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The reproductive biology of two small southern African mammals, the spiny mouse, *Acomys spinosissimus* (Rodentia: Muridae) and the Eastern rock elephant-shrew, *Elephantulus myurus* (Macroscelidea: Macroscelididae)

by

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Table of Contents

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List of tables

© University of Pretoria

List of figures

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CHAPTER 4: RESPONSES TO PHOTOPERIOD IN MALE *E. MYURUS*

CHAPTER 5: KISSPEPTIN-IMMUNOREACTIVITY IN *A. SPINOSISSIMUS*

CHAPTER 6: KISSPEPTIN-IMMUNOREACTIVITY IN *E. MYURUS*

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Declaration

The experimental work described in this thesis was carried out in the Department of Zoology and Entomology, University of Pretoria, South Africa, and in the School of Biomedical and Health Sciences, King's College London, United Kingdom, from 2007 to 2010. I, Katarina Medger, declare that this thesis, which I hereby submit for the degree Doctor of Philosophy (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.

Katarina Medger _____ Date _____

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SUMMARY

Since the energy available to an animal for cell growth, thermoregulation, reproduction and other physiological functions is highly dependent on seasonal environmental changes many small mammals breed seasonally during times when environmental conditions are most favourable for growth and survival of the young. In the tropics and sub-tropics, seasonal rainfall appears to be the main reason for seasonal breeding. In order to maximize fitness, it is important for an animal to be able to anticipate these seasonal changes and to trigger reproductive events at the correct time. Photoperiod is used to time reproduction in many temperate mammalian species, but is also used by several seasonally breeding sub-tropical and tropical mammals. The neuroendocrine system is crucial in relaying these environmental signals to the reproductive system. A number of recent studies have suggested that kisspeptin may play a major part in the regulation of the hypothalamo-pituitary-gonadal axis and ultimately reproduction. The exact role of kisspeptin signalling in seasonal breeders is, however, unclear.

The present study investigated the seasonality of reproduction and the reproductive photoresponsiveness of two southern hemisphere species, the spiny mouse (*Acomys spinosissimus*) and the Eastern rock elephant-shrew (*Elephantulus myurus*) from South Africa. Furthermore, it compared hypothalamic kisspeptin expression in males and females of both species between the breeding and non-breeding seasons to ascertain if kisspeptin had any potential role in seasonal breeders under natural conditions.

Both *A. spinosissimus* and *E. myurus* breed seasonally in South Africa. The breeding season extended through the spring and summer months, whereas the gonads were regressed and steroid hormone levels of both sexes were low during the autumn and winter months of the southern hemisphere. Testes mass and volume of *A. spinosissimus* were smaller and plasma testosterone concentrations lower under short-day than long-day photoperiods which implies that male spiny mice are reproductively photoresponsive. In contrast, male *E. myurus* did not appear to be responsive to changing photoperiods and testes size and seminiferous tubule diameter did not differ between photoperiods. It may be possible that other

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environmental factors such as changes in food availability and/or social factors influence seasonal reproduction in *E. myurus.*

In *E. myurus* and male *A. spinosissimus*, kisspeptin-immunoreactivity was significantly lower during the non-breeding than the breeding seasons suggesting that kisspeptin may be important for the regulation of the reproductive axis to seasonal environmental changes. In contrast, kisspeptin-immunoreactivity did not differ between the breeding and non-breeding seasons in female *A. spinosissimus*, but was higher in pregnant than in non-pregnant females implying a differential regulation of the reproductive system of males and females of this species by kisspeptin.

In conclusion, seasonal environmental changes trigger similar reproductive responses in these two phylogenetically very distinct species. However, *A. spinosissimus* may be energetically more restricted than *E. myurus* because of its small body size and judging from the lack of responsiveness to photoperiod, *E. myurus* may follow a more opportunistic breeding strategy. In both species, kisspeptin seems to be important in the regulation of the reproductive system to environmental changes.

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GENERAL INTRODUCTION

Seasonal reproduction

In most regions of the world, food availability and ultimately energy availability varies on an annual basis. These changes are driven by annual cycles of either temperature or rainfall which ultimately affect primary productivity and consequently food availability for animals. In addition, other functions besides reproduction require energy and it is posited that seasonal reproduction evolved in response to the annual variation in energy availability and the competition of different physiological functions for the available resources (Nelson *et al*., 2002). The majority of mammals studied to date, display some inclination towards seasonal reproduction, with many exhibiting a distinct breeding season, even in the tropics (Bronson, 1985, 2009). Mammals breed during periods of the year when food is abundant and temperatures are mild, in contrast, they cease breeding during times when food is scarce and temperatures are low. This differential allocation of resources and breeding during favourable conditions ensures maximum growth and survival of the young and ultimately maximizes reproductive success (Bronson & Heideman, 1994).

Temperate vs. sub-tropical and tropical regions

In temperate and polar regions, seasonal variation in temperature and food is marked and the winter months put high energetic constraints particularly on small mammals (Bronson, 1985). Cold winters are especially problematic for small mammals since they lose heat readily because of their high surface to body mass ratio and have relatively small energy or fat reserves (Bronson, 2009). Consequently, in times of energy shortage, resources might be diverted from reproduction towards, for example, cellular growth and thermoregulation (Bronson & Heideman, 1994). Accordingly, in edible dormice (*Glis glis*), non-reproductive males use daily torpor to save energy, but this also impairs the development of their reproductive organs. At the same time, reproductive males cannot use torpor as low temperatures have a detrimental effect on their gonads and consequently, they pay for reproductive investment with reduced body conditions (Fietz *et al.*, 2004). In contrast, in tropical and sub-tropical regions, temperature fluctuates much less throughout the year and the seasons are marked by times of abundant rainfall and severe dry periods. In

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these areas, energy availability is often determined by water availability rather than temperature and more importantly, the dry season shows a marked decrease in biomass and hence food availability (Delany, 1972; Neal, 1986).

Food quantity and quality

Reproduction puts large energetic demands on animals (Partridge & Harvey, 1985; Wade & Schneider, 1992). In particular the costs to female mammals for activities such as ovulation, pregnancy and particularly lactation are often high (Speakman, 2008). The costs of lactation are especially pronounced in small mammals with large litters. In the cotton rat (*Sigmodon hispidus*) and white-footed mouse (*Peromyscus leucopus*), for example, it was observed that the costs of reproduction increase with larger litter size (Glazier, 1985; Randolph *et al.*, 1977). To be able to balance these energetic demands, cotton rats increase their food intake by 66 % during periods of lactation (Randolph *et al*., 1977) indicating that food availability is the most important factor for mammalian reproduction and in turn dictates if an animal can or cannot reproduce. Bronson (1985) also emphasized the importance of food availability as a major environmental factor controlling reproduction in mammals. In addition to general energetic costs, small mammals also endure higher demands for protein, calcium and other micronutrients during reproduction (Speakman, 2008). Consequently, protein content of the diet (Field, 1975) and calcium concentrations (Batzli, 1986; Bernard *et al*., 1996) were established to influence reproduction. Freshly germinated and green food (El-Bakry *et al.*, 1998; Nelson *et al.*, 1983) as well as dietary salinity (Wube *et al.*, 2009) may also affect reproductive development. In addition, a secondary plant compound with oestrogenic properties, 6-methoxybenzoxazolinone, has been proposed as a proximate factor which enables rodents to accurately time the approaching periods of high availability of green grass (Sanders *et al*., 1981).

Seasonal* vs. *opportunistic breeding strategies

In temperate regions, mammals with extended longevities have distinct breeding seasons and the young are usually born during the time of year when food availability is at its maximum (Bronson, 1985). Kawamichi (1997) reported that the period of lactation and first foraging in the young of the Japanese giant squirrel (*Petaurista leucogenys*) coincides with the months when food is readily available. In addition,

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reproduction in the southern hairy-nosed wombat (*Lasiorhinus latifrons*) is timed such that the young emerge from the pouches when the conditions are most favourable and maximum survival of the young is guaranteed (Taggart *et al*., 2005). In contrast, many short-lived (i.e. ≤ 6 months) small mammals have to reproduce at every possible time to maximize reproductive success. Breeding in these species only ceases if conditions are too harsh for reproduction and survival of both young and adults is unlikely if breeding is attempted (Bronson, 1985). Such an opportunistic breeding strategy is even exhibited in species occurring in the polar regions where harsh winters are recurrent. Consequently, the meadow vole (*Microtus pennsylvanicus*) (Beer & MacLeod, 1961) as well as the deer mouse (*Peromyscus maniculatus*) (Scheffer, 1924) breed throughout the year including mid-winter if conditions allow it.

Opportunistic as well as strongly seasonally breeding small mammals are also found in sub-tropical and tropical parts of the world. For example, the four-striped field mouse (*Rhabdomys pumilio*) is a small southern African mammal which exhibits reproductive opportunism. While this rodent breeds continuously in habitats with mild winters, it was documented that females cease breeding during winter in the harsh environments within their distributional range, although males continue to be reproductively active (Jackson & Bernard, 2006). Food availability appears to be the main factor dictating these differential responses as a 10 % reduction of food quantity was observed to inhibit reproduction in male and female four-striped field mice, although the extent of reproductive inhibition was dependent on the body fat reserves of an individual (Jackson & Bernard, 2001). Furthermore, the grass rat (*Arvicanthis spp.*) breeds opportunistically in East Africa and interestingly, in this small mammal, water stress might be the reason for seasonal reproduction in one population (Neal, 1968). Other sub-tropical species appear to be strict seasonal breeders, for example the bushveld gerbil, *Gerbilliscus leucogaster* (formerly *Tatera leucogaster*; see Musser & Carleton, 2005) (Neal, 1991; Perrin & Swanepoel, 1987), the Namaqua rock mouse, *Micaelamys namaquensis* (formerly *Aethomys namaquensis*; see Skinner & Chimimba, 2005) (Muteka *et al.*, 2006a) and the Tete veld rat, *Aethomys ineptus* (Muteka *et al.*, 2006b).

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Other environmental factors

Small mammals occur in a wide range of habitats ranging from the cold polar regions of Siberia and Alaska through to very dry deserts with marked daily variations in temperature. Just as they occupy a diversity of environments so they also exhibit a wide array of reproductive and social strategies. As a consequence, reproduction is not just regulated by the availability of food, or energy, but also by a range of other environmental and social factors which interact synergistically with food availability. Some of these are snow cover (Beer & MacLeod, 1961), rainfall or water availability (Leirs *et al.*, 1996; Nelson *et al.*, 1995; Sicard *et al.*, 1993; Taylor & Green, 1976), ambient temperature (Jackson & Bernard, 2001; Nelson *et al.*, 1989), social cues (Drickamer, 1982; Hegstrom & Breedlove, 1999; Pyter *et al.*, 2005), exercise (El-Bakry *et al.*, 1998) and population density (Leirs *et al.*, 1997). Rainfall is particularly important for the onset of reproduction in many African species where reproduction is regulated by dry and rainy seasons and the first rainfall after the dry season facilitates rapid growth of vegetation triggering the start of reproduction in many small mammals (Makundi *et al.*, 2006; Neal, 1977; Taylor & Green, 1976). Interestingly, this relationship between rainfall, vegetative growth and the onset of reproduction has also been found in South American forest rodents (Bergallo & Magnusson, 1999; Dubost *et al.*, 2005). Water availability can, however, affect reproduction independently of food quality (Nelson *et al*., 1995). In the Nile grass rat (*Arvicanthis niloticus*), humid conditions or low temperatures stimulate reproduction, whereas dry conditions and high temperatures inhibit breeding and the stimulatory effects on reproduction are most pronounced when humid conditions and low temperatures are combined (Sicard *et al.*, 1993). The availability of green food in this study of the Nile grass rat was limited and it is possible that green food may counteract the effects of dry conditions in desert rodents. This may have been the reason why water-availability did not influence reproduction in Shaw's jird (*Meriones shawi*) as green food was also provided when free water was restricted (El-Bakry *et al.*, 1999). In contrast to the Nile grass rat, low instead of high ambient temperatures suppressed gonadal size in the prairie vole (*Microtus ochrogaster*) indicating that temperature may affect reproduction in different ways (Nelson *et al*., 1989).

Social cues, especially pheromones and also tactile cues, have been observed to accelerate puberty and gonadal growth and are, therefore, important for

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the regulation of reproductive function and possibly seasonal reproduction. In house mice, male urine accelerated female puberty, whereas female urine had an inhibitory effect (Drickamer, 1982). Furthermore, housing of males together with females increased testosterone concentrations and testes mass compared to singly housed males in *Peromyscus leucopus* and *P. aztecus* (Demas & Nelson, 1998; Pyter *et al.*, 2005). In the white-footed mouse, social environment only affects the male reproductive system when males are subjected to long-day compared to short-day photoperiods, suggesting that social cues may enhance photoperiodic effects on reproduction (Pyter *et al*., 2005). Social cues may, therefore, be important for the regulation of seasonal reproduction especially in tropical species (Pyter *et al*., 2005).

Photoperiod

Photoperiod and other proximate factors are used by an animal to anticipate impending annual changes in their environment (Bronson, 1985). These factors are particularly helpful in environments where changes are highly predictable and environmental events are imminent (Bradshaw & Holzapfel, 2007). The ability to envisage upcoming environmental events increases the survival and fitness of an individual and consequently, proximate factors are widely exploited (Bradshaw & Holzapfel, 2007). However, annual changes in photoperiod are less pronounced at lower than they are at higher latitudes and, therefore, many sub-tropical and tropical species do not rely on photoperiod as a cue to time reproductive events (Bronson, 1985). This is surprising since it appears that even temperate small mammals are able to respond to photoperiodic changes typical of 5° and 10° latitude (Heideman & Bronson, 1993). However, the small day to day changes in photoperiod at the lower latitudes may not be reliable enough and consequently tropical species may benefit more from other cues. It has been reported that populations of the white-footed mouse (Lynch *et al.*, 1981) and the Nile grass rat (Nunes *et al.*, 2002; Sicard *et al.*, 1993) exhibit reproductive photoresponsiveness at high latitudes, whereas populations nearer to the equator appear to be less or totally non-responsive to changing photoperiod.

In contrast, this effect was not found between different species of *Peromyscus* where the degree of reproductive photoresponsiveness was not related to latitude of origin (Trainor *et al.*, 2006). Even in a single population, photoresponsive as well as

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non-responsive morphs can occur. In the red-backed vole (*Myodes rutilus*), for example, some individuals are photoperiodically non-responsive which enables these individuals to breed in mild winters and, thus, increase their fitness, whereas the responsive individuals may miss this breeding opportunity (Stevenson *et al.*, 2009). These differences between species, populations and individuals in reproductive responsiveness to photoperiod are an adaptation to the different life history traits of different species and the diversity of environmental challenges faced and they are likely to occur in temperate as well as sub-tropical and tropical species. Photoresponsiveness is predicted to be less common in tropical and sub-tropical animals due to the subtle changes in photoperiod throughout the year (Trainor *et al*., 2006). Photoperiod has dramatic affects on puberty, with short-days delaying and long-days accelerating gonadal development in rodents (Forger & Zucker, 1985; Nelson *et al.*, 1997). Furthermore, short-day lengths may even retard reproductive ageing (Place *et al*., 2004).

Photoperiod does not only influence the reproductive system, but it also acts on a wide range of other physiological and behavioural functions allowing an individual to fully adjust to the upcoming changes in environmental conditions. Short day-length was found to induce a reduction in body mass, oxygen consumption, carbon dioxide production, feeding behaviour and locomotory activity (Warner *et al*., 2010), but enhanced immune-function (Nelson *et al.*, 1998) and resulted in an increased thickness and density of the fur (Nelson *et al.*, 1989).

Seasonal reproduction and particularly the role of photoperiod as a proximate cue to regulate reproductive responses to on-coming environmental changes has been the focus of many studies over several decades. Sadly, most studies have focused on mammals in temperate regions and these have been thoroughly investigated, whereas our knowledge about seasonal reproduction in tropical and also sub-tropical mammals is scant. The tropics have the highest species diversity of mammals and it is, therefore, crucial to investigate the biology of these species more thoroughly to initially, better understand the evolution and ecological significance of seasonal reproduction in these species and also in mammals as a whole and secondly, to reduce possible future threats such as climate change to the survival of these species (Bronson, 2009). In addition, the neuroendocrine pathways which

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regulate seasonal responses of the reproductive system to environmental factors have been studied comprehensively, but many questions remain unanswered and studies especially in natural and semi-natural settings are needed to determine the role of the neuroendocrine system in seasonal reproduction (Greives *et al*., 2008).

The hypothalamo-pituitary-gonadal axis and kisspeptin

In order to determine the appropriate time for reproduction and to maximize reproductive success, it is essential for an animal to translate the environmental cues into neuronal signals. In this regard, the hypothalamo-pituitary-gonadal (HPG) axis plays a major role in seasonal reproduction and regulation of reproductive function. The release of melatonin by the pineal gland is important for the perception of changing day-length and, therefore, for the mediation of the reproductive function through photoperiod (Goldman, 2001). Gonadotrophin-releasing hormone (GnRH) is secreted by hypothalamic neurons which in turn stimulate the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary. LH and FSH, in turn, induce the production of the steroids testosterone, oestrogen and progesterone which enhance gametogenesis (Everett, 1994). However, the mechanisms which are responsible for the interpretation of external and internal cues as well as the transfer of this information to the GnRH system are largely unknown.

A newly discovered group of neuropeptides, the kisspeptins, have recently been implicated in the regulation of reproductive functions through the HPG axis. Kisspeptins are the products of the *Kiss1* gene and a natural ligand to the G protein coupled receptor 54 (Kotani *et al*., 2001; Ohtaki *et al*., 2001). The original product of the *Kiss1* gene is a 145-amino acid protein, but four biologically active peptides with different amino acid length were identified, kisspeptin-54, -14, -13 and -10 (Kotani *et al.*, 2001; Oakley *et al.*, 2009). The shorter peptides may be derived from the longer kisspeptin-54 which may be unstable (Kotani *et al*., 2001). Oakley *et al.* (2009) proposed more standardized terms for the abbreviations of the neuropeptide, its receptor and their genes. Accordingly, Kiss1r, *Kiss1* and *Kiss1r* for the receptor, the kisspeptin gene and receptor gene, respectively, will be used throughout the text.

In 2003, two research groups discovered that a mutation in *Kiss1r* caused autosomal recessive idiopathic hypogonadotropic hypogonadism (IHH) in humans

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and mice (Funes *et al*., 2003; Seminara *et al.*, 2003). The *Kiss1r*-deficient mice showed reduced reproductive organ size and weight, lack of sexual maturation and the inability to reproduce which is strikingly similar to IHH in humans and which is characterized by low concentrations of circulating gonadotrophins. As a result, it was speculated that *Kiss1r* may play a key role in the regulation of puberty. In later studies, it was found that a central administration of kisspeptin advanced puberty in immature female rats through a precocious activation of the HPG axis (Navarro *et al*., 2004). It was further observed that kisspeptin stimulates the release of LH and FSH through an influence on GnRH (Navarro *et al.*, 2005a; Navarro *et al.*, 2005b; Thompson *et al.*, 2004). Through examining cFos expression in GnRH neurons, which indicates neuronal activation, and by blocking GnRH secretion with the help of an antagonist, it was established that kisspeptin acts directly on GnRH neurons possibly through its receptor Kiss1r (Gottsch *et al*., 2004; Irwig *et al*., 2004). This was further confirmed when a co-localization of *Kiss1r* mRNA with GnRH neurons was detected (Messager *et al*., 2005).

Another important role of kisspeptin and *Kiss1r* in the regulation of the reproductive system is the mediation of positive oestrogen feedback mechanisms to the HPG axis and triggering the GnRH/LH surge necessary for ovulation (Kinoshita *et al*., 2005). The rostral periventricular area of the third ventricle and especially the anteroventral periventricular nucleus (AVPV) have been implicated in the actions of kisspeptin for the activation of the LH surge and furthermore, oestrogen acts probably directly on kisspeptin neurons as these neurons co-express oestrogen receptor α (Clarkson *et al.*, 2008; Smith *et al.*, 2006b). Kisspeptin is most likely also regulated by progesterone and testosterone and positive as well as negative feedback actions on kisspeptin production by all steroids are recognized. In the AVPV, positive, amplifying effects of oestrogen, progesterone and testosterone on *Kiss1* expression were observed, whereas in the arcuate nucleus (Arc), *Kiss1* expression is inhibited by these same hormones (Roa *et al.*, 2008; Smith *et al.*, 2005; Smith *et al.*, 2006b). These negative feedback mechanisms of the steroids on kisspeptin may be important for the regulation of the pulsatile GnRH release through kisspeptin by stimulating kisspeptin/GnRH secretion when steroid levels are low and decreasing secretion when steroid concentrations are high (Smith *et al.*, 2006a).

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Furthermore, Lehman *et al.* (2010) suggested that a cluster of neurons in the Arc that co-localize three neuropeptides (kisspeptin, neurokinin B and dynorphin), which are all important for the steroid feedback control of GnRH release from the hypothalamus, is of major significance in the regulation of tonic release of GnRH/LH. This is interesting since this cell cluster is conserved across numerous mammalian species from rodents to humans (Lehman *et al*., 2010). In addition, further studies, which used either *Kiss1* and *Kiss1r* knockout mice or a new kisspeptin antagonist, confirmed the importance for kisspeptin and Kiss1r in the regulation of reproductive development and the preovulatory LH surge (Chan *et al*., 2009; Pineda *et al.*, 2010).

In addition to these functions, kisspeptin has also been connected to the seasonal regulation of the reproductive system and the coordination and transfer of environmental signals to the reproductive axis. Kisspeptin appears to be important for mediating photoperiodic signals, probably through a melatonin dependent mechanism, to the reproductive axis in seasonally breeding rodents as well as sheep (Clarke *et al*., 2009; Simonneaux *et al*., 2009). However, kisspeptin appears to differentially control the reproductive system in different species and many of these mechanisms of kisspeptin are still unclear or unknown. Most studies undertaken so far to investigate the effects of kisspeptin on the reproductive axis have been performed on the standard laboratory species such as rats, mice and hamsters (*Mesocricetus auratus* and *Phodopus sungorus*) and mostly under laboratory conditions.

