de Bruin, P.R., et al., The pattern of ovulation in the southern African spiny mouse (Acomys spinosissimus). Mammal. Biol. (2014), http://dx.doi.org/10.1016/j.mambio.2014.05.003

The pattern of ovulation in the southern African spiny mouse (*Acomys spinosissimus*)

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Running title: Ovulation in *Acomys spinosissimus*

Abstract: The pattern of ovulation in mammals is generally considered to be either spontaneous or induced by coitus. The present study aimed to assess the pattern of ovulation in the southern African spiny mice (*Acomys spinosissimus*). Females were divided into three treatments differing in the degree of contact with a male. Control females had no contact with males, separated females had only chemical, auditory and visual contact with a male as the sexes were separated with wire mesh and paired females had full contact with a vasectomized male and copulations were possible. Each treatment consisted of seven females and the ovarian mass, the number of primary, secondary/tertiary and Graafian follicles as well as presence of corpora lutea were compared between the three treatments. Faecal progestagen metabolite (FPM) concentrations were analysed for every second day throughout the experiment and they were used to determine luteal phases and oestrous cycles. Corpora lutea were found in both the control and the paired treatment indicating that ovulation also occurred in the absence of coitus. There was also no effect of treatment on ovarian mass or follicle numbers. In contrast, only females in the separated and paired treatments exhibited luteal phases and oestrous cycles. Especially at the beginning of the experiment, FPM concentrations were higher in those two groups than the control. The results indicate that *A. spinosissimus* appears to ovulate spontaneously, although physical as well as olfactory male cues appear to be of great importance to enhance reproductive efforts of females.

Keywords: *Acomys spinosissimus*, spontaneous ovulation, induced ovulation, progestagens, corpora lutea

Introduction

Ovulation, the rupture of the follicle and subsequent release of an oocyte, is a complex process that is critical for reproduction of animals. Two modes of ovulation, namely spontaneous and induced or reflex ovulation, have been recognised, but it has also been suggested that these two modes are extremes of a continuum (Conaway, 1971). Spontaneous ovulation is characterised by a continuous

recurring cycle of follicular development and release of the mature ovum. An increased release of oestrogen by the Graafian follicle results in a positive feedback on gonadotrophin releasing hormone neurons (GnRH), which in turn results in a surge of GnRH and luteinising hormone (LH) and subsequent rupture of the follicle. In induced ovulators, the positive feedback actions of steroid hormones appear to be absent and the surge of GnRH/LH and ultimately ovulation are elicited by copulation. It has long been thought that mounting and stimulation of the vagina and cervix by penile intromission are adequate stimuli for ovulation (Bibeau et al., 1991, Fernandez-Baca et al., 1970), but more recent studies show that an ovulation-inducing factor in the semen also prompts the LH surge and ovulation (Bogle et al., 2011; Silva et al., 2011). In both spontaneous and induced ovulators, progesterone is released by the corpus luteum that forms in the place of the ruptured Graafian follicle. Progesterone aids in implantation and maintenance of pregnancy, but in conjunction with oestrogen, it also reduces the release of GnRH and LH (Goodman et al., 1981).

The tactile stimulation of the vagina during coitus in induced ovulators appears to be facilitated by modifications of the male reproductive organs such as cornified penile spines (Bakker and Baum, 2000; Zarrow and Clark, 1968). Penile spines were first described in the rat and appear to be present in a number of mammalian orders (reviewed in Zarrow and Clark, 1968). Nevertheless, elaborate ornamentations seem to be more common in induced than spontaneous ovulators. In the African mole-rats (Bathyergidae), for example, induced ovulating species have a large number of spines along their penis whereas the penis of spontaneously ovulating species has ridges but no other ornamentations (Parag et al., 2006). Besides for tactile stimulation through penile spines, the ovulation-inducing factor in the seminal fluid appears to aid ovulation. Both the factor and its receptor seem to be conserved across mammals and are found in both spontaneous and induced ovulators (Bogle et al., 2011). The factor works in a dose-dependent manner in induced ovulators, but its role in spontaneous ovulators is unknown (Bogle et al., 2011).

There are numerous hypotheses put forward as to why the two ovulation modes may have evolved, but ovulation patterns differ markedly between species and a better understanding of the development of the two primary ovulatory patterns will only be possible with more knowledge on the modes of ovulation in many more species. So far the available studies suggest that solitary, non-gregarious and seasonally breeding species exhibit induced ovulation more often than social and aseasonally breeding species (Kauffman and Rissmann, 2006; Zarrow and Clark, 1968). As chances of acquiring a mate are limited in asocial and seasonally breeding species, induced ovulation may be more beneficial as it allows for successful fertilization of the female to occur in the short window when the sexes come into contact. This hypothesis is supported by studies on the African mole-rats (Faulkes et al., 2010; Parag et al., 2006) as well as North American carnivores (Larivière and Ferguson, 2003). The ovulation pattern of voles (*Microtus spp.*) has been under investigation for some time and although they are primarily induced ovulators, some species appear to need less male stimulation for successful ovulation than others (Breed, 1972; Gray et al., 1974). Roberts et al. (1999) suggest that the mating system may play a role with females of promiscuous species requiring more stimulation than those of monogamous species (see also Lidicker and Yang, 1986). In general, induced ovulation appears to be the more ancestral state from which spontaneous ovulation evolved and within mammals induced ovulation seems to have evolved on several separate occasions (Faulkes et al., 2010; Kauffman and Rissmann, 2006).

