The pattern of ovulation in females and effect of food restriction on male testicular development in the South African spiny mouse (*Acomys spinosissimus*)

By

Phillippus Rudolf de Bruin

Submitted in partial fulfilment of the requirements for the degree

MSc Zoology

In the Faculty of Natural and Agricultural Sciences

University of Pretoria

Pretoria

June 2013

The pattern of ovulation in females and effect of food restriction on male testicular development in the South African spiny mouse (*Acomys spinosissimus*)

Student: Ruan de Bruin[1]

Supervisors: Prof. Nigel C. Bennett[1]
Dr. Katarina Medger[1]

Affiliation: [1]Mammal Research Institute (MRI)
Department of Zoology and Entomology
University of Pretoria
Private Bag X20
Hatfield
0028
South Africa

Degree: Master of Science specializing in Zoology

Declaration

I, Phillippus Rudolf de Bruin, declare that my thesis, which I hereby submit for the degree Masters of Science with specialization in Zoology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature: ……

Date: ……

Disclaimer

This thesis consists of a series of chapters that have been prepared as stand-alone manuscripts for publication purposes. Consequently, unavoidable overlaps and/or repetitions may occur between chapters.

General abstract

Reproduction is the process whereby an animal ensures the continuous existence of their genes in the population by procreation. Reproduction presents a series of obstacles for both males and females. Males have to ensure they are in peak physical condition in order to establish dominance and compete for the attention of the opposite sex. Females need enough energy to support their bodily needs whilst supplying energy to the growing foetuses and suckling young. The current thesis investigated the pattern of ovulation in female as well as the effect of photoperiod and food restriction on male gonadal development in wild caught South African spiny mice (*Acomys spinosissimus*) from the Limpopo province in South Africa by using faecal samples to measure hormone concentrations. A novel method, using faecal samples to monitor reproductive function in *Acomys spinosissimus* was validated during this study. It was shown that reproductive function can reliably be monitored in both sexes using enzyme immunoassays for 17-oxo-androgens in males and 20-oxopregnanes in females, respectively. Females were randomly assigned to one of three treatments. Seven females were housed completely separated from any male stimuli and represented the control group. The two experimental groups were each made up of seven females. The separated treatment was housed in visual and olfactory contact with intact males, separated by wire mesh. The paired treatment was housed with vasectomized males, allowing full contact between the two sexes. Females from all three treatment groups underwent normal follicular development with corpora lutea of ovulation recorded for one female from the control and one female from the paired treatment. Progesterone concentrations were compared between the different treatments using faecal hormone metabolite levels. The progesterone concentrations were not affected by the different treatments; however, the day of faecal sample collection influenced progesterone levels. The findings from the ovarian histology and faecal progestagens strongly suggest a spontaneous pattern of ovulation. To investigate the effects of photoperiod and food restriction, males were randomly assigned to one of four treatment groups. The first two groups, consisting of six males each, were subjected to a 14L: 10D (LD) photoperiod. Within the LD treatment, one group was fed *ad libitum* (NR) whilst the other group was subjected to a 10% food restriction (R). The remaining two cohorts were subjected to the same feeding regime as mentioned above, but they were kept on a 10L: 14D

(SD) photoperiod. Male spiny mice exposed to a long photoperiod had significantly greater testes volume and seminiferous tubule diameters when compared to the males exposed to a short photoperiod. Total body fat did not differ significantly when compared between the different treatments. Males exposed to the long photoperiod also had significantly higher testosterone concentrations when compared to the males exposed to the short photoperiod. Feeding regime did not have any significant effect on any of the reproductive parameters investigated in this study. During this study it was concluded that *Acomys spinosissimus* is a spontaneous ovulator that is strongly photoperiodic with the availability of food resources enhancing the photoperiodic effect.

Keyword: spiny mouse, seasonal breeding, ovulation, spontaneous, photoperiodic, food restriction, testes

Acknowledgments

Most of all, I would like to thank my supervisors, Prof Nigel Bennett and Dr Katarina Medger. Their continuous encouragement, guidance and words of wisdom helped me to broaden my knowledge and stimulated my curious, scientific mind. Without their support this project would not have been possible. Babsie Potgieter, you are a blessing to us all! You were always there, greeting me with a smile and ready to help! When I was freaking out because there was no more wax in the histology lab, no more microscope slides in the cabinet or alcohol in the mole-rat lab, your calming voice and gentle words assured me that everything will be alright.

I would like to thank the National Research Foundation and the University of Pretoria for their financial support during completion of my degree as well as the facilities that was available to me during this time.

During the course of my degree, I could always rely on you, Claire Relton! Whether we were collecting traps in the early mornings, watching the sun rise over the mountains, setting the traps at night or taking a sun-downer drive with popcorn, you were always by my side making memories that will last a lifetime. From the mid-morning tea and rusks to the late night TT parties, all of these are special memories I shall treasure for always. I would also like to thank you for your assistance during the last two years.

To my best friend, Roan Fraser, our coffees and weekly discussions of everyone walking past the ramp was a greatly needed escape from the fumes of histology lab and the overwhelming statistical process.

Thank you to two great friends, Carol Hoole and Gerhard Göldner, who were always available for quick coffee under the tree, ending up longer than anticipated. Carol, shocked looks from passers-by became the norm as they joined in our extremely insightful smoke-break conversations.

Thank you to my family! To Mom and dad, for your motivating words when morale was low and shared excitement when things went according to plan. My sister for all the times she kept me company in the histology lab and the hot drinks during the crisp July mornings.

Table of Contents

List of Figures (Verbatim as in text and page number)

Chapter 1

Chapter 2

Chapter 3

List of Tables (Verbatim as in text and page number)

Chapter 1

Note

Chapter 1 has been submitted as a paper to *Reproductive Biology and Endocrinology*; hence the different format of the chapter

Introduction

Mammals are capable of breeding either continuously throughout a calendar year or their reproductive period may be restricted to a specific period of the year which is known as opportunistic and seasonal reproduction, respectively. Opportunistic breeding occurs when reproduction takes place throughout the year such as the breeding pattern exhibited by the Hottentot golden mole (*Amblysomus hottentotus*). Adult females exhibit ovulation and the production of corpora lutea of ovulation and pregnancy throughout the year with males having viable sperm during the same time period. The Hottentot golden mole can produce offspring throughout an entire calendar year (Schoeman *et al.* 2004). Similarly, the Natal mole-rat (*Cryptomys hottentotus natalensis*) has been found to reproduce throughout the year (Oosthuizen *et al.* 2008). It shares the same ecosystem as the Hottentot golden mole and thus we find an elegant example of where two phylogenetically distinct species share the same breeding pattern which is crafted by the environment in which they occur (Oosthuizen *et al.* 2008). Aseasonally breeding mammals are not usually constrained by their food resources and as a consequence procreation and subsequent production of offspring is possible at any time during the year. Seasonally breeding mammals time their reproduction in such a way that the birth of the offspring coincides with times of maximum resource availability, most often spring and summer, in order to ensure the survival of the mother and the young (Reiter 1980; Fitzgerald and McManus 2000). Animals use seasonal predictors to prepare for these periods when reproductive success will be optimised (Bronson and Perrigo 1987).

Many small mammals in southern Africa are found in environments where resources are available on a seasonal basis and consequently reproduce seasonally (White and Bernard 1996). The Namaqua rock mouse (*Micaelamys namaquensis)* from South Africa is a strongly seasonal breeder with breeding taking place from mid-spring to the onset of autumn (Muteka *et al.* 2006). Pregnant and lactating females are only found between October and March with no reproductively active females recorded from April through to September. Gonadal histology as well as the patterns of plasma progesterone and testosterone concentrations throughout the year supported the conclusion that both males and females of the Namaqua rock mouse breed only during the summer period of the southern hemisphere (Muteka *et al.* 2006). Female spinifix hopping mice (*Notomys alexis*) and sandy inland mice (*Pseudomys hermannsburgensis*) from

central Australia are aseasonal breeding species (Breed 1990). Museum specimens of wild caught females from the two species were found to be pregnant throughout an entire calendar year even though litter sizes were larger during the summer period (Breed 1990). Opportunistic breeding is proposed for the study population of *R. pumilio* from South Africa as an adaptive strategy to the highly unpredictable environment of the Eastern Cape making breeding possible when the energy deficit is low (Jackson and Bernard 2006). Two study populations were compared over a period of three years from different regions in the Eastern Cape, South Africa. Jackson and Bernard (2006) found that breeding was continuous through the winter seasons in one population, whilst in the other; the winter conditions inhibited female reproduction while males continued to produce spermatozoa. Some alternative breeding patterns have been identified in other South African mammals. The Cape spiny mouse (*Acomys subspinosus*), for example, has been found to breed opportunistically, with the time of breeding coinciding with the time that *Protea humiflora* flowers and the animals can feed on the available seeds (Fleming and Nicolson 2002). Aseasonal reproduction is another breeding strategy that has been proposed for the pouched mouse (*Saccostomus campestris*) with pregnant females being recorded throughout the year (Westlin and Ferreira 2000). It has been suggested that pouched mice are able to breed throughout the year because females can induce foetal resorption as late as 72 hours prior to parturition depending on the resources available, thus giving rise to great variability in litter size in this species (Westlin and Ferreira 2000; Skinner and Chimimba 2005).

Photoperiod is derived from the Greek words *photos* meaning light and *periodos* meaning to go around and is used to describe the number of hours of light and darkness in each day made up of 24 hours (Beck 1963). Photoperiod acts on organisms and is determined in mammals by the amount of melatonin produced by the pineal gland and is closely linked with the length of the night (Scherbarth and Steinlecher 2010; Yoshimura 2010). The light information is received through the eye and transmitted to the pineal gland via the retinal-hypothalamic pathway where it is integrated by the suprachiasmatic nucleus into a neural signal and subsequently translated into melatonin secretion (Beck 1963). Photoperiod is constant; making it the predominant factor used by murids to time their reproductive processes however, seasonal regulation of reproduction involves a complex interaction of dietary and environmental factors such as food availability,

rainfall, temperature and humidity (Bronson and Perrigo 1987). In the marsh rice rat (*Oryzomys palustris*), multiple cues have been proposed to affect the reproductive development, resulting in a restricted breeding season (Edmonds *et al.* 2003). Food availability is the most important limiting factor for physiological processes and is governed by energy availability (Nelson *et al.* 1983; Bronson and Perrigo 1987; Sicard *et al.* 1993).

Seasonal reproduction is also known to influence other aspects of the reproductive physiology of mammals, for example, the pattern of ovulation. In seasonally breeding species, recrudescence of ovarian activity and subsequent ovulation has to be initiated in the ovaries of females. In this regard, there are two modes of ovulation that can be seen in most species. Spontaneous ovulation is the continuous cycle of follicular development where primordial follicles on the edge of the ovary develop sequentially through a series of follicular stages into Graafian follicles and rupture, releasing the egg, with the remnants of the ruptured follicle forming the corpus luteum (Zuckerman and Weir 1977). Species which undergo induced ovulation express identical follicular growth and maturation, however, they require stimulation of the vagina and cervix during copulation in order to induce the secretion of GnRH resulting in the LH surge and subsequently causing the release of a mature egg from the Graafian follicle (Milligan 1980).

Many studies have focused on the ovulation pattern in the subterranean mole-rats from the family Bathyergidae in South Africa. The solitary, seasonally breeding Cape mole-rat (*Georychus capensis*) has been found to show induced ovulation permitting mating to take place as soon as a male and female meet during the restricted breeding season (van Sandwyk and Bennett 2005). On the other hand, in the eusocial Damaraland mole-rats (*Fukomys damarensis*) males are in constant contact with the breeding females making spontaneous ovulation the more logical pattern of ovulation in this species (Snyman *et al*. 2006). During a study on the reproductive biology of three native Australian murids, Breed (1989) concluded that even though there was some variation in the reproductive biology of the hopping mouse (*Notomys alexis*), the Australian plains mouse (*Pseudomys australis*) and the dusky rat (*P. nanus*), they all exhibited

spontaneous ovulation. Both the hopping mouse and plains mouse are social species that occur in groups (Breed and Ford 2007). Contrary to the above two species, the dusky rat is solitary during the breeding season with male home ranges found to overlap with the home ranges of various females. During the winter period, they become social with males and females huddling together to survive the snow covered winters (Breed and Ford 2007). It would thus be advantageous for solitary animals to exhibit an induced pattern of ovulation whereas for social species spontaneous ovulation would be the most favourable mode of ovulation. Solitary species will not waste an ovum in situations when no males are around, but will be able to successfully ovulate when a male is present (Larivière and Ferguson 2003).

The study of reproduction in non-domesticated species has proven to be challenging, since repeated blood sampling is often not possible, resulting in the sampling of urinary and faecal metabolite products (Schwarzenberger *et al.* 1996). This non-invasive method of monitoring reproductive activity has become a well-established approach in many mammal species and has been used in domestic, captive and wild species as well as species that are too small for repeated blood sampling (Schwarzenberger *et al.* 1996; Ganswindt *et al.* 2002; Wittemyer *et al.* 2007; Laver *et al.* 2012). In the field of reproductive physiology some of the uses for faecal steroid analysis include determining oestrous cycle lengths, differentiating between induced and spontaneous ovulators as well as monitoring pregnancy (Schwarzenberger 2007). This non-invasive method has been used only rarely on murid rodents with most studies focusing mainly on glucocorticoid metabolites (Kuznetsov *et al.* 2004; Schwarzenberger 2007). Faecal metabolites have been used in the Egyptian spiny mouse (*Acomys cahirinus*), where Frynta *et al.* (2009) designed and tested an apparatus that allowed collection of faecal matter from individual animals within a social group without causing any additional stressors that could influence the results. Faecal glucocorticoid metabolites were also used to investigate the effect of various factors in the same species in order to get a better understanding of their population dynamics (Nováková *et al.* 2008).

Within the Muridae family, the genus *Acomys* comprises 15 species that are found in a vast array of different habitats such as rocky country, semi-desert areas, dry woodlands and savannahs (Nowak 1999). Nine *Acomys* species are restricted to Africa (*A. cineraceus*, *A. ignites*, *A. kempi*, *A. louisae*, *A. mullah*, *A. percivali*, *A. spinosissimus*, *A. subspinosus* and *A. wilsoni*) and three are restricted to Eurasia (*A. cilicicus*, *A. minous* and *A. nesiotes*) (Bates 1994). The remaining three species (*A. cahirinus*, *A. dimidiatus* and *A. russatus*) are found across both continents (Bates 1994). *Acomys* are more commonly known as spiny mice stemming from the coarse hair on their backs that are modified into spines (Nowak 1999; Skinner and Chimimba 2005). Their tails are dark on top, becoming lighter towards the bottom and covered with visible scales and they also have large, erect ears (Mills and Hes 1997). Spiny mice are omnivorous, but are found to mainly feed on grain and grasses (Mills and Hes 1997). In general, *Acomys* species have a gestation period ranging between five and six weeks, after which between one and five young are born, with older females having larger litters (Nowak 1999). The only species for which a gestation period has been defined in the literature is the common spiny mice (*A. cahirinus*) where between one and four pups are born after a gestation period of approximately 39 days (Young 1976; Peitz 1981). Some *Acomys* species have been shown to reach sexual maturity at around three months of age with a life span of up to three years (Peitz 1981; Nowak 1999). There has not been much work done in earlier years, but recently, the genus *Acomys* has become well studied with most work focussing on the physiological and behavioural mechanisms in *A. cahirinus*, *A. dimidiatus* and *A. russatus* leaving the remaining 11 species relatively unexplored (Shargal *et al.* 2000; Shanas and Haim 2004; Frynta *et al.* 2011). Wube *et al.* (2008) investigated the reproductive response of two *Acomys* species adapted to a xeric or a mesic environment. Females of the xeric *A. russatus* showed no effect of photoperiod on their reproduction, with only the males affected by short photoperiods (Wube *et al.* 2008). The same authors also found that both sexes of the mesic *A. cahirinus* were unaffected by the photoperiod length with reproductive activity continuing on a long and short photoperiod (Wube *et al.* 2008).

Two species of *Acomys* are found in South Africa. The Cape spiny mouse and the spiny mouse (*Acomys spinosissimus)* found in the north-eastern part of South Africa as well as Botswana, Mozambique, Zimbabwe, Malawi and Tanzania (Skinner and Chimimba 2005).

Acomys spinosissimus is a small rodent with an average body mass and length of 19.45 ± 3.36g and 80.95 ± 4.32mm, respectively (de Bruin, this study). They are reddish-brown in colour with a white underside. They are nocturnal rodents (Hoole *et al.* 2012) that are confined to rocky areas that can provide shelter and protection. Their social structure is uncertain, with specimens found solitary, in breeding pairs or in groups (Skinner and Chimimba 2005). *Acomys spinosissimus* has been found to breed during the South African summer with hormone levels, testicular and ovarian development being greatest during September through January with pregnancies also recorded during the same period (Medger *et al.* 2010; Medger *et al.* 2012a). There was no reproductive activity shown by the females for the remaining months of the year with the males starting to increase sexual development two months prior to the onset of the breeding season (Medger *et al.* 2010, Medger *et al*. 2012a). During another study, Medger *et al*. (2012b) subjected male *A. spinosissimus* to different photoperiods and concluded that the males exposed to a long photoperiod had greater testicular mass, volume and seminiferous tubule diameters as well as testosterone concentrations than the males exposed to a short photoperiod (Medger *et al.* 2012b). These recent studies have brought about some questions regarding spiny mouse reproduction such as how the ultimate factors like food availability and salinity interact with photoperiod in controlling the reproductive processes in this species as well as the physiological processes present in the females of this species during the reproductive season (Bukovetzky *et al*. 2012a,b,c).

This study investigated the pattern of ovulation found in *A. spinosissimus* as well as looked at the effects of photoperiod and food availability on the gonadal development of males. A faecal steroid assay was also validated during this study in order to compare faecal progesterone and testosterone metabolites between the respective treatments. Based on studies on other *Acomys* species, I predict that *A. spinosissimus* will show spontaneous ovulation and that photoperiod combined with food availability will regulate seasonal reproduction in the males from this species. The findings of this study will provide a better understanding of the reproductive biology of the species that could be implemented in the conservation of the species in South Africa.

References

Bates, P.J.J. 1994. The distribution of *Acomys* (Rodentia: Muridae) in Africa and Asia. *Israel Journal of Zoology*. **40,** 199 – 214.

Beck, S.D. 1963. Photoperiodism in Mammals. *Animal Photoperiodism* (1st Edition). Holt, Rinehart and Winston, Inc., United States of America, New York. **2,** 17 – 37.

Breed, W.G. 1989. Comparative studies on the reproductive biology of three species of laboratory bred Australian conilurine rodents (Muridae: Hydromyinae). *Journal of Zoology* (London). **217,** 683 – 699.

Breed, W.G. 1990. Comparative study on the timing of reproduction and foetal number in six species of Australian conilurine rodents (Muridae: Hydromyinae). *Journal of Zoology* (London). **221,** 1 – 10.

Breed, W.G. and Ford, F. 2007. Native mice and rats. *CSIRO Publishing.* Australia. **8,** 125 – 138.

Bronson, F.H. and Perrigo, G. 1987. Seasonal regulation of reproduction in Muroid rodents. *American Zoologist*. **27,** 929 – 940.

Bukovetzky, E., Schwimmer, H., Fuad, F. & Haim, A. 2012a. Photoperiodicity and increasing salinity as environmental cues for reproduction in desert adapted rodents. *Hormones and Behavior*. **61,** 84 - 90.

Bukovetzky, E., Fuad, F. Schwimmer, H. & Haim, A. 2012b. Reproductive and metabolic responses of desert adapted common spiny male mice (*Acomys cahirinus*) to vasopressin treatment. *Comparative Biochemistry & Physiology (A)*. **162,** 349 - 356.

Bukovetzky, E., Fuad, F. & Haim, A. 2012c. Metabolic and endocrine responses of desert-adapted mice reproductive system to increased salinity. *Journal of Life Sciences*. **6,** 454 - 459.

Fitzgerald, B.P. and McManus, C.J. 2000. Photoperiodic versus metabolic signals as determinants of seasonal anestrus in the mare. *Biology of Reproduction*. **63,** 335–340.

Fleming, P.A. and Nicolson, S.W. 2002. Opportunistic breeding in the Cape spiny mouse (*Acomys subspinosus*). *African Zoology*. **37,** 101 – 105.

Frynta, D., Nováková, M., Kutalová, H., Palme, R. and Sedláček, F. 2009. Apparatus for collection of faecal samples from undisturbed spiny mice (*Acomys cahirinus*) living in a complex social group. *Journal of the American Association for Laboratory Animal Science*. **48,** 196 – 201.

Frynta, D., Fraňková, M., Čížková, B., Skarlandtová, H., Galeštoková, K., Průšová, K., Šmilauer, P. and Šumbera, R. 2011. Social and life history correlates of litter size in captive colonies of precocial spiny mice (*Acomys*). *Acta Theriologica*. **56,** 289 – 295.