Study objectives

In the present study, I investigated the reproductive biology of two small mammal species from South Africa, the spiny mouse (*Acomys spinosissimus*) and the Eastern rock elephant-shrew (*Elephantulus myurus*). A rodent and an elephant-shrew were chosen because rodents are commonly the focus of studies on reproduction, which is justified by their high abundance, although other species may shed more light on the evolution of the reproductive system. However, a comparison of the reproductive ecology and physiology between different small mammal species is made difficult due to the lack of information on non-rodent species. *Acomys spinosissimus* and *E. myurus* occur sympatrically in rocky habitats in South Africa. As a result, both species experience the same seasonal environmental changes particularly in

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temperature and rainfall. However, their different phylogenetic status and diet (granivorous *vs*. insectivorous) may result in varying ways in which these species react to the annual environmental changes. The objective of the present study is, therefore, to establish how these differences are manifested in their reproductive biology. In addition, this study aims to increase the currently limited knowledge on the general and reproductive biology of these two data pauperate species from the southern hemisphere where relatively few such studies have been undertaken in comparison to the northern hemisphere.

Study species

The spiny mouse, Acomys spinosissimus

The spiny mouse (*Acomys spinosissimus*; Fig. 1) is a small rodent with a characteristic spiny coat which is particularly pronounced at the back. The fur is a reddish-brown at the back and head and white ventrally (Skinner & Chimimba, 2005). The spiny mouse is relatively widespread in Africa south of the equator and is found in Tanzania, the Democratic Republic of Congo (DRC), Zambia, Malawi and in parts of southern Africa (Skinner & Chimimba, 2005). Both the higher- and species-level taxonomy of the genus *Acomys* is currently uncertain. The genus is currently placed under the subfamily Deomyinae and forms a clade with *Uranomys* and *Lophuromys* (Skinner & Chimimba 2005). Its previous allocation to the subfamily Murinae based on dental morphology has been questioned through the use of molecular data that suggest a closer relationship with gerbils (Gerbillinae) than with true mice (Murinae; Chevret *et al*., 1993). This was also confirmed by other molecular and biochemical studies (Chevret & Hänni, 1994). Furthermore, Fraguedakis-Tsolis *et al.* (1993) even questioned the position of *Acomys* within the Muridae. At the species level, Janecek *et al.* (1991) reported that *A. spinosissimus* is distinct from other species of *Acomys* from northern Africa and Asia as well as from its geographically close congener the Cape spiny mouse (*A. subspinosus*) that is restricted to the Western Cape Province, South Africa. Furthermore, morphology and molecular data suggest the monophyly of *A. spinosissimus* (Barome *et al.*, 2001).

The general biology of *A. spinosissimus* is largely unknown despite that other species of *Acomys*, especially the Northeast African spiny mouse (*A. cahirinus*) and the golden spiny mouse (*A. russatus*), have been thoroughly studied. *Acomys*

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spinosissimus inhabits rocky outcrops and also riverine areas where it may be found under and amongst shelters such as boulders, termite mounts and roots (Fitzherbert *et al.*, 2006; Pienaar *et al.*, 1980; Sheppe, 1973). This rodent is granivorous, but may supplement its diet with insects, such as termites and ants (Skinner & Chimimba, 2005). In addition, *A. spinosissimus* is nocturnal (C. Hoole, unpublished data). The social structure of *A. spinosissimus* is uncertain as they have been found alone, in pairs as well as in family parties and large groups (Skinner & Chimimba, 2005). The reproductive biology of this species is almost entirely unknown although modest available data suggests that *A. spinosissimus* reproduces seasonally during the wet and warm summer months in South Africa and Botswana (Pienaar *et al.*, 1980; Smithers, 1971). Other species of *Acomys* such as the Eastern spiny mouse (*A. dimidiatus*) and *A. subspinosus* give birth to precocial or semi-precocial young, respectively (Dempster *et al.*, 1992; Dieterlen, 1961) and the litter size of *Acomys* species is relatively small (Neal, 1983). The mean litter size of *A. spinosissimus* is about three and ranges from two to five (Sheppe, 1973; Smithers, 1971). As a result, *A. spinosissimus* and other *Acomys* species may follow a *K*-selected rather than an *r*-selected life history strategy (Neal, 1983).

Figure 1. Spiny mouse, *Acomys spinosissimus*

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***The Eastern rock elephant-shrew,* Elephantulus myurus**

The monophyletic order Macroscelidea encompasses four genera in two subfamilies: Rhynchocyoninae with the only genus *Rhynchocyon* and Macroscelidinae with the genera *Petrodromus*, *Macroscelides* and *Elephantulus* (Corbet & Hanks, 1968). A total of 15 species, which are all endemic to the African continent, were recognised until recently (Skinner & Chimimba, 2005). In 2008, two new species were described: one within the genus *Rhynchocyon* (Rovero *et al*., 2008) which now consists of four species and one within *Elephantulus* (Smit *et al*., 2008) which includes eleven species. These recent descriptions also indicate the lack of knowledge about these unique small mammals and emphasize the importance for continued studies of their biology. The macroscelidean fossil record dating from the Eocene and Oligocene indicates an early diversity in this group and provides new arguments for the origin of the Macroscelidea (Simons *et al.*, 1991).

The family Macroscelidae was previously placed within the order Insectivora (Nowak & Paradiso, 1983) and furthermore, elephant-shrews were considered to be related to lagomorphs and rodents (Novacek, 1992). Recent molecular and fossil data, however, suggest that the elephant-shrews should be placed within the super-cohort Afrotheria, which also comprises aardvarks, golden moles, elephants, sirenians and hyraxes (Stanhope *et al*., 1998; Tabuce *et al*., 2001). Although the relationships within the Afrotheria are uncertain, elephant-shrews appear to be more closely related to the aardvark (*Orycteropus afer*) than to any of the other orders (Robinson *et al*., 2004). In addition, it has been suggested that the elephant-shrews and the aardvark are related to elephants and hyraxes placing them together in the Paenungulate clade (Madsen *et al*., 1997). This has, however, been disputed by a subsequent study (Murata *et al*., 2003). To avoid confusion with true shrews, which are not related to elephant-shrews, the term *sengi* instead of elephant-shrew is more often used in the literature (Skinner & Chimimba, 2005).

The Eastern rock elephant-shrew (*Elephantulus myurus;* Fig. 2) occurs in southern and eastern Zimbabwe, eastern Botswana and western Mozambique and has a wide distribution in South Africa from the Limpopo Province to eastern Northern Cape, northern Eastern Cape and north-western KwaZulu-Natal Provinces (Skinner & Chimimba, 2005). Like most elephant-shrews, *E. myurus* has large ears

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and eyes and a long nose which is highly mobile (Skinner & Chimimba, 2005). The pelage of *E. myurus* is grey which gets paler towards the flanks and darker towards the rump and the under parts are white. The tail is just longer than the body and sparsely covered with hair (Skinner & Chimimba, 2005). These animals are very vigilant and easily disturbed and their long hind-legs permit rapid flight (Skinner & Chimimba, 2005; van der Horst, 1946). The habitat of the Eastern rock elephant-shrew is predominantly rocky and *E. myurus* occurs where boulders and vegetation permit cover (Skinner & Chimimba, 2005). *Elephantulus myurus* does not use or maintain trails as has been observed in other elephant-shrews (Rathbun, 1979). The diet of *E. myurus* consists predominantly of insects particularly termites and ants, but plant material is also eaten (Churchfield, 1987). This elephant-shrew is active particularly in the morning, but bouts of activity also occur during the day, evening and at night (Woodall *et al.*, 1989).

Elephantulus myurus is monogamous and males appear to stay together with only one female even when their territories overlap with several females, however, males are never seen to assist in raising the young (Ribble & Perrin 2005). The reproductive biology of these small mammals is distinctive. *Elephantulus myurus* almost always gives birth to twins which are highly precocial. In addition, only two embryos implant in the uterine horns and always a single embryo occurs in each horn (van der Horst & Gillman, 1941). Despite the small litter size, female *E. myurus* polyovulate and an average of 49 (25 – 89) corpora lutea per ovary were counted in one study (Tripp, 1971). In addition, *E. myurus* appears to be a spontaneous ovulator (Tripp, 1972). The testes of male *E. myurus* are abdominally situated, just caudal to the kidneys and the penis is very long and runs underneath the skin towards the sternum immediately before which it emerges (Woodall, 1995). Several studies have investigated the seasonality of reproduction in *E. myurus* and the data from gonad and reproductive gland histology implies that *E. myurus* reproduce seasonally during the wet and warm summer months in South Africa (van der Horst 1946; Stoch 1954; Woodall & Skinner 1989). However, some of these results are contradictory and the environmental factors regulating this seasonal reproduction are unknown.

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Figure 2. Eastern rock elephant-shrew, *Elephantulus myurus*

Thesis organisation

This study attempts to obtain a comprehensive understanding of the reproductive systems of *A. spinosissimus* and *E. myurus* by utilizing a wide array of methods. Morphological measurements, histology and endocrinological methods, such as hormone assays and immunohistochemistry were carried out to enable a crucial understanding of the reproductive biology of these species at all levels. It should also be noted, that this study is the first which investigates the influence of kisspeptin on the reproductive system in a seasonally breeding species under natural conditions and by doing so, aims to increase the information on the regulation of the neuroendocrine axis by kisspeptin in seasonal breeders.

All chapters are prepared as stand-alone manuscripts as they have been published or are being prepared for future submission. As a consequence, there may be repetition between chapters. I did not write the thesis in the first person, but used the term “we” throughout all chapters because all chapters were planned as individual manuscripts and I was unable to undertake the research, especially the field work, alone.

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Chapter one (seasonality in female *A. spinosissimus* published as Medger *et al.*, 2010, *Journal of Zoology (London)*) investigates the seasonality of reproduction in males and females of *A. spinosissimus* by examining the gonads morphologically and histologically and by determining the concentrations of reproductive hormones monthly over a 12-month period. It was predicted that *A. spinosissimus* breeds seasonally during the warm and wet spring and summer months (September – February) of the southern hemisphere. However, regression of the gonads of both males and females and a decline in hormone concentrations was expected during the cold and dry autumn and winter months (March – August). *Chapter two* deals with the seasonal reproduction in *E. myurus* and similar predictions as for *A. spinosissimus* were formulated.

Chapters three and *four* examine the response of the reproductive system of male *A. spinosissimus* and *E. myurus*, respectively, to changes in photoperiod. Short-day photoperiods were expected to decrease testes size (mass and volume) and circulating plasma testosterone concentrations in both species. In contrast, it was predicted that long-day photoperiods should stimulate the reproductive system in both species resulting in a recrudescence of testes size and circulating plasma testosterone concentrations compared to the short-day photoperiod.

The last two chapters focus on the hypothalamic distribution of kisspeptin-immunoreactivity of wild-caught *A. spinosissimus* (*Chapter 5*) and *E. myurus* (*Chapter 6*) during the breeding and non-breeding seasons. In addition, these chapters investigate possible differences in kisspeptin expression between males and females of both species. It was predicted that kisspeptin-immunoreactivity would be largely limited to the AVPV and Arc, with kisspeptin expression being higher in these two regions during the breeding season when compared to the non-breeding season. While no difference in kisspeptin expression between males and females was expected in the Arc, kisspeptin expression was assumed to be higher in the AVPV of females than of males. The *general conclusion* synthesises the main findings of the chapters, compares the reproductive system of the two species in relation to life history traits and presents directions for future research.

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CHAPTER 1

SEASONAL REPRODUCTION IN THE MALE AND FEMALE SPINY MOUSE (*ACOMYS SPINOSISSIMUS*) FROM SOUTH AFRICA

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Abstract

Many mammal species reproduce seasonally due to annual fluctuations in temperature, rainfall and photoperiod in often nutritionally-challenging habitats. The reproductive biology of many southern African small mammals is largely unknown and in critical need of study. We investigated the breeding pattern of the spiny mouse (*Acomys spinosissimus*) from South Africa. We examined the ovarian and testicular development, follicular growth, circulating plasma testosterone and progesterone concentrations, and the reproductive status of wild-caught adult male and female spiny mice sampled over a 12-month period, while also correcting for body mass and age. From these data, we conclude that *A. spinosissimus* breeds seasonally. The main breeding season of the spiny mouse is between September and January with plasma testosterone and progesterone concentrations being elevated, gonadal development and primary, secondary, tertiary and Graafian follicle numbers as well as corpora body number being highest and pregnancies occurring during this period. Females were reproductively inactive from February through to August, whereas males started to increase their reproductive activity about one to two months earlier and were reproductively inactive from February until about June/July. The breeding season coincides with the onset of the rainy season in the habitat which starts in September and ends in April. Rainfall in association with an increase in primary productivity and hence higher food availability may be the most important factor shaping reproduction in the spiny mouse.

Keywords: Muridae, follicular growth, seminiferous tubules, rainfall, food availability, southern hemisphere

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Introduction

In seasonally changing environments, both temperate and tropical mammals often breed during the most favourable times of the year (Bronson, 1985). This ensures a higher survival and growth of young and maximizes reproductive success as reproduction is energetically costly (Gittleman & Thompson, 1988). In the tropical unstriped grass rat (*Arvicanthis*), Neal (1981) observed a shift from continuous breeding in an aseasonal environment to a seasonal pattern of reproduction in an environment with seasonally changing rainfall combined with changing nutritional quality and quantity of food. These factors appear to be the primary cause for seasonal reproduction in numerous tropical and sub-tropical species of rodents (Makundi *et al.*, 2006; Muteka *et al.*, 2006b; Perrin & Swanepoel, 1987).

The effect of both quality and quantity of food and even of secondary plant compounds on reproduction has been studied in the laboratory and found to be of significance in regulating reproduction in a number of species (Batzli, 1986; Heideman *et al.*, 1998; Wube *et al.*, 2009). Numerous other factors may affect reproduction such as water availability (Nelson *et al.*, 1995), temperature (Nelson *et al.*, 1989), social factors (Trainor *et al.*, 2006), snow cover (Beer & MacLeod, 1961) and exercise (El-Bakry *et al.*, 1998). Moreover, many mammals use the seasonally changing day-night cycle as a proximate cue to anticipate seasonal changes (Prendergast *et al.*, 2001). However, equatorial populations of many species do not respond to changes in photoperiod, while populations from higher latitudes are highly photoresponsive (Nunes *et al.*, 2002). Photoperiod can interact with a number of other factors such as temperature and food availability to enhance reproductive responses (Kriegsfeld *et al.*, 2000; Nelson *et al.*, 1992).

The reproductive biology and environmental cues, in particular the affect of photoperiod, have been investigated in a large number of small mammals, but these studies have mainly been on species from the northern hemisphere such as hamsters (Prendergast *et al.*, 2001), a number of *Peromyscus* species (Trainor *et al.*, 2006) and voles (Stevenson *et al.*, 2009). The reproductive biology and life history patterns of many southern African small mammals are still largely unknown and there is an increasing need for their investigation. The present study focuses on the

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reproductive seasonality of the spiny mouse, *Acomys spinosissimus,* from South Africa.

The spiny mouse is a nocturnal, terrestrial rodent which occurs mainly in rocky habitats and is distinguished by thick and hard spine-like hairs at the back which is characteristic of the genus (Pienaar *et al.*, 1980; Sheppe, 1973; Skinner & Chimimba, 2005). *Acomys spinosissimus* is relatively widespread in Africa south of the equator and can be found in Tanzania, the Democratic Republic of Congo (DRC), Zambia, Malawi and in southern Africa (Mozambique, Botswana and the north-eastern parts of South Africa) (Skinner & Chimimba, 2005). The little available data suggest that this species breeds seasonally during the warm and wet summer months in Zambia (Sheppe, 1973) and southern Africa (Pienaar *et al.*, 1980; Smithers, 1971).

In this study, we investigated the seasonality of reproduction in *A. spinosissimus* by examining the histology of the gonads and monitoring the monthly circulating concentrations of progesterone and testosterone in the blood of females and males, respectively from April 2007 until August 2008**.** We hypothesized that reproduction of the spiny mouse is highly dependent on rainfall in combination with an increase in food availability and, therefore, predicted that *A. spinosissimus* would breed seasonally with increased gonadal and follicular growth and pregnancies occurring mainly during the warm and wet spring (September–November) and summer months (December–February), whereas ovaries and testes would be regressed and progesterone and testosterone concentrations would be low during the cold and dry winter months (June–August) of the southern hemisphere. We also investigated the influence of age on the reproductive biology of this species.

Materials and Methods

General

Up to nine male and eight female *A. spinossismus* were captured at the end of every month from April 2007 to August 2008 with a total of 65 males and 67 females being captured over the whole study period (Table 1). All spiny mice were trapped over-night around the rocky out-crops of the Goro Game Reserve in the Soutpansberg region, Limpopo Province, South Africa (22°58’S, 22°57’S; 29°25’E, 29°24’E). Animals were collected under permit number CPM-333-00002 from the CITES and

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Permit Management Office, Department of Environmental Affairs, Limpopo Province. Specimens were trapped using Sherman live traps (H. B. Sherman Traps, Inc. Tallahassee, Florida, U.S.A.) baited with a mixture of peanut butter, oats and fish oil. The body mass (g) of each individual was recorded to the nearest 0.001 g using a digital balance (Scout Pro SPU123, Ohaus Corporation, Pine Brook, New Jersey, U.S.A.) immediately after capture and rounded to 0.1 g for subsequent analyses.

Spiny mice were kept in polyurethane cages during transportation and in the laboratory and were maintained under the guidelines of the animal ethics committee of the University of Pretoria, Pretoria, South Africa (ethics clearance number: A003-07). All cages were provided with wood shavings as bedding and paper towel for additional shelter. Water was provided *ad libitum* and mouse pellets, carrots and apples were provided as food. Monthly rainfall data (mm) were kindly provided by the Goro Game Reserve. Ambient temperature (°C) was measured to the nearest 0.01 °C by two iButton digital temperature data loggers (Maxim Integrated Products, Dallas Semiconductor, U.S.A.) for 2007 and 2008. The iButtons were placed near the ground and protected from direct sunlight and measurements were taken every two hours.

Animals were housed in the laboratory for at least one day for acclimatisation prior to euthanasia by an overdose of halothane. Blood was collected from males and females by exsanguination from the heart and centrifuged at 3000 rpm for 15 min. The plasma fraction was separated from blood cells and stored at -35 °C until hormone analysis. Testes were dissected out in males. Female bodies were examined for swollen and elongated teats which were used as an indication of lactation. Ovaries with uterine horns and foetuses were dissected out and the number of foetuses was recorded. In addition, the number of placental scars was counted during autopsy to determine if the animals had given birth prior to capture. All gonads were fixed in Bouin's fluid for approximately 20 hrs before being rinsed and stored in 70 % ethanol. Standard museum techniques (DeBlase & Martin, 2001) for small mammals were used to prepare skulls which were subsequently used to determine the relative age of an individual using the degree of maxillary molar tooth-wear and eruption as defined under the "Relative age classes" section below.

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Voucher specimens will be deposited in the mammal reference collection of the Ditsong National Museum of Natural History, Pretoria, South Africa.

Histology

After removal of all excess tissue, the length (mm) and the width (mm) of the fixed gonads were measured to the nearest 0.01 mm using a pair of digital calipers (Sylvac Opto RS 232, Ultra Praezision Messzeuge GmbH, Germany). These dimensions were in turn used to calculate ovarian and testicular volume (mm^3) using the formula for the volume of an ellipsoid: $V = 4/3\ \pi ab^2$ where a represents half the maximum length and b half the maximum width (Woodall & Skinner, 1989). The volume was averaged for the two ovaries or testes per female or male, respectively. Testes were weighed to the nearest 0.0001 g using a high precision scale (Ohaus Corp. Pine Brook, N.Y., U.S.A.).

Gonads were sequentially dehydrated in increasing concentrations of ethanol baths and embedded in a cube of paraffin wax. Testes were sectioned at 8 µm widths and ovaries were serially sectioned at 5 µm widths in their totality with a rotary microtome (820 Spencer, American Optical, Scientific Instrument Division, Buffalo, N.Y., U.S.A.). The tissue sections were mounted on microscope slides with gelatin as an adhesive and subsequently dried in an oven at 36 °C for about 48 hrs. The sections were finally stained with Ehrlich's haemotoxylin and counter-stained with eosin (see Drury & Wallington, 1967). Testicular sections were checked for round seminiferous tubules with a light microscope (Diaplan, Ernst Leitz Wetzlar GmbH, Germany) at ×10 magnification. Seminiferous tubules were then photographed 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 diameter of 100 seminiferous tubules (µm) were measured with Motic Images Plus 2.0ML (Motic China Group, LTD., Xiamen, P.R. China). All ovarian sections were examined consecutively for stages of follicular growth under a light microscope (Vickers Instruments, U.K.) at magnifications of ×200 and ×400.

The identification and classification of follicles followed Bloom & Fawcett (1964). Primordial follicles have a large round ovum which is separated from the interstitial tissue by one layer of follicular cells and are primarily found at the

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periphery of the ovary (Fig. 1A). Follicular growth is characterized by enlargement of the ovum and nucleus and multiplication of follicular cells. The primary follicles are differentiated by multiple layers of follicular cells around the ovum (Fig. 1B). Later multiple irregular space, which are filled with the clear liquor folliculi, appear; follicles with just one small liquor filled space were identified as secondary follicles (Fig. 1C), while follicles with multiple spaces were categorized as tertiary follicles (Fig. 1D). Graafian follicles are differentiated by a large liquor filled space which results in the ovum being moved to one side of the follicle. The increase in liquid in the follicle leads to a further increase in size of the follicle and a capsule, the theca folliculi, emerges around the Graafian follicle (Fig. 1E). After rupture of the follicle, the corpus luteum (Fig. 1F) appears with corpus hemorrhagica just being a newly ruptured corpus luteum which is characterized by an accumulation of blood. Later the corpus luteum is reduced to a scar, the corpus albicans. The total number of primary, secondary, tertiary and Graafian follicles as well as corpora lutea, corpora hemorrhagica and corpora albicans were counted throughout the entire ovary. Corpora lutea, corpora hemorrhagica and corpora albicans (hereafter referred to as the corpora bodies) were subsequently combined for further analyses to accommodate for small monthly sample sizes. Primordial follicles were counted in every tenth section through the entire ovary. The number of primordial follicles, therefore, only represents a sub-sample of the total number of primordial follicles in the entire ovary. For analysis, the mean number of follicles and corpora bodies was calculated for both ovaries in an individual to give the relative number of follicles/corpora bodies per female. It was not possible to examine some of the ovarian sections because of logistical problems encountered during the processing of the ovarian tissue (n = 62; see Table 4 for mean monthly sample sizes).