The present study aims to investigate the ovulation pattern of a small rodent from South Africa, the southern African spiny mouse (*Acomys spinosissimus*). Spiny mice are widespread throughout Africa and the Middle East and inhabit primarily rocky habitats with scant and often seasonally variable resource availability (Fitzherbert et al., 2006; Medger et al., 2010; Wube et al., 2008). Within the genus, studies have mainly focused on the Middle Eastern and North African species such as the common spiny mouse (*Acomys cahirinus*), golden spiny mouse (*Acomys russatus*) and the eastern spiny mouse (*Acomys dimidiatus*) (e.g. Cohen et al., 2010; Frynta et al., 2011; Wube et al., 2009), but little is known about the biology of spiny mice species from south of the equator. A number of recent studies have concentrated on the reproductive physiology of *A. spinosissimus*. Medger et al. (2010; 2012b) established that *A. spinosissimus* breeds seasonally during the warm and wet spring and summer months in South Africa. In addition, the males are reproductively photoresponsive and show increased reproductive development under long-day photoperiods (Medger et al., 2012a). *Acomys spinosissimus* is widespread in southern Africa and occurs in the north-eastern part of South Africa as well as Zimbabwe, Botswana and Mozambique (Skinner and Chimimba, 2005). This rodent is also strictly nocturnal (Hoole et al., 2012), but its social structure is currently uncertain as they have been found to occur solitary as well as in pairs and groups (Skinner and Chimimba, 2005); however, most *Acomys* species appear to be social (Shargal et al., 2000). The only *Acomys* species for which the ovulation pattern is known is the common spiny mouse, which ovulates spontaneously (Peitz, 1981). Nevertheless, it suggests a spontaneous ovulatory pattern for the southern African spiny mouse, although induced ovulation cannot be excluded due to the seasonal reproduction and less well known social structure of this species. In the present work, we tested if the southern African spiny mouse is a spontaneous or induced ovulator. In addition, we investigated if faecal progesterone concentration is elevated in relation to ovulation and we characterized the penile ornamentation of the southern African spiny mouse.

Materials and Methods

Subjects and sample collection

Twenty-one female (17.23 ± 2.30g; Range: 12.52 – 20.88g) and 14 male spiny mice (19.33 ± 3.76g; Range: 15.73 – 29.02g) were trapped at the Goro Game Reserve (22°58'S, 22°57'S, 29°25'E, 29°24'E) in the Limpopo Province, South Africa. The animals were trapped overnight using Sherman live traps (H. B. Sherman Traps, Inc. Tallahassee, Florida, U.S.A.). The traps were baited with a mixture of fish, peanut butter and oats. Males were captured in May 2011, four months before the onset of the experiments. Females were captured in July 2011 and kept separate from any males for a further five weeks before the start of the experiments to eliminate any possibility of male induced reproductive activity. All animals were housed in individual cages with wood shavings and paper towel provided as bedding and housing, respectively. They were fed with mouse pellets, bird seeds and sliced apples and carrots. Water was provided *ad libitum*. Males and females were kept in temperature controlled rooms at 25°C and on a 14:10 h light-dark cycle simulating the photoperiod during the South African summer. The project was approved by the animal ethics committee of the University of Pretoria (EC008-11) and collection permits were granted by the Departments of Nature Conservation in the Limpopo and Gauteng Provinces.

For the experiment, the females were randomly assigned to one of three experimental treatments consisting of seven females each. For the first treatment (control), the females were housed individually and separately from any males in their own temperature controlled room. This guaranteed

that any signs of reproductive function were not induced by male cues. For the second treatment (separated), females were housed in cages separated from the males by a wire mesh, which allowed visual, auditory and olfactory communication between the two sexes, but prevented mounting and copulation. For the third treatment (paired), seven females were individually kept in a cage together with a vasectomized male in order to allow physical contact between the sexes. The experiments were conducted over a period of 35 days.

All females were weighed at the beginning and at the end of the experiments. Faecal samples for measuring progestagen metabolite (FPM) concentrations were collected every second day from all females. The females were placed into a urine collection chamber which assured an easy collection of any faeces without contamination. They were provided with apple to ensure food and water intake during the collection time. The chambers were checked for any faecal matter every three hours. Collection was conducted over nine hours after which the females were returned to their home cages. All faecal matter was immediately frozen and stored at -20°C until analysis. At the end of the experiments, the animals were sacrificed by exposing them to an overdose of halothane. Their final body mass was determined and the entire reproductive tract including the ovaries was dissected out. The reproductive tract was fixed in Bouin's fluid for ten hours and then stored in 70 % ethanol.

A qualified veterinarian vasectomized seven males one month after capture. Anaesthesia was mask-induced using 5% isofluorane gas and then maintained using 2 – 2.5% isofluorane gas for the duration of the procedure. Meloxicam (0.5mg/kg) was used as a method of pain control after the procedure. Males were placed together with the females three months later to ensure sufficient time for the recovery from the procedure and to prevent any spermatozoa being present in the vas deferens when placed in direct contact.