Ganswindt, A., Heistermann, M., Borragan, S. and Hodges, J.K. 2002. Assessment of testicular endocrine function in captive African elephants by measurement of urinary and fecal androgens. *Zoo Biology*. **21,** 27 – 36.

Hoole, C., Oosthuizen, M.K., Chimimba, C.T. and Bennett, N.C. 2012. The locomotory activity rhythm of the spiny mouse, *Acomys spinosissimus* from southern Africa: light entrainment and endogenous circadian rhythms. *Journal of Zoology (*London). **288,** 93 – 102.

Jackson, C. and Bernard, R.T.F. 2006. Variation in the timing of reproduction of the four-striped field mouse, *Rhabdomys pumilio,* in the Eastern Cape Province, South Africa. *African Zoology*. **41,** 301 - 304.

Kuznetsov, V.A., Tchabovsky, A.V., Kolosova, I.E. and Moshkin, M.P. 2004. Effect of habitat type and population density on the stress level of midday gerbils (*Meriones meridianus* Pall.) in free-living populations. *Biology Bulletin*. **31,** 628 – 632.

Larivière, S. and Ferguson, S.H. 2003. Evolution of induced ovulation in North American carnivores. *Journal of Mammalogy*. **84,** 937 – 947.

Laver, P., Ganswindt, A., Ganswindt, S. and Alexander, K. 2012. Non-invasive monitoring of glucocorticoid metabolites in banded mongooses (Mungos mungo) in response to physiological and biological challenges. *General and Comparative Endocrinology***. 179,** 178 – 183.

Milligan, S.R. 1980. Pheromones and rodent reproductive physiology. *Symposia of the Zoological Society of London*. **45,** 251 – 275.

Mills, G. and Hes, L. 1997. The complete book of southern African mammals. Struik Publishing group (Pty) Ltd. Cape Town, South Africa.

Medger, K., Chimimba, C.T. and Bennett, N.C. 2010. Seasonal reproduction in the female spiny mouse from South Africa. *Journal of Zoology* (London). **282,** 163-170.

Medger, K., Chimimba, C.T. and Bennett, N.C. 2012a. Seasonal changes in reproductive development in male spiny mice (*Acomys spinosissimus*) from South Africa. *Mammalian Biology*. **77,** 153 – 159.

Medger, K., Chimimba, C.T. and Bennett, N.C. 2012b. Reproductive photoresponsiveness in male spiny mice from South Africa. *Journal of Zoology* (London). **286,** 243 – 249.

Muteka, S.P., Chimimba, C.T. and Bennett, N.C. 2006. Reproductive seasonality in *Aethomys namaquensis* (Rodentia: Muridae) from South Africa. *Journal of Mammalogy*. **87,** 67 – 74.

Nelson, R.J., Dark, J. and Zucker, I. 1983. Influence of photoperiod, nutrition and water availability on the reproduction of male California voles (*Microtus californicus*). *Journal of Reproduction and Fertility*. **69,** 473 – 477.

Nováková, M., Palme, R., Kutalová, H., Janský, L. and Frynta, D. 2008. The effects of sex, age and commensal way of life on levels of fecal glucocorticoid metabolites in spiny mice (Acomys cahirinus). *Physiology & Behavior*. **95,** 187–193.

Nowak, R.M. 1999. Walker's mammals of the world, 6thedition, Volume II. The Johns Hopkins University Press, Baltimore.

Oosthuizen, M.K., Lutermann, H., Coen, C.W. and Bennett, N.C. 2008. Reproductive suppression in subordinate Natal mole-rats (*Cryptomys hottentotus natalensis*.): the pituitary response to exogenous GnRH. *General Comparative Endocrinology* **159**, 236-240.

Peitz, B. 1981. The oestrous cycle of the spiny mouse (*Acomys cahirinus*). *Journal of Reproduction and Fertility*. **61,** 453 – 459.

Reiter, R.J. 1980. Seasonal reproduction: an expedient and essential artifice. *Seasonal Reproduction in Higher vertebrates*. Buchdruckerei 'Der Bund', Bern, Switzerland. Edited by Reiter, R.J. and Follett, B.K. **5,** 1 – 4.

Scherbarth, F. and Steinlechner, S. 2010. Endocrine mechanisms of seasonal adaptation in small mammals: from early results to present understanding. *Journal of Comparative Biology B*. **180,** 935-952.

Schoeman, S., Bennett N.C., van der Merwe, M and Schoeman, A.S. 2004. Reproduction in the Hottentot golden mole, *Amblysomus hottentotus* (Afrosoricida: Chrysochloridae). *African Zoology* **39,** 41-46.

Schwarzenberger, F. 2007. The many uses of non-invasive faecal steroid monitoring in zoo and wildlife species. *International Zoo Yearbook*. **41,** 52 – 74.

Schwarzenberger, F., Möstl, E., Palme, R. and Bamberg, E. 1996. Faecal steroid analysis for non-invasive monitoring of reproductive status in farm, wild and zoo animals. *Animal Reproduction Science*. **42,** 515 – 526.

Shanas, U. and Haim, A. 2004. Diet salinity and vasopressin as reproduction modulators in the desert-dwelling golden spiny mouse (*Acomys russatus*). *Physiology and Behavior*. **81,** 645 – 650.

Shargal, E., Kronfeld-Schor, N. and Dayan, T. 2000. Population biology and spatial relationships of coexisting spiny mice (*Acomys*) in Israel. *Journal of Mammalogy.* **81,** 1046 – 1052.

Sicard, B., Fuminier, F., Maurel, D. and Boissin, J. 1993. Temperature and water conditions mediate the effects of day length on the breeding cycle of a Sahelian rodent, *Arvicanthis niloticus*. *Biology of Reproduction.* **49,** 716 – 722.

Skinner, J.D. and Chimimba, C.T. 2005. The mammals of the southern African subregion (3rd edition).Cambridge: Cambridge University Press.

Snyman, P.C., Jackson, C.R. and Bennett, N.C. 2006. Do dispersing non-reproductive female Damaraland mole-rats, *Cryptomys damarensis* (Rodentia: Bathyergidae) exhibit spontaneous or induced ovulation? *Physiology and Behavior*. **87,** 88 – 94.

van Sandwyk, J.H. and Bennett, N.C. 2005. Do solitary, seismic signalling Cape mole-rats

(*Georychus capensis*) demonstrate spontaneous or induced ovulation? *Journal of Zoology*, London. **267**, 75–80.

Westlin, L.M. and Ferreira, S.M. 2000. Do pouched mice alter litter size through resorption in response to resource availability? *South African Journal of Wildlife Research*. **30,** 118 – 121.

White, R.M. and Bernard, R.T.F. 1996. Secondary plant compound and photoperiod influences on the reproduction of two southern African rodent species, *Gerbillurus paeba* and *Saccostomus campestris*. *Mammalia*. **60,** 639 – 649.

Wittemyer, G., Ganswindt, A. and Hodges, K. 2007. The impact of ecological variability on the reproductive endocrinology of wild female African elephants. *Hormones and Behavior*. **5,** 346–354.

Wube, T., Haim, A. and Fares, F. 2008. Effect of increased dietary salinity on the reproductive status and energy intake of xeric and mesic populations of the spiny mouse, *Acomys*. *Physiology and Behavior*. **96,** 122 – 127.

Yoshimura, T. 2010. Neuroendocrine mechanism of seasonal reproduction in birds and mammals. *Animal Science Journal*. **81,** 403-410.

Young, D.A.B. 1976. Breeding and fertility of the Egyptian spiny mouse, *Acomys cahirinus*: effect of different environments. *Laboratory animals*. **10,** 15 – 24.

Zuckerman, P.L. and Weir, B.J. 1977. The Ovary II, Physiology (2nd edition). New York: Academic Press, New York. **6,** 235 – 259.

Chapter 1

Non-invasive assessment of reproductive function in southern African spiny mice (*Acomys spinosissimus*) by measurement of faecal progestagens and androgens

This chapter is essentially in the format required by *Reproductive Biology and Endocrinology* to which this chapter has been submitted and is currently under review for publication purposes.

Non-invasive assessment of reproductive function in southern African spiny mice (*Acomys spinosissimus*) by measurement of faecal progestagens and androgens

Ruan de Bruin[1,§], Katarina Medger[1], Nigel C. Bennett[1], André Ganswindt[1,2,3]

[1]*Mammal Research Institute, Department of Zoology and Entomology, University of Pretoria, Pretoria 002, RSA*

[2]*Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, RSA*

[3]*Current address: Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, RSA*

[§]*Corresponding author*

Email addresses:
RdB: prdebruin@zoology.up.ac.za
KM: kmedger@zoology.up.ac.za
NCB: ncbennett@zoology.up.ac.za
AG: aganswindt@zoology.up.ac.za

Abstract (max. 350 words)

Background: The genus *Acomys* raises increasing interest in science, as spiny mice show a comparatively long oestrus cycle for an animal of such small size, raising immediately questions regarding their reproductive strategies. Unfortunately, detailed long-term data on reproductive function and especially endocrine correlates is still lacking, as limited blood volumes make frequent collections of individual plasma samples for hormone monitoring impractical. To provide the necessary tools in order to fill this gap, the overall aim of this study was to examine the suitability of two group-specific enzyme immunoassays (EIAs), detecting 17-oxo-androgens

(EA) and 20-oxopregnanes (5ß-20-one), respectively, for monitoring male and female reproductive function in the southern African spiny mouse (*Acomys spinosissimus*) based on faecal hormone analysis.

Methods: Fourteen non-pregnant and one pregnant, adult female and 24 adult male spiny mice were wild-caught and subsequently housed under controlled laboratory conditions. Females were kept in olfactory, visual, and physical contact with vasectomised males. Study males were subjected to either a long or short photoperiod. Faecal samples for hormone monitoring were collected every second day for up to six weeks. Minimum sample mass for hormone monitoring required as well as the rate of respective hormone metabolism post-defecation was additionally investigated using separately collected faecal material.

Results: Thirteen out of the 14 monitored females revealed elevated faecal 5ß-20-one concentrations with eight out of 14 individuals showing indications of a luteal phase. Two females showed two post-ovulatory luteal phases with estimated cycle lengths of 16 and 18 days, respectively. The pregnant female showed an elevation of 231% in mean faecal 5ß-20-one levels compared to the overall mean baseline 5ß-20-one concentration determined for the 14 non-pregnant females. Males exposed to a long photoperiod, simulating breeding season, exhibited a 47.8% increase in faecal EA levels compared to males exposed to a short photoperiod.

Conclusions: Male and female spiny mice reproductive function can be reliably assessed non-invasively using enzyme immunoassays for 17-oxo-androgens and 20-oxopregnanes, respectively. The established method now provides a solid basis for more specific studies designed to examine the factors regulating reproductive activity in this charismatic mammal.

Key words:
Rodents; luteal activity; oestrous cycle; male reproductive activity; faecal hormones; non-invasive monitoring; faecal androgen metabolites; faecal progestagen metabolites; hormone stability post-defecation.

Background

The genus *Acomys* has become an important group of rodents for behavioural and physiological studies as they are found across a wide range of habitats ranging from rocky country to semi-desert areas, dry woodlands and savannahs (1-4). Spiny mice are especially well adapted to areas with very limited water supply such as deserts, raising the question on how they survive in these harsh environments (5; 6). In this regard, information about species-specific reproductive processes would be most helpful, but even basic reproductive knowledge, especially in terms of endocrine correlates of reproductive function, is still limited for the entire genus, with the common spiny mouse (*Acomys cahirinus*) and golden spiny mouse (*Acomys russatus*) being the only two out of 14 *Acomys* species for which e.g. an oestrous cycle has been described (1; 7; 8). For these two species an oestrous cycle length of about 11 – 18 days has been revealed, which is considered comparatively long for a species of such small size, as the average cycle length of mice is defined between five and seven days (7-9).

The southern African spiny mouse (*Acomys spinosissimus*) is confined to the rocky areas of the north-eastern parts of South Africa and can be found as far north as Mozambique, Zimbabwe and Tanzania (10). Medger *et al*. (11; 12) concluded that the onset of the breeding season of *Acomys spinosissimus* coincides with the respective summer rainy season in the area. Females were found to be pregnant only from September until January with accompanying higher plasma progesterone levels and greater follicular development during this time of the year (11). When exposed to a long photoperiod, simulating summer, spiny mice males had greater testicular mass and volume, as well as elevated plasma testosterone concentrations compared to males subjected to a simulated winter situation (13).

Currently, no long-term data on the endocrine correlates of reproductive function exists for *Acomys spinosissimus*, or any other *Acomys* species for that matter, as limited blood volumes make it impossible for continuous collection of plasma on an individual basis (K. Medger, pers. observation). However, repeated sampling for hormone analysis is often mandatory in order to accurately predict and monitor reproductive processes such as seasonality, ovulation, cycle length, or pregnancy (14; 15).

As continuous collection of blood for hormone analysis has proved challenging especially in free-ranging non-domesticated species, as well as small animals like many rodents including *Acomys*, alternative sampling methods have been developed for species where repeated sampling is not feasible (16; 17). One well established approach to monitor reproductive function in small, non-domesticated species is through the use of faeces as a hormone matrix, because faeces can be collected frequently and animals are usually not disturbed during sample collection (18; 19). Therefore, faeces have been used in order to characterise reproductive activity in terms of determining oestrous cycle length, identify spontaneous or induced ovulation and monitor pregnancy as well as seasonal patterns of reproduction in a variety of rodents (16; 20-22). However, in terms of *Acomys*, faeces were so far only used to monitor the effect of various intrinsic and extrinsic factors on glucocorticoid metabolite output in common spiny mice (19).

The sex steroids, androgens and progestagens, are primarily secreted by the testes and corpus luteum, respectively, and metabolised in the liver before being excreted via urine or faeces; in the latter case typically in an unconjugated form (18; 23). However, due to the species-specific differences in hormone metabolite excretion (21; 24) and the presence of numerous different steroid metabolites in the excreta of even closely related species (e.g. 25-27), respective assays need to be carefully validated in terms of their applicability for the hormone matrix of interest to ensure a reliable quantification of respective hormone metabolites (15).

The overall aim of this study was to examine the suitability of two enzyme immunoassays (EIAs), detecting faecal 17-oxo-androgens and 20-oxopregnanes, respectively,

for monitoring reproductive function in *Acomys spinosissimus*. More specifically, the aims of this study were four-fold: 1) defining the minimum sample mass required in order to accurately determine hormone metabolite concentrations in spiny mice faeces; 2) investigating the effect of storage by determining the rate of metabolism of faecal androgens and progestagens post-defecation; 3) monitoring luteal activity and defining ovarian cyclicity, and 4) comparing faecal androgen metabolite levels in reproductively active and inactive males.

Methods

Study animals

During May and July 2011, 14 non-pregnant female (17.10 ± 2.52g; mean ± SD) and 24 male spiny mice (19.33 ± 3.76g) 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, while a single pregnant female was additionally captured in September 2011 in the same area. Sherman live traps (H. B. Sherman Traps, Inc. Tallahassee, Florida,U.S.) were baited with a peanut butter, oats and fish mixture before being set out in the late afternoon for a total of 20 days. The traps were left over night and collected before sunrise the next morning to ensure that the animals did not undergo any unnecessary stress due to over-heating or starvation. The animals were subsequently sexed and kept in individual cages prepared with wood shavings and paper towel as bedding and cover respectively. The mice were fed a combination of standard mouse pellets, carrots, apples and bird seeds, and water was available *ad libitum*. Within seven days, all animals were transported back to small mammal research facility at the University of Pretoria. The study was conducted with permission of the Animal Use and Care Committee (EC008-11) of the University of Pretoria, South Africa.

Experimental Design

In the laboratory, the 14 females were kept in individual cages under controlled conditions at 25ºC and on a light cycle of 14 hours light and 10 hours dark (14L:10D) for six weeks to ensure that no individuals were pregnant during the time of capture. At the beginning of September 2011, the females were placed in olfactory, visual, and partly physical contact with vasectomised males to potentially stimulate ovarian endocrine activity. For 32 days, individual faecal matter was collected every other day from all females by temporarily placing the animals in respective collection chambers (UNO, Zevenaar, Holland) for nine hours. The chambers were checked for faeces every three hours and the respective material was combined for each female. Afterwards all animals were returned to their cages. All sampled material was frozen at -20 ºC immediately after collection and stored until analysis.

A single pregnant female was housed under the same laboratory conditions, but without male contact and the respective faecal material was collected and stored as mentioned above. Six samples were obtained over 12 days before parturition after which sample collection was halted to avoid unnecessary stress to the mother and young, therefore no post-pregnancy samples could be obtained.

The 24 males were initially housed as described above. From beginning of December 2011 onwards, 12 males were subjected to a short photoperiod cycle (10L:14D), while the remaining 12 males were continuously kept on a 14L:10D light cycle, representing a long photoperiod cycle. The different light cycles simulate the breeding and non-breeding season of *Acomys spinosissimus*, respectively, as defined by Medger *et al.* (13). The following six weeks, individual faecal samples were collected every other day, by cleaning the individual cages in the morning and subsequently collecting freshly produced faeces every two hours for six hours in total. The material was collected and stored as mentioned above until further processing.

Effects of storage

The stability of hormone metabolites post defecation was investigated by following the procedure described by Laver *et al.* (28). Fresh faecal matter from one female spiny mouse was collected over a period of 12 days, homogenised, and divided into 12 subsamples, which were stored at room temperature. Subsequently, three subsamples were frozen at -20 °C after 0, 1, 2, and 3 hours post defecation, respectively.

Sample processing and extraction

To reveal information regarding the minimal appropriate mass for hormone measurement (29), fresh faecal matter from six spiny mice was additionally collected prior to the main experiments, homogenised, and divided five times into equal subsamples of 10, 20, 30, 40, 50, and 75mg. The resulting 30 samples were extracted as described below and respective extracts subsequently measured for immunoreactive glucocorticoid metabolites using an 11-oxoetiocholanolone enzyme immunoassay (30), which has been shown to reliably reflect adrenocortical function in spiny mice (19).

The frozen faecal samples were lyophilized and the resulting dried faecal matter pulverized. Between 25 - 50mg of the dry faeces was then extracted with 1ml of 80% ethanol in water by vortexing for 15 minutes. Following centrifugation for 2 min at 1500g, supernatants were transferred into micro-centrifuge tubes and stored at -20 °C until hormone analysis.

Hormone analysis

Resulting extracts were measured for immunoreactive 17-oxo-androgens and 20-oxopregnanes using enzyme immunoassays for epiandrosterone (EA; 31) and 5ß-pregnane-3a-ol-20-one (5ß-

20-one, 14), respectively. Sensitivities (90% binding) of the assays were of 3 pg/well for both assays. Serial dilutions of extracted faecal samples gave displacement curves that were parallel to the respective standard curves in both assays. Intra- and inter-assay coefficients of variation, determined by repeated measurements of high and low value quality controls ranged between 8.6% and 14.7% for EA, and 8.6% and 17.3% for the 5ß-20-one measurements. Assays were performed on microtiter plates as described by Ganswindt *et al.* (32).

Data analysis

To determine the minimal appropriate faecal mass for hormone analysis, we tested for differences in the distribution of hormone metabolite concentrations among sample subsets using Friedman's rank sum test (whereby each subset consists of the above mentioned five subsamples with equal faecal mass for hormone extraction). Furthermore, the variability in hormone metabolite levels within each subset was determined by calculating the respective coefficient of variations. Differences in relative degradation rate between samples stored at t = 0h and of faeces stored at 1, 2, and 3 hours post defecation, respectively, were examined using one-way repeated measures ANOVA, followed by a post hoc analysis making use of Bonferroni t-test, with the application of Bonferroni correction. The method described in Brown *et al.* (33) was followed to deduce individual baseline 5ß-20-one values. For that, all 5ß-20-one concentrations of an individual data set exceeding the mean + 1.5 standard deviations (SD) were excluded, the average successively recalculated, and the elimination process repeated until no values exceeded the mean + 1.5 SD. The remaining values yielded the baseline 5ß-20-one concentrations. More than one consecutive 5ß-20-one value above the baseline concentration was taken as the onset of a luteal phase of an oestrous cycle and thus of ovulation and the post-ovulatory formation of a corpus luteum (8). Oestrous cycle lengths were calculated as the interval in days between the onsets of two luteal phases resulting in cycle lengths only being recorded for females that experienced at least two post-ovulatory luteal phases. Revealed faecal 5ß-20-one concentrations from the monitored pregnant female were averaged and compared to the overall mean 5ß-20-one baseline concentration revealed for the other 14 monitored females. Differences in median faecal EA levels from males kept either under short or long day conditions were examined by t-test. All

tests were two tailed, with the α level of significance set at 0.05. The programs Jandel Sigma Stat, version 2.0 and KyPlot, version 2.0 beta 13, were used for statistical analyses.