Hormone analysis

Coat-a-count hormone kits (Siemens Medical Solutions Diagnostics, Los Angeles, USA) were used to determine plasma progesterone and plasma testosterone concentrations. These kits use a solid phase radioimmunoassay by which the progesterone or testosterone from the plasma of the individual spiny mouse competes with ^{125}I-labelled progesterone/testosterone for antibody sites on the wall of a tube. The competition is simply enforced by decanting the tube which contains both sample and labelled hormone (Abraham, 1977). The tube is then put in a

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gamma counter where the amount of bound ^{125}I-labelled progesterone or testosterone is counted. The hormone concentration in the sample was obtained using a calibration curve and for more accurate measurements, individual plasma hormone concentrations were counted in two separate tubes. To validate the plasma progesterone and plasma testosterone concentration for *A. spinosissimus*, the slopes of a serial dilution curve and the calibration curve were tested for parallelism with a General Linear Model (GLM) after log-logit-transformation (Chard, 1978). The dilution percentages were used as covariates and the type of curve was employed as a random factor.

Plasma progesterone concentration (nmol/L) was determined in female *A. spinosissimus* for every month throughout the sampling period. Due to the small body mass and, therefore, small blood volume of the spiny mouse, we could not obtain enough plasma for the hormone analysis from some individuals (V = 2 × 100 µl). Two individuals sampled in April and one individual sampled in July were, therefore, excluded from the analyses (n = 64). The assay could be validated for *A. spinosissimus* as there was no significant difference between the serial dilution curve of plasma progesterone with that of the calibration curve ($F_{1,5}$ = 0.37; n = 4; P = 0.57). The intra-assay coefficient of variation for repeated determination of quality control was 5.3 % and the sensitivity of the assay was 0.36 nmol/L.

Plasma testosterone concentration (nmol/L) was determined for 59 male *A. spinosissimus* over the whole study period. Due to the small plasma volume in *A. spinosissimus*, the plasma testosterone concentration could not be established for three males in April, one male in September and two males in December. There was no significant difference between the serial dilution curve of plasma testosterone and the calibration curve ($F_{1,3}$ = 5.87; n = 3; P = 0.09) and the testosterone assay could be validated for *A. spinosissimus*. The sensitivity of the assay was 1.39 nmol/L and the intra-assay coefficient of variation was 1.1 %.

Relative age classes

All males and females were aged using the degree of maxillary molar tooth-wear and eruption. Each individual was initially placed in one of five tooth wear classes as described and illustrated by Dippenaar and Rautenbach (1986) for *A. spinosissimus*.

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None of our animals showed incompletely erupted cheek teeth (i.e., tooth-wear class 1 as defined by Dippenaar and Rautenbach, 1986) and we, thus, only considered the four remaining consecutive tooth-wear classes herein re-categorised as: 1) Tooth-wear class 1 - minimal wear, 2) Tooth-wear class 2 - obvious wear, 3) Tooth-wear class 3 - extensive wear, and 4) Tooth-wear class 4 - severe wear. We can, therefore, be certain that we do not have sub-adult animals in our sample (some of the females with tooth wear class 1 were found to be pregnant). To accommodate for the small monthly sample sizes, we combined tooth-wear classes 1 and 2 and tooth-wear classes 3 and 4 into two broad tooth-wear classes comprising relatively young and relatively old individuals, respectively. Monthly sample sizes for the different relative age classes are presented for males and females separately in Table 1.

Data analysis

Data for same months over the two-year sampling period were combined into one universal 12-month dataset for all subsequent analyses. A GLM was applied to assess differences in log-transformed ovarian volume and square root-transformed data of primordial and primary follicles for the two relative age class groupings and over the 12-month period. Differences between the two relative age class groupings and over the 12 months in all other variables including body mass measured for females and males were evaluated by a Generalized Linear Model (GZLM). Relative age class and month of the year were used as factors and body mass was used as a covariate for all GLM and GZLM analyses. Sex, relative age class and month of the year were applied as factor codes for the body mass analysis. Adjusted R^2 (GLM) or Akaike's Information Criterion (GZLM) were applied as a measure of fit for the model. Tukey's and least significant difference (LSD) *post-hoc* tests followed every GLM and GZLM, respectively. In case of a relationship between a dependent variable and body mass, a Pearson's correlation was performed on parametric data, while a Spearman's correlation was used for non-parametric data. All statistical analyses were performed using the *Statistical Package for the Social Sciences* (SPSS) Statistics version 17.0 (Polar Engineering and Consulting 1993-2007). The results herein are presented as mean ± 1 standard error (SE) and were found to be significant at $P \leq 0.05$.

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Results

Climate data

The rainy season lasted from September until April with the highest rainfall in December (146 mm), while the dry season started in May and terminated in August. Ambient temperatures were highest from September until March with mean monthly temperatures above 20 °C and February being the warmest month (23.5 ± 0.2 °C). Mean monthly temperatures below 20 °C were recorded between April and August, and July was the coldest month (16.2 ± 0.2 °C). Data on rainfall and ambient temperature are shown in Figure 3.

Body mass

There was no significant difference in body mass between males and females (Wald $\chi^2 = 1.21$; $df = 1$; $n = 132$; $P = 0.27$; males: 19.1 ± 0.4 g, females: 19.8 ± 0.5 g). Body mass was, however, significantly different between the relative age classes (Wald $\chi^2 = 39.45$; $df = 1$; $n = 132$; $P < 0.001$) with relatively old individuals (males: 22.0 ± 0.8 g, females: 22.4 ± 0.8 g) being heavier than relatively young individuals (males: 18.2 ± 0.4 g, females: 18.8 ± 0.3 g). Male and female body mass was significantly different between the 12 months (Wald $\chi^2 = 68.50$; $df = 11$; $n = 132$; $P < 0.001$). Female as well as male body mass decreased significantly from January towards March (LSD; males: $P < 0.04$, females: $P < 0.001$), both were lowest in May and increased significantly from June to September (LSD; males: $P < 0.02$, females: $P < 0.01$; Table 1). Female body mass was greatest in December and also significantly larger than male body mass in this month (LSD; $P < 0.001$; Table 1).

Males

Testicular mass and volume, diameter of seminiferous tubules and plasma testosterone concentration was significantly different between the 12 months (Wald $\chi^2 > 144.62$; $df = 11$; $n = 65$; $n_{Testosterone} = 59$; $P < 0.001$). There was a significant interaction between the 12 months and the relative age classes for testicular volume and mass and seminiferous tubule diameter (Wald $\chi^2 > 26.16$; $df = 7$; $n = 65$; $P < 0.001$; Fig. 2A - C) as well as plasma testosterone concentration (Wald $\chi^2 = 37.49$; $df = 6$; $n = 59$; $P < 0.001$; Fig. 2D). Testicular volume and mass as well as seminiferous tubule diameter started to increase significantly from June to July (LSD; relatively young males: $P \leq 0.02$, relatively old males: $P < 0.001$), were highest from about

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September (peak) until December and decreased significantly from January to February (LSD; relatively young males: $P < 0.001$; Fig. 2A - C). Plasma testosterone concentration was low from January until July, increased significantly from July to August in relatively older males (LSD; $P = 0.01$) and from July to September in relatively younger males (LSD; $P < 0.05$), peaked in September (relatively young males) and dropped significantly from December to January (LSD; all: $P \leq 0.01$; Fig. 2D). Plasma testosterone concentration did not differ significantly between relatively young and relatively old males (Wald $\chi^2 = 0.69$; $df = 1$; $n = 59$; $P = 0.41$), whereas testes mass and volume and seminiferous tubule diameter were significantly different between the relative age classes (Wald $\chi^2 > 4.52$; $df = 11$; $n = 65$; $P < 0.04$) being significantly larger in relatively old males than in relatively young males (LSD; $P < 0.001$). In relatively young males, testicular mass and volume were 58.1 ± 11.2 mg and 53.6 ± 10.7 mm³, respectively and 165.8 ± 21.8 mg and 147.4 ± 20.7 mm³, respectively in relatively old males. Diameter of seminiferous tubules was 113.3 ± 7.9 µm in relatively young males and 177.1 ± 11.6 µm in relatively old males. Plasma testosterone concentration was not dependent on body mass (Wald $\chi^2 = 2.88$; $df = 1$; $n = 59$; $P = 0.09$), but there was a significant relationship between male body mass and testicular mass and volume as well as seminiferous tubule diameter (Wald $\chi^2 > 35.80$; $df = 1$; $n = 65$; $P < 0.001$). Testicular mass and volume as well as the diameter of the seminiferous tubules increased with increasing male body mass ($\rho > 0.72$; $n = 65$; $P < 0.001$).

Females

Pregnant females were detected from September through to January, while no pregnant females were encountered from February until August. Females were found to have two to four embryos (mean = 3.3) and between two to six placental scars (mean = 3.5) were found in the uterine horns (Table 2). Females with placental scars were collected mainly from September through to March (Table 2). Evidence of lactation was observed in some female *A. spinosissimus* from October until April (Table 2).

Ovarian volume was significantly different across the 12 months ($F_{11,53} = 9.28$; $n = 67$; $P < 0.001$), but it did not differ significantly between the relative age classes ($F_{11,53} = 0.17$; $n = 67$; $P = 0.68$). There was a significant relationship between ovarian

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volume and female body mass ($F_{1,53}$ = 5.27; n = 67; P = 0.02). Ovarian volume increased with increasing body mass (r = 0.68; n = 67; P < 0.001). The standard residuals for ovarian volume were calculated to account for the relationship between ovarian volume and body mass and a Tukey's post-hoc test was performed on these. Ovarian volume was significantly greater in October than in February, March and April (Tukey's test; P < 0.01) and it decreased significantly from January to March (Tukey's test; P = 0.002; Fig. 3).

There was a significant difference in plasma progesterone concentration across the 12 months (Wald χ^2 = 82.64; df = 11; n = 64; P < 0.001) and an interaction between the month of the year and relative age classes (Wald χ^2 = 44.19; df = 8; n = 64; P < 0.001). There was, however, no significant difference in plasma progesterone concentration between the relative age classes alone (Wald χ^2 = 0.62; df = 1; n = 64; P = 0.43). LSD tests demonstrated a significant rise in progesterone concentration from the levels found between February and August to October (P < 0.05), while plasma progesterone concentration decreased significantly from October to December and from January to March (P < 0.05; Fig. 4). In September, relatively old females were found to have higher plasma progesterone levels than the relatively young females, whereas in December, the opposite was observed (LSD; $P \leq 0.05$; Fig. 4). However, these results may be a reflection of the small sample sizes as mainly relatively old pregnant females were collected in September, while predominantly relatively young pregnant females were collected in December (Table 1). Plasma progesterone concentration was statistically directly proportional to body mass (Wald χ^2 = 5.56; df = 1; n = 64; P = 0.02).

Body mass significantly influenced the numbers of primordial ($F_{1,48}$ = 8.11; n = 62; P < 0.01) as well as Graafian follicles (Wald χ^2 = 4.00; df = 1; n = 62; P < 0.05), but not the number of primary ($F_{1,48}$ = 1.42; n = 62; P = 0.29), secondary and tertiary follicles, and corpora bodies (Wald χ^2 < 2.56; df = 1; n = 62; P > 0.11). Except for primordial follicles, the numbers of all other follicles and corpora bodies were significantly different between the 12 months (Table 3). The numbers of primordial, primary and secondary follicles were significantly different between relative age classes, while the number of tertiary and Graafian follicles as well as corpora bodies were not significantly different between relative age classes (Table 3). The number of

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primordial follicles decreased with increasing body mass (r = -0.63; n = 62; P < 0.001) and relatively young females had significantly more primordial follicles than relatively old females (young individuals: 413.5 ± 29.8, old individuals: 138.9 ± 16.8; Fig. 5).

The number of primary follicles was significantly higher from October to January compared with February to May (Tukey's test; P < 0.05) and increased significantly between May and June (Tukey's test; P < 0.05; Table 4). The number of secondary follicles was lowest in April and highest in October (Table 4). The number of primary and secondary follicles was higher in relatively young than in relatively old females (primary/young: 33.8 ± 2.9, primary/old: 28.2 ± 3.9 and secondary/young: 2.6 ± 0.4, secondary/old: 2.1 ± 0.6). There was a significant difference in the number of tertiary follicles from April to June compared with July to January (LSD; P < 0.05) with no tertiary follicles being recorded in April and the highest number being recorded in January (Table 4). The number of tertiary follicles decreased significantly from January to February (LSD; P < 0.05) and increased significantly from June to July (LSD; P < 0.05; Table 4). The number of Graafian follicles was positively correlated with body mass (ρ = 0.46; n = 62; P < 0.001). No Graafian follicles were recorded from March to June and most Graafian follicles were found in December (Table 4). We did not find any corpora bodies between May and August and the numbers were significantly lower in March and April compared to September through to January (LSD; P < 0.01), while most corpora bodies were recorded in January (Table 4). None of the follicle types or corpora bodies demonstrated an interaction between month of the year and relative age classes (Table 3).

Discussion

The present study confirms and extends previous evidence that *A. spinosissimus* breeds seasonally with the onset of reproduction at the beginning of the rainy season in September and reproduction terminating with the last pregnancies in January. The breeding season reaches its peak in October with the highest incidence of pregnancies, largest ovarian volume and highest plasma progesterone concentrations recorded in females. In males, however, the breeding season peaks one month earlier, in September, when the testes and seminiferous tubules reached their maximum size and plasma testosterone concentration was highest.

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Furthermore, an increase in testes size was already observed from July onwards. Male reproduction also terminated earlier than female reproduction with testes being significantly smaller in February and a significant decrease in testosterone concentration being observed in January compared to the previous months. Ovarian volume, on the other hand, decreased slowly from January towards March and plasma progesterone concentration in females dropped from January to February and was very low from February until August which coincided with an absence of gravid females in our data. Lactating females were, however, found from October until April and no evidence of lactation was observed from May until September with one exception in June. The lactating female recorded in June was found to have placental scars in the uterine horns implying that it may have given birth earlier in June. The numbers of primary, secondary, tertiary and Graafian follicles as well as corpora bodies corresponded with the seasonal reproduction of *A. spinosissimus.* The slight shift of reproduction in males compared to females may be due to the time it takes for the males to achieve maximum growth of the testes (to almost 18 times the testes size in September compared to March) which has to be completed by the time females are ready to reproduce.

Our data on seasonality of reproduction correspond partly with the results found by Sheppe (1973) for *A. spinosissimus* in Zambia and Smithers (1971) in Botswana. Both studies demonstrated a breeding season for *A. spinosissimus* during the warm and wet summer months, however, both recorded pregnant females until March and April and Sheppe (1973) also observed some breeding males until March. This difference to our results might be explained by the more northern populations examined by Sheppe (1973) and Smithers (1971) where a prolonged reproductive season may be achieved through increased temperatures and/or prolonged rainy seasons and longer vegetation periods. In addition, we collected lactating females until April indicating that females might have been giving birth up until early April and it is probable that we just did not sample pregnant females during these months despite their presence in the population. The significant decrease of ovarian volume, primary, tertiary and Graafian follicle numbers as well as corpora bodies from January to February/March, however, indicates a decline in reproductive activity after January. We used swollen teats as an indicator of lactation which may have been

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misleading, since they still might have been pronounced although the females may not have been lactating anymore.

The seasonal reproductive pattern of *A. spinosissimus* is similar to that found in another *Acomys* species north of the equator. *Acomys minous* from Crete breeds during the northern hemisphere spring (March–May) and summer months (June-August) (Dieterlen, 1978). The Northeast African spiny mouse, *Acomys cahirinus*, from Sudan has also been found to breed seasonally with the breeding season commencing about one to three months after the rain (Happold, 1966). In contrast, *A. percivali* and *A. wilsoni* from central Kenya breed throughout the year, although they occur in a strongly seasonal habitat with two distinct rainy seasons (Neal, 1983). Interestingly, *A. subspinosus* from the Western Cape Province of South Africa, the closest geographical congener of *A. spinosissimus*, is an opportunistic breeder that is able to reproduce during the winter months (June-August) by utilizing the flowering *Protea humiflora* as a food source (Fleming & Nicolson, 2002).

The mean litter size of 3.3 (range = 2-4) found in this study, is slightly higher than that recorded in other studies on *A. spinosissimus*. Mean litter size of *A. spinosissimus* was 3.0 (range = 2-5) in Botswana (Smithers, 1971) and 2.6 (range = 2-5) in Zambia (Sheppe, 1973). This small litter size is unusual for rodents, but still higher than that reported for other *Acomys* species. The mean number of embryos was determined to be 1.97 in *A. wilsoni* and 1.54 in *A. percivali* (range = 1-5; Neal, 1983) and the mean litter size recorded for *A. minous* and *A. dimidiatus* is 2.4 for both species (range = 2-4 for *A. minous*, and 1-5 for *A. dimidiatus*; Dieterlen, 1961, 1978). The larger litter size in *A. spinosissimus* may be due to their young being born more altricial (K. Medger, personal observation) compared to other spiny mice such as *A. dimidiatus* and *A. minous* (Dieterlen, 1961, 1978). In addition, *A. subspinosus* has been found to have a litter size of three and gives birth to semi-precocial young similar to *A. spinosissimus* (Dempster *et al.*, 1992). Although the mean number of placental scars (3.5; range = 2-6) was only slightly higher than the mean litter size in this study, some female *A. spinosissimus*, especially at the end of the breeding season (March/April) had a larger number of placental scars which indicates that these animals may possibly have had up to two litters during the reproductive season. The semi-precociality of the young, small litter size and two litters per year

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altogether suggest that *A. spinosissimus* is a *K*-selected species as has also been described for other *Acomys* species (Neal, 1983).

Acomys spinosissimus initiated breeding exactly in synchrony with the onset of the rainy season and, therefore, the main environmental factor influencing reproductive seasonality of this small mammal in South Africa appears to be rainfall, probably combined with an increase in primary productivity and higher food quantity and quality. Many small tropical and sub-tropical mammals reproduce during the rainy season when there is the highest abundance of food resources. In the French Guiana rainforests, two large rodent species, the acouchy (*Myoprocta exilis*) and the agouti (*Dasyprocta leporine*), reproduce during the time when most fruits are available which is linked to rainfall even in a rainforest (Dubost *et al.*, 2005). Neal (1977) established the importance of rainfall for reproduction in the Natal multimammate mouse (*Mastomys natalensis*) from Uganda. However, food abundance can also influence reproduction independently of rainfall (Neal, 1991). In South Africa, seasonal rainfall in combination with food abundance has been found to influence the breeding of a number of rodent species. Indeed, the Namaqua rock mouse, *Micaelamys namaquensis* (formerly *Aethomys namaquensis*; Skinner & Chimimba, 2005) (Muteka *et al.*, 2006b) as well as the bushveld gerbil, *Gerbilliscus leucogaster* (formerly *Tatera leucogaster*; Musser & Carleton, 2005) (Perrin & Swanepoel, 1987) breed during the rainy summer months in the northern parts of South Africa.

As rainfall in combination with food quality and quantity might be the main factors regulating seasonal reproduction in *A. spinosissimus*, other factors such as temperature and photoperiod might also be important. *Acomys spinosissimus* is a very small rodent with a mean body mass of 19.5 ± 0.3 g (this study) and we also found that male and female body mass is significantly reduced during autumn and winter months (March – May). Therefore, the low environmental temperatures of below 20 °C recorded from April to August might put high energetic constraints on this small mammal which, in turn, might prevent it from breeding during the winter period when more energy is needed for thermogenesis, cellular maintenance and immune functions (Klein & Nelson, 1999). In the Drakensberg Mountains of the KwaZulu-Natal Province, South Africa, some rodent populations at higher altitudes

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start breeding later than at lower altitudes due to lower temperatures, although reproduction is otherwise dependent on the rainy season and the subsequent increase in food availability (Rowe-Rowe & Meester, 1982). Furthermore, photoperiod has been recorded to influence breeding in South African rodent species (Muteka *et al.*, 2006a) and there are initial indications that male *A. spinosissimus* may be reproductively photoresponsive (Chapter 3).

Primordial follicle number was the only variable recorded which did not differ throughout the year. However, primordial follicle numbers were higher in relatively younger than in relatively older females and decreased with increasing body mass which might be a result of older females being heavier than younger females. In the female human, monkeys, and rodents, it has been reported that the numbers of germ cells decrease substantially with female age and new follicles are only recruited from an initial follicle pool which proliferates around the time of birth (Broekmans *et al.*, 2009; Nozaki *et al.*, 1997; Peters *et al.*, 1962). The gradual decrease in fertility with the shrinking follicle pool finally results in menopause or reproductive senescence. Bristol-Gould *et al.* (2006) developed models to explain the phenomenon of follicle number decrease in female mammals and compared these to findings in primordial, primary, and secondary follicle number reduction in mice. They found that a fixed pool model, meaning all germ cells are present at the time of birth and no new ones are recruited afterwards, best explains the observed decrease of primordial, primary and secondary follicle numbers. This may also explain the slight effect that age has on the primary and secondary follicles in this study. Therefore, as no new primordial follicles are recruited in the course of the year, no monthly differences in primordial follicle numbers should be observed, even within a seasonally breeding mammal species.

In conclusion, *Acomys spinosissimus* reproduces seasonally during the warm and wet summer months in South Africa. The most important factor influencing reproduction in this small rodent appears to be rainfall that brings about a concomitant increase in food quality and quantity. A number of other factors such as temperature and photoperiod are additional factors which may influence seasonal reproduction, both on their own and in combination with rainfall and food availability.

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The number of primordial follicles is, however, not affected by the seasons, but is dependent on the age of the female spiny mouse.

This study has raised a number of questions as to the potential cues that may be influencing seasonal reproduction in *A. spinosissimus* and to gain insights into the seasonality of reproduction in this rodent and other small mammals from tropical and sub-tropical regions more information is required. Although *A. spinosissimus* seems to be common and is listed as a species of Least Concern (LC) in terms of conservation in the *Red Data Book of the Mammals of South Africa* (Friedmann & Daly, 2004), this species is not very common throughout its range and is restricted to mountainous habitat. The fragmented habitat (Friedmann & Daly, 2004), anthropogenic influences and deficient knowledge on its general biology and ecology might pose future threats to the conservation of this species. It is thus important to learn as much as possible about this species to be able to avoid future threats to its survival especially in the southern African subregion.

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Tables

Table 1. Monthly sample sizes (*n*), sample sizes for two relative age classes (relatively young and relatively old individuals) and mean body mass (g) ± 1 standard error (*SE*) and body mass ranges (g) of female and male *Acomys spinosissimus* from South Africa over 12 months between 2007 and 2008.