Histology

The uterine horns and any connective tissue were removed from the ovaries and the mass of both ovaries per female was determined to the nearest 0.01 mg using a Sartorius 1213MP scale (Sartorius AG, Göttingen, Germany). At first, the ovaries were dehydrated by exposing them to a series of ethanol baths of increasing concentrations and then embedded in paraffin wax (Drury and Wallington, 1967). The entire ovary was then cut into 5 µm sections using a microtome (820 Spencer Microtome, American Optical) and immediately mounted on microscope slides using gelatine. The ovarian sections were stained with Ehrlich's haematoxylin and eosin. The slides were examined for the different follicular stages at 200X magnification with a light microscope. Primary, secondary, tertiary and Graafian follicles as well as corpora lutea were identified according to Bloom and Fawcett (1964). Primary follicles were identified by multiple layers of follicular cells around the ovum and secondary and tertiary follicles were characterized by one or more irregular spaces between the follicular cells. Graafian follicles were categorized by a large space which resulted in the ovum being moved to one side of the follicle. The mean number of each follicular stage was recorded per female. Due to the degree of similarity and only subtle differences in size, the secondary and tertiary follicles were grouped together in order to minimise error during classification.

Hormone analysis

The minimal appropriate mass for hormone measurement (Millspaugh and Washburn, 2004) was determined in a preceding study (de Bruin et al., 2014), and revealed that 25 to 55 mg of faecal matter is required to provide reliable results. In many cases, we were unable to obtain the required amount of individual faeces during the nine hours of collection. As a consequence only 241 of the 358 samples

collected could be used for the subsequent hormone analysis, resulting in individual data sets of four to 15 samples per female. Faecal samples were freeze-dried, pulverized, and between 25 - 50 mg were extracted by adding 1 ml of 80% ethanol and subsequent vortexing for 15 min. Extracts were centrifuged for 2 min at 1500*g* after which the supernatant were decanted into micro-centrifuge tubes and frozen at -20°C until analysis. Faecal extracts were measured for immunoreactive progestagen metabolite (FPM) concentrations using an enzyme immunoassay for 5ß-pregnane-3a-ol-20-one (Schwarzenberger et al., 1996). The assays were performed on microtiter plates according to the procedure described by Ganswindt *et al.* (2002). The suitability of the assay for the use in *A. spinosissimus* was confirmed by de Bruin et al. (2014). The Inter- and Intra-Assay Coefficient of Variation (%), determined by repeated measurements of high and low value quality controls, ranged between 12.73 – 17.28% and 8.61 – 11.54%, respectively.

Penile morphology

The penises of four euthanized males were dissected out and preserved in 70% ethanol. For electron microscopy, all penises were fixed in 10% formalin and rinsed three times for ten minutes each with 0.075 M phosphate buffer. Subsequently, they were fixed in 0.5% osmium tetraoxide for approximately two hours, where after the samples were rinsed again using 0.075 M phosphate buffer. The final dehydration was done by exposing the samples to a series of ethanol solutions of increasing concentration for ten minutes each. The dehydrated samples were then dried to a critical point using a critical point drier (Bio-Rad E 3000, Watford, England) with liquid CO_2 and mounted on a stub and sputtered with gold using a Sputter coater (Emitech K550X, Ashford, England). A scanning electron microscope (SEM) (JEOL JSM-5800LV, JEOL, Tokyo, Japan) was used to determine the presence of penile modifications. SEM pictures were taken at 37X, 40X and 110X magnifications.

Statistical analysis

All dependent variables were tested for normal distribution and homogeneity of variance. Ovarian mass and Graafian follicle number were log- and sqrt-transformed, respectively, to obtain normally distributed data. The number of corpora lutea was not analysed statistically, because of the small numbers observed. Body mass was analysed with a repeated measures Analysis of Variance (RM-ANOVA) with the day of measurement as within-subject variable and treatment as factor. An Analysis of Covariance was utilized to compare ovarian mass between the three treatments and the body mass from the end of the experiment was added as a covariate to account for possible body mass effects. The numbers of all follicle types were compared between treatments using ANOVAs.

Individual baseline FPM values were evaluated using the method described in Brown et al. (1999). The mean + 1.5 standard deviation (SD) from an individual's data set was calculated and all concentrations that exceeded this value were excluded. The mean + 1.5 SD was then recalculated and the procedure repeated until no concentrations exceeded the mean + 1.5 SD. Subsequently, the baseline FPM concentration was calculated from the remaining values. Following van Aarde and Haim (1999), more than one consecutive FPM concentration above baseline was used as an indication for the onset of a luteal phase of an oestrous cycle. Consequently, this indicated ovulation and the post-ovulatory formation of a corpus luteum and oestrous cycles could be evaluated for females with at least two post-ovulatory luteal phases (de Bruin et al., 2014). The number of FPM concentrations above baseline was compared between the treatments with a Generalized linear model (GZLM) fitted with a Poisson distribution and log linear function. To account for the differences in the number of samples per

individual, sample number was included as a covariate. We further analysed FPM concentrations over the entire sampling period (17 sampling days) and compared it between treatments by using a Generalized Estimating Equation (GEE) with a gamma distribution with log-link function. Both the GZLM and the GEE were followed by least significant difference post-hoc tests (LSD). IBM SPSS 20 (IBM Corp., 2012) was used for all statistical analyses. *P*-values of ≤ 0.05 were considered to be significant and results are presented as mean ± standard error (SE).