Results

Utilized sample mass

No significant changes in faecal glucocorticoid metabolite (FGM) concentrations were found between the six analysed sample subsets (χ^2 = 3.60; df = 5; p = 0.61; Fig. 1). However, FGM levels vary within each subset, with highest coefficient of variations (CV) found for sample subsets with comparatively low faecal matter (Fig. 1). Subsequently, only samples with an available minimum amount of 25mg dry faecal matter were further processed for the remaining experiments, excluding 359 out of 787 collected samples.

Storage experiment

Mean faecal 5ß-20-one levels decreased by 14.6% and 27.7% in samples frozen 1 and 2h post-defecation, respectively, before reaching 97.1% of the original hormone concentration after 3h post-defecation (Fig. 2A). A statistically significant difference was found between 0h and 2h post-defecation samples (F = 8.42; p = 0.014; post hoc analysis: p = 0.012).

The revealed pattern of faecal EA concentrations post-defecation was quite similar to the determined 5ß-20-one levels, but with an overall higher variability in EA levels even for the sample triplet stored at t = 0 (Fig. 2B). Mean faecal EA levels decreased by 7.0% and 22.4% in samples frozen 1 and 2h since defecation, respectively, and subsequently reached an averaged 84.5% of the original concentration after 3h post-defecation (Fig. 2B). No statistically significant difference was found between the samples stored at t = 0h and of faeces stored at 1, 2, and 3 hours post-defecation (F = 1.49; p = 0.31).

Female reproductive activity

Thirteen out of the 14 monitored females revealed elevated faecal 5ß-20-one concentrations with eight out of 14 individuals showing indications of a luteal phase (Table 1). Two females (A35 and A97) showed two post-ovulatory luteal phases with estimated cycle length of 16 and 18 days, respectively (Fig. 3).

The verified pregnant female showed an elevation of 231% in mean faecal 5ß-20-one levels (mean ± SD: 2.39 ± 0.91 µg/g DW; n = 6) compared with the overall mean baseline 5ß-20-one concentration (0.72 µg/g DW) revealed for the 14 non-pregnant females.

Male reproductive activity

For 23 of the 24 monitored males 2 - 5 samples with a total mass ≥ 25mg dry faecal matter were obtained. There was a statistically significant difference between faecal EA levels of males exposed to a short photoperiod when compared to the EA concentrations of males exposed to a long photoperiod ($t = -2.27$; $p = 0.034$) with long day males having on average 47.8% higher faecal EA values (Fig. 4).

Discussion

The results of this study demonstrate that changes in faecal androgens and progestagens can be accurately monitored in the spiny mouse (*Acomys spinosissimus*) using enzyme immunoassays for epiandrosterone (EA) and 5ß-pregnane-3a-ol-20-one (5ß-20-one), respectively. We, therefore, examined the accompanying sample collection and hormone extraction procedure by investigating the effect of storage on the rate of hormone metabolism post-defecation and defined the minimum sample mass required for hormone extraction. We further validated the reliability of the two assays mentioned, by identifying luteal activity via respective changes in faecal 5ß-20-one levels of female spiny mice, as well as by comparing faecal EA levels of reproductively active and inactive males as a form of biological validation.

Modification by bacteria has been made responsible for alterations in steroid metabolite levels in unpreserved faecal samples (34), as microbial activity could alter hormone metabolite composition in the matrix, which might then be reflected in respective changes of hormone metabolite concentrations detected, depending on the specificity of the antibody used (29; 35). In this regard, freezing faecal samples directly after defecation seems to be a reliable way of slowing down or even preventing microbial activity and the breakdown of steroid hormone metabolites post-defecation (36). In our study, mean faecal progestagen and androgen levels decreased by 27.7% (5ß-20-one) and 22.4% (EA), respectively, after storing freshly produced spiny mouse faeces for two hours unpreserved at room temperature. This trend is in line with findings from other studies determining changes in faecal glucocorticoid metabolite concentrations post-defecation (e.g. 28; 37). Interestingly, faecal 5ß-20-one and EA concentrations start to increase afterwards, reaching 97.1% and 84.5% of the initial hormone concentration after three hours of unpreserved storage, respectively. This subsequent increase rather supports reported trends for post-defecation changes in faecal glucocorticoid metabolite levels for e.g. pigtailed macaques (*Macaca nemestrina*) and the African buffalo (*Syncerus caffer*) (38; 39). The distinct fluctuation in steroid metabolite concentrations discovered in spiny mouse faeces over just three hours post-defecation is even to some extent comparable with the described long-term changes in glucocorticoid metabolite concentrations in grizzly bears (*Ursus arctos horribilis*) and African elephant (*Loxodonta africana*) faeces, stored unpreserved at room temperature for up to two years (40). Our findings therefore underline once more the importance of controlling for the interval between defecation, sampling, and freezing of the collected material to minimize the potential risk of inadvertent changes in steroid metabolite composition due to active bacterial enzymes present in the faeces (29; 41).

As in other mice and rats (42), spiny mice faecal samples vary greatly in size and mass with sometimes very low overall quantities (P.R. de Bruin, pers. observation). In this regard, Millspaugh and Washburn (29) examined the influence of sample mass used for hormone extraction and showed that comparatively small faecal samples (< 0.02g) result in proportionately higher hormone concentrations, which subsequently biased the findings. Our

results partly confirm the described pattern, as faecal material of less than 0.03g used for hormone extraction showed greater variability in respective hormone metabolite levels. As a possible explanation for the revealed pattern, it was hypothesized that small sample size may result in a biased representation of hormone metabolite data because of microbial activity resulting in a higher metabolite concentration per gram mass (29; 43). To overcome the problem of insufficient sample mass, which might be an expected issue for studies involving small animals like mice, material could be pooled to a certain extent. Suedkamp Wells *et al*. (44) have shown that pooling data can result in accurate and reliable findings when using faecal hormone analyses. However, the option of pooling samples obviously depends on the research question, as pooling could dampen short fluctuations in hormone secretion which would be a disadvantage if the data is necessary for describing exactly these short-term alterations in hormone concentration. Visual estimates for the degree of variability in the mass-dependent results reported by Millspaugh and Washburn (29) also indicate a proportionally slightly higher variance in faecal glucocorticoid metabolite values when a comparatively large faecal mass ($\geq$ 125mg) was used for hormone extraction. This is again in line with our results, as we also found a slightly higher coefficient of variation for the sample set including masses of 75mg faeces for extraction. Although the reason for this increase in variability is unknown, a comparatively less-than-ideal hormone extraction procedure for faecal samples of larger masses is conceivable.

So far, the length of the oestrous cycle in spiny mice has only been examined in common and golden spiny mice (7; 8) by investigating vaginal smears and plasma progesterone levels. However, the described cycle length of 11-18 days for the two *Acomys* species (7; 8) is comparable to length of the two oestrous cycles (16 and 18 days, respectively) identified in this study. Compared to other mouse species, however, the oestrous cycle of the three investigated spiny mouse species is approximately three times longer in duration (9). A possible explanation for this could be a difference in social organization, although respective data for *A. spinosissimus* is lacking (45). If spiny mice are solitary as already hypothesized for *Acomys russatus* from Southern Sinai (46) the comparatively long oestrous cycle could enhance the possibility for the female to be visited by a male within the fertile window. It is also possible that maturation of the follicles takes much longer than in other species, resulting in an extended oestrous cycle.

Therefore, investigations into the time required for the ovarian follicles to develop into Graafian follicles ready to rupture could aid in the explanation of the comparatively long oestrous cycle observed in this genus. The reason for only identifying two cycles out of a possible 14 may be due to the chosen sampling regime in this study, with samples being collected only every second day. Ideally, faecal samples should be collected continuously over a >20 day period in order to precisely determine the luteal activity and subsequently the oestrous cycles for this species. However, an alternative biological explanation for the low number of oestrous cycles identified in this study may be the age of the females involved. Although age could not be determined precisely in our study since females were wild caught, it is conceivable that some of the females were sexually immature or already too old to undergo any ovarian activity. However, because we were able to reveal luteal activity in eight out of the 14 study animals, the explanation of a suboptimal sampling regime seems more realistic.

The common spiny mouse is the only *Acomys* species for which a gestation period has been described and with approximately 39 days its duration is comparable with some rodent species but generally longer than for most rodents of equal body size (7; 47). In our study 5-β-20-one levels were over 200% higher in a confirmed pregnant female, which again demonstrates the validity of the EIA used for monitoring reproductive function in *A. spinosissimus* females. However, samples could only be obtained 12 days prior to parturition, and further research would be necessary in order to examine the applicability of our established method to determine pregnancy during the first two-thirds of gestation. Therefore, future studies should frequently monitor female reproductive activity from the day of copulation in order to reveal detailed longitudinal hormone profiles, which could subsequently be used to determine the duration of pregnancy for *Acomys spinosissimus*.

The males exposed to a long photoperiod, simulating the austral summer, had elevated faecal EA levels compared to the males that were exposed to the short photoperiod, simulating the austral winter, which confirmed the findings of Medger *et al*. (12) regarding an existing seasonal pattern in plasma testosterone concentrations for *Acomys spinosissimus* males. Based on

their findings, Medger *et al*. (11) concluded that spiny mice are seasonal breeders with the breeding season coinciding with the summer months when the rainfall is maximal in the Limpopo province of South Africa. The revealed differences in EA levels in reproductively active and inactive males demonstrate the suitability of the EIA used to determine the reproductive state of males in the wild as has been shown for various other species such as Eurasian lynx (*Lynx lynx*), African elephants (*Loxodonta africana*) and Moustached tamarins (*Saguinus mystax*) (48-50).

Conclusion

Acomys spinosissimus is a small rodent making continuous blood sampling for hormone monitoring impossible and the small amount of urine usually produced by the species adds another constraint on using this matrix for monitoring reproductive activity. Faecal samples have been used in various rodent species to monitor male and female reproductive activity (18; 51; 52), and our study adds to the list by demonstrating that reproductive function can be monitored in male and female spiny mice by measuring respective faecal hormone metabolites. The established techniques could prove invaluable as continuous sampling is now possible on an individual level making long term studies more feasible, simultaneously improving the ethical basis for respective experiments as animals would not necessarily have to be euthanized for sample collection.

List of abbreviations

EIA = Enzyme Immunoassay

EA = 17-oxo-androgens (Epiandrosterone)

5ß-20-one = 5ß-pregnane-3a-ol-20-one (20-oxopregnanes)

ANOVA = Analysis of Variance

FGM = Faecal glucocorticoid metabolites

CV = Coefficient of Variation

DW = Dry weight

Competing interests

The authors declare that they have no competing interest.

Author contribution

RDB was responsible for data collection, analysis, interpretation, and drafting of the manuscript. AG coordinated the laboratory analysis of faecal samples and helped to draft the manuscript. KM and NCB assisted in designing and coordinating the study as well as in interpreting the data. All authors read and approved the final manuscript.

Acknowledgements

Collection of animals occurred under permits granted by the Department of Nature Conservation in the Limpopo and Gauteng Provinces, respectively. The project was funded by the SARChI Chair for Mammal Behavioural Ecology and Physiology as well as the National Research Foundation to N.C. Bennett. P.R. de Bruin acknowledges a Masters bursary from the NRF and K. Medger a postdoctoral bursary from the University of Pretoria. We would like to thank Dave Dewsnap from Goro Game Reserve for allowing us to work on the premises and Claire Relton for her assistance during the experimental period.

References

1. Bates P.J.J: **The distribution of *Acomys* (Rodentia: Muridae) in Africa and Asia.** *Isr J Zool* 1994, **40:**199–214.
2. Nowak, R.M. Walker's mammals of the world. The Johns Hopkins University Press: Baltimore; 1999.
3. Shargal E, Kronfeld-Schor N, Dayan T: **Population biology and spatial relationships of coexisting spiny mice (*Acomys*) in Israel.** *J Mammal* 2000, **81:**1046–1052.
4. Nováková M, Palme R, Kutalová H. Janský L, Frynta D: **The effects of sex, age and commensal way of life on levels of fecal glucocorticoid metabolites in spiny mice (*Acomys cahirinus*).** *Physiol Behav* 2008, **95:**187–193.
5. Shanas U, Haim A: **Diet salinity and vasopressin as reproduction modulators in the desert-dwelling golden spiny mouse (*Acomys russatus*).** *Physiol Behav* 2004, **81:**645–650.
6. Wube T, Haim A. Fares F: **Reproductive response of xeric and mesic populations of the spiny mouse *Acomys* to photoperiod acclimation.** *J Arid Environ* 2008, **72:**440–447.
7. Peitz B: **The oestrous cycle of the spiny mouse (*Acomys cahirinus*).** *J Reprod Fertil* 1981, **61:**453–459.
8. van Aarde R, Haim A: **The influence of urinary and fecal odors on ovarian function in coexisting *Acomys* species.** *Isr J Zool* 1999, **45:**261-265.
9. Parkes AS: **Observations on the oestrous cycle of the albino mouse.** *Proc R Soc Lond B Biol Sci* 1926, **100:**151-170.
10. Mills G, Hes L: The complete book of southern African mammals: Struik Publishing group (Pty) Ltd; 1997.
11. Medger K, Chimimba CT, Bennett NC: **Seasonal reproduction in the female spiny mouse from South Africa.** *J Zool* (Lond) 2010, **282:**163-170.
12. Medger K, Chimimba CT, Bennett NC: **Seasonal changes in reproductive development in male spiny mice (*Acomys spinosissimus*) from South Africa.** *Mamm Biol* 2012a, **77:** 153-159.

13. Medger K, Chimimba CT, Bennett NC: **Reproductive photoresponsiveness in male spiny mice from South Africa.** *J Zool (Lond)* 2012b, **286:** 243-249.
14. Schwarzenberger F, Tomášová K, Holečková D, Matern B, Möstl E: **Measurement of fecal steroids in the black rhinoceros (*Diceros bicornis*) using group-specific enzyme immunoassays for 20-oxo-pregnanes.** *Zoo Biol* 1996, **15:** 159-171.
15. Hodges K, Brown J, Heistermann M: **Endocrine monitoring of reproduction and stress.** In Wild mammalsin captivity: Principles and techniques for zoo management. 2nd Edition. Edited by Kleiman DG, Thompson KV, Kirk Baer C. Chicago: The University of Chicago Press; 2010: 447-468.
16. Cavigelli SA, Monfort SL, Whitney TK, Mechref YS, Novotny M, McClintock MK: **Frequent serial fecal corticoid measures from rats reflect circadian and ovarian corticosterone rhythms.** *J Endocrinol* 2005, **184:**153-163.
17. Valades GB, Ganswindt A, Annadale H, Schulman ML, Bertschinger HJ: **Non-invasive assessment of the reproductive cycle in free-ranging female African elephants (*Loxodonta africana*) treated with a gonadotropin-releasing hormone (GnRH) vaccine for inducing anoestrus.** *J Reprod Biol Endocrinol* 2012, **10:** 63.
18. Schwarzenberger F: **The many uses of non-invasive faecal steroid monitoring in zoo and wildlife species.** *Int Zoo Yearb* 2007, **41:** 52–74.
19. Frynta D, Nováková M, Kutalová H, Palme R, Sedláček F: **Apparatus for collection of fecal samples from undisturbed spiny mice (*Acomys cahirinus*) living in a complex social group.** *J Am Assoc Lab Anim* 2009, **48:** 196-201.
20. Kuznetsov VA, Tchabovsky AV, Kolosova IE, Moshkin MP: **Effect of habitat type and population density on the stress level of midday Gerbils (*Meriones meridianus* Pall.) in free-living populations.** *Biol Bull* 2004, **6:** 628-632.
21. Busso JM, Ponzio MF, Dabbene V, de Cuneo MF, Ruiz RD: **Assessment of urine and fecal testosterone metabolite excretion in *Chinchilla lanigera* males.** *Anim Reprod Sci* 2005, **86:** 339-351.
22. Busso JM, Ponzio MF, Dabbene V, de Cuneo MF, Ruiz RD: **Noninvasive monitoring of ovarian endocrine activity in the chinchilla (*Chinchilla lanigera*).** *Gen Comp Endocrinol* 2007, **150:** 288-297.

23. Touma C, Palme R: **Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation.** *Ann NY Acad Sci* 2005, **1046:** 54-74.
24. Palme R, Fischer P, Schildorfer H, Ismail MN: **Excretion of infused ^{14}C-steroid hormones via faeces and urine in domestic livestock.** *Anim Reprod Sci* 1996, **43:** 43-63.
25. Berkeley EV, Kirkpatrick JF, Schaffer NE, Bryant WM, Threlfall WR: **Serum and fecal steroid analysis of ovulation, pregnancy, and parturition in the black rhinoceros (*Diceros bicornis*).** *Zoo Biol* 1997, **16:** 121-132.
26. Brown JL, Bellem AC, Fouraker M, Wildt DE, Roth TL: **Comparative analysis of gonadal and adrenal activity in the black and white rhinoceros in North America by noninvasive endocrine monitoring.** *Zoo Biol* 2001, **20:** 463-486.
27. Schwarzenberger F, Rietschel W, Vahala J, Holeckova D, Thomas P, Maltzan J, Baumgartner K, Schaftenaar W: **Fecal progesterone, estrogen, and androgen metabolites for noninvasive monitoring of reproductive function in the female Indian rhinoceros, *Rhinoceros unicornis*.** *Gen Comp Endocrinol* 2000, **119:** 300-307.
28. Laver PN, Ganswindt A, Ganswindt SB, Alexander KA: **Non-invasive monitoring of glucocorticoid metabolites in banded mongooses (Mungos mungo) in response to physiological and biological challenges.** *Gen Comp Endocrinol* 2012, **179:** 178-183.
29. Millspaugh JJ, Washburn BE: **Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation.** *Gen Comp Endocrinol* 2004, **138:** 189-199.
30. Möstl E, Maggs JL, Schrötter G, Besenfelder U, Palme E: **Measurement of cortisol metabolites in faeces of ruminants.** *Vet Res Commun* 2002, **26:** 127-137.
31. Palme R, Möstl E: **Biotin-streptavidin enzyme immunoassay for the determination of oestrogens and androgens in boar faeces.** In *Procedings of the 5th Symposium on the Analysis of steroids*: 1993; Hungary. Edited by S. Görög. 1994: 111-117.
32. Ganswindt A, Heistermann M, Borragan S, Hodges JK: **Assessment of testicular endocrine function in captive African elephants by measurement of urinary and fecal androgens.** *Zoo Biol* 2002, **21:** 27-36.

33. Brown JL, Schmitt DL, Bellem A, Graham LH, Lehnhardt J: **Hormone secretion in the Asian elephant (*Elephas maximus*): characterization of ovulatory and anovulatory luteinizing hormone surges.** *Biol Reprod* 1999, **61:** 1294-1299.
34. Goymann W: **On the use of non-invasive hormone research in uncontrolled, natural environments: the problem with sex, diet, metabolic rate and the individual.** *MEE* 2012, **3:** 757-765.
35. Lexen E, El-Bahr SM, Palme R, Möstle E: **Monitoring disturbance in sheep by measuring glucocorticoid metabolites in faeces.** *Wien Tierärztl Monat* 2008, **95:** 64-71.
36. Pettitt BA, Wheaton CJ, Waterman JM: **Effect of storage treatment on fecal steroids hormone concentrations of a rodent, the Cape ground squirrel (*Xerus inauris*).** *Gen Comp Endocrinol* 2007, **150:** 1-11.
37. Ludwig C, Wachter B, Silinski-Mehr S, Ganswindt A, Bertschinger H, Hofer H, Dehnhard M: **Characterisation and validation of an enzyme-immunoassay for the non-invasive assessment of faecal glucocorticoid metabolites in cheetahs (*Acinonyx jubatus*).** *Gen Comp Endocrinol* 2013, **180:** 15-23.
38. Wasser SK, Risler L, Steiner RA: **Excreted steroids in primate feces over the menstrual cycle and pregnancy.** *Biol Reprod* 1988, **39:** 862-872.
39. Ganswindt A, Tordiffe ASW, Stam E, Howitt MJ, Jori F: **Determining adrenocortical activity as a measure of stress in African buffalo (*Syncerus caffer*) based on faecal analysis.** *Afr Zool* 2012, **47:** 261-269.
40. Hunt KE, Wasser SK: **Effects of long term preservation methods on fecal glucocorticoid concentrations of grizzly bear and African elephant.** *Physiol Biochem Zool* 2003, **76:** 918-928.
41. Möstl E, Messmann S, Bagu E, Robia C, Palme R: **Measurement of glucocorticoid metabolite concentrations in faeces of domestic livestock.** *J Vet Med A* 1999, **46:** 621-631.
42. **New Mexico Department of Health**
[http://nmhealth.org/ERD/HealthData/documents/DomesticRatsand Mice_001.pdf]
43. Tempel DJ, Gutièrrez RT: **Factors related to fecal corticosterone levels in California spotted owls: implications for assessing chronic stress.** *Conserv Biol* 2004, **18:** 538-547.