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Females												
n	5	7	7	8	6	4	6	5	4	6	3	6
Young	3	6	7	6	6	3	6	4	1	2	2	5
Old	2	1	0	2	0	1	0	1	3	4	1	1
Mean	23.5	20.7	18.1	18.2	15.7	16.6	16.5	17.5	22.6	22.3	23.1	25.4
SE	1.1	1.4	0.6	1.2	0.8	1.2	0.9	0.7	1.7	1.3	4.4	1.1
Range	20 - 26	15 - 25	16 - 20	15 - 24	14 - 19	14 - 20	14 - 20	16 - 20	19 - 27	19 - 26	16 - 31	22 - 28
Males												
n	6	5	5	8	8	9	3	4	5	3	1	8
Young	5	5	5	7	6	7	1	2	2	0	1	5
Old	1	0	0	1	2	2	2	2	3	3	0	3
Mean	20.8	18.7	17.9	17.0	17.4	18.0	20.1	19.7	22.6	19.9	20.1	20.5
SE	1.3	0.8	0.3	1.4	0.6	0.8	1.9	1.7	2.0	0.6		0.6
Range	18 - 24	17 - 22	17 - 19	12 - 24	15 - 21	15 - 22	16 - 22	16 - 24	18 - 28	19 - 21		19 - 24

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Table 2. Monthly number of females with embryos and placental scars, average number of embryos and placental scars and number of lactating females of *Acomys spinosissimus* from South Africa sampled over a 12-month period between 2007 and 2008.

	Summer		Autumn			Winter			Spring			Summer
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Females with embryos	1	0	0	0	0	0	0	0	1	4	2	3
Average number of embryos	3	0	0	0	0	0	0	0	2	3.5	3.5	3.3
Females with placental scars	4	4	4	1	0	1	0	0	1	2	0	3
Average number of placental scars	3.3	3.8	3.8	6	0	2	0	0	3	2	0	3.7
Lactating females	3	6	4	1	0	1	0	0	0	6	2	6

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Table 3. Results of general (*F*-value) and generalized linear models (Wald χ^2) for numbers of primordial, primary, secondary, tertiary and Graafian follicles, and corpora bodies between months, age classes and the interaction between month and age classes for *Acomys spinosissimus* sampled over a 12-month period in 2007 and 2008. Variables which were evaluated by generalized linear model are indicated with an asterisk (*). Significant *P*-values are highlighted in bold.

	Months			Age classes			Months*age classes		
	F or Wald χ^2	*df*	*P*	*F* or Wald χ^2	*df*	*P*	*F* or Wald χ^2	*df*	*P*
Primordial follicles	1.38	11,48	0.21	7.15	1,48	**0.01**	0.84	7,41	0.56
Primary follicles	7.12	11,48	**< 0.001**	7.20	1,48	**0.01**	1.16	7,41	0.34
Secondary follicles*	63.08	11	**< 0.001**	7.28	1	**0.007**	6.50	7	0.48
Tertiary follicles*	77.39	11	**< 0.001**	0.52	1	0.47	5.79	7	0.56
Graafian follicles*	57.71	11	**< 0.001**	2.47	1	0.12	7.03	7	0.43
Corpora bodies*	155.50	11	**< 0.001**	0.13	1	0.71	9.03	7	0.25

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Table 4. Monthly sample sizes (*n*), mean numbers and standard errors (*SE*) for primary, secondary, tertiary and Graafian follicles and corpora bodies (corpora lutea, corpora albicans and corpora hemorrhagica combined) of *Acomys spinosissimus* from South Africa over 12 months in 2007 and 2008.

Follicle	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
n	5	7	6	8	4	3	5	5	4	6	3	6
Primary												
Mean	37.5	20.1	20.8	13.6	17.8	32.5	39.6	39.8	31.9	47.6	41	58
SE	4.7	2.1	4	3.7	3.3	0.5	8.3	8	5.1	8.2	10.6	9.3
Secondary												
Mean	2.4	2	1.1	0.9	1.1	2.2	1.6	1.7	1.3	6	4	5.1
SE	0.8	1	0.5	0.6	0.1	0.2	0.3	0.6	0.5	1.6	1.2	1
Tertiary												
Mean	4.9	2	0.7	0	0.1	0.3	2	3.5	2.8	3.5	2.8	2.8
SE	1.1	0.7	0.6	0	0.1	0.3	0.5	0.4	1.2	1.2	1.7	0.6
Graafian												
Mean	0.9	0.4	0	0	0	0	1.1	0.6	0.8	1.3	1.5	1.6
SE	0.3	0.3	0	0	0	0	0.4	0.2	0.3	0.5	0.8	0.2
Corpora bodies												
Mean	2.7	1.6	0.2	0.2	0	0	0	0	1	2	1.5	2.6
SE	0.3	0.3	0.2	0.2	0	0	0	0	0.4	0.1	0.3	0.4

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Figure legends

Fig. 1. Stages of follicular growth in the ovaries of *Acomys spinosissimus*. A: primordial follicles, B: primary follicle, C: secondary follicle, D: tertiary follicle, E: Graafian follicle, F: corpora lutea. The images were taken with magnifications of ×40 (A, B), ×16 (C, D) and ×10 (E, F).

Fig. 2. Box-plots showing mean monthly testicular mass (mg) and volume (mm^3), diameters of seminiferous tubules (µm) and plasma testosterone concentration (nmol/L) of male *Acomys spinosissimus* from South Africa sampled over 12 months between 2007 and 2008. All variables are presented separately for relatively young and relatively old males. A: testicular mass, B: testicular volume, C: seminiferous tubule diameter, D: plasma testosterone concentration.

Fig. 3. Standard residuals for ovarian volume (mm^3) by body mass (g) of *Acomys spinosissimus* from South Africa averaged over 12 months in 2007 and 2008. Values are mean ± 1 standard error (*SE*). Statistical significance: * = $P < 0.05$; ** = $P < 0.01$. The insert illustrates total rainfall (mm) and mean monthly ambient temperature (°C) over the same 12 months.

Fig. 4. Box-plot showing monthly plasma progesterone concentration (nmol/L) of female *Acomys spinosissimus* from South Africa averaged over 12 months in 2007 and 2008. Plasma progesterone concentration is displayed separately for relatively young and relatively old females.

Fig. 5. Relationship between the number of primordial follicles and body mass (g) of female *Acomys spinosissimus* from South Africa. Values are presented separately for relatively young and relatively old females.

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Figures

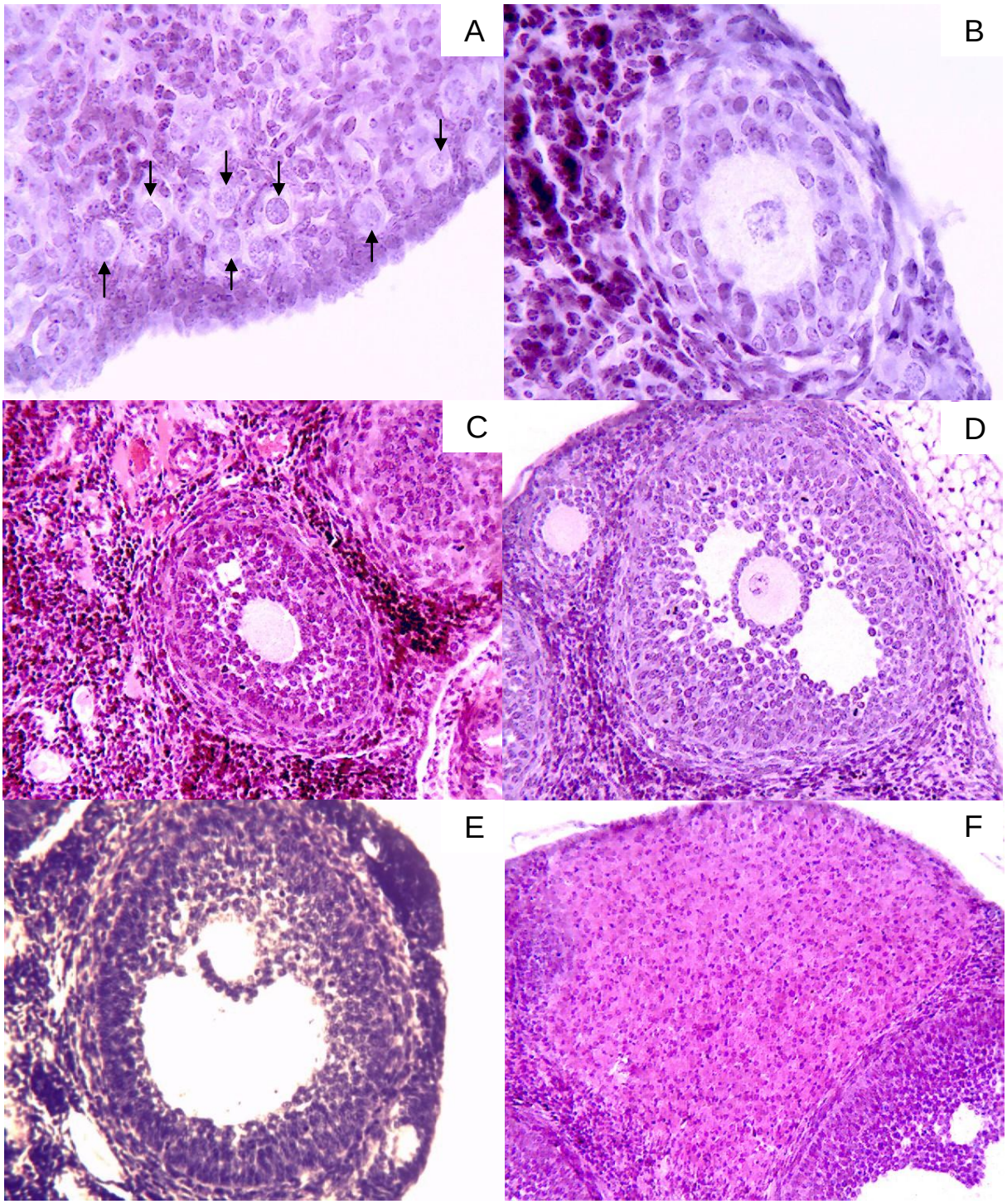

Figure 1.

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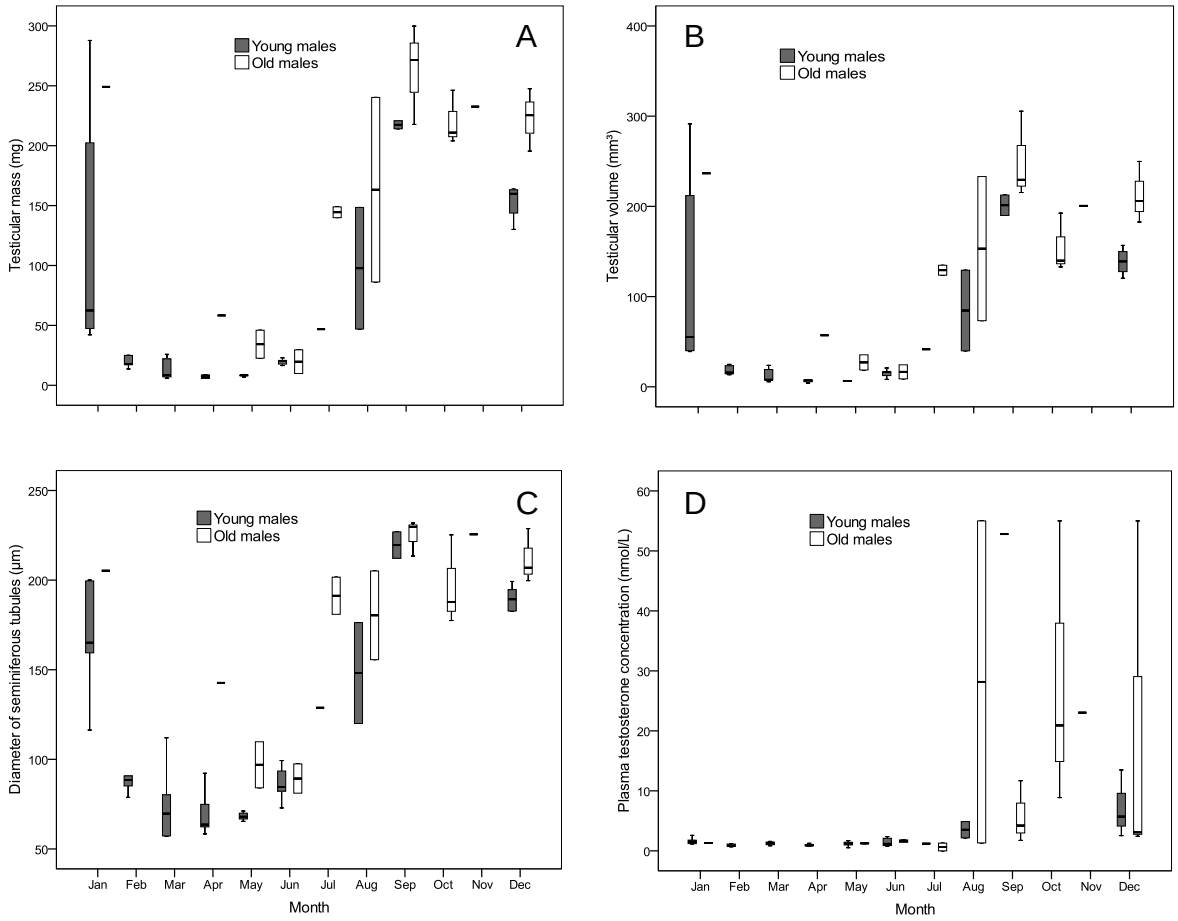

Figure 2.

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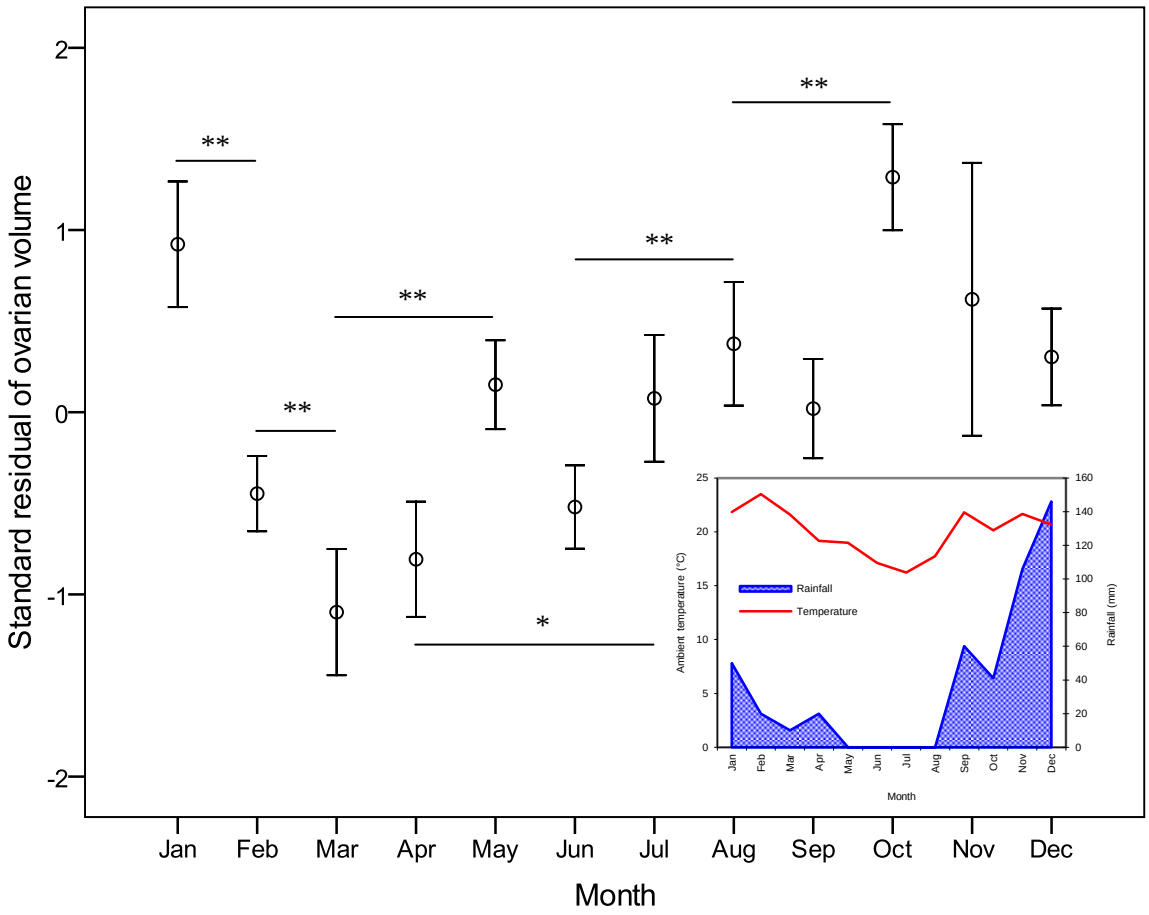

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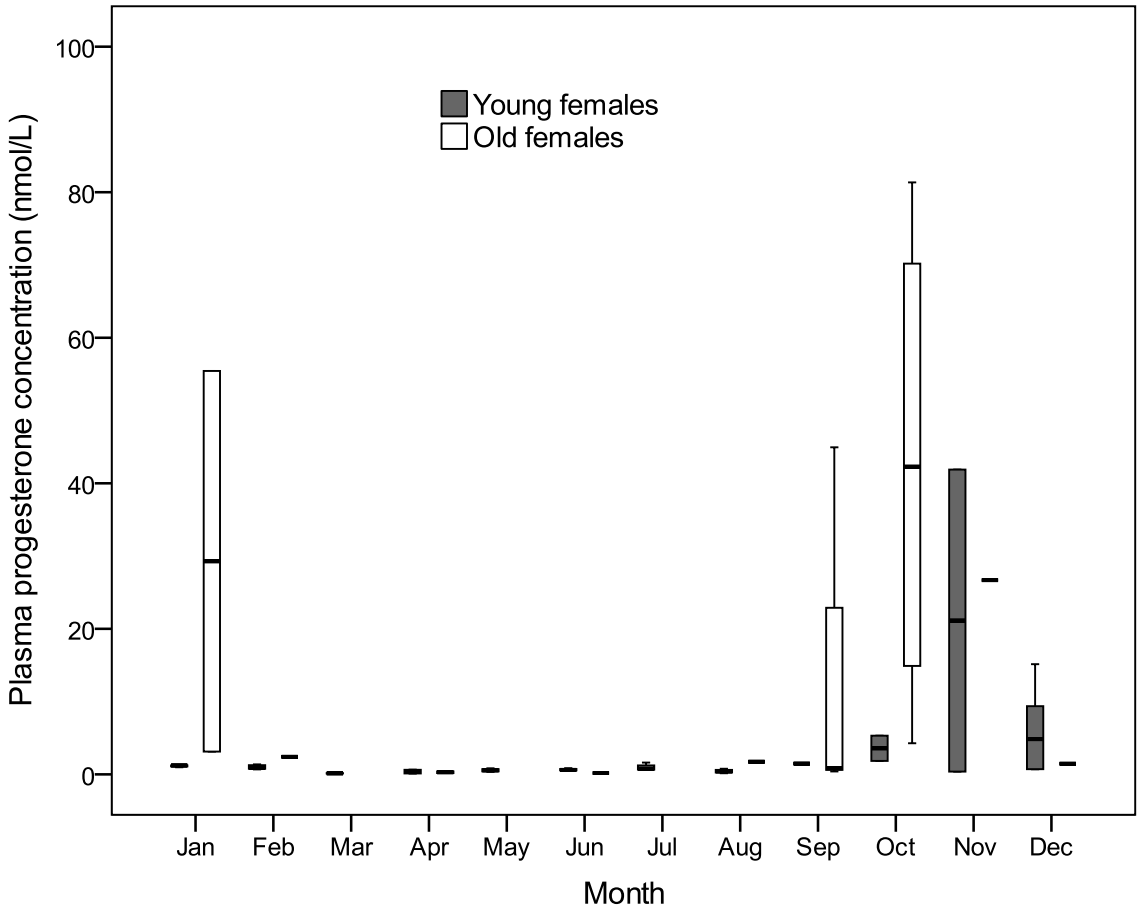

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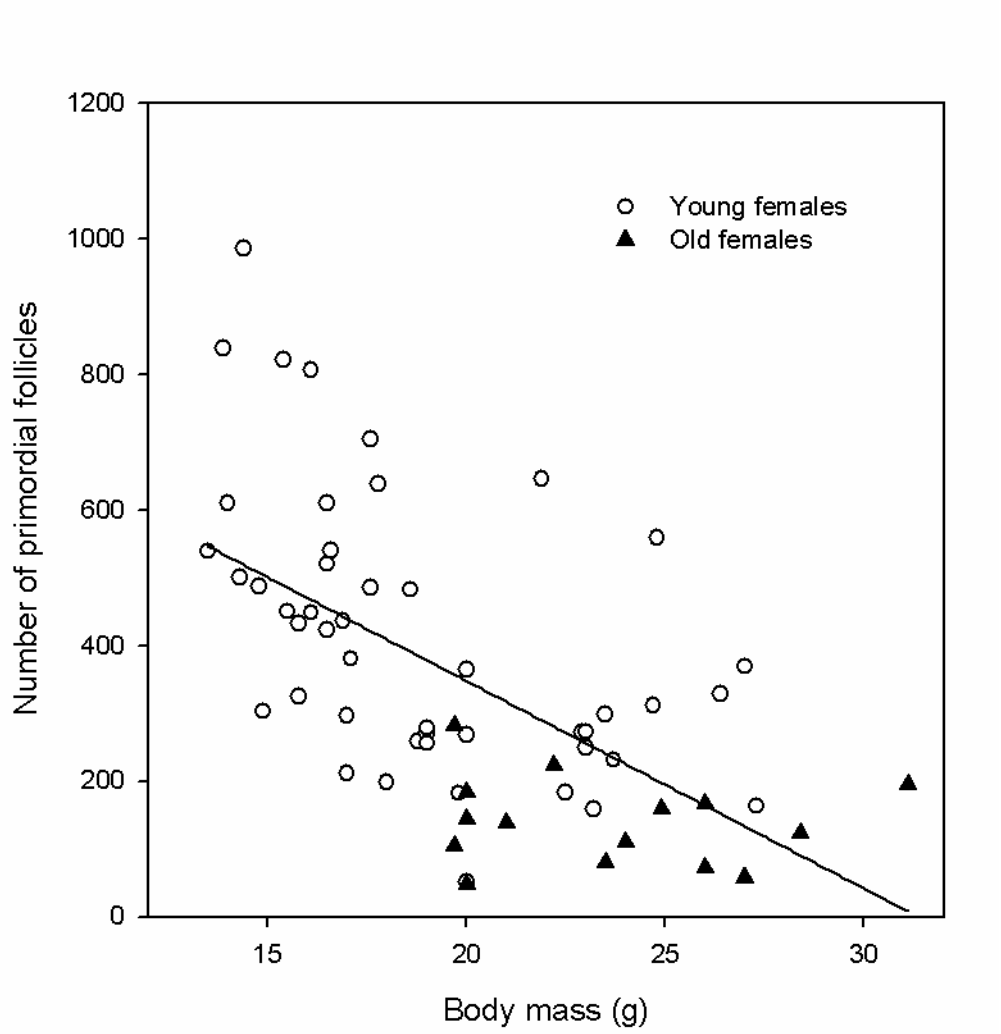

Figure 5.