Results

RM-ANOVA showed no difference in body mass between the start and end of the experiment ($F_{1,18}$ = 0.84, p = 0.37). There was also no difference in body mass between the three treatments overall ($F_{2,18}$ = 0.02, p = 0.98) and body mass was similar for all treatments at the beginning and at the end of the experiment ($F_{2,18}$ = 0.12, p =0.89). Ovarian mass was not dependent on female body mass ($F_{1,17}$ = 2.94, p = 0.11) and was also not different between control (5.6 ± 1.5 mg), separated (4.0 ± 1.0 mg) and paired females (5.4 ± 1.0 mg; $F_{2,17}$ = 0.88, p = 0.43). Moreover, the number of any of the follicle types was not correlated with ovarian mass ($F_{1,17}$ < 0.73, p > 0.40). There was also no difference in primary, secondary/tertiary and Graafian follicle numbers between the treatments ($F_{2,17}$ < 2.76, p > 0.09; Fig. 1). *Figure 1 to be placed here* One corpora lutea was present in one control female and two corpora lutea were found in a single paired female.

The number of samples with FPM concentrations above baseline (elevated FPM values) was positively related to the number of samples analysed per individual (Wald χ^2 = 10.30, df = 1, p = 0.001). Nevertheless, the number of samples with elevated FPM concentrations differed significantly between treatments (Wald χ^2 = 11.59, df = 2, p = 0.003). LSD tests showed that more samples with elevated FPM values were detected in the separated and paired treatment groups compared to the control group ($p \leq 0.002$; Table 1). The number of samples with elevated FPM concentrations was similar in the separated and paired treatments (LSD: p = 0.19; Table 1). Correspondingly, luteal phases were only observed in females of the separated and paired treatments and not in any of the control females (Table 1). *Table 1 to be placed here* Representative FPM profiles for individual females from each treatment are shown in Figure 2. Complete oestrous cycles were found only in one female of the separated and one female of the paired treatment with cycle lengths of 16 and 18 days, respectively (Fig. 2). *Figure 2 to be placed here* We further analysed FPM concentrations over the entire sample period using a GEE to see at which time during the experimental period changes could be seen. Using this approach, FPM concentrations were not different between the three treatments (Wald χ^2 = 0.64, df = 2, p = 0.73). There was, however, a difference in FPM concentrations between the 17 collection days overall (Wald χ^2 = 583.28, df = 16, p < 0.001) and between and within treatments (Wald χ^2 > 1000, df = 20, p < 0.001, Fig. 3). FPM concentrations exhibited a peak a few days after the start of the experiment with the highest concentration measured on day 6 in comparison to any other day (LSD: p < 0.05). When the different treatments were compared, this pattern was only observed in the separated treatment and in the paired treatment, although the increase was less marked in the latter (Fig. 3). For the separated treatment, FPM concentration at day 6 was significantly increased compared to all other days (LSD: $p \leq 0.01$) except days 8, 10 and 22 (LSD: $p \geq 0.08$) and was also significantly higher than FPM values measured on day 6 for the control treatment (LSD: p < 0.03). For the paired treatment, FPM concentration was higher at day 6 compared to days 4, 8 and 12 to 24 (LSD: $p \leq 0.03$). For the control treatment, FPM levels were much more uniform over the experimental period and the peak around day 6 was not that pronounced (Fig. 3). *Figure 3 to be placed here*

Male *A. spinosissimus* have small penile spines that are mainly confined to the distal part of the penis around the glans penis with the more proximal part of the penis appearing smooth (Fig. 4A and B). Furthermore, we observed an elongated structure, possibly the urethral process, which extends past the tip of the glans penis (Fig 4B and C). *Figure 4 to be placed here*

Discussion

The discovery of corpora lutea in a female of the control group, where no male contact was possible, suggests that ovulation occurred spontaneously. However, it should be noted that under our experimental conditions, it is likely that the analysed females were not at the same stage of the oestrous cycle and therefore, the observation of corpora lutea occurred at random. The notion that *A. spinosissimus* is a spontaneous ovulator is further substantiated by the similar ovarian mass and follicular development of the females in all three treatments. In contrast, the existence of luteal phases and oestrous cycles in the separated and paired treatments, but not the control, indicate that the presence of a male benefits reproductive function and ovulation in female *A. spinosissimus.* The small penile spines found on the glans penis of male *A. spinosissimus* may, therefore, aid in the stimulation of the female and help to increase the chance of ovulation. This indicates that *A. spinosissimus* expresses some of the characteristics of an induced ovulatory. Considering, however, that FPM concentrations, luteal phases and oestrous cycles were similar in both separated and paired treatments, the presence of other male cues appears to be enough and copulation does not seem to be necessary for an increase in reproductive function and ovulation. Besides for stimulation of the female, the penile spines may help to establish a short copulatory lock as it has been found in *A. cahirinus* (Dewsbury & Hodges, 1987). The elongated structure on the tip of the glans penis is most likely the urethral process, but further studies are needed to confirm this. The urethral process may be used to spread semen around the cervix during copulation in goats (McEntee, 1990) and could have a similar function in *A. spinosissimus*. Unfortunately, there is no information available on the mating behaviour of *A. spinosissimus* and further studies are required to understand the function of these penile structures.