44. Suedkamp Wells KM, Washburn BE, Millspaugh JJ, Ryan MR, Hubbard MW: **Effects of radio-transmitters on fecal glucocorticoid levels in captive dickcissels.** *The Condor* 2003, **105:** 805-810.
45. Skinner JD, Chimimba CT: The mammals of the southern African subregion. Cambridge: Cambridge University Press; 2005.
46. Haim A: **Behavior patterns of cold-resistant Golden spiny mouse *Acomys russatus.*** *Physiol Behav* 1991, **50:** 641-643.
47. Yom-Tov Y: **The reproductive rates of Australian rodents.** *Oecologia (Berl)* 1985, **66:** 250-255.
48. Göritz F, Neubauer K, Naidenko SV, Fickel J, Jewgenow K: **Investigations on reproductive physiology in the male Eurasian lynx (*Lynx lynx*).** *Theriogenology* 2006, **66:** 1751-1754.
49. Ganswindt A, Rasmussen HB, Heistermann M, Hodges JK: **The sexually active states of free-ranging male African elephants (*Loxodonta africana*): defining musth and non-musth using endocrinology, physical signals, and behavior.** *Horm Behav* 2005, **47:** 83-91.
50. Huck M, Löttker P, Heymann EW, Heistermann M: **Characterization and social correlates of fecal testosterone and cortisol excretion in wild male *Saguinus mystax*.** *Int J Primatol* 2005, **26:** 159-179.
51. Billitti JE, Lasley BL, Wilson BW: **Development and validation of a fecal testosterone biomarker in *Mus musculus* and *Peromyscus maniculatus*.** *Biol Reprod* 1998, **59:** 1023-1028.
52. Busso JM, Ruiz RD: **Excretion of steroid hormones in rodents: an overview on species differences for new biomedical animal research models.** In *Contemporary Aspects of Endocrinology*. Edited by Diamanti-Kandarkis E. ISBN: 978-953-307-357-6, InTech, DOI: 10.5772/17051. 2011: 375-396.

Figure 1: Faecal glucocorticoid metabolite (FGM) levels from the six sample subsets representing the different faecal mass from spiny mice (10, 20, 30, 40, 50 and 75mg, respectively) used for hormone analysis. The coefficient of variation (CV) for each sample subset (n = 5) is given on top.

Figure 2: Relative changes (%) of faecal 5ß-20-one (A) and EA levels (B) (mean ± SEM) in spiny mouse faeces over time (0, 1, 2 and 3 hours since defecation). * indicates significant difference between samples.

Figure 3: Profiles of faecal 5ß-20-one immunoreactivity throughout a period of 35 days in two female spiny mice (*Acomys spinosissimus*). Dotted line indicates the individual threshold values for faecal 20-oxopregnanes.

Figure 4: Individual mean faecal EA concentrations for males exposed to a short (SD) or a long photoperiod (LD) over the 6 week experimental period. * indicates significant difference between samples.

Table 1: Individual baseline and elevated faecal 5ß-20-one concentrations (mean ± SD) of 14 captive spiny mice females monitored for 35 days

Female code	Faecal 5ß-20-one levels (µg/g DW)		Indications of luteal phase
	Baseline (mean +/-SD)	Elevated (mean +/-SD)	
A1	0.88 +/- 0.16 (n=11)	1.69 +/- 0.39 (n=2)	No
A5	0.77 +/- 0.14 (n=10)	1.32 +/- 0.34 (n=4)	Yes
A7	0.78 +/- 0.20 (n=8)	1.49 +/- 0.31 (n=4)	Yes
A9	0.80 +/- 0.18 (n=7)	1.49 (n=1)	No
A10	0.53 +/- 0.06 (n=7)	1.26 +/- 0.60 (n=4)	Yes
A21	0.66 +/- 0.15 (n=9)	1.08 +/- 0.10 (n=5)	Yes
A35*	0.41 +/- 0.06 (n=8)	0.96 +/- 0.45 (n=7)	Yes
A36	0.84 +/- 0.22 (n=9)	1.96 +/- 0.50 (n=3)	Yes
A41	0.80 +/- 0.10 (n=6)	-	No
A49	0.65 +/- 0.08 (n=12)	1.02 +/- 0.07 (n=3)	No
A90	0.85 +/- 0.26 (n=10)	1.40 (n=1)	No
A97*	0.62 +/- 0.09 (n=7)	1.27 +/- 0.38 (n=7)	Yes
A108	0.59 +/- 0.08 (n=7)	1.02 +/- 0.26 (n=3)	Yes
A110	0.93 +/- 0.09 (n=11)	1.15 +/- 0.07 (n=2)	No

* Females with two identified post-ovulatory luteal phases

Figure 1

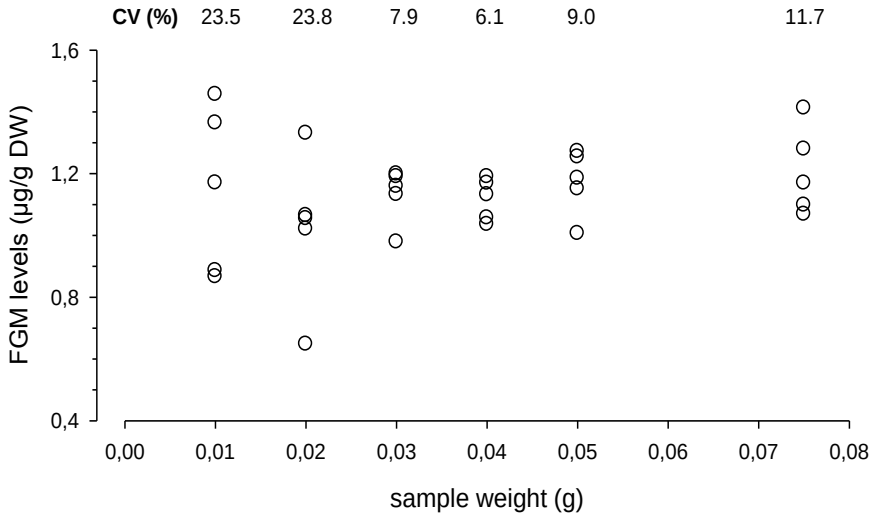


Figure 2

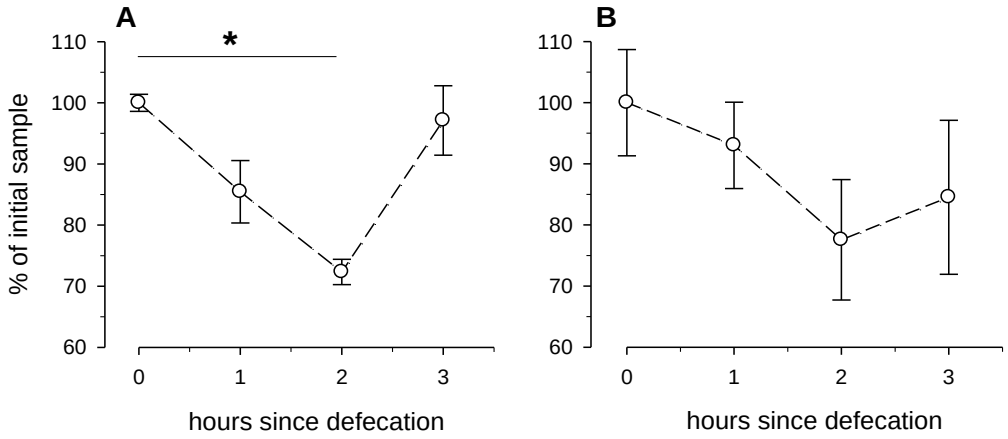


Figure 3

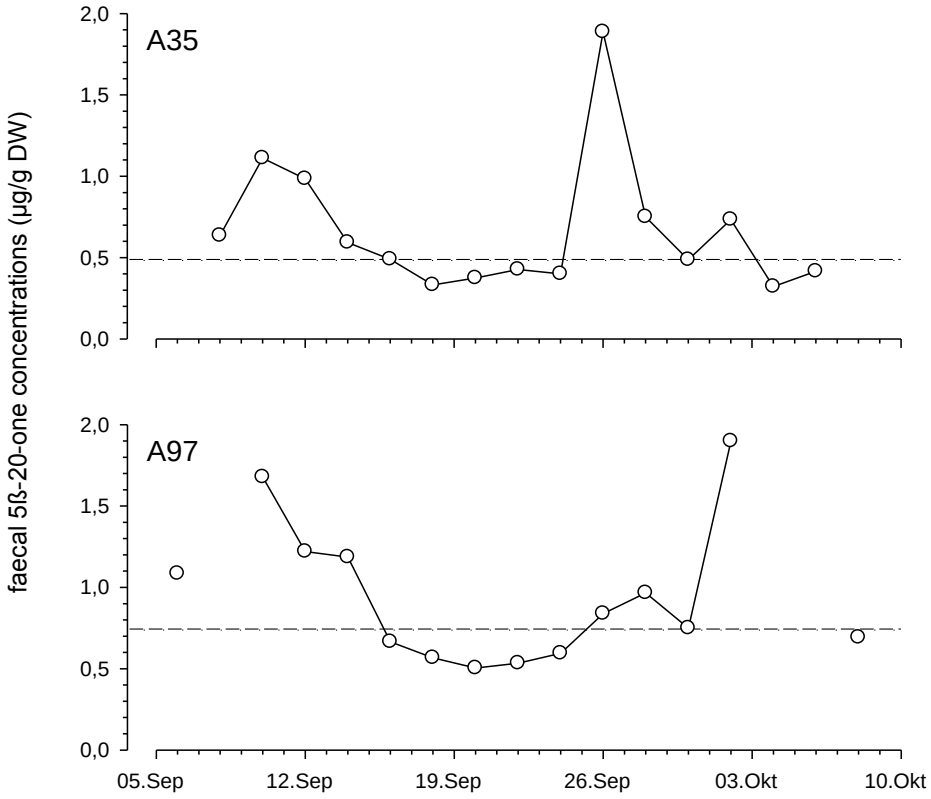
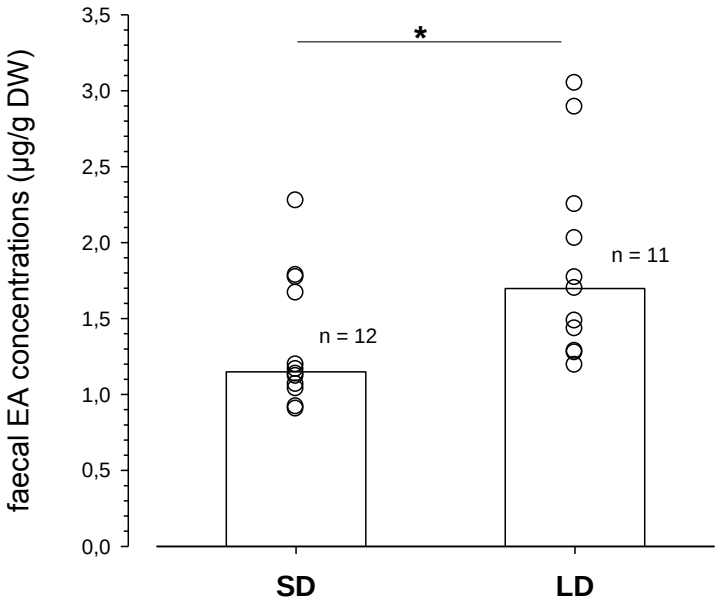


Figure 4



Chapter 2

The pattern of ovulation in the spiny mouse (*Acomys spinosissimus*) from South Africa

Abstract

The pattern of ovulation in mammals is generally considered to be either spontaneous or induced, being brought about by the act of coitus. This study aimed to assess whether female spiny mice (*Acomys spinosissimus*) from South Africa exhibit spontaneous or induced ovulation. Females were divided into three treatments differing in the degree of contact with a male. Seven control females had no contact with males; seven females were separated from males by wire mesh so that they only had chemical, auditory and visual contact with a male, while seven females had intermittent periods of full contact with a vasectomized male. Follicular type and quantity, presence of corpora lutea and faecal progesterone measurements were compared between the three treatments. Corpora lutea were found in one female each from the control and the paired treatments. Progesterone profiles did not differ significantly between the three experimental treatments but were affected by the day of collection. The ovarian histology along with the progesterone profiles proves conclusively that *Acomys spinosissimus* is a spontaneous ovulator.

Keywords: *Acomys spinosissimus*, spontaneous ovulation, progesterone, corpora lutea.

Introduction

Ovulation is a complex process that is critical for reproductive success (Kauffman and Rissman 2006) and is defined as the process whereby hormones are responsible for triggering the rupturing of the ovum and the subsequent release of an oocyte (Hickman *et al.* 2006). Females can express either one of two modes of ovulation (Milligan 1980; Larivière and Ferguson 2003; Jackson and Bennett 2005) in order to maximize their reproductive success.

Spontaneous ovulation is the continuous cycle of follicular development where primordial follicles on the edge of the ovary develop sequentially through a series of follicular stages into Graafian follicles and rupture, releasing the egg, with the remnants of the ruptured

follicle forming the corpus luteum (Zuckerman and Weir 1977a). Gonadotropin releasing hormone (GnRH) is an important neuropeptide which plays a crucial role in vertebrate reproduction (Bakker & Baum 2000). GnRH is secreted from the hypothalamus and subsequently stimulates the secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary. The increased levels of LH cause the rupturing of the Graafian follicle and subsequent formation of the corpus luteum (Zuckerman and Weir 1977b). The corpus luteum secretes progesterone, which has a negative effect on GnRH and gonadotropin secretion (Crighton *et al.* 1978). If pregnancy does not result, the corpus luteum eventually degenerates, bringing about a reduction in the secretion of progesterone and this in turn feeds back to the pituitary, causing the cycle to be repeated (Hilliard 1973).

Species which undergo induced ovulation express identical follicular growth and maturation; however, they require stimulation of the vagina and cervix during copulation. This stimulation brings about increased release of oxytocin which in turn brings about the secretion of GnRH resulting in the LH surge and subsequently causing the release of a mature egg from the Graafian follicle (Milligan 1980).

Thus, spontaneous ovulation is a predictable cycle (Conaway 1971) and the chemical or physical presence of a male is not necessary for the female to ovulate (Jackson and Bennett 2005; Kauffman and Rissman 2006). In contrast, females exhibiting induced ovulation show normal growth and development of the primordial follicles up unto the Graafian follicle stage in the ovary, but despite follicle maturation, ovulation does not occur (Zuckerman and Weir 1977b). Induced ovulation lacks the positive feedback of oestrogen (Bakker and Baum 2000) and requires the physical presence of a male and the act of coitus. The tactile stimulation of the vagina is the main cause of ovulation during coitus and has resulted in modifications of the male reproductive organs with ornamentation such as cornified penile spines (Zarrow and Clark 1968; Bakker and Baum 2000; Parag *et al.* 2006) that stimulates the vaginal and uterine lining and triggers a surge of GnRH from the hypothalamus, which in turn stimulates the immediate release of LH from the pituitary (Bakker and Baum 2000; Kauffman and Rissman 2006) resulting in

ovulation within a few hours. This is especially beneficial for solitary species occupying large home ranges (Larivière and Ferguson 2003) where the chances of acquiring mates are limited.

Induced ovulation allows the female to be fertilized by a male as soon as they come in contact (Weir and Rowlands 1973). It has been speculated that induced ovulation is the more ancestral state amongst mammals with spontaneous ovulation being derived (Conaway 1971; Kauffman and Rissman 2006). If this is the case, we would expect non-mammalian vertebrates to exhibit an induced method of ovulation, however, spontaneous ovulation has been identified across all genera (Bakker and Baum 2000; Kauffman and Rissman 2006). Further complicating the investigation into which is the ancestral trait is the fact that induced ovulation cannot be isolated to a specific group, but has been confirmed across many mammalian orders such as rodents (Clulow and Mallory 1970; Gray *et al.* 1974) and carnivores (Dixson 1995). Larivière and Ferguson (2003) investigated the evolution of induced ovulation and concluded that sexual selection is the driving force responsible for this phenomenon. In males it ensures that sperm will fertilize the egg during the limited time period of pair bonding and in females, post-copulatory mate choice is based on the level of stimulation (Larivière and Ferguson 2003). Rodents represent about one third of all the mammals on earth (Weir and Rowlands 1973) and can be found in almost all habitats. Within the order Rodentia, both methods of ovulation have been identified (Conaway 1971; Zuckerman and Weir 1977a).

Within the order Rodentia, the genus *Acomys* has received little attention over the years, but more recently a number of studies have been directed at unravelling the general biology as well as reproduction of a variety of species of *Acomys* (Shargal *et al.* 2000; Shanas and Haim 2004; Nováková *et al.* 2008; Wube *et al.* 2009; Medger *et al.* 2010; Frynta *et al.* 2011; Medger *et al.* 2012a,b). The work has mainly focused on *A. cahirinus* and *A. dimidiatus* (Frynta *et al.* 2011) as an experimental model in physiological and behavioural studies (Nováková *et al.* 2008). Spiny mice are widespread throughout Africa and the Middle East and can be found in high spatio-temporal varied areas (Frynta *et al.* 2011). The genus owes its name to the characteristic modified hairs on their backs that give a spiny appearance (Skinner and Chimimba 2005).

There are two species of *Acomys* found in South Africa, the opportunistic breeding Cape spiny mouse (*A. subspinosus*), confined to the Western Cape (Mills and Hes 1997; Fleming and Nicolson 2002) and the South African spiny mouse (*A. spinosissimus*) found in the north-eastern part of South Africa (Mills and Hes 1997). *Acomys spinosissimus* is a small, terrestrial, strictly nocturnal (Hoole *et al*. 2012) rodent that can be found in rocky areas that provide shelter and protection against predators (Skinner and Chimimba 2005). The sociality of *A. spinosissimus* is not well known and individuals have been found to be solitary, in pairs and in family groups (Skinner and Chimimba 2005). Several other species of *Acomys* have been shown to be social with *A. russatus* from Southern Sinai proposed to be solitary (Haim 1991; Shargal *et al.* 2000; Frynta *et al.* 2011). *Acomys spinosissimus* is found in areas with one annual rainy season in South Africa (September – April) (Medger *et al.* 2010). Medger *et al.* (2010, 2012a) concluded that *A. spinosissimus* is a seasonal breeder and reproduction commences at the onset of the rainy season with the last offspring being born by the end of January. Despite the recent findings by Medger (2010, 2012a,b), there is still very little known about *A. spinosissimus* in general and, more specifically on their reproduction and the pattern of ovulation. Peitz (1981) investigated the oestrous cycle of the common spiny mouse, *Acomys cahirinus* while Dewsbury and Hodges (1987) investigated the copulatory behaviour of the same species. Both studies concluded that *A. cahirinus* is a spontaneous ovulator. The aim of this study was to determine the pattern of ovulation present in *A. spinosissimus* by examining the ovarian histology and the secretion of progesterone by corpora lutea (Zuckerman and Weir 1977b). Faecal samples were used in order to compare progesterone levels between the different treatments. Based on the findings of Peitz (1981) and Dewsbury and Hodges (1987) and the sociality of *A. cahirinus*, and *A. dimidiatus*, I predict that *A. spinosissimus* will exhibit a spontaneous pattern of ovulation.

Materials and Methods

Subjects and sample collection

Twenty-one female (mean ± SD, 17.23 ± 2.30g; range: 12.52 – 20.88g) and 14 male spiny mice (19.33 ± 3.76g; range: 15.73 – 29.02g) were collected during May and July 2011 from the Goro Game Reserve (22°58'S, 22°57'S, 29°25'E, 29°24'E) in the Limpopo Province, South Africa 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 and were set out in the late afternoon and left over night. Traps were checked before sunrise to make sure that the animals did not undergo any unnecessary thermal and starvation stress. The captured animals were sexed before being placed into individual cages with wood shavings and paper towel provided as bedding and housing, respectively. Water and mouse pellets were provided *ad libitum*, with apple, carrot and bird seed as supplementary feed. In the lab, males and females were kept in separate temperature controlled rooms at 25 °C and on a light cycle of 14 hours light and 10 hours dark (14L:10D) simulating the photoperiod during the South African summer.

The males were captured four months prior to the onset of the experiment and a qualified veterinarian vasectomized seven males one month later to allow sufficient time for recovery from the procedure and prevent any spermatozoa being present in the vas deferens when placed in direct contact with a female. 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. The vasectomies ensured that any corpora lutea present in the ovaries would be those of ovulation and not of pregnancy.