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CHAPTER 2

SEASONAL REPRODUCTION IN MALE AND FEMALE EASTERN ROCK ELEPHANT-SHREWS (*ELEPHANTULUS MYURUS*) FROM SOUTH AFRICA

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Abstract

Environmental conditions vary throughout the year in most regions and, therefore, many small mammals reproduce at a specific time of the year to maximize reproductive success. In the tropics and sub-tropics, the breeding season is usually determined by the extent of the dry and rainy season and in many species, it was found that rainfall combined with the increase in food quantity and quality determines seasonal reproduction. We investigated the seasonality of reproduction in male and female Eastern rock elephant-shrews (*Elephantulus myurus*) from South Africa over a 12-months period by histological examination of the gonads and by measuring plasma testosterone and progesterone concentrations. In addition, animals were categorized into six relative age classes using the degree of maxillary molar tooth-row eruption and wear. Pregnant females were collected from August through to January and ovary size and plasma progesterone started to increase from July to August, peaked in October and regressed progressively thereafter. Follicular growth corresponded to this seasonal pattern of reproduction and Graafian follicles as well as corpora hemorrhagica, corpora lutea and corpora albicans were found from August until January, but not in the remaining months. Testes mass and volume, seminiferous tubule diameter and plasma testosterone concentration increased significantly from May and were highest from August until October after which they decreased. The distribution of relative age classes throughout the year confirmed that *E. myurus* is a seasonal breeder. In conclusion, *E. myurus* breeds seasonally during the warm and wet spring and summer months and stops breeding during the cold and dry winter months of the southern hemisphere. We suggest that seasonal reproduction evolved in *E. myurus* because of the severe seasonal changes in rainfall and ultimately food availability.

Keywords: Macroscelidea, follicular growth, food availability, rainy season, relative age, southern hemisphere

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Introduction

Reproduction is costly (Partridge & Harvey, 1985) and, therefore, most mammals breed only during a specific time of the year when environmental conditions are favourable. However, many small mammals are opportunistic and may breed during harsh conditions as long as prolonged or year-round breeding offsets the costs and ultimately maximizes reproductive success (Beer & MacLeod, 1961; Scheffer, 1924). In the tropics, many mammals breed throughout the year because environmental conditions are more stable than in temperate regions. Seasonal reproduction has, however, also been reported and often occurs in conjunction with seasonal variation in rainfall (Delany, 1971; Delany & Neal, 1969). For example, the multimammate mouse (*Mastomys natalensis*) was found to breed during the rainy season, but to cease breeding during the dry season in several countries throughout Africa such as Uganda, Tanzania, Swaziland and South Africa (Bronner *et al.*, 1988; Delany & Neal, 1969; Leirs *et al.*, 1996; Monadjem, 1998; Neal, 1977).

Many temperate species, particularly *Peromyscus* species (Trainor *et al.*, 2006), hamsters (Hegstrom & Breedlove, 1999; Prendergast *et al.*, 2001) and voles (Kerbeshian *et al.*, 1994) have been traditionally used to unravel the mechanisms of seasonal reproduction. On the other hand, the reproductive strategies of many tropical and sub-tropical mammals are still largely unknown (Bronson, 2009) and although a number of African rodent species have been investigated, many other species have been largely ignored. In southern Africa, seasonal as well as aseasonal and opportunistic breeding has been reported in small mammals. For example, the Namaqua rock mouse (*Micalaemys namaquensis*) and the Tete veld rat (*Aethomys ineptus*) breed during the spring and summer months (Muteka *et al.*, 2006b; Muteka *et al.*, 2006c), whereas the four-striped field mouse (*Rhabdomys pumilio*) breeds year-round in environments with mild winters and seasonally under harsh conditions (Jackson & Bernard, 2006).

Many different factors have been attributed to influence and shape seasonal reproduction. Photoperiod, the seasonally changing day/night cycle, is the most reliable at latitudes above 30° and is, therefore, mostly utilized by small mammals from temperate regions as a cue to predict events distant in time (Bradshaw & Holzapfel, 2007), but also sub-tropical rodents have been found to be reproductively

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photoresponsive (Muteka *et al.*, 2006a; Chapter 3). However, rainfall in conjunction with an increase in food quantity and quality appears to be the major factor which controls seasonal reproduction at sub-tropical and tropical latitudes especially in habitats with distinct wet and dry seasons (Taylor & Green, 1976; Tinney *et al.*, 2001). Ambient temperature, although less investigated, has likewise been found to influence reproduction. In the Nile grass rat (*Arvicanthis niloticus*), low temperatures stimulate testicular activity, whereas breeding is inhibited by high temperatures (Sicard *et al.*, 1993)

The members of the family Macroscelidea, the elephant-shrews or sengis, are unique among the small mammals of Africa and are endemic to this continent (Skinner & Chimimba, 2005). Most elephant-shrew species have been found to be monogamous (FitzGibbon, 1997; Rathbun & Rathbun, 2006; Ribble & Perrin, 2005; Schubert *et al.*, 2009), give birth to precocial young and have distinct male and female reproductive systems (Tripp, 1971; van der Horst, 1951; van der Horst & Gillman, 1941a; Woodall, 1995). Seasonal as well as aseasonal reproduction has been reported in various species of elephant-shrews (Neal, 1995). The Eastern rock elephant-shrew (*Elephantulus myurus*) has been documented to breed seasonally (Stoch, 1954; van der Horst, 1946, 1954; Woodall & Skinner, 1989). However, these studies, although often extensive, have either not taken environmental factors into account, pooled data from different localities and months or have not investigated reproduction over an entire year which makes it difficult to interpret the reproductive patterns of this species. We, therefore, investigated the seasonality of reproduction of *E. myurus* on a finer scale and over an entire year and also measured environmental factors such as temperature and rainfall in its habitat. In doing so, the present study attempts a first understanding of the ecological significance and the evolution of seasonal reproduction in this elephant-shrew. We further aim to characterize environmental factors which may shape seasonal reproduction in *E. myurus.*

The habitat of *E. myurus* is characterized by seasonal rainfall with the rainy season in the spring and summer months and a severe dry period in autumn and winter. We hypothesized that *E. myurus* breeds seasonally during the warm and wet spring and summer months (September – February) and ceases breeding during the cold and dry months of the year (March – August) of the southern hemisphere when

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conditions are less advantageous for reproduction and reproductive success may be low.

Materials and Methods

General

The study was carried out at Goro Game Reserve in the Soutpansberg region of the Limpopo Province, South Africa (22°58'S, 22°57'S; 29°25'E, 29°24'E). The elephant-shrews were trapped with Sherman live traps (H. B. Sherman Traps, Inc. Tallahassee, Florida, U.S.A.) baited with a mixture of oats, peanut butter and fish and set overnight around the rocky outcrops of the reserve. Eastern rock elephant-shrews were captured at the end of every month from September 2007 until August 2008. With the exception of September when only four males and six females were collected and November when only three individuals of both sexes were collected, five males and five females were collected each month. Hence a total of 59 females and 57 males were captured over the entire study period. On the day of capture, all animals were weighed to the nearest 0.001 g using a digital balance (Scout Pro SPU123, Ohaus Corporation, Pine Brook, New Jersey, U.S.A.) and body mass (g) was subsequently rounded to 0.1 g for further analyses. During transport and in the laboratory, elephant-shrews were housed in polyurethane cages containing wood shavings and fed with Pronutro (high protein cereal; Pioneer Foods Ltd., Bokomo Foods, Cape Town, South Africa), canned dog food (Promeal Ltd., Dassenberg, South Africa) and grated apples or carrots. Fresh food and water were provided daily in open dishes. In addition, meal worms were fed, especially in cases where animals did not want to feed. Animals were collected under permit number: CPM-333-00002 from the CITES and Permit Management Office, Department of Environmental Affairs, Limpopo Province. This study was approved by the animal ethics committee of the University of Pretoria (ethics clearance number: EC028-07).

Monthly rainfall (mm) and ambient temperature (°C) were recorded throughout the study period. Rainfall data were provided by the Goro Game reserve and two iButton digital temperature data loggers (Maxim Integrated Products, Dallas Semiconductor, U.S.A.) were used to measure ambient temperature to the nearest 0.01 °C. The iButtons were placed near the ground and protected from direct sunlight

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and measurements were taken every two hours, from which the mean monthly ambient temperature was calculated.

All elephant-shrews were euthanized with an overdose of halothane after they were kept in the laboratory for one to three days. The gonads of both males and females were dissected out and blood was obtained by exsanguinations from the heart. The blood was centrifuged at 3000 rpm for 15 mins and the blood plasma was stored at -35 °C until hormone analysis. The entire female reproductive tract was removed and any embryos or placental scars in the uterine horns were recorded. Female bodies were also examined for milk in the mammary glands because swollen teats, as a sign for lactation, were difficult to observe. The gonads were fixed in Bouin's fluid for approximately 20 hrs and then stored in 70 % ethanol. Skulls were prepared by boiling and subsequent removal of any tissue with tweezers and finally used to determine the relative age of an individual using the degree of maxillary molar tooth-wear and eruption (DeBlase & Martin, 2001) (see "Relative age classes" section below). All specimens will be deposited in the mammal reference collection at the Ditsong National Museum of Natural History, Pretoria, South Africa.

Histology

After the removal of fat and connecting tissue, fixed ovaries and testes were weighed to the nearest 0.0001 g using a high precision scale (Ohaus Corp. Pine Brook, N.Y., U.S.A.) and the length and width were measured (mm) using a pair of digital calipers (Sylvac Opto RS 232, Ultra Praezision Messzeuge GmbH, Germany). Gonadal length and width were used to calculate ovarian and testicular volume (mm^3) by using the formula for the volume of an ellipsoid as follows: $V = 4/3\ \pi ab^2$ where a represents half the maximum length and b half the maximum width (Woodall & Skinner, 1989). The gonads were subjected to a series of histological preparations, including dehydration and embedding in wax. Subsequently, both testes and ovaries were cut into 7 µm-thick sections with a rotary microtome (820 Spencer, American Optical, Scientific Instrument Division, Buffalo, N.Y., U.S.A.); while only a few sections were cut from the testes, each ovary was cut in its entirety. Sections were mounted in consecutive order on microscope slides with gelatin and dried in an oven at 36 °C for approximately 48 hrs and thereafter stained with Ehrlich's haemotoxylin and counter-stained with eosin (Drury & Wallington, 1967). Testicular sections were investigated

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for round seminiferous tubules with a light microscope (Diaplan, Ernst Leitz Wetzlar GmbH, Germany) and subsequently photographed at a magnification of ×10 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. The programme Motic Images Plus 2.0ML (Motic China Group, LTD., Xiamen, P.R. China) was later used to measure the diameter of 100 seminiferous tubules (µm).

The ovarian sections were examined serially for stages of follicular growth with a light microscope (Vickers Instruments, U.K.) at magnifications of ×100, ×200 and ×400. Follicular stages were identified and classified according to Bloom and Fawcett (1964). Primordial follicles are mainly seen on the periphery of the ovary where they are usually found in large numbers. Primordial follicles are the smallest follicular types and are mainly distinguished by their large round ovum (Fig. 1A). Primary follicles are characterized by one layer of follicular cells around the ovum and are also larger than primordial follicles (Fig. 1B). The follicles are mainly growing through cell division of the follicular cells surrounding the ovum and when different layers of follicular cells appear, the follicle is identified as a secondary follicle (Fig. 1C). Tertiary follicles are formed when irregular spaces begin to appear between the follicular cells (Fig. 1D). The follicle continues to grow especially through rapid mitosis of the cells and a continuous accumulation of follicular liquid in the spaces which also start to merge. Graafian follicles are distinguished by a large liquid-filled space, thin outer wall and the ovum being pressed against one side of the follicle (Fig. 1E). After rupture of the follicle, the corpus luteum is left which can become a corpus luteum of pregnancy if fertilization occurs (Fig. 1F). The corpus luteum, which is left by a newly ruptured follicle, is called the corpus hemorrhagica and is characterized by a large accumulation of blood. Corpus albicans is the scar left after the corpus luteum has perished. However, to accommodate for small sample sizes, corpus luteum, corpus hemorrhagica and corpus albicans were combined for analyses (hereafter referred to as corpora bodies). Primordial follicles were counted in every tenth section only and the number of primordial follicles, therefore, represents a sub-sample instead of the total number for the entire ovary. The total number for all other follicular stages was counted throughout each ovary.

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Hormone analysis

Coat-a-count hormone kits (Siemens Medical Solutions Diagnostics, Los Angeles, U.S.A.) were used to measure progesterone and testosterone concentrations in the plasma of female and male elephant-shrews, respectively. This method uses a solid phase radioimmunoassay which is based on a radioactive labelled hormone (i.e., ^{125}I-labelled progesterone) and the hormone in the sample competing for antibody sites in a test tube (Abraham, 1977). After reaction time, the amount of bound ^{125}I-labelled hormone in the tube is counted with a gamma counter. A calibration curve with known hormone concentrations is generated with the same method and used to obtain the hormone concentration of the sample. All assays were carried out according to the manufacturer's protocol. The slopes of a serial dilution curve, which was generated by serially diluting a sample from an individual with high hormone concentrations (i.e., pregnant female), and the calibration curve were tested for parallelism with a General Linear Model (GLM) after log-logit-transformation (Chard, 1978) to validate this method for the plasma progesterone and testosterone concentrations of female and male *E. myurus*, respectively. The dilution percentages were used as covariates and the type of curve was employed as a random factor.

Plasma progesterone concentrations (nmol/L) were evaluated in 58 female *E. myurus* (December: n = 4, see above for n of all other months) throughout the 12-months period. The assay could be validated for *E. myurus* as there was no significant difference between the serial dilution curve of plasma progesterone of female *E. myurus* and the calibration curve ($F_{1,5}$ = 0.01; n = 4; P = 0.93). The intra-assay coefficient of variation for repeated determination of quality control was 11.2 % and the sensitivity of the assay was 0.36 nmol/L.

Plasma testosterone concentration (nmol/L) was determined for a total of 56 male *E. myurus* because one male from September (n = 3) was excluded from the analysis. The testosterone assay could be validated for male *E. myurus* as there was no significant difference between the serial dilution curve of plasma testosterone and the calibration curve ($F_{1,3}$ = 0.23; n = 3; P = 0.66). The sensitivity of the assay was 1.39 nmol/L and the intra-assay coefficient of variation was 3.6 % and 3.5 % and the inter-assay coefficient was 3.1 %.

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Relative age classes

In order to be able to distinguish between young and old animals, all males and females were separated in relative age classes using the eruption patterns and the degree of maxillary tooth wear. Only the premolar and molar on the right side of the maxilla were analyzed for the degree of tooth wear and eruption. The following six relative age classes could be characterized by this method:

(1) Age class 1:

All premolars completely erupted and a cavity present where M^1 is about to appear. No signs of tooth wear and cusps are sharp with very deep grooves on the surface (Fig. 2A).

(2) Age class 2:

M^1 completely erupted and a cavity, where the M^2 is about to emerge, is present. Very little sign of tooth wear evident and the cusps are pointed with deep grooves in between. Deciduous dentition is still present (Fig. 2B).

(3) Age class 3:

All individuals exhibit a change from deciduous to permanent dentition. Deciduous teeth are loosely attached to the emerging permanent teeth or have fallen out, in which case, the new permanent teeth are present underneath, emerging from the cavity. All teeth are fully erupted (Fig. 2C).

(4) Age class 4:

Minimal tooth wear is evident with the grooves between cusps being slightly rounded, but still narrow. Premolar and molar cusps are sharp showing little signs of wear (Fig. 2D).

(5) Age class 5:

Obvious premolar and molar tooth wear apparent. Grooves between cusps are obviously worn and curved out and some dentine is exposed. Cusps with some signs of wear being more rounded than sharp, but still distinct (Fig. 2E).

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(6) Age class 6:

All teeth are severely worn and completely deformed. Teeth are entirely smooth with no discernible grooves on the surface. Only remnants of cusps on the side of the teeth are visible or the cusps are almost indistinguishable from the rest of the tooth. Large amount of dentine is present on the premolar and molar surfaces (Fig. 2F).

Elephant-shrews reach their adult body size and sexual maturity before their permanent cheek teeth have erupted (Asher & Olbricht, 2009) and it is, therefore, very difficult to conclude on their reproductive status based on dentition. For this reason, a Principal Components Analysis (PCA) on the reproductive variables and body mass of *E. myurus* was performed to determine if some of the relative age classes could either be grouped together or excluded from the analysis because of their uncertain reproductive status in comparison to individuals of the other relative age classes. The PCA was performed separately for male and female *E. myurus* resulting in the analysis of five variables for males and ten for females (see Table 1). The principal components (PC) scores of the first two PC axes explained most of the variation to the total variance (Table 1). The derived scatter-plots from these two PC axes showed that most of the six relative age classes were grouped together in both males and females. Nevertheless, relative age class (1) was separated from all other relative age classes in both sexes (Fig. 3) suggesting the uncertainty of the reproductive status of this relative age class with reference to dentition. Because individuals of this relative age class may represent juveniles, they were excluded from further analysis of reproductive parameters, but were, however, included in the analysis involving body mass.

Data analysis

The number of individuals per relative age class was compared between the 12 months using a Chi-square (χ^2) test. A GLM was applied to assess differences in body mass between the 12 months, the six relative age classes and the sexes (factors) and adjusted R^2 was applied as a measure of fit for the model.

The means of the different variables measured, which were obtained twice per male or female *E. myurus* (e. g. testicular and ovarian mass and volume and number per follicular stage), was calculated per individual. Due to small sample sizes, all

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reproductive parameters of both male and female *E. myurus* were analyzed using Generalized Linear Models (GZLM). For all GZLMs, months and relative age classes were used as factors, while body mass was applied as a covariate. Akaike's Information Criterion was used to verify the fit of the model for all GZLM. Tukey's and least significant difference (LSD) *post-hoc* tests followed GLM and GZLM, respectively where significant differences were detected. Linear regressions were performed in cases when a relationship between body mass and a dependent variable was assumed during a GZLM and the data were parametric. A sequential Bonferroni correction for multiple comparison (SqBc) (Rice, 1989) was applied for all analyses for males and females separately. All statistical analyses were performed using the *Statistical Package for the Social Sciences* (SPSS) Statistics version 17.0 (Polar Engineering and Consulting 1993-2007). The results are presented as mean ± 1 standard error (*SE*) and were found to be significant at $P < 0.05$.

Results

General

The rainy season in the study area began in September and lasted until April with December having the highest rainfall (146 mm). From May until August no rain was recorded with these months spanning the dry season. Mean monthly ambient temperatures above 20 °C were recorded from September to March with February being the warmest month (23.5 ± 0.2 °C). The coldest period of the year was from April until August with temperatures below 20 °C and July was the coldest month (16.2 ± 0.2 °C). Data on rainfall and ambient temperature are shown in Figures 6a and 8a.

There was a significant difference in the number of individuals caught per relative age class between the 12 months ($\chi^2 = 121.74$; $df = 55$; $n = 116$; $P < 0.001$). Animals of relative age class (5) and to some extent (6), comprising relatively old animals, were caught throughout the year and in September and October only animals of these age classes were collected. Relatively younger animals of the relative age classes (1) to (4) were collected only during a few months of the year (Fig. 4). Elephant-shrews of relative age class (4) were caught from February until August, individuals of relative age class (3) were caught from December until March and a few individuals of relative age class (2) were sampled in November, December

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and February (Fig. 4). Only one male of relative age class (1) was collected in January and one female was collected in March (Fig. 4). Very small and young animals were, however, caught, but released again in October and December. Sample sizes for relative age classes for males and females are presented in Table 2.

Body mass

Body mass was not significantly different between male and female elephant-shrews ($F_{1,63}$ = 2.29; n = 116; P = 0.14; males: 50.8 ± 0.7 g, females: 51.9 ± 0.8 g; Fig. 5). However, body mass differed significantly between the six relative age classes ($F_{5,63}$ = 2.9; n = 116; P = 0.02) and between the 12 months ($F_{11,63}$ = 2.18; n = 116; P < 0.03). Body mass was lowest in August and increased significantly during October and November (Tukey's test; P < 0.05), when it was greatest (Fig. 5). The youngest elephant-shrews from relative age class (1) had the lowest body mass and were significantly smaller than the oldest individuals of relative age classes (5) and (6) (Tukey's test; P < 0.05; Table 2). There was a significant interaction between sex and relative age classes ($F_{4,63}$ = 2.67; n = 116; P = 0.04; Table 2) with females of relative age class (5) being heavier than males of the same age class (LSD: P = 0.001; Table 2). In addition, there was a significant interaction between relative age classes and months ($F_{19,63}$ = 2.02; n = 116; P = 0.02). The body mass of animals of relative age classes (5) and (6) decreased significantly from June/July to July/August (LSD: P < 0.05) and increased significantly from August to October/November (LSD: P < 0.05; see LSD for month only). No significant interaction was found between sex and months ($F_{11,63}$ = 1.04; n = 116; P = 0.42; Fig. 5).

