired with a vasectomised male.

Figure 3 (a) Penis of Ansell's mole-rat *Fukomys anselli* (b) surface of penis of Ansell's mole-rat *F. anselli*

Figure 1

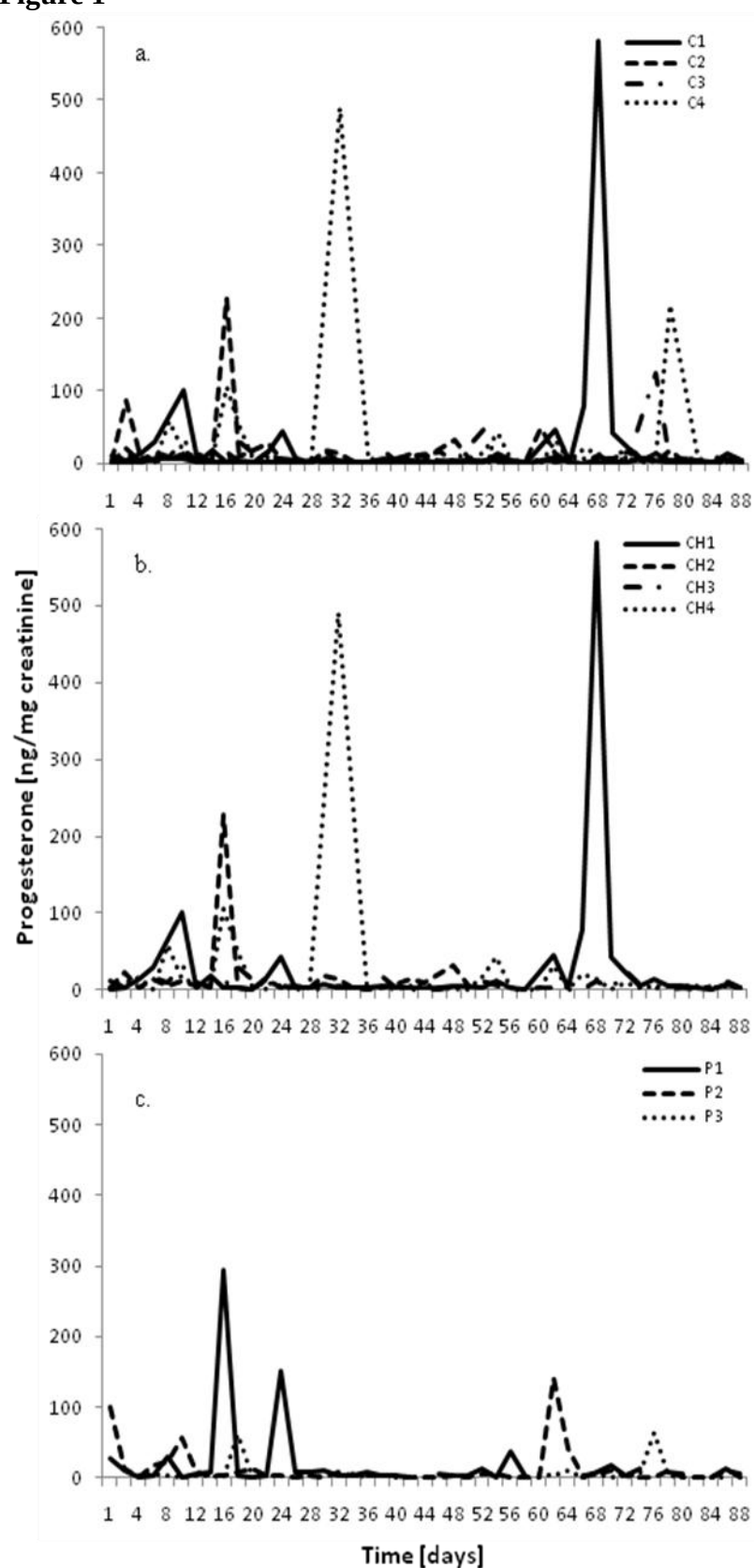


Figure 2

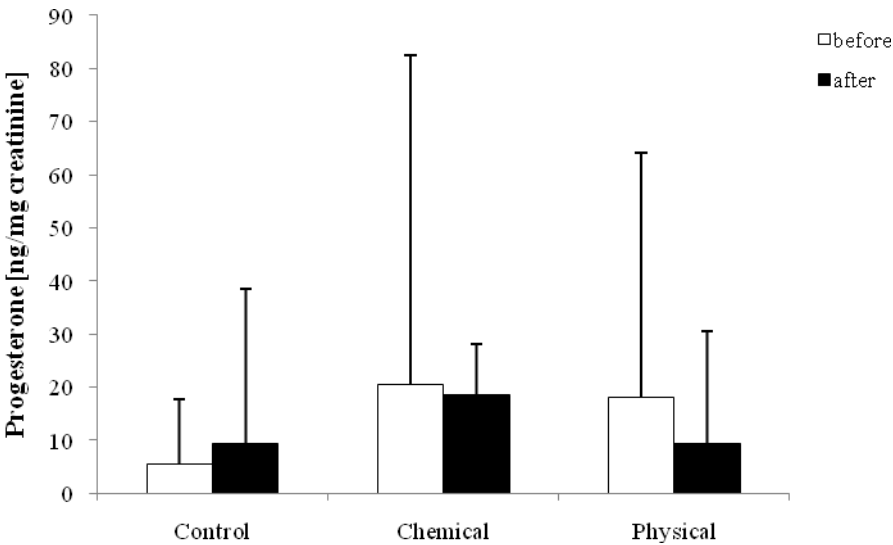
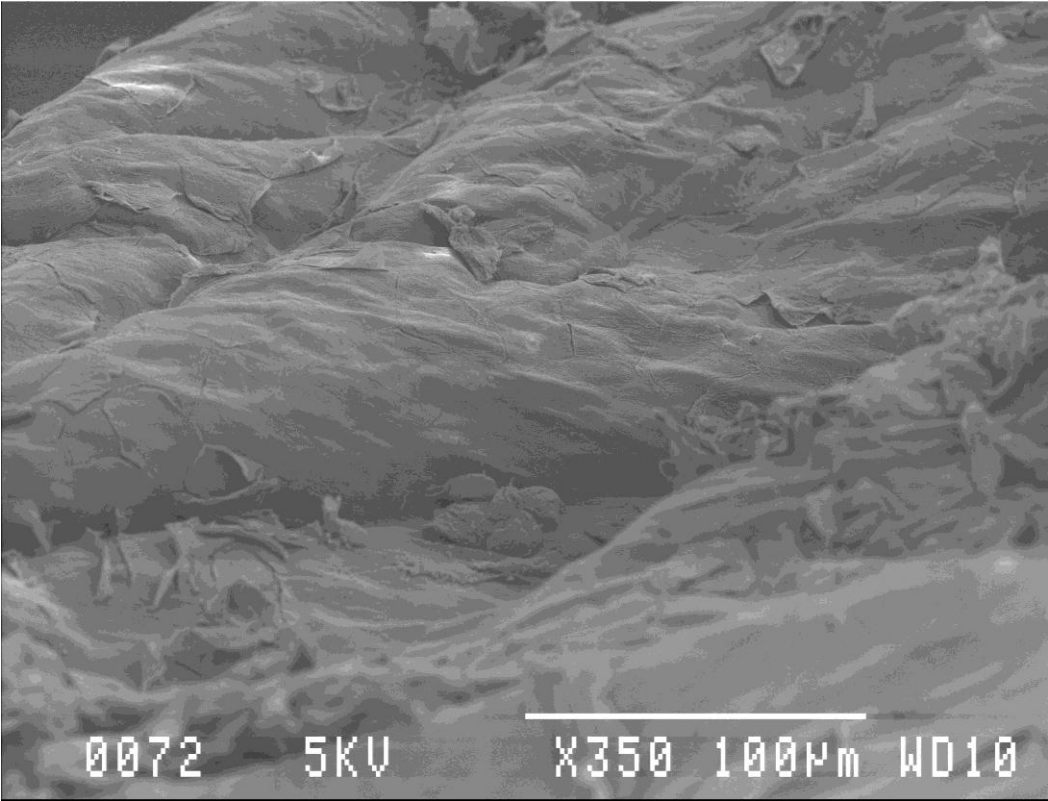
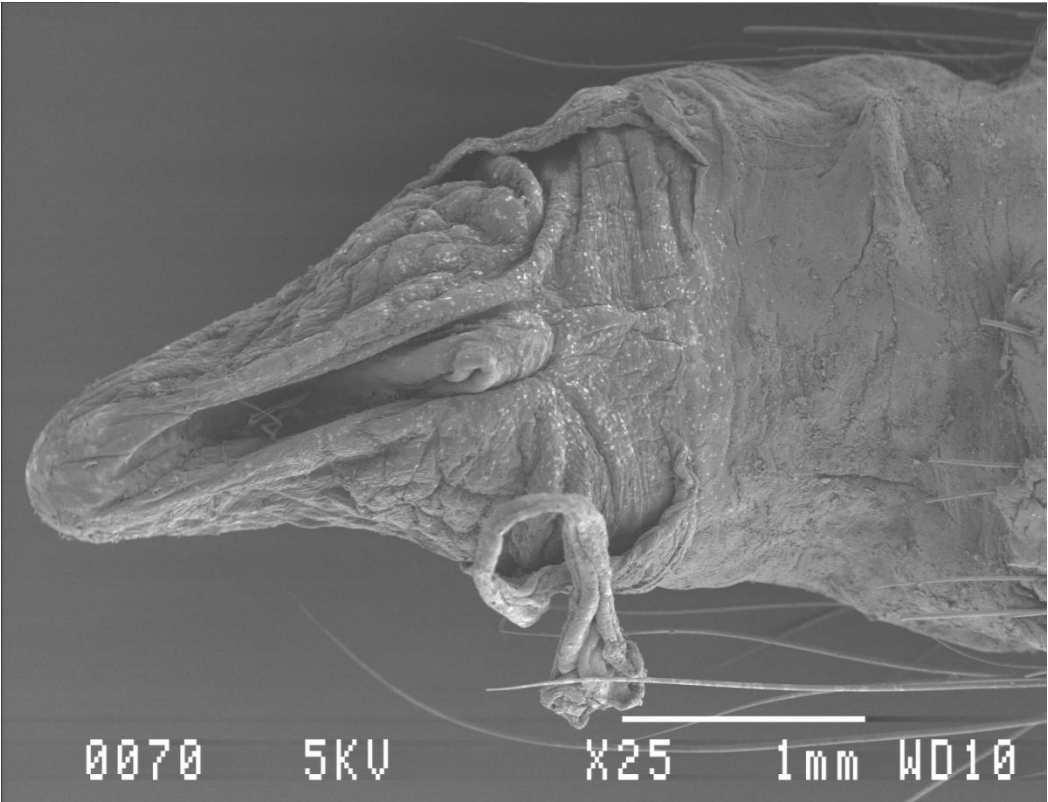