Before the onset of the experiment, female mice were kept separate from the males for an initial period of five weeks in order to prevent any male induced reproductive activity. The

females were then randomly assigned to one of three experimental treatments after their body mass had been determined. The first treatment consisted of seven females housed alone, without any male contact in a temperature controlled room as mentioned above. These females represented the control group. The second treatment of seven females was housed in cages separated from the males by a wire mesh, that allowed visual, auditory and olfactory communication between the two sexes, but no physical contact was possible. For the third experimental group, seven females were each kept in a cage together with a vasectomized male for physical contact to occur between the sexes. Faecal samples were collected every second day for each female during the five week experimental period by placing them into collection chambers. This allowed the collection of female faeces only which were used to analyse progesterone concentrations. The faecal samples were collected every three hours over a nine hour period. The faecal samples were stored at -30 °C until analysed. At the end of the experimental procedure, the animals were sacrificed by exposing them to an overdose of halothane. The final body mass was determined and the ovaries dissected out, fixed in Bouin's fluid for 10 hours and then stored in 70% ethanol before being prepared for histological analysis.

Histological procedure

The mass of the ovaries was determined to the nearest 0.01mg using a Sartorius 1213MP scale (Sartorius AG, Gottengen, Germany). The ovaries were dehydrated by exposing each sample 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 5µm sections using a microtome (820 Spencer Microtome, American Optical). Ehrlich's haematoxylin and eosin were used as stains. The samples were mounted on microscope slides with gelatine. The slides were examined for the different follicular stages using a light microscope with 200X magnification. Bennett (1994) was used to classify each follicle as primary, secondary, tertiary, Graafian follicles or corpora lutea and the total number of each follicular stage was recorded for every 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.

Progesterone assay

The faecal samples were used to compare the progesterone (Pg) concentrations between the different treatments. The procedure followed is described in Chapter 1 of this thesis. The inter and intra Assay Coefficient of Variation (%) for 20-oxopregnanes was 12.73 – 17.28% and 8.61 – 11.54% respectively. In total, 241 samples were used during the analysis, 73 samples made up the control treatment with the separated and paired treatment having 79 and 89 samples respectively. After correcting for mass, there were on average, 11 – 12 samples used per female.

Penile morphology

The penises of four males were dissected out after euthanasia and preserved in 70% ethanol. The penises were fixed in 10% formalin and rinsed three times for ten minutes each with 0.075M phosphate buffer. The penises were then fixed in 0.5% osmium tetraoxide for approximately two hours. Thereafter, the samples were rinsed again using 0.075M phosphate buffer. The final dehydration was done by exposing the samples to a series of ethanol solutions of increasing concentration, for ten minutes at each concentration. 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 see if there were any penile modifications present which may promote ovulation if the species is found to be an induced ovulator. SEM pictures were taken at 37X, 40X and 110X magnifications.

Statistical analysis

IBM SPSS 20 (IBM Corporation 1989, 2011) was used for statistical analysis. Primary, secondary and tertiary follicle numbers had a normal distribution. After log-transformation ovarian mass was also normally distributed. A Levene's test showed that all variables were homogeneous. A General Linear Model (GLM) was used to compare start and final body mass and ovarian mass between the different females with the treatment as the factor. Body mass was used as covariate for the analysis of ovarian mass. Primary, secondary and tertiary follicle numbers were also compared between the three different treatments using a GLM with ovarian mass as the covariate. Graafian follicle numbers were compared between the different treatments using a Generalized Linear Model (GZLM) with a gamma distribution with log-link function; because it was not normally distributed, ovarian mass was used as the covariate for all GZLMs. Because of technical difficulty, only 19 females were used to compare follicle numbers between the different treatments. A log transformation was done on the Pg data in order to obtain a normal distribution after which a General Estimating Equation with a linear distribution was done to compare the Pg concentrations between the different treatments and sample days. The results are presented as mean ± 1 standard deviation (SD).

Results

The starting body mass of the females did not differ significantly between the control (17.18 ± 2.88g), separated (17.01 ± 2.32g) or paired treatments (17.49 ± 1.96g, $F_{2, 18} = 0.07$, $p = 0.93$). There was no significant difference in final body mass between the control (17.81 ± 3.05g), separated (17.67 ± 2.33g) or paired females (17.62 ± 1.75g, $F_{2,18} = 0.11$, $p = 0.99$). Ovarian mass was not affected by final body mass of the females ($F_1 = 3.68$, $p = 0.07$). Ovarian mass did not differ significantly between the control (5.57 ± 3.91mg), separated (4.03 ± 2.66mg) or paired females (5.36 ± 2.74mg, $F_{2,18} = 0.88$, $p = 0.43$). There was no significant difference between the number of primary follicles in the control (13.57 ± 6.50), separated (12.40 ± 2.51) or paired females (13.14 ± 11.31, $F_{2,16} = 0.03$, $p = 0.97$) as was the case when the secondary/tertiary

follicles were compared between the control (12.14 ± 6.62), separated (7.40 ± 3.65) and paired females (9.43 ± 4.24, $F_{2,16}$ = 1.28, p = 0.36). The Graafian follicles also showed no significant difference between the control (2.71 ± 1.89), separated (1.29 ± 1.49) and paired treatments (1.57 ± 2.30, χ^2 = 2.73, df = 3, p = 0.10, Fig. 1). The number of primary follicles was not affected by ovarian mass (χ^2 = 1.20, df = 1, p = 0.27). The same was found for secondary/tertiary follicles (χ^2 = 2.64, df = 1, p = 0.11) and Graafian follicles (χ^2 = 0.296, df = 1, p = 0.59). One and two corpora lutea were found in one female from the control treatment and one female from the paired treatment, respectively. No statistical analysis was done on the number of corpora lutea because of the small sample size. When compared between the different treatments, the Pg concentrations did not differ significantly between the control (0.85 ± 0.31µg/g dry weight), separated (0.87 ± 0.43µg/g dry weight) or paired treatment (0.88 ± 0.29µg/g dry weight, χ^2 = 0.12, df = 2, p = 0.92, Fig. 2). Collection day, however, did have a significant effect on the progesterone concentration of the different females (χ^2 = 389.77, df = 16 p < 0.01, Fig. 2). The interaction between collection day and treatment was significant (χ^2 = 1.6 x 10^{13}, df = 19 p = 0 < 0.01). Luteal activity was also identified in eight females from the separated and paired treatments with no luteal activity shown by the control group.

Penile morphology

Male *A. spinosissimus* have penile spines, mainly confined to the first half of the glans of the penis with the second half of the penis appearing more smooth (Fig. 3A and B, red arrows). They have an elongated structure on the ventral side of the penis reaching back from the tip (Fig. 3B and C, yellow arrows).

Discussion

Similar follicular growth was recorded in all *A. spinosissimus* individuals throughout the three treatments. The only corpora lutea of ovulation recorded was in a single female from the control treatment and in a single female from the paired treatment. The presence of the corpora lutea in the control female provides strong evidence that the species exhibits spontaneous ovulation. These findings are supported by the faecal progesterone profiles as there was no difference in progesterone concentrations between the different treatments, whether it was for the control treatment where there was no male contact, separated treatment where olfactory cues played a role or the paired treatment where the different sexes were able to mate.

The family Muridae is considered to be one of the largest mammalian families (Pfau *et al*. 1999) with many murids showing spontaneous ovulation (Voss 1979). It has been proposed that spontaneous ovulation is the most common pattern of ovulation found in the family Muridae (Bradley and Terman 1979). Laboratory rats and mice are spontaneous ovulators (Bronson *et al.* 1968); however, certain strains of mice will not ovulate unless coitus has taken place (Zarrow & Clark 1968). In a laboratory study done by Breed (1989) on three native Australian murids, *Notomys alexis*, *Pseudomys australis* and *P. nanus*, spontaneous ovulation was found in all three species. Many reproductive experiments are carried out on captive bred animals from established colonies (Peitz 1981; Dewsbury and Hodges 1987; Breed 1989; Frynta *et al.* 2011). Our study focussed on the ovulation of wild caught individuals and thus gives a more clear indication of how *Acomys spinosissimus* reproduces in the wild.

The body mass and the age of a female are important factors governing the reproductive process (Bronson 1998). Medger *et al.* (2010) showed that the breeding season for spiny mice coincides with the warm summer months with adequate rainfall events (September – January). In the current study, we collected females in May and July so as to avoid capturing juvenile and sub-adult individuals. The general time considered for female spiny mice to reach sexual

maturity is around 45 days (Peitz 1981). Based on these results, it is suffice to conclude that all the experimental females used in this study were adult. Peitz (1981) investigated the pattern of ovulation in *Acomys cahirinus* and found that females first showed vaginal openings around 45 days of age, however, the first litters were produced at an age of around 103 days. Only adult females were used for this study which ensured that if the females did not ovulate, it cannot be attributed to the fact that they were juveniles, thus, ensuring accurate findings.

Steinman (2012) found that females subjected to restricted diets on short day photoperiods showed reduced body mass as well as underdeveloped reproductive tracts, illustrating the importance of body mass and nutrition for females in order to reproduce. The mice were fed *ad libitum* and kept on a photoperiod of 14 hours light and 10 hours dark. The photoperiod simulated the South African summer and the high quality food represented the high food available during the summer months, when breeding occurs in the wild (Medger *et al.* 2010). The average body mass of the females from the different treatments was apportioned such that they did not differ significantly; ensuring that body mass did not affect the reproductive status of the females. In this study, it was shown that the physical presence of a male might enhance ovarian activity as is the case in the Namaqua rock mouse (*Micaelamys namaquensis*), a murid that occurs in the same habitat as *A. spinosissimus*, where the presence of a male significantly influenced ovarian size, structure and function (Relton 2011). Even though there were no differences in the progesterone profiles from females from different treatments, I was able to determine luteal activity in the separated and paired treatments.

Penile ornamentation is closely linked to the degree of sociality, along with ovulation pattern as can be seen in the African mole-rat species. The Cape mole-rat (*Georychus capensis*) is a solitary, highly xenophobic species showing induced ovulation (van Sandwyk and Bennett 2005) with noticeable spines on the shaft as well as the glans of the penis. These spines may assist the males in forming a lock during copulation. The common mole-rat (*Cryptomys hottentotus),* highveld mole-rat (*C. h. pretoriae*) and Natal mole-rat (*C. h. natalensis*) are three social mole-rat species that also exhibit induced ovulation (Malherbe *et al*. 2004; Jackson and

Bennett 2005). However, these species exhibit rounded, raised structures across the penile body rather than penile spines. Living in a colony presents a more stable environment and increases the chances of encountering a potential mate for copulation allowing for the less extreme penile ornamentation present in these species. The two eusocial species, Damaraland mole-rat (*Fukomys damarensis*) and naked mole-rat (*Heterocephalus glaber*) show absolutely no noticeable penile ornamentation but rather smooth ridges spanning the entire penile body. Spontaneous ovulation has been identified in both these species (Faulkes *et al.* 1990; Snyman *et al*. 2006) and might be a consequence of natal philopatry and little mating opportunity. There is thus no selective advantage to inducing ovulation and no need for elaborate penile ornamentation or modifications (Parag *et al*. 2006). Horner and Taylor (1968) investigated the reproductive behaviour and growth rate of spontaneous ovulating (Taylor 1968) southern grasshopper mice (*Onychomys torridus longicaudus*) and observed that after an extensive courtship ritual, several matings occurred that lasted from a few seconds to about one minute. The males have curved spines that are used as a locking mechanism during copulation, ensuring that the sperm from the male is the sperm that fertilises the female (Horner and Taylor 1968). *Acomys spinosissimus* lack extreme penile spines, but some degree of penile ornamentation was found, mostly confined to the head of the penis. The penile protrusions present might be too small to induce ovulation in the females, but rather act as a locking mechanism to ensure fertilisation by the male. This study did not specifically investigate the function of the penile spines, so future studies could shed more light on the exact need and use of the penile spines of spiny mice.

In conclusion, based on our findings where ovarian mass did not differ between females from the different treatments, normal follicular growth was recorded with corpora lutea present in the control treatment as well as in the paired treatment and the fact that progesterone profiles showed absolutely no difference whether a female had no contact with a male, only olfactory contact, or was able to mate with vasectomized males, we can sufficiently conclude that *Acomys spinosissimus* exhibits spontaneous ovulation.

Acknowledgements

This project was approved by the ethics committee of the University of Pretoria (EC008-11) and collection occurred under permits granted by the Departments of Nature Conservation in the Limpopo and Gauteng Provinces. The project was funded by the SARChI Chair for Mammal Behavioural Ecology and Physiology as well as the National Research Foundation to N.C. Bennett. P.R. de Bruin acknowledges a Master's bursary from the NRF. I would like to thank Dave Dewsnap from Goro Game Reserve for allowing us to work on the premises, Dr Dorianne Elliott from the Bird and Exotic animal hospital at Onderstepoort for conducting the vasectomies and Claire Relton for her assistance during the experimental period.

References

Bakker, J. and Baum, M.J. 2000. Neuroendocrine regulation of GnRH release in induced ovulators. *Frontiers in Neuroendocrinology*. **21,** 220 – 262.

Bennett, N.C. 1994. Reproductive suppression in social *Cryptomys damarensis* colonies: a lifetime of socially-induced sterility in males and females. *Journal of Zoology* (London). **234,** 25 – 39.

Bradley, E.L. and Terman, C.R. 1979. Ovulation in *Peromyscus maniculatus bairdi* under laboratory conditions. *Journal of Mammalogy*. **60,** 543 – 549.

Breed, W.G. 1989. Comparative studies on the reproductive biology of three species of

laboratory bred Australian conilurine rodents (Muridae: Hydromyinae). *Journal of Zoology*, London. **217,** 683 – 699.

Bronson, F.H. 1998. Energy balance and ovulation: small cages versus natural habitats. *Reproduction Fertility and Development*. **10,** 127 – 137.

Bronson, F.H., Dagg, C.P. and Snell, G.D. 1968. Reproduction. *Biology of the laboratory mouse*. (2nd Ed). Dover Publications, Inc, New York. **11,** 1 – 26.

Clulow, F.V. and Mallory, F.F. 1970. Oestrus and induced ovulation in the meadow vole, *Microtus pennsylvanicus*. *Journal for Reproduction and Fertility.* **23,** 341 – 343.

Conaway, C.H. 1971. Ecological adaptation and mammalian reproduction. *Biology of Reproduction*. **4,** 239-247.

Crighton, D.B., Haynes, N.B., Foxcroft, G.R. and Lamming, G.E. 1978. The feedback effects of ovarian steroids on gonadotropin secretion. *Control of Ovulation.* South Africa, Butterworth and Co (South Africa) (Pty) Ltd. **3,** 29 – 49.

Dewsbury, D.A. and Hodges, A.W. 1987. Copulatory behavior and related phenomena in spiny mice (*Acomys cahirinus*) and hopping mice (*Notomys alexis*). *Journal of Mammalogy*. **68,** 49 – 57.

Dixson, A.F. 1995. Baculum length and copulatory behavior in carnivores and pinnipeds (Grand Order Ferae). *Journal of Zoology* (London). **235,** 67 – 76.

Drury, R.A.B. and Wallington, E.A. 1967. Carleton's histological technique: Oxford University Press, London.

Faulkes, C.G., Abbott, D.H. and Jarvis, J.U.M. 1990. Social suppression of ovarion cyclicity in captive and wild colonies of naked mole-rats, *Heterocephalus glaber*. *Journal for Reproduction and Fertility*. **88,** 559 – 568.

Fleming, P.A. and Nicolson, S.W. 2002. Opportunistic breeding in the Cape spiny mouse (*Acomys subspinosus*). *African Zoology*. **37,** 101-105.

Frynta, D., Fraňková, M., Čížková, B., Skarlandtová, H., Galeštoková, K., Průšová, K., Šmilauer, P. and Šumbera, R. 2011. Social and life history correlates of litter size in captive colonies of precocial spiny mice (*Acomys*). *Acta Theriologica*. **56,** 289 – 295.

Grey, G.D., Davis, H.N., Zerylnick, M. and Dewsbury, D.A. 1974. Oestrus and induced ovulation in montane voles. *Journal for Reproduction and Fertility*. **38,** 193 – 196.

Haim, A. 1991. Behavior patterns of cold-resistant Golden spiny mouse *Acomys russatus*. *Physiology and Behaviour*. **50,** 641 - 643.

Hickman, C.P., Roberts, L.S., Larson, A., I'Anson, H. and Eisenhour, D.J. 2006. The reproductive process. *Integrated principles of Zoology* (13th Edition), New York, McGraw-Hill. **7,** 131 – 150.

Hilliard, J. 1973. Corpus luteum function in guinea pigs, hamsters, rats, mice and rabbits. *Biology of Reproduction*. **8,** 203-22.

Hoole, C., Oosthuizen, M.K., Chimimba, C.T. and Bennett, N.C. 2012. The locomotory activity rhythm of the spiny mouse, *Acomys spinosissimus* from southern Africa: light entrainment and endogenous circadian rhythms. *Journal of Zoology*, London. **288,** 93 – 102.

Horner, B.E. and Taylor, J.M. 1968. Growth and reproductive behavior in the southern grasshopper mouse. *Journal of Mammalogy*. **4,** 644 – 660.

Jackson, C.R. and Bennett, N.C. 2005. Is the natal mole-rat (*Cryptomys hottentotus natalensis*) a spontaneous or induced ovulator? *Journal of Mammalogy*. **86,** 1 – 6.

Kauffman, A.S. and Rissman, E.F. 2006. Neuroendocrine control of mating-induced ovulation. *Knobil and Neill's Physiology of Reproduction* (3rd Edition), Virginia, Elsevier. **42,** 2283 – 2318.

Larivière, S. and Ferguson, S.H. 2003. Evolution of induced ovulation in north american carnivores. *Journal of Mammalogy.* **84,** 937 – 947.

Malherbe, G.P., Schoeman, A.S. and Bennett, N.C. 2004. Is the Highveld mole-rat *Cryptomys hottentotus pretoriae* (Rodentia: Bathyergidae) an induced or spontaneous ovulator? *Journal of Zoology* (London). **263,** 159 – 165.

Medger, K., Chimimba, C.T. and Bennett, N.C. 2010. Seasonal reproduction in the female spiny mouse from South Africa. *Journal of Zoology* (London). **282,** 163-170.

Medger, K., Chimimba, C.T. and Bennett, N.C. 2012a. Seasonal changes in reproductive development in male spiny mice (*Acomys spinosissimus*) from South Africa. *Mammalian Biology*. **77,** 153 – 159.

Medger, K., Chimimba, C.T. and Bennett, N.C. 2012b. Reproductive photoresponsiveness in male spiny mice from South Africa. *Journal of Zoology* (London). **286,** 243 – 249.

Milligan, S.R. 1980. Pheromones and rodent reproductive physiology. *Symposia of the Zoological Society of London*. **45,** 251 – 275.

Mills, G. and Hes, L. 1997. The complete book of southern African mammals. Struik Publishing group (Pty) Ltd. Cape Town, South Africa.

Nováková, M., Palme, R., Kutalová, H., Janský, L. and Frynta, D. 2008. The effects of sex, age and commensal way of life on levels of fecal glucocorticoid metabolites in spiny mice (*Acomys cahirinus*). *Physiology and Behavior*. **95,** 187 – 193.

Parag, A., Bennett, N.C., Faulkes, G.C. and Bateman, P.W. 2006. Penile morphology of African mole rats (Bathyergidae): structural modification in relation to mode of ovulation and degree of sociality. *Journal of Zoology*. **270,** 323-329.

Peitz, B. 1981. The oestrous cycle of the spiny mouse (*Acomys cahirinus*). *Journal for Reproduction and Fertility*. **61,** 453 – 459.

Pfau, R.S., van den Bussche, R.A., McBee, K. and Lochmiller, R.L. 1999. Allelic diversity at the MHC-DQA locus in cotton rats (*Sigmodon hispidus*) and a comparison of DQA sequences within the family Muridae (Mammalia: Rodentia). *Immunogenetics*. **49,** 886 – 893.

Relton, C.E. 2011. The mode of ovulation in the Namaqua rock mouse, *Micaelamys namaquensis*. Honours thesis, University of Pretoria, Pretoria.

Shanas, U. and Haim, A. 2004. Diet salinity and vasopressin as reproduction modulators in the desert-dwelling golden spiny mouse (*Acomys russatus*). *Physiology and Behavior*. **81,** 645 – 650.

Shargal, E., Kronfeld-Schor, N. and Dayan, T. 2000. Population biology and spatial relationships of coexisting spiny mice (*Acomys*) in Israel. *Journal of Mammalogy*. **81,** 1046 – 1052.

Skinner, J.D. and Chimimba, C.T. 2005. The mammals of the southern African subregion (3rd edition). Cambridge: Cambridge University Press.

Snyman, P.C., Jackson, C.R. and Bennett, N.C. 2006. Do dispersing non-reproductive female Damaraland mole-rats, *Cryptomys damarensis* (Rodentia: Bathyergidae) exhibit spontaneous or induced ovulation? *Physiology and Behavior*. **87,** 88 – 94.

Steinman, M.Q., Knight, J.A. and Trainor, B.C. 2012. Effects of photoperiod and food restriction on the reproductive physiology of female California mice. *General and Comparative Endocrinology*. **176,** 391 – 399.