Testicular volume and mass showed a significant positive increase with increasing body mass (Wald $\chi^2 \leq 17.13$; df = 1; n = 56; P < 0.002) and, therefore, the standard residuals for testicular mass and volume by body mass were calculated. Plasma testosterone concentration and seminiferous tubule diameter were not dependent on body mass (Wald $\chi^2 \leq 0.87$; df = 1; $n_{\text{seminiferous tubules}}$ = 56; $n_{\text{testosterone}}$ = 55; $P \geq 0.35$). In addition, all reproductive parameters measured for female elephant-shrews were not dependent on body mass after a SqBc (Wald $\chi^2 \leq 6.57$; df = 1; n = 58; $n_{\text{progesterone}}$ = 57; $P \geq 0.01$; secondary follicle: $P_{\text{Bonferroni}}$ = 0.006).

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Months

Testicular volume and mass, seminiferous tubule diameter as well as plasma testosterone concentration were significantly different between the 12 months (Wald $\chi^2 \geq 93.58$; $df = 11$; $n = 56$; $n_{testosterone} = 55$; $P < 0.001$). Volume and mass of the testes was smallest in April and increased significantly during May (LSD; $P = 0.02$), reaching its peak in August and decreased significantly from October to December (LSD; $P < 0.01$) and January to March (LSD; $P < 0.001$; Fig. 6A). Seminiferous tubule diameter was smallest in March and increased significantly from April to May (LSD; $P < 0.001$). It peaked in September and October and decreased significantly towards November/December (LSD; $P < 0.001$) and January to February (LSD; $P = 0.04$; Fig. 6B). Although only very low levels of plasma testosterone were observed in general, testosterone concentration was lowest from November/December until April and significantly increased between April and June (LSD; $P < 0.04$; Fig. 7A). Plasma testosterone concentration was highest in July and August and declined significantly from October to November (LSD; $P < 0.04$; Fig. 7A).

All reproductive parameters recorded for female elephant-shrews were significantly different between the 12 months (Wald $\chi^2 \geq 27.98$; $df = 11$; $n = 58$; $n_{progesterone} = 57$; $P \leq 0.003$), except for the number of primordial follicles, which was not significantly different over the 12-month period (Wald $\chi^2 = 12.98$; $df = 11$; $n = 58$; $P = 0.3$). Ovarian mass and volume increased significantly from July to August and August to September (LSD; $P \leq 0.05$) until both reached their maximum in October and decreased significantly from October towards January (LSD; $P < 0.001$; Fig. 8). Mass and volume of the ovaries was lowest from about February until June (Fig. 8). A significant rise in plasma progesterone concentration was observed from July to September (LSD: $P <0.01$; Fig. 7B). Plasma progesterone concentration was highest in October, then decreased significantly towards November (LSD: $P < 0.001$) and was smallest from December until July (Fig. 7B). Most primary follicles were found in May and from then on, the number of these follicles decreased progressively towards December with a significant difference in the number of primary follicles between May and October (LSD; $P = 0.04$; Table 3). The number of primary follicles was similar from December and January to April the following year, but was significantly different from May (LSD; $P \leq 0.03$; Table 3). The number of secondary, tertiary and Graafian follicles reached their maximum one month later than the previous follicular

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stage, with the number of secondary follicles peaking in July, tertiary follicle numbers peaking in August and Graafian follicles being highest in September (Table 3). The number of secondary follicles was significantly lower from July to August (LSD; $P < 0.02$) and reached their minimum in March/April (Table 3). The number of tertiary follicles decreased significantly between September/October and December (LSD; $P < 0.05$) and was lowest from February to May (Table 3). The number of Graafian follicles was significantly lower in November and December than in September and October (LSD; $P < 0.04$) and no Graafian follicles were present from February until July (Table 3). No corpora bodies were found from March until July. The number of corpora bodies started to increase significantly from July to August (LSD; $P < 0.001$) and August to September (LSD; $P < 0.01$), were highest in November and decreased significantly towards December (LSD; $P = 0.001$; Table 3).

All females that were found to be pregnant ($n = 17$), had two embryos in the uterine horns and females with placental scars ($n = 14$) had mostly two placental scars with the exception of one female that had four. Pregnant females were collected in every month from August until January. Females with placental scars were found throughout the year (Fig. 9). Lactating females ($n = 7$) were caught in September and December until March (Fig. 9).

Relative age classes

Testes mass and seminiferous tubule diameter did not show any significant difference between the relative age classes (Wald $\chi^2 \leq 8.55$; $df = 4$; $n = 56$; $P \geq 0.07$; Table 4). However, testicular volume and plasma testosterone concentration were significantly different between the relative age classes (Wald $\chi^2 \geq 14.31$; $df = 4$; $n_{volume} = 56$; $n_{testosterone} = 55$; $P \leq 0.006$; Table 4). Testes volume was smallest in males of relative age class (4), significantly increased towards relatively older individuals of age classes (5) and (6) and relatively younger individuals of age class (2) (LSD; $P < 0.001$) and was significantly largest in individuals of relative age class (6) (LSD; $P < 0.03$; Table 4). The plasma of individuals of relative age class (4) showed the highest testosterone concentrations (1.26 ± 0.74 nmol/L), being significantly elevated in comparison to all other relative age classes (LSD; $P < 0.05$) with the exception of relative age class (6) (LSD; $P > 0.05$; Table 4).

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Primordial and tertiary follicles as well as corpora bodies demonstrated a significant difference between the relative age classes (Wald $\chi^2 \geq 17.09$; $df = 11$; $n = 58$; $P \leq 0.002$), while the other follicle types and female reproductive variables were not significantly different after a SqBr (Wald $\chi^2 \leq 10.93$; $df = 11$; $n = 58$; $n_{progesterone} = 57$; $P \geq 0.03$, primary follicle: $P_{Bonferroni} < 0.02$; Table 4). Relative age class (4) exhibited the largest number of primordial follicles being significantly different from relative age classes (3), (5) and (6) (Table 4). The number of primordial follicles decreased after relative age class (4) and was significantly lower for relative age class (6) in comparison to all the other relative age classes (LSD; $P < 0.04$) except for relative age class (3) (LSD; $P = 0.11$; Table 4). The number of tertiary follicles was significantly different between relative age classes (3) and (6) and age class (5) with females of age class (5) having the largest number of tertiary follicles (LSD; $P < 0.05$; Table 4). The number of corpora bodies was significantly lower in females of the relative age class (4) than in any of the other relative age classes (LSD; $P < 0.02$; Table 4). The number of corpora bodies increased towards relatively older females as well as towards relatively younger females from relative age class (4) (Table 4). The number of corpora bodies was significantly greater in relative age class (6) compared to those of relative age classes (3), (4) and (5) (LSD; $P <0.001$; Table 4).

Month and relative age class

The results of the interactions between the 12 months and relative age classes were most likely an artefact of the small sample sizes for several relative age classes and are, therefore, not presented. Nevertheless, relative age classes (4), (5) and (6) showed important seasonal variations in reproductive parameters which are presented here.

Significant interactions of months with relative age classes were found for seminiferous tubule diameter, ovarian volume and mass, plasma progesterone concentration, secondary and tertiary follicles as well as corpora bodies (Wald $\chi^2 \geq 33.89$; $df = 13$; $n_{males} = 56$; $n_{females} = 58$; $n_{progesterone} = 57$; $P \leq 0.001$). All other reproductive parameters for both males and females did not exhibit a significant interaction after a SqBr (males: Wald $\chi^2 \leq 24.06$; $df = 13$; $n = 56$; $n_{testosterone} = 55$; $P \geq 0.03$, testes mass: $P_{Bonferroni} < 0.02$; females: Wald $\chi^2 < 25.27$; $df = 13$; $n = 58$; $P \geq 0.02$, Graafian follicle: $P_{Bonferroni} < 0.02$). Seminiferous tubule diameter increased

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significantly in individuals of relative age classes (4) and (5) from April to May (LSD; $P < 0.001$) and decreased significantly from October to November for males of relative age class (5) (LSD; $P < 0.01$). A significant increase of both ovarian mass and volume was detected for animals of relative age class (4) from July to August (LSD; $P < 0.05$) and for relative age class (6) from August to September (LSD; $P < 0.001$) and a significant decrease of the two variables was detected for relative age class (5) from October to November - January (LSD; $P \leq 0.02$). Females of relative age classes (5) and (6) follow a similar pattern of follicle and corpora body numbers over the 12-month period as was established between the 12 months alone. For example, the number of secondary follicles was highest in July for both relative age classes (5) and (6) and in females of relative age class (6) decreased significantly from July to August (LSD; $P < 0.001$) and August to September (LSD; $P < 0.02$), while the number of secondary follicles of relative age class (5) were significantly reduced from August to September (LSD; $P < 0.01$).

Discussion

Elephant-shrews exhibit a number of breeding strategies ranging from aseasonal to seasonal breeding. The Rufous elephant-shrew (*Elephantulus rufescens*) from Kenya, the Short-snouted elephant-shrew (*E. brachyrhynchus*) from Zimbabwe and Tanzania and the Round-eared elephant-shrew (*Macroscelides proboscideus*) from South Africa have been reported to breed throughout the year although all species occur in highly seasonal habitats (Bernard *et al.*, 1996; Leirs *et al.*, 1995; Neal, 1982, 1995). *Elephantulus brachyrhynchus* and *M. proboscideus*, however, displayed a reduction in pregnancies during the southern African winter (Bernard *et al.*, 1996; Neal, 1995). In contrast, a number of elephant-shrew species have been found to breed only during the spring and summer months of the year and to entirely halt breeding during winter. The elephant-shrew with the most northerly distribution, the North African elephant-shrew (*E. rozeti*), breeds from January until August and thus, largely during the spring and summer months of the northern hemisphere (Séguignes, 1989). In southern Africa, *E. myurus* and the Bushveld elephant-shrew (*E. intufi*) only breed during the warm and wet periods of the year (Skinner & Chimimba, 2005; Smithers, 1971; van der Horst, 1946, 1954; Woodall & Skinner, 1989) although it has been suggested that *E. intufi* breeds year-round in Namibia (reviewed in Skinner and Chimimba, 2005).

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In the present study, it was confirmed that *E. myurus* is a seasonal breeder with its breeding period being predominantly confined to the warm and wet spring and summer months of northern South Africa. The breeding season was shown to begin in August and end in January which is the time when pregnant females were found although lactating females were collected up until March. The significant increase in ovarian size and progesterone concentrations from July to August indicates that the reproductive system altered drastically during this period and the occurrence of corpora lutea at the end of August, but not in July, indicates that ovulation and fertilization must have occurred at the beginning of August rather than in July. Van der Horst (1954) similarly observed, in extensive histological studies, that *E. myurus* comes out of anoestrus in July. We found that the female breeding season peaked in October after which ovarian size, progesterone concentration, and the number of Graafian follicles and corpora bodies declined rapidly. The end of the breeding season was reached at the end of summer which was marked by lower progesterone concentrations and lower numbers of tertiary and Graafian follicles as well as corpora bodies in February and March compared to the previous months. A number of studies have, however, established a longer breeding season for *E. myurus* with pregnant females being collected until March (van der Horst, 1954 - South Africa; Woodall & Skinner, 1989 - South Africa and Botswana) and even April (Smithers, 1971 - Botswana).

Male *E. myurus* started to prepare for the breeding season several months prior to the females. This was shown by the significant increase in testicular volume and mass and seminiferous tubule diameter in May. The peak breeding season for males was found to be from August to October after which the size of the testes, seminiferous tubule diameter and testosterone concentration dropped significantly. Woodall and Skinner (1989) found a similar pattern for testes volume and seminiferous tubule diameter in *E. myurus* although they established that the peak in male breeding season occurs two months later than in the present study. In addition, in an earlier study, no reduction in testes size and seminiferous tubule diameter was encountered, while the size and activity of the accessory glands varied with the seasons (Stoch, 1954). However, both studies combined data from different months which may have led to the different results.

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Moreover, the distribution of young and old *E. myurus* across the year agrees with the results of the reproductive parameters and underlines the notion that this elephant-shrew breeds seasonally. While relatively old animals (relative age class (5)) were caught throughout the year, an influx of new and very young animals was found to occur from October until March. These very small elephant-shrews, which appeared to have just been weaned, were caught in October, but during our trapping regime were released again and it is probable that they would have been placed in an age class prior to relative age class (1). The first females probably gave birth in September because the first lactating females were found in the same month. Since the young are precocial and probably released early into the population, the occurrence of relatively large numbers of very young individuals in October is explained. It is interesting to note that the population appears to become progressively "older" from October onwards as animals of relative age class (2) were caught initially in November and animals of relative age class (3) and (4) were collected in December and February, respectively. Moreover, at the beginning of the breeding season the population is relatively old and it is possible that not many of these animals will survive until the end of the season. Individuals of relative age class (6) possessed completely worn teeth which must make feeding difficult and may be a potential explanation for the disappearance of these older animals from the population from April until June. The combined evidence suggests that the population of *E. myurus* turns over once a year and only few individuals are able to breed during two seasons (see also van der Horst, 1954).

The present study agrees with other findings on *E. myurus* that this elephant-shrew is a seasonal breeder. Nevertheless, we found some differences between the present and the other earlier studies, particularly regarding the length of the breeding season. The breeding season of *E. myurus* established in the present study is, however, similar to the breeding season of the spiny mouse (*Acomys spinosissimus*) (Chapter 1). Both species were studied in the same area at the same time although the breeding season was found to start one month later in the rodent than in the elephant-shrew (Chapter 1).

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Many mammals from high latitudes breed seasonally, whereas reproduction may shift towards aseasonality closer to the equator (Bronson, 1985). This shift from reproductive seasonality towards aseasonality at lower latitudes has also been suggested for *Elephantulus* (Neal, 1995) and possibly explains the extended breeding season of *E. myurus* found in Botswana (Smithers, 1971; Woodall & Skinner, 1989) and the aseasonal reproduction of *E. rufescens* (Neal, 1982) and *E. brachyrhynchus* closer to the equator (Leirs *et al*., 1995; Neal, 1995). However, factors timing the breeding season in *E. myurus* are unknown, although some conclusions can be drawn from studies on other species of elephant-shrews. Neal (1995) suggested that the increase in insect abundance during the warm season may be a possible reason for seasonal reproduction in *Elephantulus.* Although body mass was found to be relatively stable during the year, we observed a significant decrease in body mass of both males and females just prior to the beginning of the breeding season in August which may intimate the importance of food availability during reproduction of *E. myurus*. This drop in body mass may be explained by the high energetic demands at the beginning of the breeding season, due to energy needed for both gonadal growth as well as courtship, which could not be balanced by increasing food intake as many insects were not yet available at the end of the dry and cold winter. Rainfall and subsequent increase in food quantity and quality has been proposed to be the main factor driving seasonal reproduction in tropical as well as southern African mammals (Delany, 1972; Perrin, 1986). The cyclicity of rainfall and ambient temperature combined with varying availability of food throughout the year may, therefore, be the main cause for the seasonal reproduction exhibited by *E. myurus.* However, rainfall is not the proximate factor which triggers the onset of reproduction in *E. myurus*, since this species already initiates breeding before the start of the rainy season. Furthermore, Neal (1995) suggested that photoperiod may be the proximate cause for the control of reproduction in *E. myurus* and *E. rozeti* since both species start breeding just after the winter solstice, but recent work (Chapter 4) suggests that *E. myurus* might not be reproductively photoresponsive.

Although the end of the breeding season varies by a number of months in *E. myurus*, there appears to be less variation in the onset of breeding suggesting that there may be a factor which regulates the initiation of the reproductive season in *E. myurus*. Bernard *et al.* (1996) suggested that a connection between an increase in

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bone calcium concentration and the higher efficiency of calcium assimilation from fresh green plants at the beginning of the rainy season may be the main trigger for an increase in pregnancies of the omnivorous *M. proboscideus*. *Elephantulus myurus* is largely insectivorous and with only small amounts of plant material in its diet (Churchfield, 1987). However, this study was conducted during winter and because other elephant-shrews have been found to take plant material in varying degrees during the year (Kerley, 1995; Leirs *et al*., 1995), it may be possible that plants make up a larger component of the diet of *E. myurus* than previously thought. If this should be the case, plant compounds such as those described in *M. proboscideus*, might then be used as a cue for the onset of reproduction in this species of elephant-shrew. In addition, ambient temperature may also be an important factor affecting reproduction in this species, especially since *E. myurus* has been observed to frequently go into torpor, particularly during the coldest part of the night (Mzilikazi & Lovegrove, 2005).

In most mammals, a fixed pool of follicles is deposited in the ovary at the time of birth and from then on the numbers continuously decrease as the animal ages without further supplementation which ultimately results in reproductive senescence (Bristol-Gould *et al*., 2006). *Elephantulus myurus* revealed a similar pattern of ovarian aging with the number of primordial follicles decreasing significantly from middle-aged individuals (relative age class (4)) through to relatively old individuals (relative age class (6)). The oldest individuals of relative age class (6) had the lowest quantity of primordial follicles compared to all other age classes with as few as 64 counted in one ovary. However, young elephant-shrews of the relative age classes (2) and (3) were also found to have significantly less primordial follicles than females of relative age class (4). These results could possibly be a consequence of the small sample sizes for these two age classes although another explanation is possible. Johnson *et al.* (2004) discovered that at least some mice strains are still recruiting new follicles from germ cells in the epithelial cell layer around the ovary after birth. These mice maintained a larger follicle pool for longer than would have been possible without addition of new cells (Johnson *et al*., 2004). We, therefore, propose that *E. myurus* may also retain the ability to recruit new follicles for some time after birth and furthermore, may even be able to fill up its follicle pool through this mechanism. The effect of age on tertiary follicles and corpora bodies may be a remnant of older

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females being more reproductively active than younger females although even females from the relative age classes (2) and (3) exhibited reproductive activity. Females of relative age class (4) were largely caught outside the breeding season and were the least reproductively active individuals as revealed by the small numbers of corpora bodies found. A similar pattern was observed in males, with older males having larger testes and higher plasma testosterone concentrations. In addition, the higher testosterone concentrations in males of relative age class (4) may well be a result of an earlier start of reproduction in males compared to females.

Evidence from the number of embryos and placental scars found, suggests that *E. myurus* always carries twins with each uterine horn holding only a single embryo (van der Horst & Gillman, 1941b). The occurrence of four placental scars in one female during December suggests that females may become pregnant at least twice during one season, indeed, it has been proposed that up to three litters per reproductive season are possible (van der Horst, 1946). However, the breeding season terminated earlier in males than in females (this study) and fertilization may, thus, be less likely at the end of the breeding season and, therefore, three litters per season may be more an exception than the rule.

In conclusion, *E. myurus* is a seasonal breeder which reproduces during the warm and wet spring and summer months and is reproductively regressed during most of the cold and dry autumn and winter months of South Africa. The life expectancy of *E. myurus* in the wild does not appear to be much more than 12 months which results in the renewal of the entire population once a year during the breeding season. Some attempts have been made to uncover factors which influence seasonal reproduction in *E. myurus.* Rainfall with the simultaneous increase in food quantity and quality appears to be the main factor influencing seasonal reproduction in this species. In addition, a number of other factors may also be responsible for shaping reproduction in this elephant shrew and may trigger the onset of reproduction in August. The habitat of *E. myurus* is ecologically challenging and seasonal reproduction may have evolved to enable this species to cope with these severe environmental changes.

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Tables

Table 1. Principal components analysis (PCA) of five variables measured for male and ten variables measured for female Eastern rock elephant-shrews (*Elephantulus myurus*). Eigenvalues and cumulative variance (%) are presented separately for males and females.

Sex	Variable	Component	
		1	2
Males	Body mass (g)	0.35	0.91
	Testicular volume (mm³)	0.96	-0.01
	Testicular mass (mg)	0.97	0.002
	Seminiferous tubule diameter (µm)	0.86	-0.26
	Testosterone (nmol/l)	0.35	-0.24
	Eigenvalues	2.85	0.96
	Cumulative variance (%)	57.0	76.2
Females	Body mass (g)	0.44	-0.58
	Ovarian mass (mg)	0.95	0.11
	Ovarian volume (mm³)	0.96	0.10
	Progesterone (nmol/L)	0.89	0.08
	Primordial follicle	-0.29	0.74
	Primary follicle	0.18	0.81
	Secondary follicle	0.09	0.78
	Tertiary follicle	0.74	0.07
	Graafian follicle	0.76	0.14
	Corpora bodies	0.71	-0.27
	Eigenvalues	4.57	2.28
	Cumulative variance (%)	45.7	68.5

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Table 2. Sample sizes (*n*) for six relative age classes from (1) (relatively young) to (6) (relatively old) and mean body mass (g) ± 1 standard error (*SE*) and range of body mass for each relative age class are presented separately for male and female Eastern rock elephant-shrews (*Elephantulus myurus*) from South Africa.

Relative age classes		Females				Males			
		n	Mean	*SE*	Range	*n*	Mean	*SE*	Range
relatively young individuals	1	1	39.6	-	-	1	36.4	-	-
↓	2	3	43.1	0.5	47 - 49	4	45.8	1.1	44 - 49
	3	4	52.0	3.8	46 - 62	6	49.2	1.8	44 - 56
	4	14	48.0	1.2	40 - 54	10	51.7	1.2	44 - 56
	5	30	53.6	0.9	44 - 65	22	49.8	1.0	40 - 58
relatively old individuals	6	7	54.8	2.8	44 - 65	14	54.8	1.3	47 - 63

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Table 3. Number of primordial, primary, secondary, tertiary and Graafian follicles and corpora bodies (corpora lutea, corpora hemorrhagica and corpora albicans combined) and standard errors (*SE*) of the Eastern rock elephant-shrew (*Elephantulus myurus*) from South Africa over 12 months between 2007 and 2008.