Although the FPM profiles appear to follow a similar trend to ovarian mass and follicular development, we found differences between the three treatments. In general, separated and paired females showed more often elevated FPM concentrations than control females and luteal phases and oestrous cycles were only observed in these two treatments and not the control. This suggests that the presence of a male may increase the chance of successful ovulation and reproduction in *A. spinosissimus*. As there was no apparent difference between separated and paired treatments, visual, auditory and pheromonal contact was sufficient for males to reproductively stimulate the females. Copulation was not necessary and also did not further enhance the effect. Although we cannot exclude that visual and auditory contact played a role in stimulation, it is most likely that the olfactory and pheromonal contact was of primary importance as has been observed in many mammal species and in spontaneous as well as induced ovulators (e.g. Rekwot et al., 2001). Relton et al. (2013), for example, determined that male contact, olfactory as well as physical, significantly influenced ovulation and female reproductive development of the Namaqua rock mouse from South Africa. Most studies implicate the accessory olfactory system, which is located in the vomeronasal organs, as the place where male odours such as pheromones are registered and trigger female reproductive behaviour and development even in the physical absence of a male. If and how these signals are detected, often depends on the oestrous cycle of the female and is facilitated by oestrogen, whereas progesterone decreases the ability of the female to detect

male odours (Moffatt, 2003). In the spontaneously ovulating mice, for example, the vomeronasal organ is important for lordosis behaviour (Keller et al., 2006). In many vole species, which are induced ovulators, the presence of male olfactory cues enhance reproductive development or is even necessary for reproductive activation although it is not always sufficient for ovulation (Gray et al., 1974; Solomon et al., 1996). The olfactory system was also found to be important for ovulation in the induced ovulating musk shrew (*Suncus murinus*) (Rissman et al., 1990). In this study, the influence of the presence of a male on FPM concentration was primarily evident at the beginning of the experiment. In both separated and paired females, FPM concentrations were elevated about six days after the start of the experiments although they were similar to the control females for most of the rest of the experiment. This was especially evident in the females that had no direct, but olfactory and auditory contact to males which further emphasises the importance of these cues for female reproductive success. Nevertheless, the effect of *A. spinosissimus* males on female ovulation appears to be fairly small although it is probably not negligible and similar pattern may also be found in other *Acomys* species (Peitz, 1981).

Corpora lutea were detected in only two (one control and one paired female) of the 21 females used in the experiment. Considering that luteal phases were detected in nine females, it is likely that corpora lutea were formed, but disintegrated quickly when no implantation and pregnancies occurred. In many females, luteal phases were observed in the beginning to middle of the experiment, which may further support this possibility. Nevertheless, neither of the females, in which we observed corpora lutea, exhibited any luteal activity. The paired female, however, showed elevated FPM concentrations on the last sampling day and we may have missed the luteal phase because faecal sampling was conducted only every second day. Body mass and age of a female are important factors governing the reproductive process (Bronson 1998). It is, however, unlikely that these affected our results. All females were of similar size at the beginning and end of the experiment and most likely mature. *Acomys cahirinus* are considered to be sexually mature at around 45 days of age and to breed successfully at about 2 months (Peitz, 1981). All female *A. spinosissimus*, which were caught in July for the present study, were most likely born during the previous breeding season and not later than January or February (Medger et al., 2010). Consequently, they were sexually mature at the time of the experiments. In addition, the experiments were conducted at the beginning of the breeding season of *A. spinosissimus* in its natural habitat (Medger et al., 2010) and the long-day photoperiod in the laboratory further ensured that females were sexually active.

Fairly little is known about the social and mating systems of *Acomys* species. *A. cahirinus* and *A. russatus* appear to be social (Shargal et al., 2000) and the same is probably true for *A. spinosissimus* as they can be found in large numbers in suitable habitat (K. Medger, personal observation). Dewsbury and Hodges (1987) found some evidence that *A. cahirinus* may be more monogamous than promiscuous, which is further supported by laboratory studies where pairs or groups of females with one male fare better than groups with multiple males (Young, 1976). Both systems favour spontaneous ovulation as access to females is relaxed and male competition is low (Larivière and Ferguson, 2003). Although most *Acomys* species show some tendency towards seasonal reproduction such as *A. spinosissimus* (Medger et al., 2010; 2012b), *A. cahirinus* (Young, 1976) and *A. minous* (Dieterlen, 1978), other species breed throughout the year (*A. subspinosus*: Fleming and Nicolson, 2002; *A. percivali* and *A. wilsoni*: Neal, 1983). This suggests that spontaneous ovulation is a general pattern for the genus, but more information is needed to

get a better understanding of the reproductive biology of these fascinating rodents.

In conclusion, the presence of corpora lutea in a female of the control treatment as well as the similar ovarian mass and follicular development of the females in all treatments suggests that *A. spinosissimus* ovulates spontaneously. In contrast, *A. spinosissimus* also expresses characteristics of an induced ovulatory such as small penile spines. In addition, the presence of a male is important for female reproduction in this species as indicated by the presence of luteal phases in only the separated and paired treatments.

Acknowledgements

The project was funded by a SARChI Chair for Mammal Behavioural Ecology and Physiology awarded to N.C. Bennett. P.R. de Bruin acknowledges an MSc bursary from the National Research Foundation of South Africa and K. Medger a postdoctoral grant from the University of Pretoria. We would like to thank Dr Dorianne Elliott from the Bird and Exotic animal hospital at Onderstepoort for conducting the vasectomies, Claire Relton for her assistance during the experiment, as well as Stefanie Ganswindt for expert help in laboratory techniques.