Taylor, J.M. 1968. Reproductive mechanisms of the female southern grasshopper mouse, *Onychomys torridus longicaudus*. *Journal of Mammalogy*. **49,** 303-309.

van Sandwyk, J.H. and Bennett, N.C. 2005. Do solitary, seismic signalling Cape mole-rats

(*Georychus capensis*) demonstrate spontaneous or induced ovulation? *Journal of Zoology*, London. **267**, 75–80.

Voss, R.S. 1979. Male accessory glands and the evolution of copulatory plugs in rodent. *Occasional Papers of the Museum of Zoology, University of Michigan.* **689,** 1 -27.

Weir, B.J. and Rowlands I.W. 1973. Reproductive strategies of mammals. *Annual Review of Ecology and Systematics.* **4,** 139 – 163.

Wube, T., Haim, A. and Fares, F. 2009. Effect of increased dietary salinity on the reproductive status and energy intake of xeric and mesic populations of the spiny mouse, *Acomys*. *Physiology and Behavior*. **96,** 122 – 127.

Zarrow, M.X. and Clark, J.H. 1968. Ovulation following vaginal stimulation in a spontaneous ovulator and its implications. *Journal of Endocrinology*. **40,** 343 – 352.

Zuckerman, P.L. and Weir, B.J. 1977a. The Ovary II, Physiology (2nd edition). New York: Academic Press, New York. **6,** 235 – 259.

Zuckerman, P.L. and Weir, B.J. 1977b. The Ovary III, Regulation of oogenesis and steroidogenesis (2nd edition). New York: Academic Press, New York. **4,** 227 – 267.

Figures

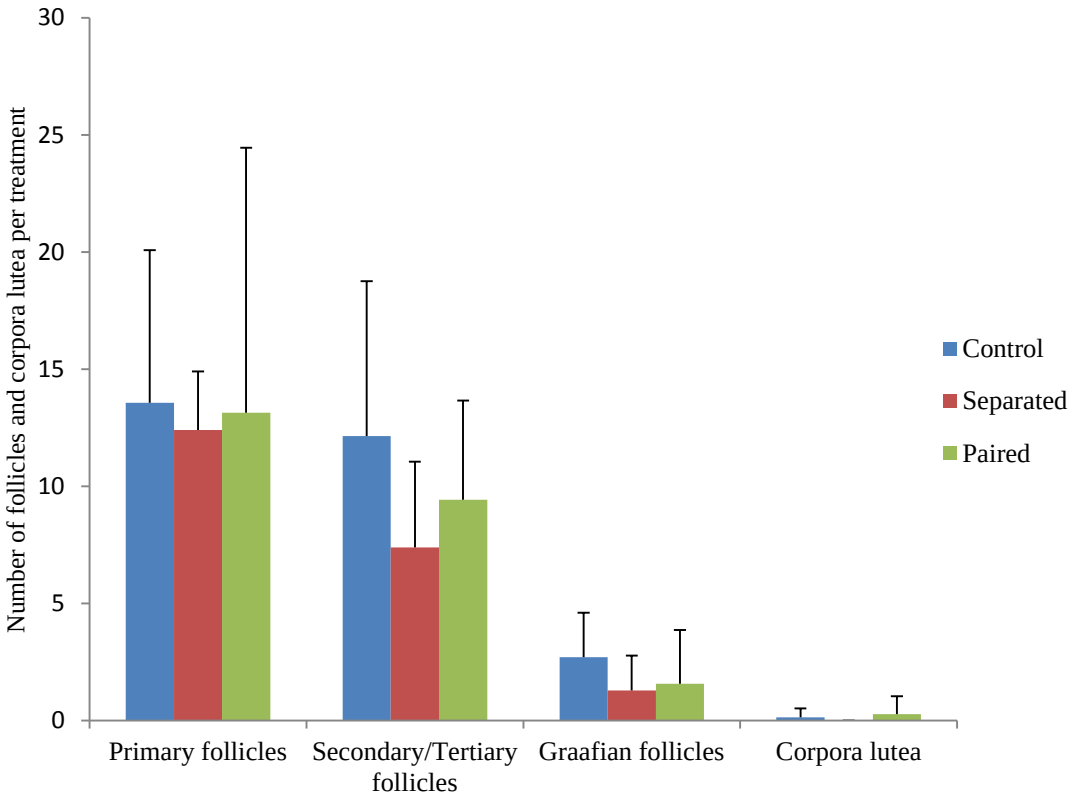


Figure 1. The mean ± SD number of primary, secondary/tertiary and Graafian follicles as well as corpora lutea per treatment for female spiny mice.

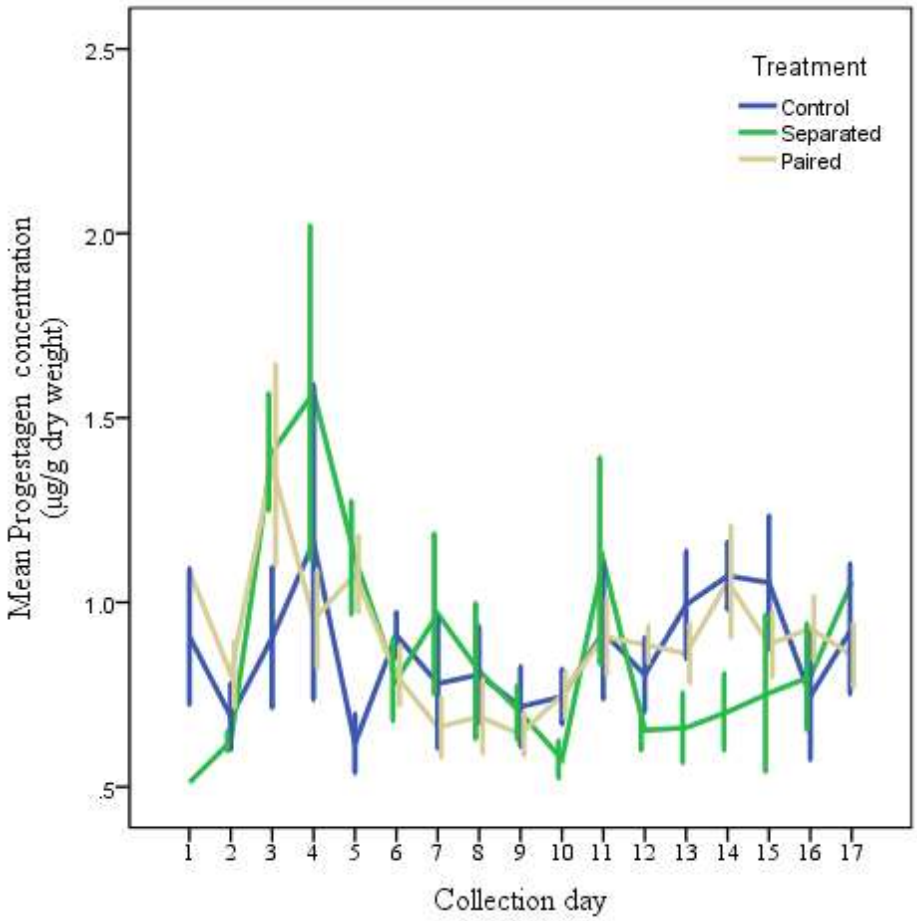


Figure 2. The mean ± SE progesterone concentration of female spiny mice for the different treatments over the experimental period.

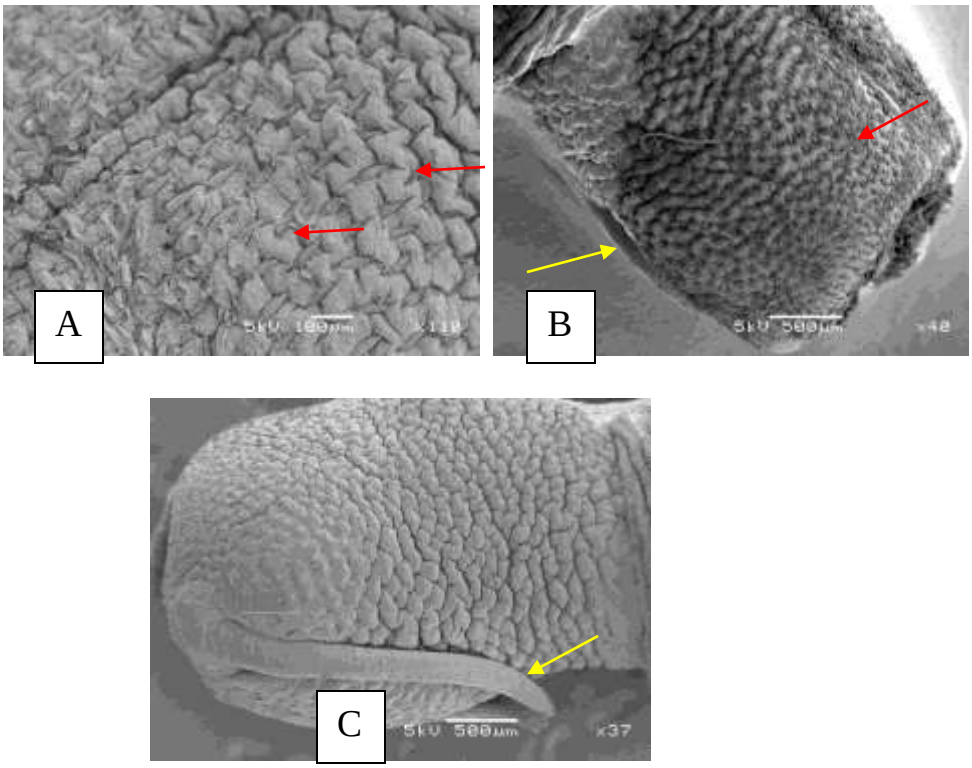


Figure 3. Scanning Electron Micrographs (A, B, C) showing the penile spines present on *Acomys spinosissimus*. The red arrows show small spines and the yellow arrow shows an elongated structure reaching from the tip of the penis backwards.

Chapter 3

The influence of photoperiod and food restriction on gonadal development in male spiny mice (*Acomys spinosissimus*) from South Africa

Abstract

Photoperiod is one of the most important signals governing the reproductive cycle in small mammal species. Factors such as food availability, rainfall and temperature might interact, enhancing the effect of photoperiod. Male spiny mice were exposed to either a long (14L:10D) or short photoperiod (10L:14D) simulating the South African summer and winter respectively. On each photoperiod, two groups of males were either fed *ad libitum* or a 10% restricted diet. The effect of each treatment was investigated by comparing the testicular volume, seminiferous tubule diameters and total body fat between each of the different treatments. The testosterone concentration of the four treatments was also compared using faecal samples. Males exposed to 14L:10D had significantly greater testicular volumes as well as larger seminiferous tubule diameters compared to males exposed to 10L:14D. The testosterone concentration of males on a long photoperiod was also higher than of the males exposed to the short photoperiod. *Acomys spinosissimus* males are strongly photoperiodic.

Key words: Photoperiod, Food restriction, Seasonal breeding, Testosterone

Introduction

It is well known that reproduction is energetically expensive (Hayward and Gillooly 2011) and due to this, most small mammal species time their reproduction in such a way that enough energy is available to support the lactating mother as well as the developing neonates (Tinney *et al*. 2001). As a result, many rodent species have evolved the ability to reproduce seasonally (Larivière and Ferguson 2003).

Photoperiod is the means by which animals measure the length of the day and set their reproductive processes (Beck 1963). Photoperiod is constant and it has been suggested that

photoperiod plays an important role in the timing of reproduction as many species use changes in daily photoperiod in order to time their reproductive activity (Gwinner *et al*. 1997; Edmonds *et al*. 2003; Muteka *et al*. 2006; Perfito and Bentley 2009; Tassino and Passos 2010; Greiner *et al*. 2011). Rodent species like *Peromyscus spp.* (Tinney *et al.* 2001), Syrian hamsters (*Mesocricetus auratus*) (Tamarkin *et al*. 1977) and Siberian hamsters (*Phodopus sungorus*) (Adam *et al.* 2000) have been used extensively in studies to investigate photoperiod in the laboratory and it has been shown that short photoperiods induce the regression of the reproductive organs and may even cause delayed reproductive development in juveniles (Nelson *et al.* 1997). Long photoperiods however, have the opposite effect, stimulating reproductive development in juveniles and bringing about reproductive recrudescence in adults (Reiter 1984). Non-tropical rodent species, such as deer mice (*Peromyscus maniculatus*) make use of short day lengths to inhibit reproduction (Tinney *et al.* 2001).

In the tropics, both photoperiodic and non-photoperiodic mammals have been identified (Rissman *et al.* 1987; Heideman and Bronson 1990). As in the tropics, species that are responsive to photoperiod and species that are not have been found in the sub-tropics. Male pouched mice (*Saccostomus campestris)* were exposed to either long or short day conditions for 14 weeks, after which reproductive development was measured (Bernard and Hall 1995). There was no difference in reproductive development between the two photoperiods and both long and short day males showed spermatogenic activity (Bernard and Hall 1995). During another experiment the oestrous cycle for the same species was determined on a short and long day photoperiod and it was concluded that photoperiod had no effect on the oestrous cycle or reproductive development of *S. campestris* (Bernard and Hall 1995). Male spiny mice from South Africa (*Acomys spinosissimus*) use photoperiod to regulate reproduction (Medger *et al.* 2012a). Males were exposed to long and short photoperiods and it was found that males exposed to long day conditions had increased testicular development as well as testosterone concentrations compared to the males exposed to short day conditions. The environment in which a species occurs is also a critical determinant of reproductive development (Shanas and Haim 2004). The xeric, golden spiny mouse (*A. russatus*) showed decreased spermatogenic activity on short photoperiods when compared to males on long photoperiods with females

exposed to long photoperiods having heavier uteri than females exposed to short photoperiods (Wube *et al.* 2008). In the same study, the common spiny mouse (*A. cahirinus*) represented a mesic species and it was found that both male and female *A. cahirinus* sustained gonadal activity when exposed to either long or short day photoperiods (Wube *et al.* 2008).

Bronson and Perrigo (1987) proposed that reproduction is not only controlled by photoperiod, but may be controlled by many other factors such as temperature, rainfall and food availability, with interactions between these factors influencing the reproduction of mammals. In the Nile grass rat (*Arvicanthis niloticus*), humid conditions along with low temperatures bring about reproductive recrudescence whereas dry conditions along with low temperatures bring about reproductive regression (Sicard *et al.* 1993). Nelson *et al.* (1983) investigated the effect of photoperiod along with the provisioning of food and water in California voles (*Microtus californicus*). Males exposed to both long and short photoperiods, but fed supplementary spinach, maintained their reproductive ability (Nelson *et al.* 1983), whereas males with restricted water access showed decreased reproductive activity (Nelson *et al.* 1983). Many studies have either focused on the effect of food restriction (Cameron and Speakman 2011; Zhao *et al.* 2011) or the effect of food restriction and photoperiod on the reproduction of mammals (Nelson *et al.* 1992; Young *et al.* 2000). The interaction between reduced food quality and/or quantity and photoperiod is most commonly responsible for suppressed reproductive function and reduced reproductive organ mass in both males and females (Edmonds *et al.* 2003; Steinmann *et al.* 2012). Nelson *et al.* (1997) exposed male *P. maniculatus* to different photoperiods but restricted the food intake of half the males on each of the photoperiods and concluded that photoperiod along with food restriction greatly affects the reproductive ability of the mice. Tinney *et al.* (2001) tested the effects that food restriction as well as low quality food has on the reproduction of male pouched mice from southern Africa. They found that both reduced food quantity and quality (high-fibre diet) brought about a decreased body mass, a reduction in body fat content and the mass of accessory glands as well as the epididymis (Tinney *et al.* 2001). In a study that provided supplementary food to *R. pumilio* in the wild, it was concluded that males that received supplementary feed had heavier testicles and epididymis than the males that did not receive supplementary feed (Jackson and Bernard 2005). During the same study, it was also noted that

females which received supplementary feed had thicker uterine walls, endometria as well as myometria than the females that had to survive on a normal winter diet. Jackson and Bernard (2005) concluded that supplying additional food in the field had a stimulatory effect on the reproductive system. Medger *et al*. (2010) suggested that an increase in food quantity and possibly quality with the onset of the rainy season are the main driving factors for seasonal reproduction in the spiny mouse (*Acomys spinosissimus*) from South Africa. In another study, the same authors established that photoperiod is important for the timing of reproduction in the spiny mouse (Medger *et al*. 2012a); however, it remains unclear what role food quantity plays.

The spiny mouse, *Acomys spinosissimus,* is a small terrestrial rodent distributed through the north-eastern parts of South Africa, Mozambique, Zimbabwe, Malawi and Tanzania (Skinner and Chimimba 2005). The spiny mouse is strictly nocturnal (Hoole *et al.* 2012) and occurs mainly in rocky areas which provide shelter. They can occur solitarily, in pairs or in family groups (Mills and Hes 1997). *Acomys spinosissimus* feeds on grass and seeds but also supplements its diet with insects (Mills and Hes 1997). The spiny mouse breeds during spring and summer (longer days) which coincides with the rainy season in its habitat and both sexes are reproductively regressed during the dry autumn and winter (shorter days) as photoperiod is the predictable environmental signal (Medger *et al.* 2010; Medger *et al.* 2012b). To compare the effects of photoperiod and food availability on the reproductive physiology of male *A. spinosissimus*, I investigated the change in body mass, the mass of fat deposits, the testicular histology as well as testosterone levels of males that received *ad libitum* food with that of males whose food was restricted and subjected the males in both food groups to a long or short photoperiod. Testosterone concentrations were observed throughout the experimental period to constantly monitor effects of food availability and photoperiod. To be able to do this in the least invasive manner and because of the small size of the study animals, faecal matter was used to measure the testosterone concentrations. Based on the findings of Medger *et al.* (2012a), I predict that a long photoperiod would stimulate testicular development of this South African rodent with food availability enhancing the effect of photoperiod.

Materials and Methods

Subjects and sample collection

Twenty-four male spiny mice (mean ± SD; 19.78 ± 3.50g, range: 14.73 – 27.29g) were collected during May and July 2011 from Goro Game Reserve (22°58'S, 22°59'S, 29°25'E, 29°24'E) in the Limpopo Province of South Africa using Sherman live traps (H. B. Sherman Traps, Inc. Tallahassee, Florida, U.S.A.). The traps were baited with a mixture of sardines, peanut butter and oats. Traps were set in the late afternoon and collected the next morning before sunrise to avoid overheating, dehydration and starvation of the captured individuals. Animals were sexed and placed into individual cages prepared with wood shavings for bedding and paper towel as housing. Water and mouse pellets were available *ad libitum* and apple, carrot and bird seed was provided as supplementary feed.

In the laboratory, the animals were kept in a temperature controlled room (25°C) with a short-day photoperiod of 10h light and 14h dark (10L:14D; SD) for a period of 40 days in order for complete gonadal regression to occur. This ensured that all males were at the same reproductive stage. Throughout the initial 40 days and the experimental period, males were only fed mouse pellets and water was provided *ad libitum.* The animals were weighed once a week for the entire 40 day period. During the last week, food intake for each individual was determined by subtracting the amount of mouse pellets left over from the initial amount that was given to the individual animal 24h prior. After this period, males were randomly assigned to one of four experimental treatments with six males assigned to each treatment. The males were either exposed to a SD photoperiod as during the initial 40 days or a long-day photoperiod of 14h light and 10h dark (14L:10D; LD). For each photoperiodic treatment, males were either fed mouse pellets *ad libitum* (not food restricted, NR) or food intake was restricted by 10% of that measured during the week before the onset of the experiments (food restricted, R). The experiment ran for eight weeks during which each male was weighed three times per week to monitor body mass. If

a R male had lost more than 20% of its body mass measured at the start of the experimental period, it was fed *ad libitum* until it had regained its initial mass (2 – 4 days). During the experimental period, faecal samples were collected every second day in order to determine testosterone concentrations. Faecal sampling was done every two hours over a six hour period. The animals were kept on paper towelling to ease faecal collection and to keep the stress to the animals to a minimum. Faecal samples were frozen at -30ºC immediately after collection and stored until testosterone analysis. At the end of the experiment, all males were sacrificed by exposing each individual to an overdose of halothane. Final body mass was determined. Body fat from fat pads between the shoulder blades, base of the back as well as lining of the gut was dissected out, combined and weighed after being dried in an oven for 5 days at 70ºC. Fat around the testes was also dissected out, dried and weighed as described above. Body fat and testicular fat was combined during the statistical analysis. The testes were dissected out and prepared for histological analysis.