Follicle		Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Primordial	Mean	2004.6	3947.8	4070.1	3425.4	4178.6	3245.3	3535.0	3506.00	1603.2	1963.5	2865.7	2187.4
	SE	618.8	901.8	919.8	1462.8	410.5	859.6	738.9	1230.9	483.7	518.1	1202.6.	755.3
Primary	Mean	132.1	137.2	133.0	130.8	269.7	262.0	221.9	216.3	182.2	174.4	185.7	132.2
	SE	20.1	12.4	28.5	35.3	29.6	33.1	25.4	39.8	37.2	52.3	49.8	23.2
Secondary	Mean	56.1	72.7	36.9	35.0	64.4	82.0	106.0	98.3	62.9	56.3	61.2	54.6
	SE	8.1	2.6	4.5	6.8	12.5	6.2	18.3	25.1	10.1	8.4	20.3	13.0
Tertiary	Mean	11.6	0.3	3.8	0.1	3.0	7.1	13.6	53.3	31.3	39.9	26.2	11.1
	SE	11.2	0.3	2.2	0.1	2.9	3.7	5.2	22.6	7.9	4.5	3.6	6.8
Graafian	Mean	2.4	0	0	0	0	0	0	13.6	20.4	16.4	4.8	0.5
	SE	2.4	0	0	0	0	0	0	7.0	12.9	7.8	4.3	0.5
Corpora bodies	Mean	20.6	10.1	0.3	0	0	0	0	32.0	50.7	38.8	72.8	19.3
	SE	11.0	10.1	0.3	0	0	0	0	5.2	7.1	2.7	8.5	7.9

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Table 4. Mean testicular volume (mm³) and mass (mg), seminiferous tubule diameter (µm) and plasma testosterone concentration (nmol/L) per relative age class for male Eastern rock elephant-shrews (*Elephantulus myurus*) from South Africa and ovarian volume (mm³) and mass (mg), plasma progesterone concentration (nmol/L) and numbers of different follicle types and corpora bodies (corpora lutea, corpora hemorrhagica and corpora albicans combined) and standard errors (*SE*) of female *E. myurus*. Relative age classes are presented from relative age class (2) (relatively young) to relative age class (6) (relatively old). Reproductive parameters, which were significantly different between the relative age classes according to a generalized linear model, are highlighted in bold.

			Relative age class				
			2	3	4	5	6
Males	**Testicular volume (mm³)**	**Mean**	**42.8**	**35.6**	**33.4**	**57.6**	**71.0**
		SE	**3.3**	**2.4**	**12.3**	**17.3**	**15.3**
	Testicular mass (mg)	Mean	50.9	42.1	42.7	69.8	85.1
		SE	4.4	1.6	4.6	4.2	5.1
	Seminiferous tubules (µm)	Mean	161.5	149.6	147.7	170.2	174.1
		SE	3.7	5.1	5.4	3.5	4.5
	Testosterone (nmol/L)	**Mean**	**0**	**0**	**1.3**	**0.6**	**0.7**
		SE	**0**	**0**	**0.7**	**0.1**	**0.2**
Females	Ovarian volume (mm³)	Mean	5.0	5.7	4.6	8.3	9.3
		SE	0.7	0.7	0.5	0.9	2.1
	Ovarian mass (mg)	Mean	4.5	5.1	4.3	7.6	8.9
		SE	0.3	0.7	0.5	0.7	2.1
	Progesterone (nmol/L)	Mean	0.3	0.3	0.6	16.4	22.2
		SE	0.04	0.1	0.1	5.0	17.7
	Primordial follicles	**Mean**	**2625.8**	**2263.3**	**4834.8**	**2776.8**	**934.6**
		SE	**1216.4**	**327.6**	**561.0**	**266.2**	**302.7**
	Primary follicles	Mean	145.7	125.8	189.4	202.7	127.6
		SE	39.5	16.9	19.8	15.8	31.5
	Secondary follicles	Mean	67.8	55.9	63.4	69.7	61.4
		SE	14.9	3.8	5.5	7.0	15.6
	Tertiary follicles	**Mean**	**5.3**	**2.9**	**7.8**	**24.4**	**16.0**
		SE	**5.3**	**1.8**	**3.9**	**5.3**	**6.5**

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Graafian follicles	Mean	0	0	2.3	6.4	11.1
	SE	0	0	2.3	2.6	9.1
Corpora bodies	**Mean**	**9.8**	**5.0**	**2.0**	**25.3**	**41.7**
	SE	**5.8**	**5.0**	**2.0**	**4.4**	**12.7**

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Figure legends

Fig.1. Stages of follicular growth in ovaries of the Eastern rock elephant-shrew (*Elephantulus myurus*). from South Africa A: primordial follicles; B: primary follicle; C: secondary follicle; D: tertiary follicle; E: Graafian follicle; F: corpora lutea. The illustrations were taken with magnifications of ×40 (A, C) and ×16 (B, D, E, F).

Fig. 2. Toothwear classes of the Eastern rock elephant-shrew (*Elephantulus myurus*) from South Africa categorized by maxillary molar tooth wear and eruption, the degrees of which are defined in the text.

Fig. 3. Scatterplots of the first two principal component scores generated by principal components analysis of five male (A) and ten female (B) reproductive parameters of the Eastern rock elephant-shrew (*Elephantulus myurus*) from South Africa. The scores are presented separately for six relative age classes. Relative age class (1) is indicated by an arrow in both illustrations.

Fig. 4. Sample sizes of the Eastern rock elephant-shrew (*Elephantulus myurus*) from South Africa per relative age class presented separately for every month over a 12-month study period. Relative age classes are shown from (1) the relatively young to (6) the relatively old animals.

Fig. 5. Monthly body mass (g) ± 1 standard error of male and female Eastern rock elephant-shrews (*Elephantulus myurus*) from South Africa over a 12-months period.

Fig. 6. Standardized residuals of testicular volume (mm^3) and mass (mg) by body mass and diameter (µm) of seminiferous tubules of male Eastern rock elephant-shrews (*Elephantulus myurus*) from South Africa presented monthly over a 12-month study period. A: standardized residuals of testicular volume and mass; B: seminiferous tubule diameters. Values are presented as mean ± 1 standard error. The insert in A represents total rainfall (mm) and mean monthly ambient temperature (°C) over the same period.

Fig. 7. Box-plots illustrating monthly plasma testosterone concentrations (nmol/L) of male Eastern rock elephant-shrew (*Elephantulus myurus*) and plasma progesterone

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concentrations (nmol/L) of female *E. myurus* from South Africa over 12 months from 2007 to 2008. A: plasma testosterone concentration; B: plasma progesterone concentration.

Fig. 8. Monthly mean ovarian volume (mm^3) and mass (mg) ± 1 standard error for female Eastern rock elephant-shrews (*Elephantulus myurus*) from South Africa over 12 months from 2007 to 2008. The insert in A illustrates total rainfall (mm) and mean monthly ambient temperature (°C) over the same 12 months.

Fig. 9. Number of pregnant and lactating females and females with placental scars in the Eastern rock elephant-shrew (*Elephantulus myurus*) from South Africa presented monthly over a 12-months period.

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Figures

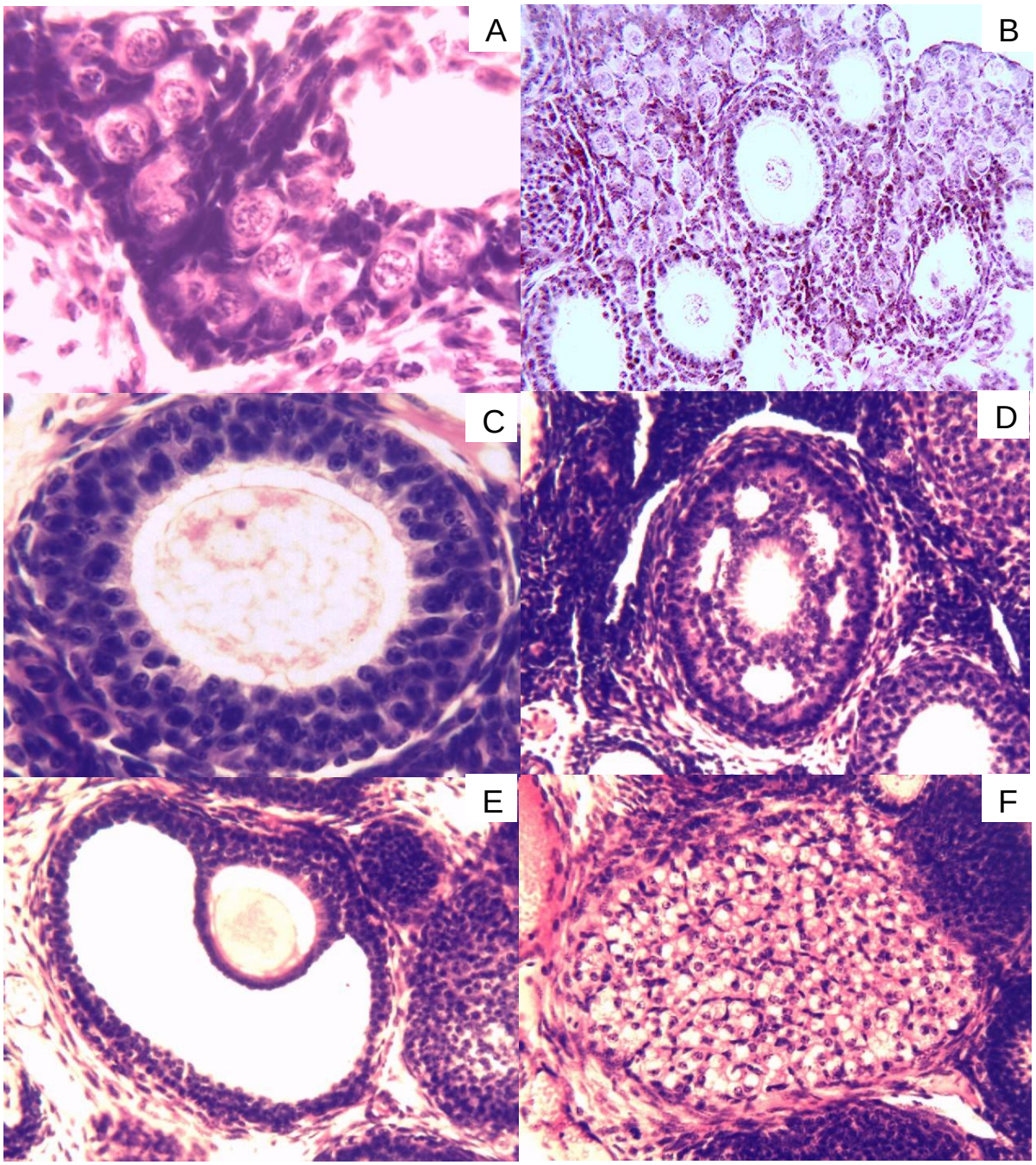

Figure 1.

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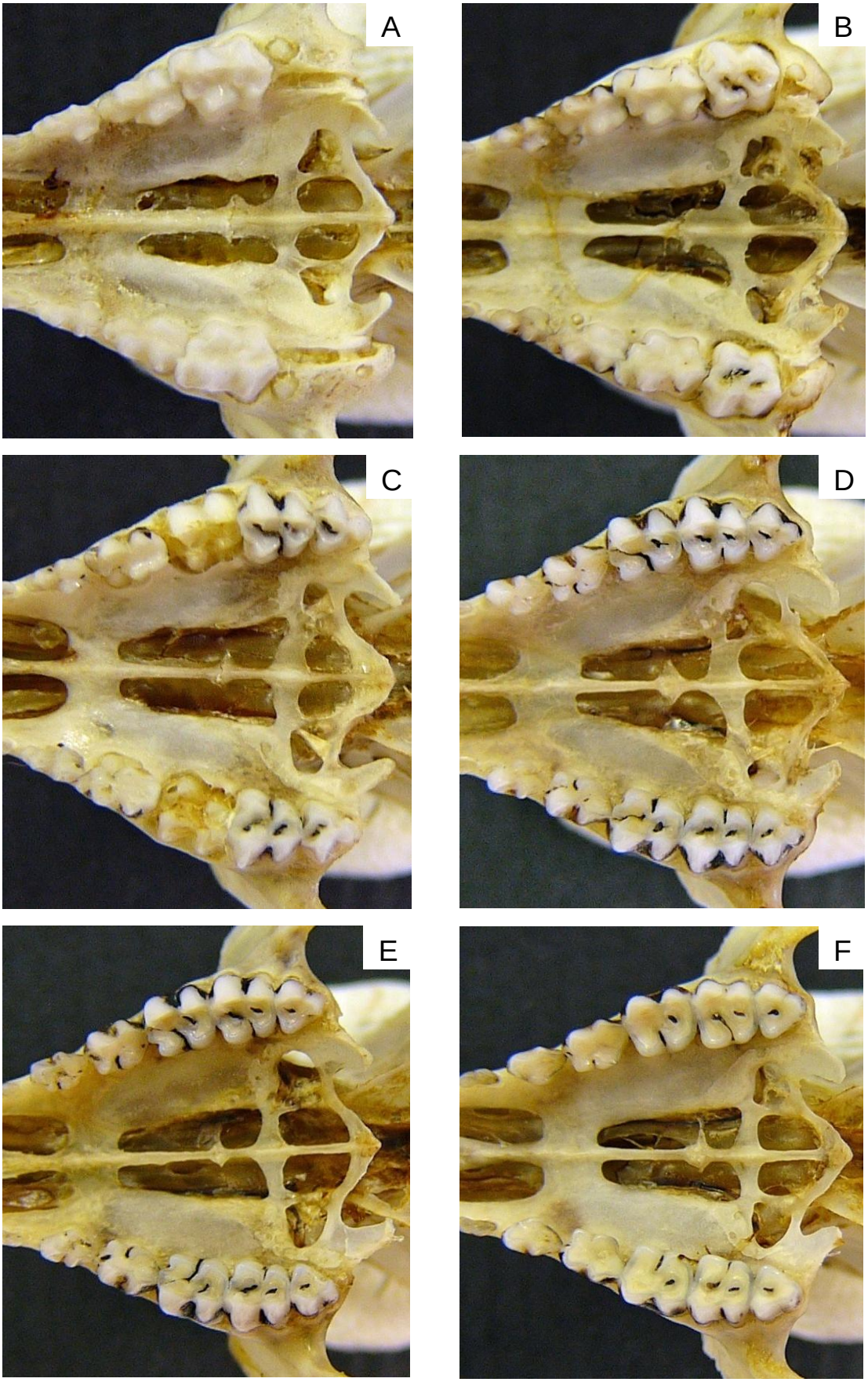

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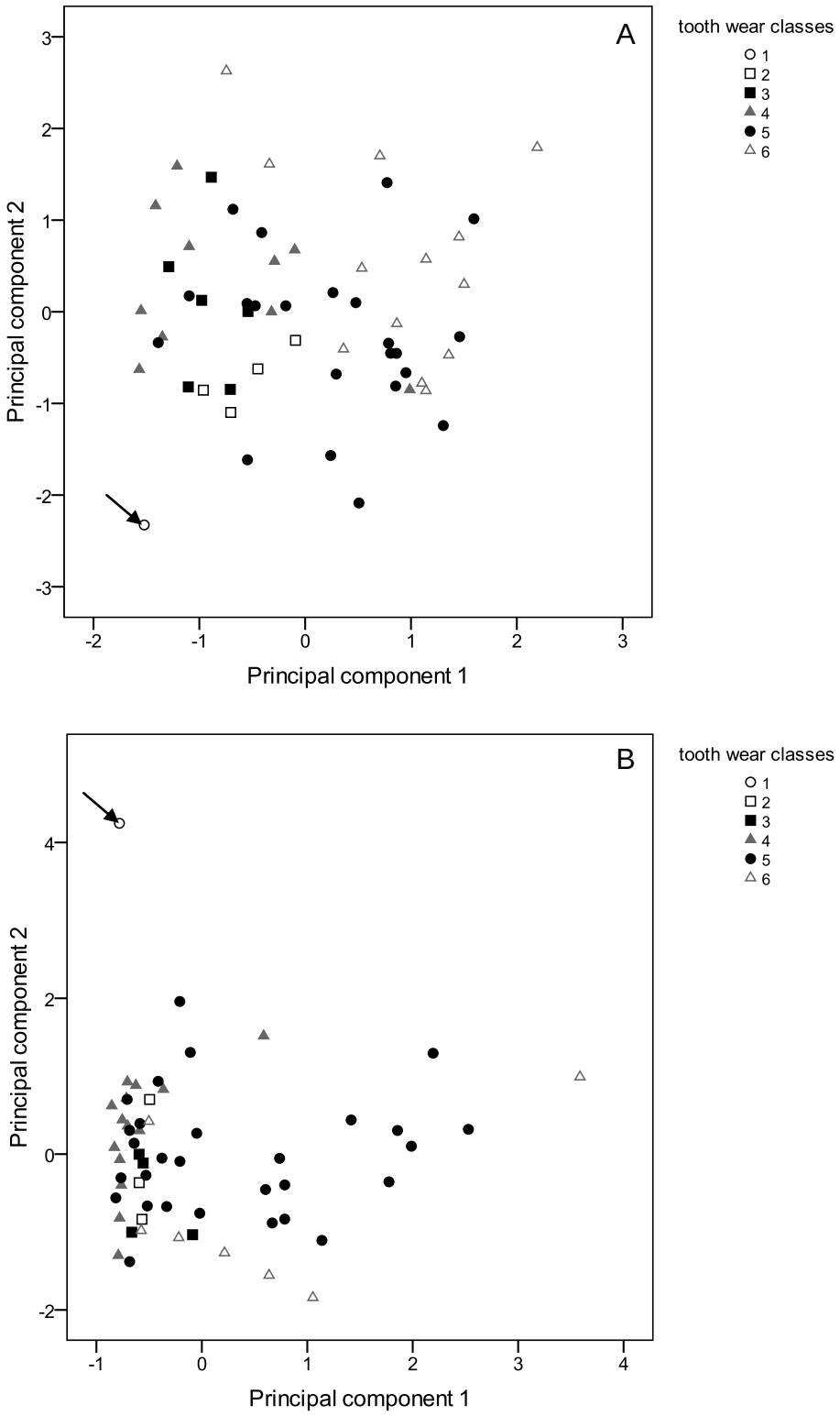

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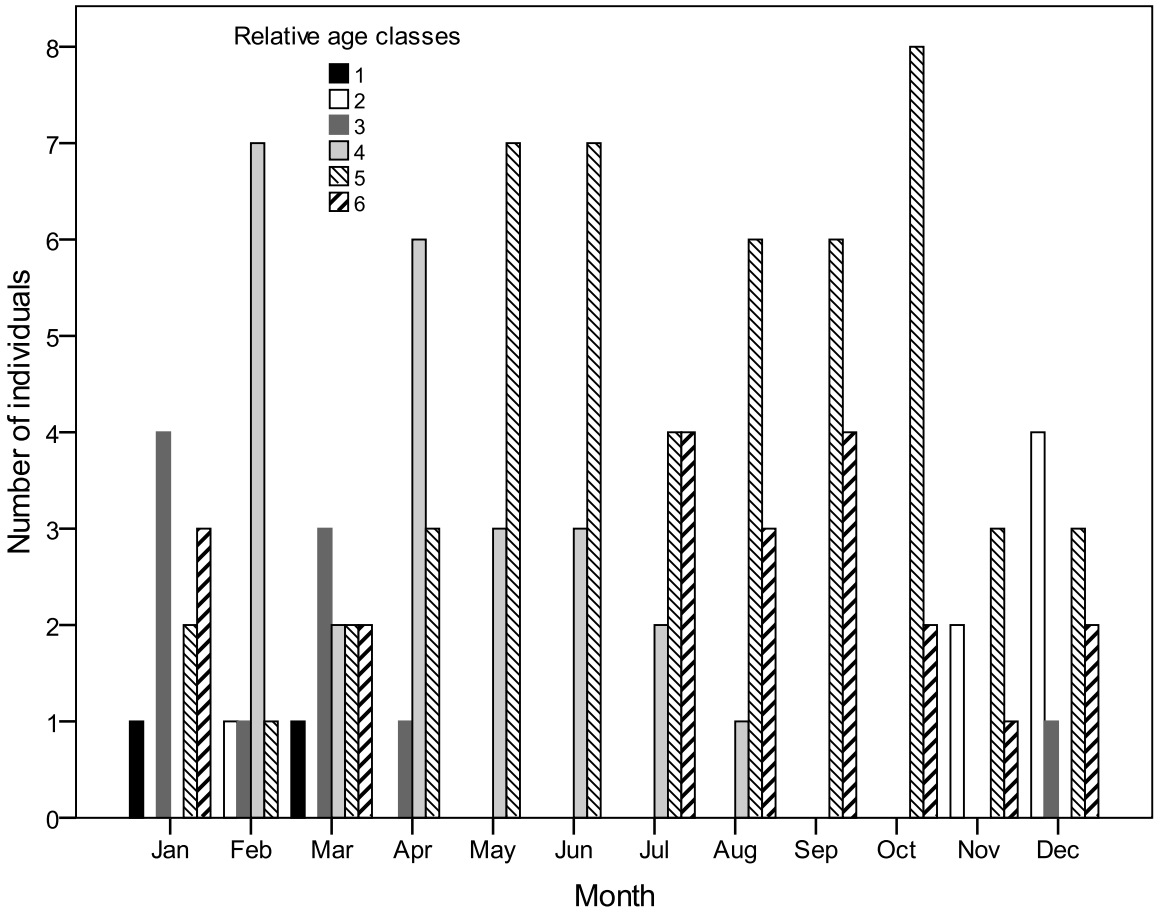

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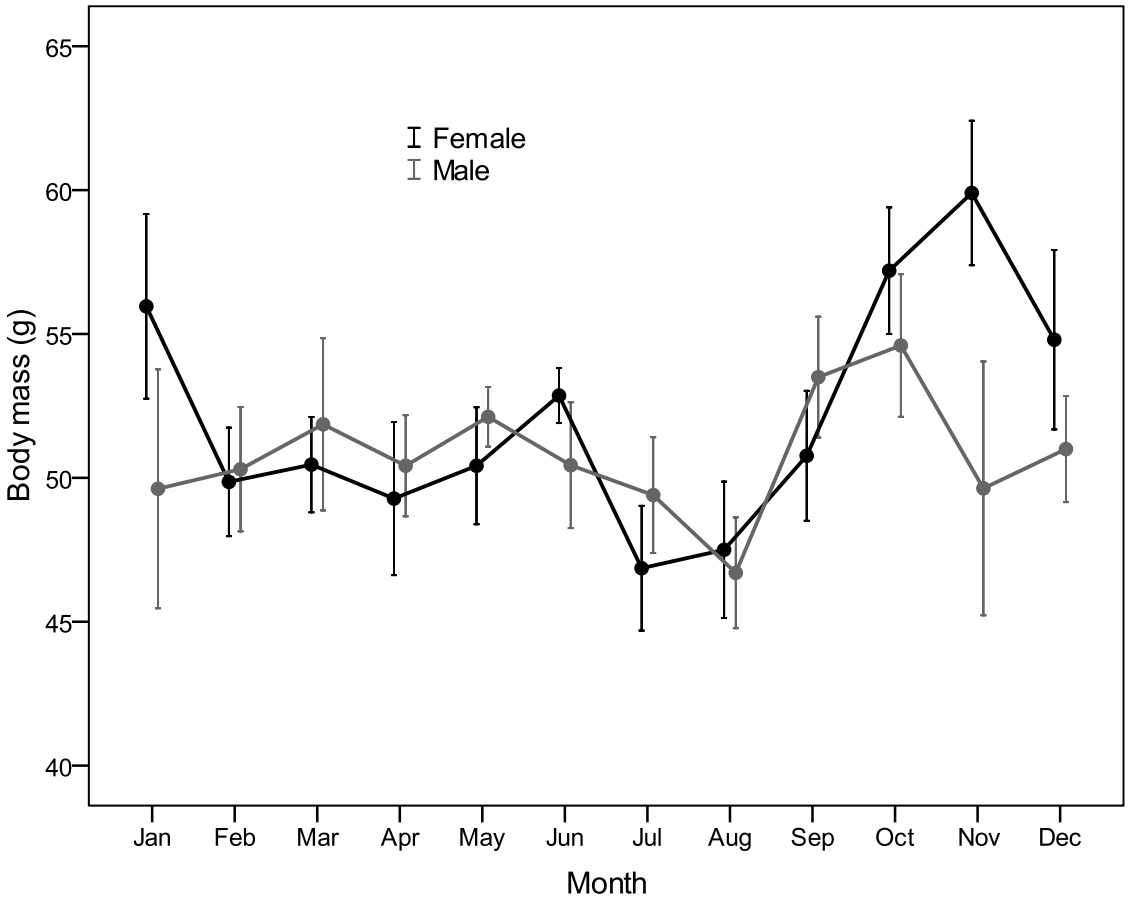