Figure 3



Bennett et al. Table 1. Social, phylogenetic and ecological characters found in bathyergids assessed for their mode of ovulation and penile structures

Species	Sociality	Genus	Habitat	Mode of ovulation	Penile ornamentation
^{a,b} Naked mole-rat	eusocial	<i>Heterocephalus</i>	arid	spontaneous	none
^{b,c} Damaraland mole-rat	eusocial	<i>Fukomys</i>	arid	spontaneous	none
^d Ansell's mole-rat	social	<i>Fukomys</i>	mesic	spontaneous	none
^e Natal mole-rat	social	<i>Cryptomys</i>	mesic	induced	rounded protrusions
^{b,f} Highveld mole-rat	social	<i>Cryptomys</i>	mesic	induced	-
^b Cape dune mole-rat	solitary	<i>Bathyergus</i>	mesic	induced	large, distinct spines
^{b, g} Cape mole-rat	solitary	<i>Georchus</i>	mesic	induced	sharpened spines

^aFaulkes , Abbott & Jarvis 1990, ^bParag et al. 2006, ^cSnyman, Jackson & Bennett 2006, ^dcurrent study, ^eJackson & Bennett 2005, ^fMalherbe, Bennett & Schoeman 2004, ^gvan Sandwyk & Bennett 2005