Histological procedure

Length and width of the testes was measured to the nearest 0.01mm using a Vernier calliper (Mitutoyo American Corporation, Aurora, Illinois) and these measurements were used to determine the volume (mm^3) of the testes using the formula for an ellipsoid, $V = 4/3\pi ab^2$. The length and width of the gonads are represented by a and b, respectively. For histology, each sample was first dehydrated using a series of increasing concentrations of ethanol baths and then embedded in paraffin wax (Drury and Wallington 1967). A microtome (820 Spencer Microtome, American Optical) was used to cut the samples into 7µm sections. The sections were mounted on microscope slides treated with gelatine. Sections were stained with Ehrlich's haematoxylin and eosin. Testes sections were examined using a microscope with a 10X magnification. Photos of the testes were taken with a digital camera (Moticam 1000 1.3 M Pixel USB 2.0, Motic China Group, LTD., Xiamen, P.R. China) attached to the microscope and the diameters of 50 randomly selected seminiferous tubules were measured (µm) for each testes per male with Motic Images Plus 2.0 (Motic China Group, LTD., Xiamen, P.R. China).

Testosterone assay

The faecal samples collected during the experiment were used to compare the testosterone concentrations between the males from the different treatments. Samples were chosen as described in Chapter 1 of this thesis. Between five and seven samples were used per male according to the faecal matter available adding up to a total of 139 samples being analysed. One male, with less than five samples, were excluded from the analysis. Inter and intra Assay Coefficient of Variation (%) for epiandrosterone was 12.98 – 14.72% and 8.64 - 10.05%, respectively.

Statistical analysis

The statistical analysis was done using IBM SPSS 20 (IBM Corporation 1989, 2011). All variables were found to be normally distributed except final body mass and testosterone concentration. Levene's test was used to test for homogeneity and only total body fat was not homogenous. Total body fat was found to be homogenous after log-transformation. Final body mass and T concentration was found not to be normally distributed, even after a log-transformation was done. To compare testicular volume and seminiferous tubule diameter between the different treatments, Generalized Linear Models (GZLM) with linear distributions were performed and final body mass and testicular volume were used as covariates, respectively. Total body fat was also compared between the different treatments using GZLMs with a linear distribution and final body mass as covariate. Body mass and T concentrations were compared over the experimental period and between the four treatments using a Generalized Estimating Equation with Gamma-Log distribution with treatment and photoperiod as the factors. All the variables were tested for normality using a Kolmogorov-Smirnov tests and Tukey HSD was used for post-hoc analysis. The results are presented as mean ± standard deviation (SD).

Results

The testicular volume was significantly different between the four treatments (F_3 = 6.29, p < 0.01). The LDNR males had greater testicular volume than the LDR males (p = 0.02), with the testicular volume of the LDR males significantly larger than the SDR males (p < 0.01). The seminiferous tubule diameter did also differ significantly between the different treatments (F_3 = 8.48, p < 0.01, Fig. 1). The seminiferous tubule diameter of the LDR males differed significantly from that of the SDR males (p < 0.01). There was no significant difference between any of the four treatments regarding total body fat (F_3 = 2.47, p = 0.09). Testicular volume and final body mass were positively correlated (χ^2 = 12.27, df = 1, p < 0.01). The correlations between seminiferous tubule diameter and testicular volume (χ^2 = 2.15, df = 1, p = 0.14) and total body fat with final body mass (χ^2 < 0.01, df = 1, p = 0.97) were not significant. There was no significant difference in body mass at the beginning of the experiments between the four treatments (χ^2 = 0.83, df = 3, p = 0.84, Fig. 2). There was no significant difference in final body mass between LDNR and LDR (p = 0.12; Fig. 2), LDNR and SDNR (p = 0.20; Fig. 2) and LDR and SDR (p = 0.70; Fig. 2), however, the final body mass between SDNR and SDR differed significantly (p = 0.01; Fig. 2) with SDNR males weighing more than SDR. The mass of LDNR males did not differ significantly at the start of the experiment compared to the final body mass at the end of the experiment (p = 0.47; Fig. 2). The LDR males weighed significantly less at the end of the experimental period compared to the start of the experiment (p < 0.01; Fig. 2) as was the case with the SDR males (p = 0.04; Fig. 2). The SDNR males weighed significantly more at the end of the experimental period than at the start of the experiment (p < 0.01; Fig. 2).

The treatment (χ^2 = 18.24, df = 3, p < 0.01) as well as the days of samples collected (χ^2 = 21.80, df = 6, p < 0.01) had a significant effect on testosterone concentration of the males. The interaction between the four treatments and the days of faecal sample collection was not significant (χ^2 = 54.85, df = 27, p = 0.67). The LDNR males had significantly higher faecal

testosterone concentrations at the end of the experimental period than at the beginning ($p = 0.03$). There was no significant difference in faecal testosterone concentration at the end of the experiment than at the beginning for either LDR ($p = 0.370$), SDNR ($p = 0.07$) or SDR ($p = 0.07$) males. There was no significant difference in the faecal testosterone concentrations of LDNR males compared to LDR males at the end of the experiment ($p = 0.15$; Fig. 3), there was also no significant difference in testosterone concentrations of LDNR males compared to SDNR males at the end of the experiment ($p = 0.29$; Fig. 3). When the testosterone concentrations of the LDR males were compared to SDR males, there was no significant difference ($p = 0.37$; Fig. 3). The testosterone concentrations of the SDNR males did not differ significantly when compared to the SDR males at the end of the experiment ($p = 0.98$; Fig. 3).

Discussion

The annual photoperiodic cycle remains constant from year to year regardless of any other change, whereas all other environmental factors such as food availability, temperature and rainfall vary seasonally and annually and are not predictable (Knobil and Neill 1988). Therefore it is advantageous for the animals to time their reproduction to coincide with the photoperiod that is most favourable to maximise their reproductive success (Bronson 1985).

Acomys cahirinus is a desert dwelling rodent whose breeding season extends from February to October (El-Bakry *et al.* 1998). An investigation into the effect of photoperiod on the reproductive status of *A. cahirinus* found that long and short photoperiods did not affect the relative or absolute testicular mass of this species (El-Bakry *et al.* 1998). Medger *et al.* (2012a) used a photoperiod of 8L:16D and 16L:8D respectively and found that male *Acomys spinosissimus* exposed to short day conditions showed reduced reproductive development compared to males exposed to long day conditions. The males exposed to short photoperiods had smaller testicular volume and seminiferous tubule diameters when compared to the males exposed to long photoperiods (Medger *et al.* 2012a). Similarly, in this study, the testicular

volume and seminiferous tubule diameters did not differ between males exposed to different feeding regimes, but rather between males exposed to different photoperiods with males exposed to short photoperiods having smaller seminiferous tubule diameters compared to males on long photoperiods. Medger *et al.* (2010; 2012b) found that male spiny mice started sexual development in mid-winter, before the females, this could explain why food restriction did not affect testicular volume and seminiferous tubule diameter. Testicular development may take several weeks (Edmonds *et al.* 2003) and to be ready to mate when the females are ready, the males need to become sexually mature before the onset of the breeding season. This indicates that males may possibly tolerate mild food restriction in order to be sexually mature at the start of the breeding season.

Excess energy is stored in the form of fat once the body's demands have been met. These reserves can then be used to support reproductive functions (Knobil and Neill 1988). Two groups of male and female *Dipodillus dasyurus* and *Gerbillus pyramidum,* both desert species, were exposed to a 10L:14D and then a 14L:10D photoperiod and the effect on body composition and reproductive functions determined (El-Bakry *et al.* 1998). The authors found that there was no difference in the fat pad mass of the SD animals when compared to the LD animals (El-Bakry *et al.* 1998). Hamilton and Bronson (1985) found that even if male house mice showed retarded body growth because of a restricted diet, their reproductive development was similar to males that were not restricted and they were still able to mate with females and successfully produce offspring. In this study, the males on the restricted diets did show some body fat depositions even though the fat content in these males was much lower than in the non-restricted males. The same was found for testicular fat and total fat with the restricted males having less testicular and total fat than the non-restricted males under both photoperiod conditions. There appears to be a conundrum here since although there was no difference between the amounts of fat mass of the different males, even though the LDR and SDR males exhibited a decreased body mass at the end of the experiment. This implies that, spiny mice may allocate some fat reserves to be used for reproduction or other functions if necessary as shown for house mice (Hamilton and Bronson 1985).

Body mass is important to initiate and sustain reproductive functions (Wootton 1987). Animals must satisfy the energy requirements for cellular maintenance, thermoregulation and locomotion, before energy can be used for reproduction (Knobil and Neill 1988). Hamilton and Bronson (1985) investigated the effect of food restriction on sexual development in wild house mice and found that females subjected to a restricted diet lost 4% of their body mass on average and did not show reproductive development compared to the females with *ad libitum* food availability, who gained 32% body mass and underwent normal development of the reproductive tract. Although males subjected to food restriction did not lose mass, they did show retarded body growth and most were still able to fertilise a female successfully (Hamilton and Bronson 1985). In the present study, the body mass of the males was similar for all treatments at the start of the experimental period. This assured that all the males started the experiment in a similar condition. Therefore any variation in testes volume may be attributed to the effect of photoperiod and/or food restriction.

The LDNR males were more active than any of the other treatments in the mornings when they were fed (P.R. de Bruin, personal observation). All the non-restricted males cached food and as a result of the increased activity, more energy was used, thereby limiting the amount of excess calories stored as fat. This may explain why the starting body mass did not differ from the final body mass of LDNR males.

Food restriction limits the amount of energy that can be consumed by an animal. In order to ensure that there is energy available for critical body functions, animals conserve energy by reducing activity (Hambly and Speakman 2005; Cameron and Speakman 2011). In Swiss mice, animals subjected to restricted diets showed lower activity and increased resting behaviour when compared to those animals whose diets was not restricted in order to compensate for the lower energy intake (Zhao *et al.* 2011). Cameron and Speakman (2011) subjected female mice to either a restricted diet or a diet of increased cellulose and found that females on restricted diets as well as females on diets with high cellulose levels showed a significant decrease in activity in order to conserve energy when compared to the control females who were fed *ad libitum*. Hambly and

Speakman (2005) found that the reduction in activity was responsible for 75% of the total energy saved when mice were exposed to limited food availability. The final body masses of LDR and SDR males were lower at the end of the experiment than at the beginning probably due to the restricted energy intake. When fed, some individuals ate the food *in situ* where it was placed, whereas others cached the food.

As with *Rhabdomys pumilio* where the males exposed to a short day photoperiod had higher body mass at the end of the experimental period when compared to the starting body mass (Jackson and Bernard 1999), SDNR males had a higher body mass at the end of the experiment than at the start. Because food availability is variable during the winter months (Knobil and Neill 1988), the animals might eat more when food is available in order to compensate for the times when food is scarce, explaining the increase in body mass seen in the SDNR males. Hoole *et al.* (2012) found that total activity counts of spiny mice decreased with the time spent in a laboratory, therefore lab conditions may cause the animals to become less active, resulting in an increased body mass; however, there was no increase in body mass in LDNR males.

Nelson *et al.* (1997) found that short day photoperiods combined with food restriction resulted in a decreased final body mass in *Peromyscus maniculatus*. At the end of the experiment, the body masses of the LDNR and SDNR males were higher than those of the LDR and SDR males. The *ad libitum* fed males had more food, and thus more energy, available to them allowing excess energy to be used for testicular development or storage, a resource the restricted males did not have. The LDR and SDR males had limited energy to use for all the necessary processes resulting in a decrease in body mass.

Testosterone is the primary androgen responsible for spermatogenesis (Austin and Short 1979). Testosterone, together with follicle stimulating hormone, interacts with the Sertoli cells in the testes to regulate the formation of sperm (Austin and Short 1979). *Oryzomys palustris* were exposed to either 16L:8D or 14L:10D photoperiods with a 25% food restriction at each

photoperiod (Edmonds *et al.* 2003). Edmonds *et al.* (2003) found no significant difference in the testosterone concentrations of the males on the restricted diet compared to non-restricted males on either photoperiod. In another study, Young *et al.* (2000) found that on a long photoperiod, the testosterone concentration did not change in non-restricted males however, in non-restricted males on a short photoperiod, the testosterone concentration decreased after six weeks with restricted males having an overall lower testosterone concentration than non-restricted males. Short photoperiod reduced the testosterone levels in *ad libitum* and restricted males, but food restriction interacted with short photoperiod and caused a significantly reduced testosterone concentration at four weeks compared to long day restricted males (Young *et al.* 2000). Male spiny mice exposed to 16L:8D had increased testosterone level when compared to males subjected to 8L:16D (Medger *et al.* 2012a). The findings of this study under a more natural photoperiod was in accordance with Medger *et al.* (2012a), with males subjected to a long photoperiod having increased testosterone levels compared to males on short photoperiods. In contrast, food availability did not affect the testosterone concentration of the males. The increased testosterone concentrations of males on long photoperiods clearly indicate that spiny mice males use photoperiod as the timing mechanism in order to synchronise their reproduction with the more favourable time of the year when food will be readily available. The increased testosterone levels might also result in high levels of spermatozoa present in the testes assuring fertilisation if a female is encountered.

The effect of food restriction on females depends on which stage of the cycle the animal was experiencing the reduced food availability (McClure 1967). Female mice experiencing food deprivation over a 48 hour period either delayed their ovulation by several days or failed to ovulate completely depending at which stage of oestrus the depravation occurred (Bronson and Marsteller 1985). The same authors found that females which experience food shortage soon after giving birth are more likely to eat their young in order to compensate for the low energy availability (Bronson and Marsteller 1985). I predict that female spiny mice will not ovulate or simply delay their ovulation depending on the degree of food shortages. However, litter size was controlled for all the females during the study by Bronson and Marsteller (1985) with females only allowed eight pups. Spiny mice are smaller and the litters range from two to five pups

(average three) (Mills and Hess 1997) so the lactational stress experienced by the mother is unknown. Lactational stress should be considered in relation to litter size as well as the body size of the species, but I predict that female spiny mice could resort to cannibalism if litters reach a certain threshold.

In most habitats, food is scarce during the winter (Knobil and Neill 1988) and the 10% food restriction used during this study may simulate food availability in the natural habitat during the winter months. Future studies could investigate the effect of a greater food restriction on the testicular volume, seminiferous tubule diameters and testosterone concentrations of male spiny mice. In females, it would be interesting to investigate the effect of photoperiod and food restriction on the ovulation cycle as well as pregnancy, in order to get a better understanding of how this South African rodent behaves in the wild. It would also be interesting to see whether females actually allow mating to occur during periods of food shortage and how the shortage of food affected pup survival.

In conclusion, males exposed to a 14L:10D photoperiod, simulating the South African summer, had both higher testicular volumes and greater seminiferous tubule diameters when compared with the males exposed to an artificial winter photoperiod, 10L:14D. Males exposed to a long photoperiod also had higher circulating testosterone concentrations than those males exposed to a short photoperiod. The feeding regime, either *ad libitum* or a 10% restriction, did not have any effect on any of the parameters investigated. Although the present study supports the findings of Medger *et al.* (2012a), the photoperiod used in this instance is more comparable to the natural photoperiod experienced by spiny mice in the wild thus giving a more accurate result of how spiny mice would react in their natural environment. The findings from the present study as well as those by Medger *et al.* (2010; 2012a,b) imply that *Acomys spinosissimus* is a highly photoperiodic species with photoperiod being the main driving force behind the seasonal reproduction of males of this species.

Acknowledgements

This project was approved by the ethics committee of the University of Pretoria (EC008-11) and permits were granted by the Department of Nature Conservation in Limpopo and Gauteng. The SARChI Chair of Mammal Behavioural Ecology and Physiology (to NC Bennett) funded the research and P.R. de Bruin acknowledges a bursary from the National Research Foundation. I would like to thank Dave Dewsnap from Goro Game Reserve for allowing us to work on the premises. I would also like to thank Claire Relton and Gerhard Göldner for their assistance during the research period.

References

Adam, C.L., Moar, K.M., Logie, T.J., Ross, A.W., Barrett, P., Morgan, P.J. and Mercer, J.G. 2000. Photoperiod regulates growth, puberty and hypothalamic neuropeptide and receptor gene expression in female Siberian hamsters. *Endocrinology*. **141,** 4349 – 4356.

Austin, C.R. and Short, R.V. 1979. The androgens. *Mechanisms of Hormone action, Reproduction in Mammals*. University Press, Cambridge, Great Brittain. **4,** 117 – 156.

Beck, S.D. 1963. Photoperiodism in Mammals. *Animal Photoperiodism* (1st Edition). Holt, Rinehart and Winston, Inc., United States of America, New York. **2,** 17 – 37.

Bernard, R.T.F. and Hall, J. 1995. Failure of the estrous cycle and spermatogenesis to respond to day length in a subtropical African rodent, the pouched mouse (*Saccostomus campestris*). *Biology of Reproduction.* **52,** 1291 – 1295.

Bronson, F.H. 1985. Mammalian reproduction, an ecological perspective. *Biology of Reproduction*. **32,** 1 – 26.

Bronson, F.H. and Marsteller, F.A. 1985. Effect of short-term food deprivation on reproduction in female mice. *Biology of Reproduction*. **33,** 660 – 667.

Bronson, F.H. and Perrigo, G. 1987. Seasonal regulation of reproduction in Muroid rodents. *American Zoologist*. **27,** 929 – 940.

Cameron, K.M. and Speakman, J.R. 2011. Reduction of dietary energy density reduces body mass regain following energy restriction in female mice. *The Journal of Nutrition*. **141,** 182 – 188.

Drury, R.A.B. and Wallington, E.A. 1967. Carleton's histological technique: Oxford University Press, London.

Edmonds, K.E., Riggs, L. and Stetson, M.H. 2003. Food availability and photoperiod affect reproductive development and maintenance in the marsh rice rat (*Oryzomys palustris*). *Physiology and Behavior*. **78,** 41 – 49.

El-Bakry, H.A., Zahran, W.M. and Bartness, T.J. 1998. Photoperiodic responses of four wild-trapped desert rodent species. *American Journal of Physiology*. **275,** 2012 – 2022.

Greiner, S., Schwarzenberger, F. and Voigt, C.C. 2011. Predictable timing of oestrus in the tropical bat *Saccopteryx bilineata* living in a Costa Rican rain forest. *Journal of Tropical Ecology*. **27,** 121-131.

Gwinner, E., Hau, M. and Heigl, S. 1997. Melatonin: Generation and modulation of avian circadian rhythms. *Brain Research Bulletin.* **4,** 439-444.

Hambly, C. and Speakman, J.R. 2005. Contribution of different mechanisms to compensation for energy restriction in the mouse. *Obesity Research*. **13,** 1548- 1557.

Hamilton, G.D. and Bronson, F.H. 1985. Food restriction and reproductive development in wild house mice. *Biology of Reproduction*. **32,** 773 – 778.

Hayward, A. and Gillooly, J.F. 2011. The cost of sex: Quantifying energetic investment in gamete production by males and females. *Plos ONE.* **6,** 1-4.

Heideman, P.D. and Bronson, F.H. 1990. Photoperiod, melatonin secretion and sexual maturation in a tropical rodent. *Biology of Reproduction*. **43,** 745 – 750.

Hoole, C., Oosthuizen, M.K., Chimimba, C.T. and Bennett, N.C. 2012. The locomotory activity rhythm of the spiny mouse, *Acomys spinosissimus* from southern Africa: light entrainment and endogenous circadian rhythms. *Journal of Zoology,* London. **288,** 93 – 102.

Jackson, C. and Bernard, R.T.F. 1999. Short day length alone does not inhibit spermatogenesis in the seasonally breeding four-striped field mouse (*Rhabdomys pumilio*). *Biology of Reproduction*. **60,** 1320 – 1323.

Jackson, C. and Bernard, R.T.F. 2005. Effects of supplementary food on winter inhibition of reproduction in male and female four-striped field mice (*Rhabdomys pumilio*). *Reproduction, Fertility and Development*. **17,** 393 – 400.

Knobil, E. and Neill, J.D. 1988. Seasonal regulation of reproduction in mammals. *The Physiology of Reproduction.* Raven Press, Ltd, New York, New York. **43,** 1831 – 1871.

Lariviere, S. and Ferguson, S.H. 2003. Evolution of induced ovulation in North American carnivores. *Journal of Mammalogy*. **84,** 937-947.

McClure, T.J. 1967. Infertility in mice caused by fasting at about the time of mating. *Journal for Reproduction and Fertility.* **13,** 393 – 403.

Medger, K., Chimimba, C.T. and Bennett, N.C. 2010. Seasonal reproduction in the male and female spiny mouse (*Acomys spinosissimus*) from South Africa. *Journal of Zoology* (London). **282,** 163-170.

Medger, K., Chimimba, C.T. and Bennett, N.C. 2012a. Reproductive photoresponsiveness in male spiny mice from South Africa. *Journal of Zoology*, London. **286,** 243 - 249.

Medger, K., Chimimba, C.T. and Bennett, N.C. 2012b. Seasonal changes in reproductive development in male spiny mice (*Acomys spinosissimus*) from South Africa. *Mammalian Biology*. **77,** 153 – 159.