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Table

Table 1. Number of samples ± SE that were found to have faecal progestagen metabolite concentrations above baseline and number of females with faecal progestagen metabolite concentrations above baseline, at least one luteal phase and oestrous cycle are shown per treatment (control: no male contact, separated: olfactory, auditory and visual contact to a male, paired: physical contact to a vasectomized male). The mean number of samples ± SE that were analysed as well as the sample size for each treatment is included. Significant differences are shown using different letters ($p \leq 0.002$).

Treatment	*n*	Samples	Above baseline	Luteal phase	Oestrous cycle
Control	7	10.4 ± 1.6	0.3 ± 0.5^{a} (2)	0	0
Separated	7	11.3 ± 0.8	3.3 ± 2.4^{b} (7)	5	1
Paired	7	12.7 ± 1.1	3.3 ± 2.1^{b} (6)	4	1

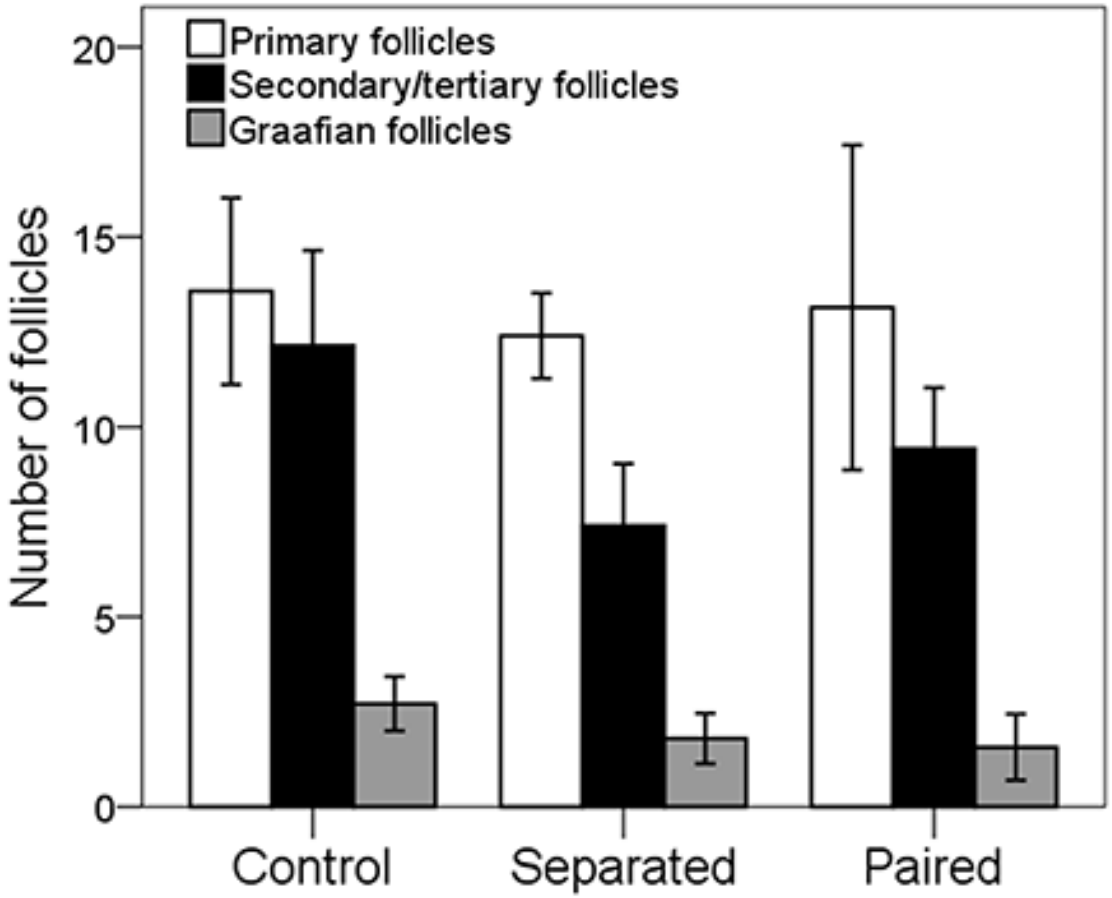

Fig. 1. Number of primary, secondary/tertiary and Graafian follicles counted in the ovaries of female spiny mice subjected to three experimental treatments including total separation from a male (control, n = 7), olfactory, auditory and visual contact (separated, n = 5) as well as full physical contact with a male (paired, n = 7). Results are presented as mean ± SE and $p > 0.05$.