Mills, G. and Hes, L. 1997. The complete book of southern African mammals. Struik Publishing group (Pty) Ltd. Cape Town, South Africa.

Muteka, S.P., Chimimba, C.T. and Bennett, N.C. 2006. Reproductive photoresponsiveness in *Aethomys ineptus* from southern Africa. *Journal of Zoology.* **268,** 225 – 231.

Nelson, R.J., Dark, J. and Zucker, I. 1983. Influence of photoperiod, nutrition and water availability on the reproduction of male California voles (*Microtus californicus*). *Journal of Reproduction and Fertility.* **69,** 473 – 477.

Nelson, R.J., Kita, M., Blom, J.M.C. and Rhyne-Grey, J. 1992. Photoperiod influences the critical caloric intake necessary to maintain reproduction among male deer mice (*Peromyscus maniculatus*). *Biology of Reproduction.* **46,** 226 – 232.

Nelson, R.J., Marinovic, A.C., Moffatt, C.A., Kriegsfeld, L.J. and Kim, S. 1997. The effect of photoperiod and food intake on reproductive development in male deer mice (*Peromyscus maniculatus*). *Physiology and Behavior.* **62,** 945 – 950.

Perfito, N. and Bentley, G.E. 2009. Opportunism, photoperiodism and puberty: Different mechanisms or variations on a theme? *Integrative and Comparative Biology*. **49,** 538-549.

Reiter, R.J. 1984. The pineal gland. Raven Press, New York.

Rissman, E.F., Nelson, R.J., Blank, J.L. and Bronson, F.H. 1987. Reproductive response of a tropical mammal, the mush shrew (*Suncus murinus*), to photoperiod. *Journal of Reproduction and Fertility*. **81,** 563 – 566.

Shanas, U. and Haim, A. 2004. Diet salinity and vasopressin as reproduction modulators in the desert-dwelling golden spiny mouse (*Acomys russatus*). *Physiology and Behavior*. **81,** 645 – 650.

Sicard, B., Fuminier, F., Maurel, D. and Boissin, J. 1993. Temperature and water conditions mediate the effects of day length on the breeding cycle of a Sahelian rodent, *Arvicanthis niloticus*. *Biology of Reproduction.* **49,** 716 – 722.

Skinner, J.D. and Chimimba, C.T. 2005. The mammals of the southern African subregion (3rd edition). Cambridge: Cambridge University Press.

Steinman, M.Q., Knight, J.A. and Trainor, B.C. 2012. Effects of photoperiod and food restriction on the reproductive physiology of female California mice. *General and Comparative Endocrinology*. **176**, 391–399.

Tamarkin, L., Hollister, C.W., Lefebvre, N.G. and Goldman, B.D. 1977. Melatonin induction of gonadal quiescence in pinealectomized Syrian hamsters. *Science*. **198,** 953 – 955.

Tassino, B. and Passos, C.A. 2010. Reproductive biology of Rio Negro tuco-tuco, *Ctenomys rionegrensis* (Rodentia: Octodontidae). *Mammalian Biology*. **75,** 253-260.

Tinney, G.M., Bernard, R.T.F. and White, R.M. 2001. Influences of food quality and quantity on the male reproductive organs of a seasonally breeding rodent, the pouched mouse (*Saccostomus campestris*), from a seasonal but unpredictable environment. *African Zoology*. **36,** 23 – 30.

Wootton, J.T. 1987. The effects of body mass, phylogeny, habitat and tropic level on mammalian age at first reproduction. *Evolution*. **41,** 732 – 749.

Wube, T., Haim, A. and Fares, F. 2008. Reproductive response of xeric and mesic populations of the spiny mouse *Acomys* to photoperiod acclimation. *Journal of Arid Environments.* **72,** 440 – 447.

Young, K.A., Zirkin, B.R. and Nelson, R.J. 2000. Testicular regression in response to food restriction and short photoperiod in white-footed mice (*Peromyscus leucopus*) is mediated by apoptosis. *Biology of Reproduction.* **62,** 347 – 354.

Zhao, Z,. Li, J., Zhang, H., Yu, R. and Zhao, Y. 2011. Body mass and behaviour in Swiss mice subjected to continuous or discontinuous food restriction and refeeding. *Acta Theriologica.* **56,** 129 – 139.

Figures

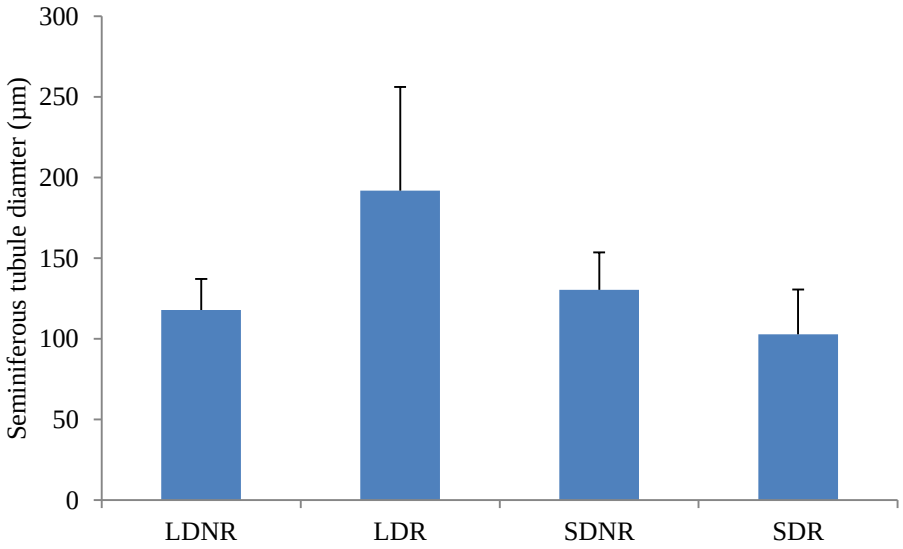


Figure 1. The mean ± SD seminiferous tubule diameter (µm) between long day not-restricted males (LDNR), long day restricted males (LDR), short day not-restricted males (SDNR) and short day restricted males (SDR).

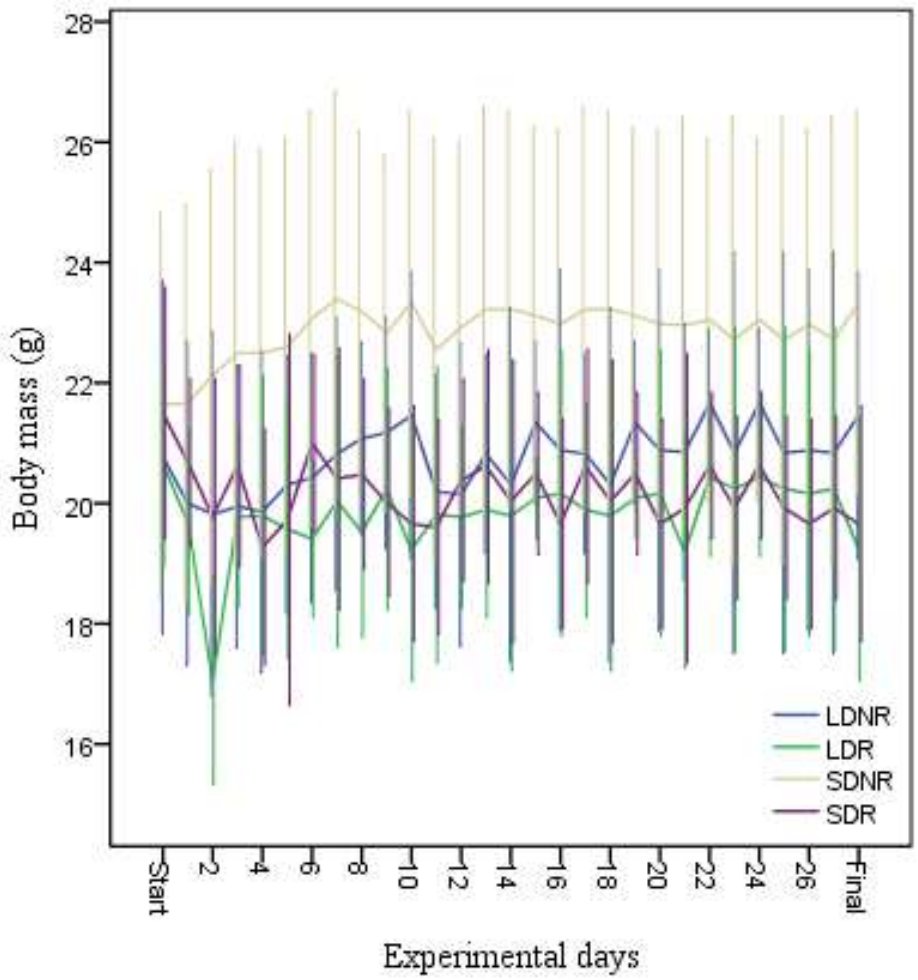


Figure 2. Mean body mass (g) ± SD of male *Acomys spinosissimus* measured every second day for eight weeks. Males were subjected to four treatments consisting of either long-day (LD) or short-day (SD) photoperiods and food restriction (R) or no restriction (NR).

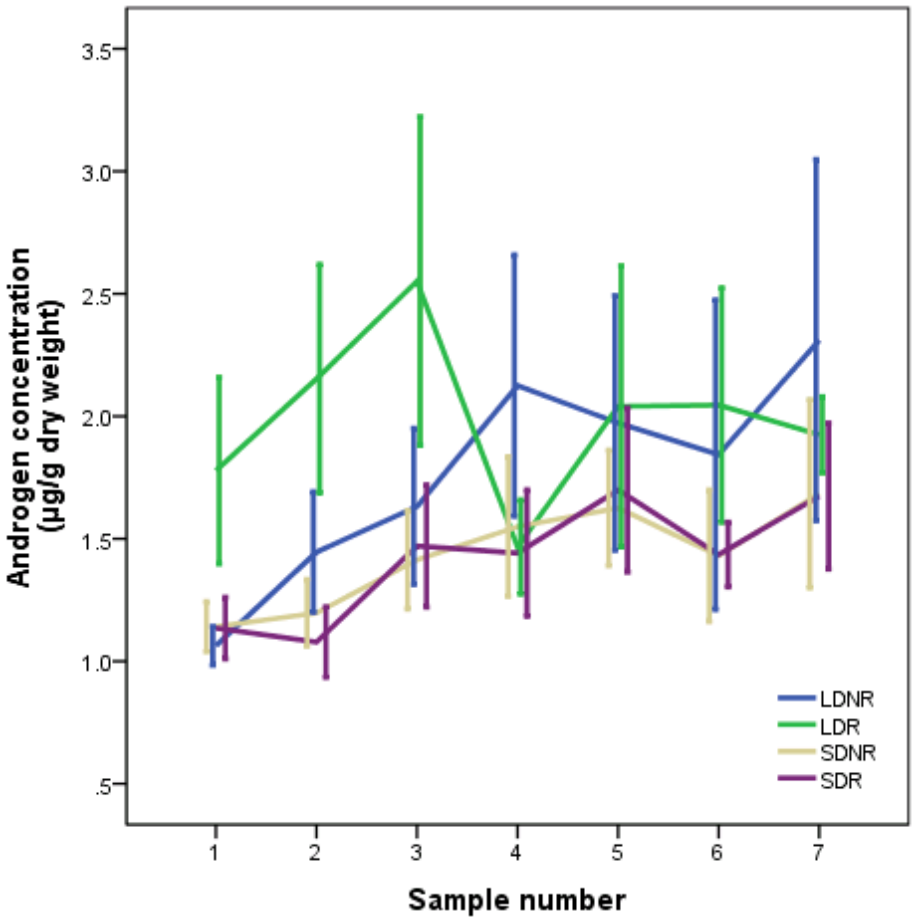


Figure 3. The mean testosterone concentration (µg/g dry weight) ± SE over the eight week experimental period for long day not-restricted males (LDNR), long day restricted males (LDR), short day not-restricted males (SDNR) and short day restricted males (SDR).

General Conclusion

Mammalian reproduction is a complicated process that has to coincide with the various environmental factors that can sustain and support the growth and development of the new generation (Bronson 1985). Seasonal variation in energy and nutrient availability is important in most environments where small mammals are dependent on predictors such as photoperiod, plant compounds or food availability which play an important role in the metabolic preparation for reproduction (MacArthur 1972; Bronson 1985). The Muridae rodents account for 25 % of all extant mammals on earth with the majority of this family exhibiting spontaneous ovulation (Voss 1979; Martin *et al.* 2001).

Until recently, there have not been many studies which have focused on the reproductive processes in the spiny mouse, *Acomys spinosissimus* (Skinner & Chimimba 2005). Medger *et al.* (2010, 2012a) has shown that the spiny mouse is a strict seasonal breeder with breeding taking place in the summer months of the southern hemisphere. Medger *et al.* (2012b) also demonstrated that reproduction in *A. spinosissimus* is under photoperiodic control with long photoperiods resulting in increased testicular activity in the males and a short photoperiod resulting in the regression of testicular activity. With my thesis, I attempted to add to the growing literature by investigating the pattern of ovulation in the female and how food restriction in combination with different photoperiods affects testicular development in the male using well established methods. It is known that doing continuous hormone measurements in small mammals is difficult as the bleeding of animals is invasive and most small mammals like mice and hamsters do not have enough blood to make this possible (Schwarzenberger *et al.* 1996; Chelini *et al.* 2005). In order to monitor the reproductive hormone levels in spiny mice during a specific experimental period, I validated a non-invasive technique using faecal progesterone and testosterone metabolites (Chapter 1). This novel method proved to work well in distinguishing between the hormone levels of the breeding and non-breeding animals and to monitor the hormone levels throughout the entire experimental period (Chapters 2 and 3). Even though I could detect a clear difference in progesterone concentration between the pregnant and non-pregnant females, I only had samples from one pregnant female during the last stage of pregnancy with no post-pregnancy samples. Improvements to this method might be made by monitoring multiple females throughout an entire pregnancy and continued sample collection post parturition after which these samples can be compared to the hormone levels of non-

pregnant females. A minimum sample mass is needed for accurate measurements, so future studies might collect samples more frequently during the experimental period in order to obtain the maximum sample mass needed. This method can be used to monitor reproductive hormone concentrations continuously in future studies on *Acomys spinosissimus*, decreasing the number of animals that has to be put down during a specific study with animals acting as their own controls. This method can also act as a guideline to validate similar techniques for other species that can be used in the lab and in the natural environment.

In the present study it was found that female spiny mice exhibit spontaneous ovulation (Chapter 2). All females in three treatments showed normal follicular development with one female from the control treatment (completely separated from any male stimuli) and one female from the paired treatment (full contact with a vasectomised male) exhibiting corpora lutea of ovulation in their ovaries at the time of histological analysis (Chapter 2). Based on the faecal progesterone results an oestrous cycle for *Acomys spinosissimus* was estimated at 17 ± 1 days (Chapter 1). Spontaneous ovulation would be of particular advantage if fertilisation did not occur during the first mating, making it possible for the female to return to oestrous in an attempt to mate again (Conaway 1971). These findings are in accordance with those of Peitz (1981) who investigated the pattern of ovulation in *Acomys cahirinus*. The overriding evidence suggests that *Acomys cahirinus* is a spontaneous ovulator (Peitz 1981).

In the males, gonadal activity was found to be under photoperiodic control with the feeding regimen having no effect on the testicular volume, seminiferous tubule diameter, fat deposition or testosterone concentration (Chapter 3). In accordance with Medger *et al.* (2012b), *A. spinosissimus* males exposed to a long day photoperiod showed significantly greater reproductive development and higher testosterone concentrations than the males exposed to a short photoperiod simulating winter in the southern hemisphere (Chapter 3). None of the males subjected to LDR or SDR showed any effect on the reproductive parameters measured when compared to the males that were fed *ad libitum* (LDNR and SDNR) on the same photoperiod (Chapter 3). Similar experiments were done on male deer mice (*Peromyscus maniculatus*) after which it was concluded that photoperiod along with feeding regimen had a significant effect on reproductive development of this species (Nelson *et al.* 1997). Contradictory to my study, males

on a short photoperiod and limited food availability did not show any reproductive development and also did not reach reproductive maturity (Nelson *et al.* 1997). In a study on marsh rice rats (*Oryzomys palustris*), food restriction and photoperiod only affected final body mass on a 16L:8D photoperiod with all the reproductive parameters measured being indistinguishable between the experimental and control groups (Edmonds *et al.* 2003). On a 12L:12D photoperiod however, food restriction caused a reduction in all the reproductive parameters measured in the experimental group compared to the control group (Edmonds *et al.* 2003).

There is very little known about the ecology and behaviour of the spiny mouse and the findings from this study could be used to get a better understanding of this species. In captivity, spiny mice seem to be social creatures even though this has not explicitly been investigated. Being spontaneous ovulators might support this hypothesis as it enhances the chances of a male being able to assess the reproductive state of a female ensuring copulation when ovulation has occurred. Food restriction might not be the most important ultimate factor as previously thought, but other factors such as temperature and rainfall/humidity might play a more important role in the ecology and reproduction of the spiny mouse. Further studies are needed however to support these statements.

Future research that could be conducted on spiny mice includes investigating the critical day length that induces reproductive activity. This could be conducted by exposing groups of spiny mice to increasing day lengths to find out which day length brings about photo-stimulation. It would be very interesting, in light of the findings of the males, to vary the provisioned food amounts available to females and recording the effect on the development of the reproductive tract and how this might influence ovulation, conception and litter mass and number of pups born. In the males, exposing the animals to a greater food restriction may have a greater effect on the reproductive development or including temperature as a third factor to investigate the effect on the male reproductive development. It would also be interesting to investigate the effect of photoperiod in combination with temperature on the development of the reproductive tract of both male and female spiny mice from the Limpopo province seeing that the increase in photoperiod coincides with increased temperatures and it is possible that temperature may act as an important proximate factor in the reproductive development of spiny mice in this area. Studies

could also focus on the effect of these seasonal cues outside of the lab in the natural environment of this species, possible giving a better representation of how this species reacts to natural changes. Determining the life span of spiny mice in the wild would also prove valuable in interpreting future findings.

References

Bronson, F.H. 1985. Mammalian reproduction: An ecological perspective. *Biology of Reproduction*. **32,** 1 – 26.

Chelini, M.O.M., Souza, N.L., Rocha, A.M., Felippe, E.C.G. and Oliveira, C.A. 2005. Quantification of fecal estradiol and progesterone metabolites in Syrian hamsters (*Mesocricetus auratus*). *Brazilian Journal of Medical and Biological Research*. **38,** 1711 - 1717.

Conaway, C.H. 1971. Ecological adaptation and mammalian reproduction. *Biology of Reproduction*. **4,** 239 – 247.

Edmonds, K.E., Riggs, L. and Stetson, M.H. 2003. Food availability and photoperiod affect reproductive development and maintenance in the marsh rice rat (*Oryzomys palustris*). *Physiology and Behavior*. **78,** 41 – 49.

MacArthur, R.H. 1972. Geographical ecology: Patterns in the distribution of species. Harper and Row, New York.

Martin, R.E., Pine, R.H. and DeBlase, A.F. 2001. A manual of mammalogy: with keys to families of the world. 3rd Edition. McGraw-Hill Book Company, Boston.

Medger, K., Chimimba, C.T. and Bennett, N.C. 2010. Seasonal reproduction in the female spiny mouse from South Africa. *Journal of Zoology* (London). **282,** 163 – 170.

Medger, K., Chimimba, C.T. and Bennett, N.C. 2012a. Seasonal changes in reproductive development in male spiny mice (*Acomys spinosissimus*) from South Africa. *Mammalian Biology*. **77,** 153 – 159.

Medger, K., Chimimba, C.T. and Bennett, N.C. 2012b. Reproductive photoresponsiveness in male spiny mice from South Africa. *Journal of Zoology*, London. **286,** 243 - 249.

Nelson, R.J., Marinovic, A.C., Moffatt, C.A., Kriegsfeld, L.J. and Kim, S. 1997. The effect of photoperiod and food intake on reproductive development in male deer mice (*Peromyscus maniculatus*). *Physiology and Behavior*. **62,** 945 – 950.

Peitz, B. 1981. The oestrous cycle of the spiny mouse (*Acomys cahirinus*). *Journal for Reproduction and Fertility*. **61,** 453 – 459.

Schwarzenberger, F., Möstl, E., Palme, R. and Bamberg, E. 1996. Faecal steroid analysis for non-invasive monitoring of reproductive status in farm, wild and zoo animals. *Animal Reproduction Science*. **42,** 515 - 526.

Skinner, J.D. and Chimimba, C.T. 2005. The mammals of the southern African subregion (3rd edition). Cambridge: Cambridge University Press.

Voss, R.S. 1979. Male accessory glands and the evolution of copulatory plugs in rodent. *Occasional Papers of the Museum of Zoology, University of Michigan.* **689,** 1 -27.