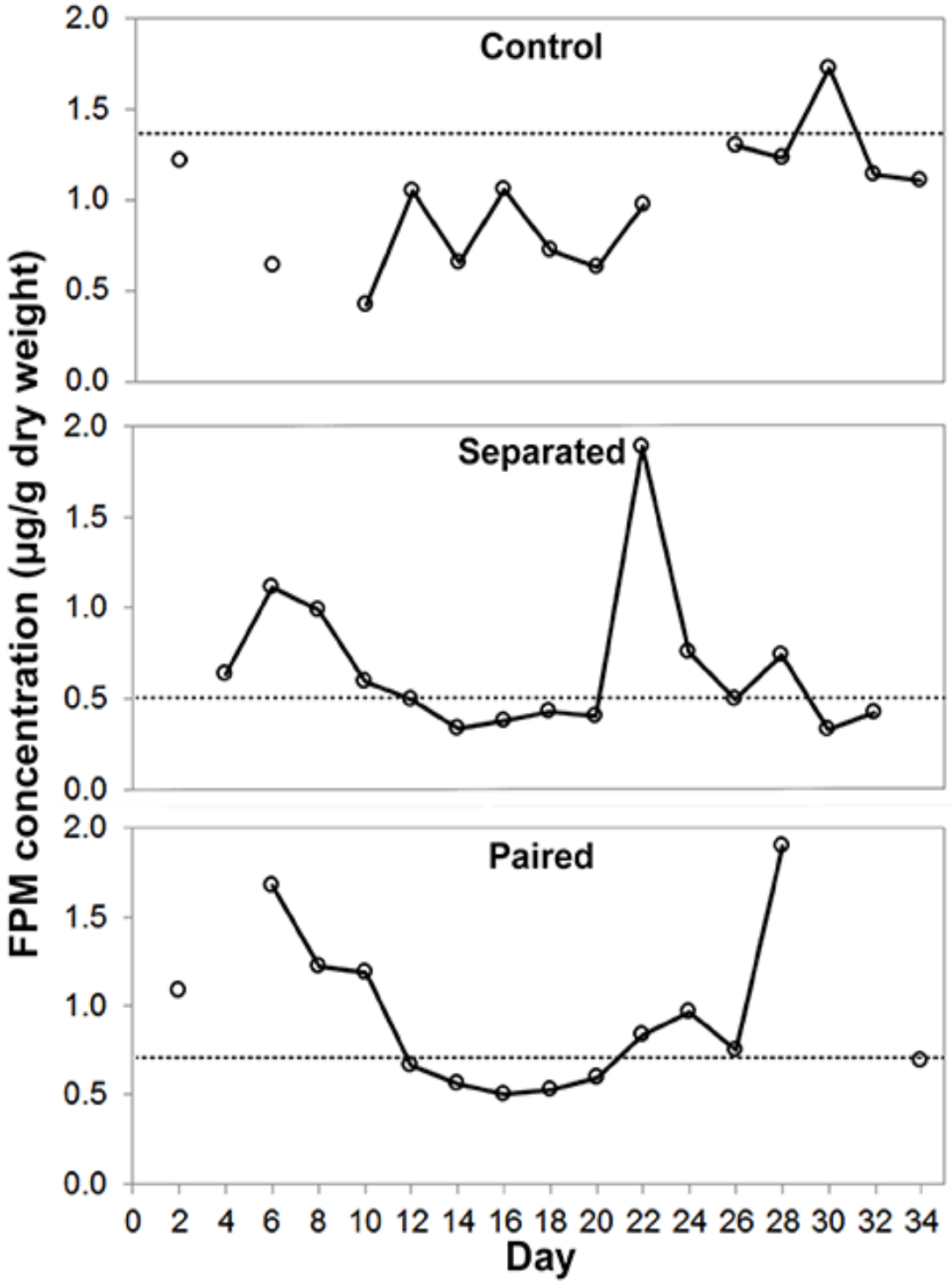

Fig. 2. Faecal progestagen metabolite (µg/g dry weight, FPM) concentrations of a representative female from each treatment: 1) control (no male contact); 2) separated (olfactory, auditory and visual contact to a male); 3) paired (full physical contact with a male). FPM concentrations were measured every second day throughout the experimental period of 35 days. Dashed lines indicate individual baseline FPM concentrations.

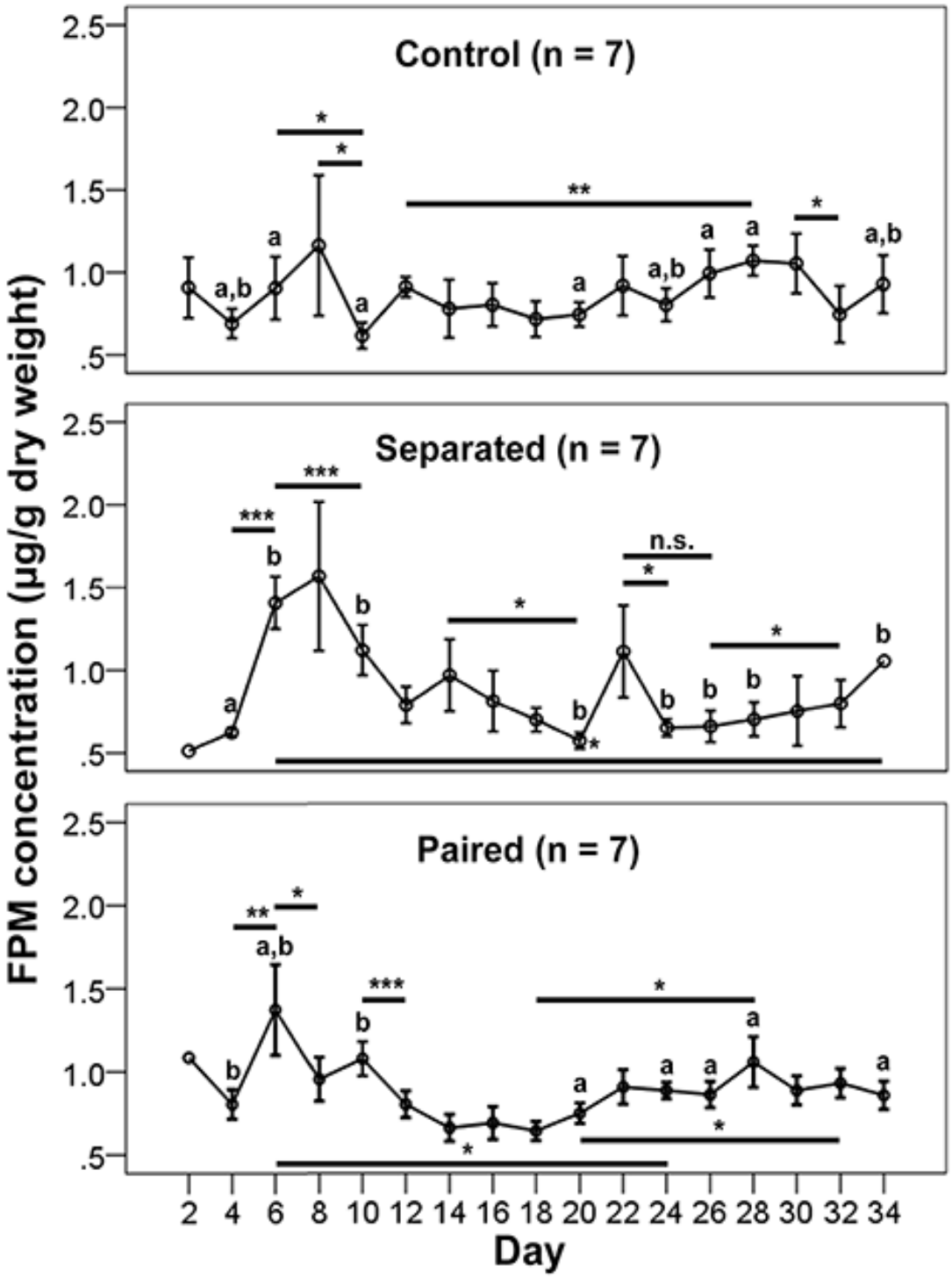

Fig. 3. Faecal progestagen metabolite (µg/g dry weight, FPM) concentrations measured in samples of female spiny mice that were subjected to three different treatments: 1) control (no male contact); 2) separated (olfactory, auditory and visual contact to a male); 3) paired (full physical contact with a male). FPM concentrations were measured every second day throughout the experimental period of 35 days. Results are presented as mean ± SE. Different letters indicate significant differences between the treatments for each sampling day. $^{n.s.}$ non-significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (for selected comparisons).

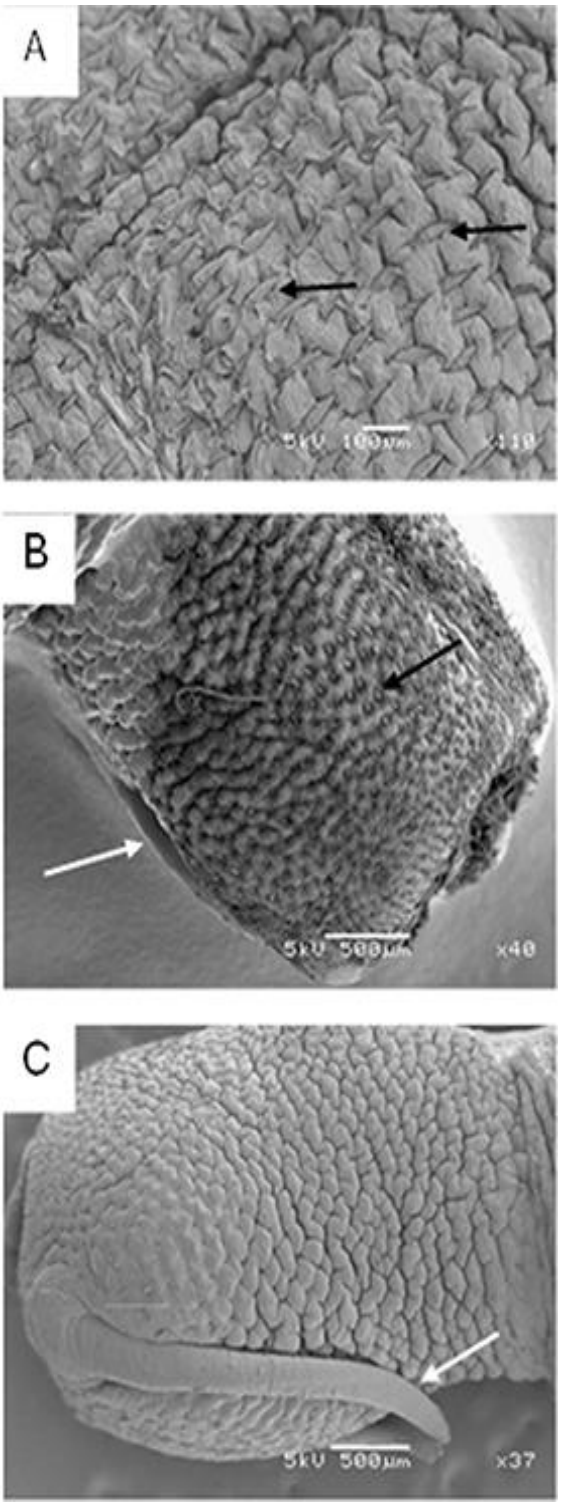

Fig. 4. Scanning electron micrographs showing the penile spines present in male spiny mice. Black arrows indicate small spines, concentrated on the glans of the penis (A and B) and the white arrows point to the urethral process reaching from the tip of the penis backwards (B and C).