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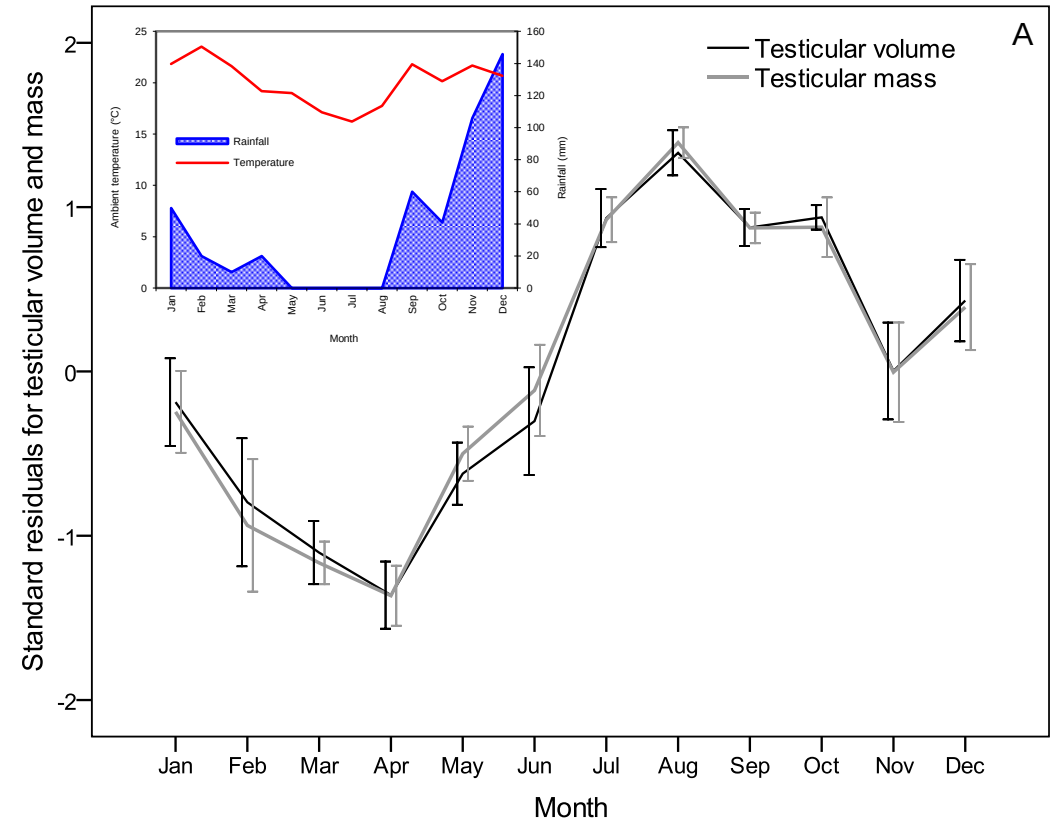

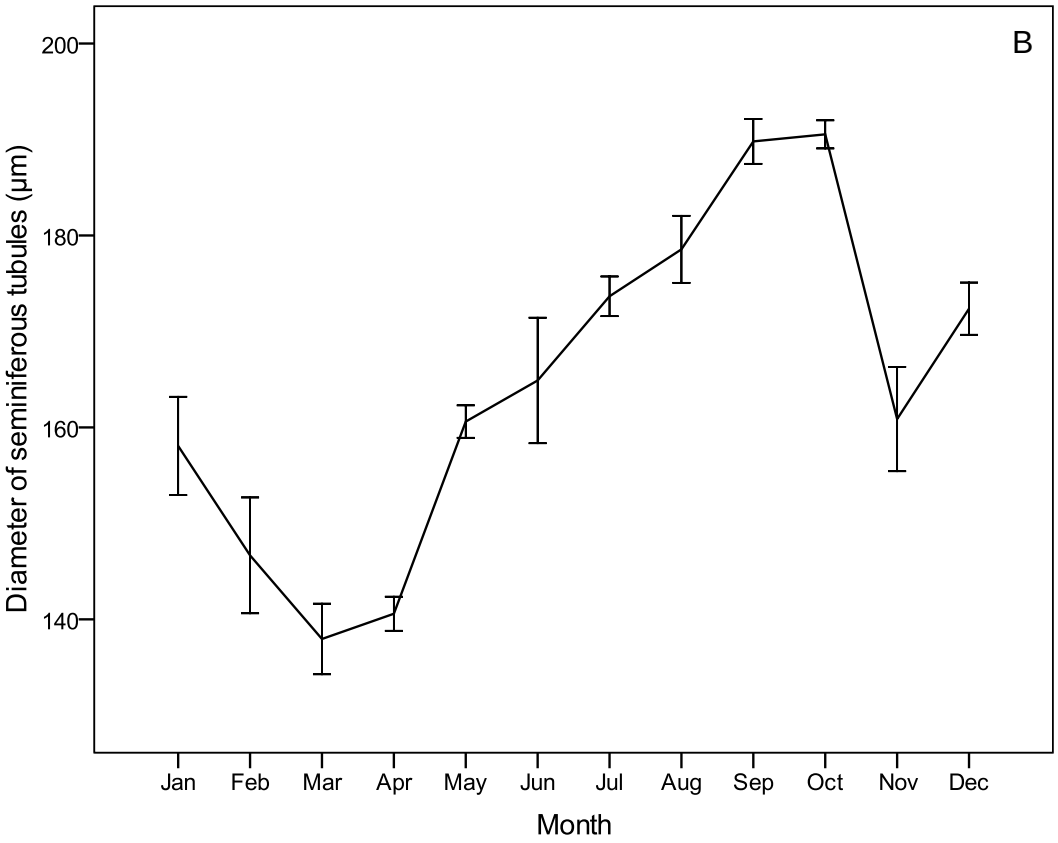

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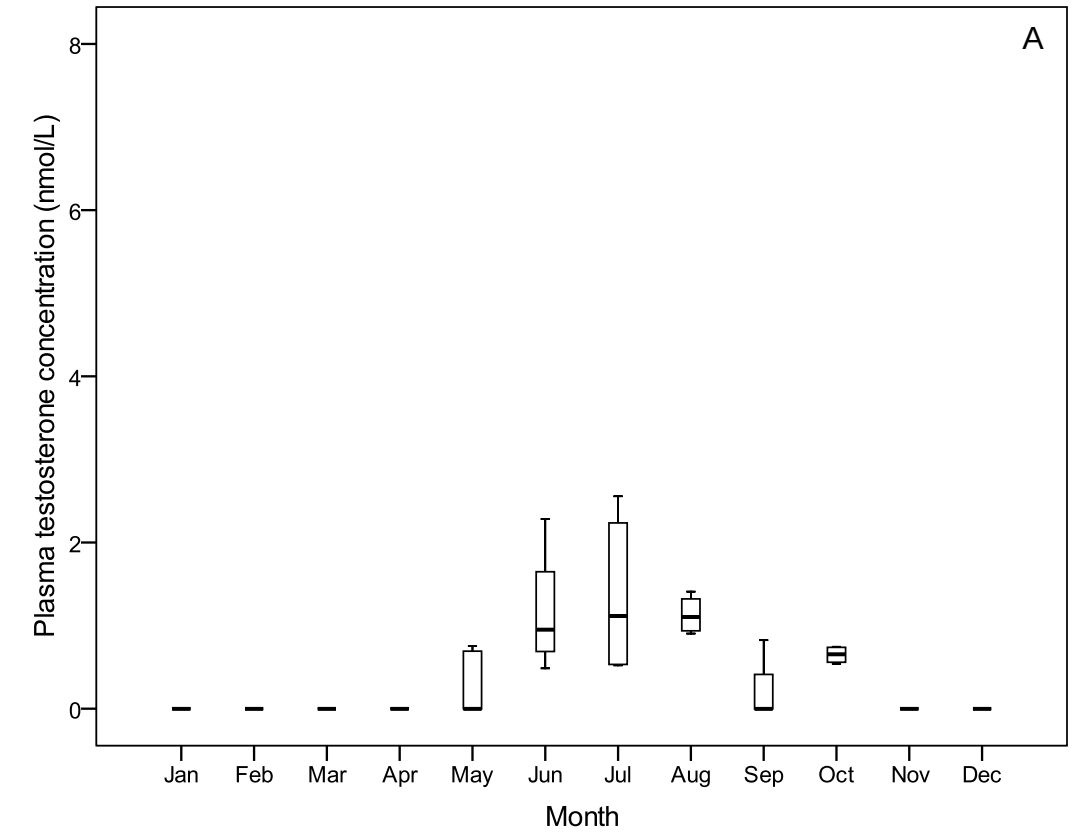

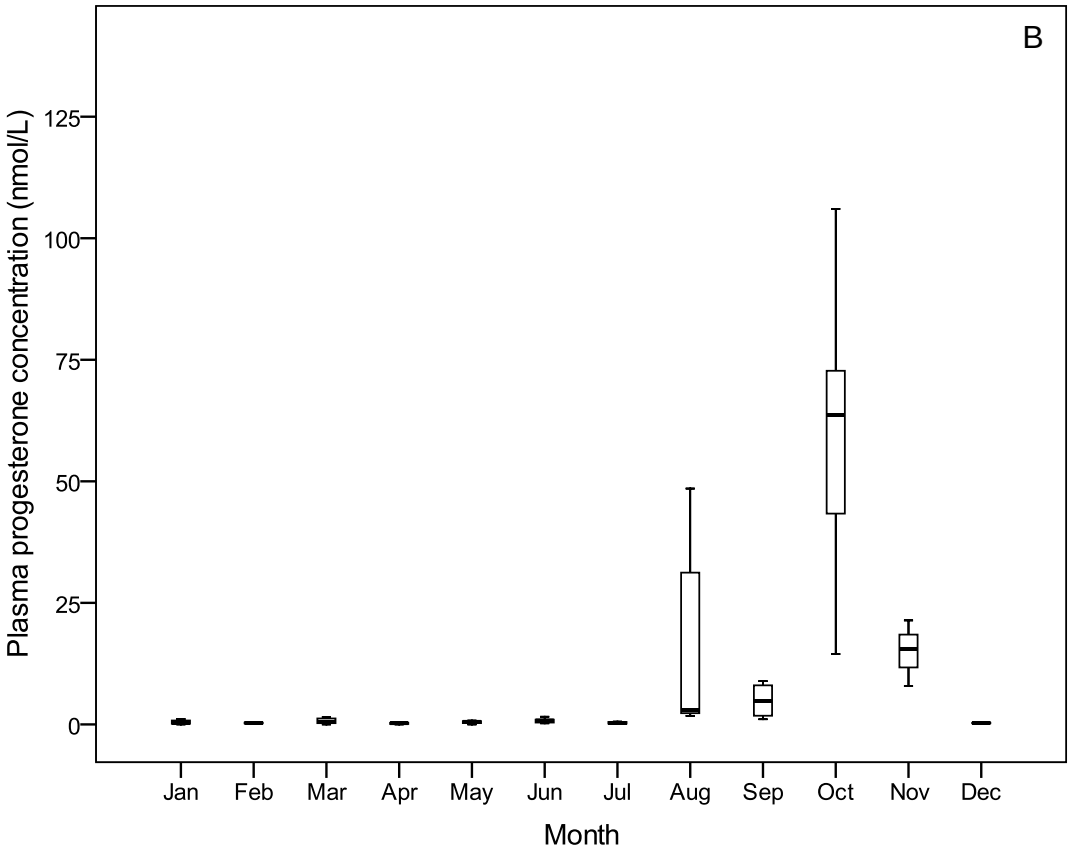

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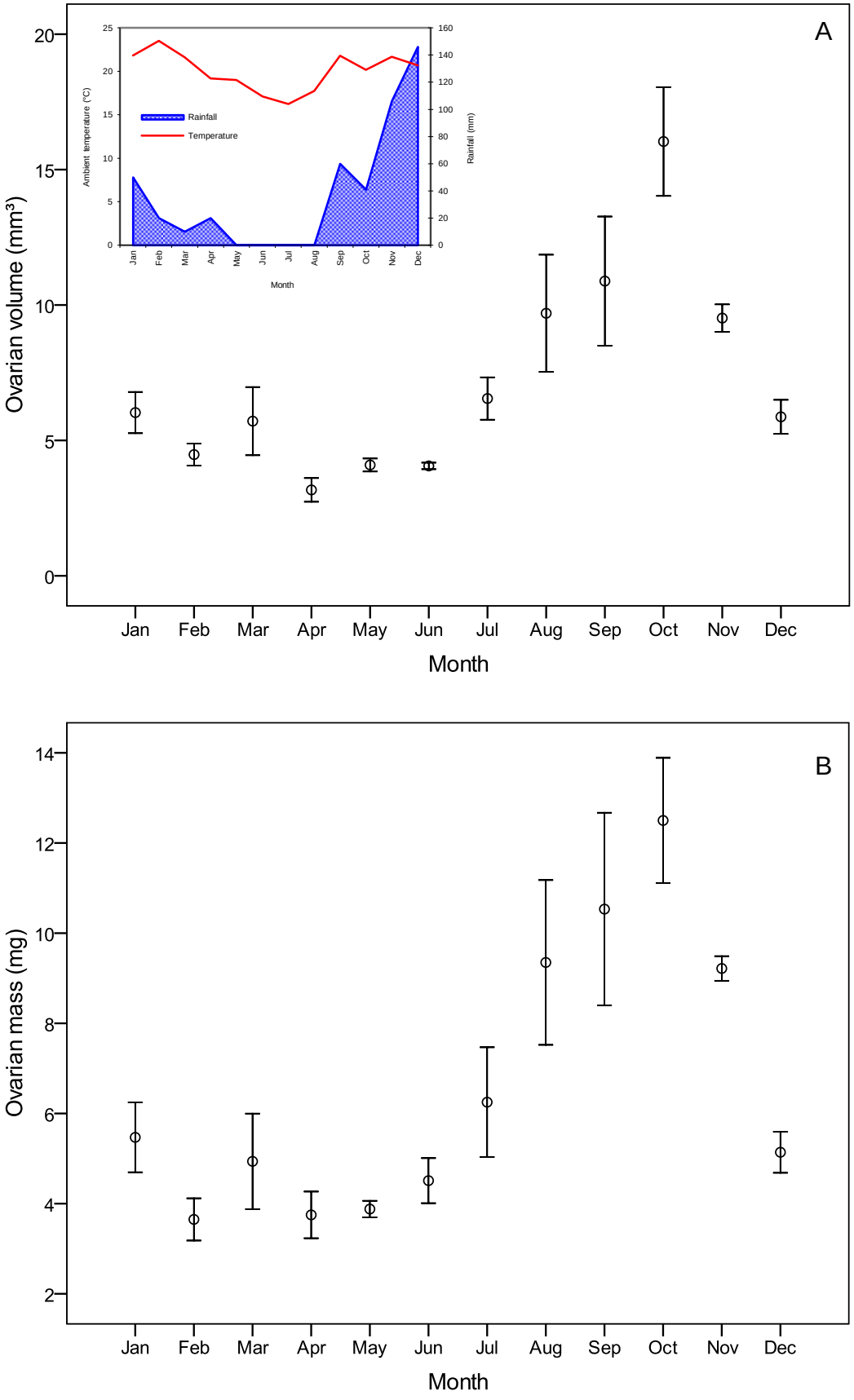

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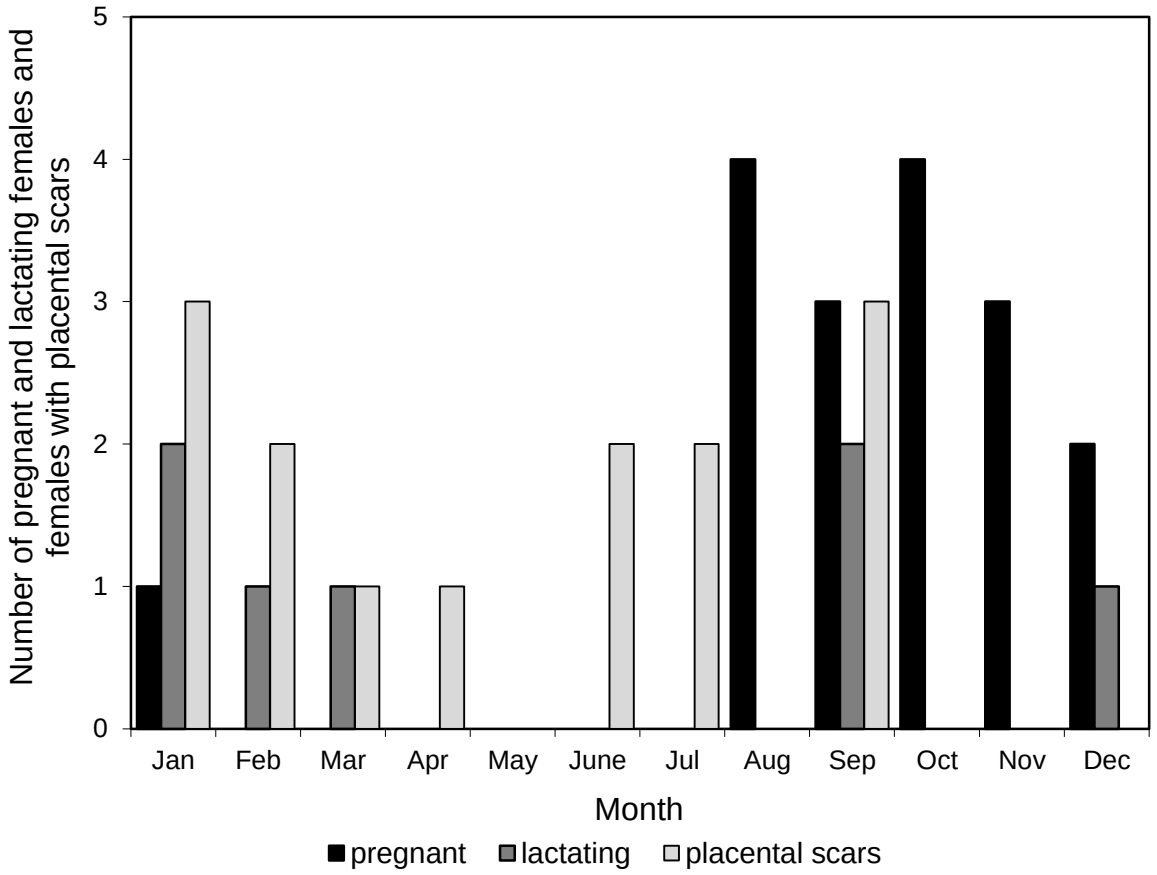

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CHAPTER 3

REPRODUCTIVE PHOTORESPONSIVENESS IN MALE SPINY MICE (*ACOMYS SPINOSISSIMUS*) FROM SOUTH AFRICA

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Abstract

Many seasonally breeding mammals use changes in photoperiod as a reliable cue to time reproduction. Photoperiodic timing assists an animal in predicting annual environmental changes in its habitat and so, enables it to accurately time reproductive events to the most favourable conditions. Changes in day length are more pronounced in the temperate regions and photoperiod is used as a cue for reproduction by most mammals above 30° latitude, however, a number of sub-tropical species also use this proximate factor to regulate their reproductive cycle. We investigated the reproductive photoresponsiveness of 14 male spiny mice (*Acomys spinosissimus*) from southern Africa to short-day (SD; 8 hrs light:16 hrs dark) and long-day (LD; 16L:8D) photoperiods. Testicular mass and volume, the diameter of seminiferous tubules and circulating plasma testosterone concentrations were significantly increased in animals subjected to LD, whereas they were regressed when the males were kept under SD. Body mass of the males was not significantly affected by the photoperiodic conditions. Although male *A. spinosissimus* seem to use photoperiod as a proximate factor to regulate reproduction seasonally, there are other environmental factors which may influence seasonal reproduction in this species such as rainfall, food quantity and quality as well as temperature. These factors may regulate reproduction of *A. spinosissimus* in concert with photoperiod. In conclusion, the present study demonstrates the significance of photoperiodic time-measuring systems in the regulation of seasonal reproduction in a sub-tropical rodent.

Keywords: photoperiod, environmental factors, seasonality, reproduction, gonadal development, southern Africa

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Introduction

Many mammals occur in habitats where seasonal changes in environmental parameters are predictable throughout the year; however, it is crucial for an animal's survival and reproductive success to be able to anticipate these changes accurately. The absolute day length as well as the direction of day length change (photoperiod) are used by a large number of mammals as proximate cues to time seasonal changes in reproduction as well as other changes in physiology and behaviour (Goldman, 2001). Changes in photoperiod are most pronounced at the higher latitudes and it has been proposed that this proximate factor is primarily used by species occurring at latitudes above 30° where the photoperiodic signal is strongest and most reliable (Bradshaw & Holzapfel, 2007). More northerly populations of the white-footed mouse (*Peromyscus leucopus*) and the deer mouse (*Peromyscus maniculatus*) were observed to be more reproductively photoresponsive than their southerly populations (Dark *et al.*, 1983; Lynch *et al.*, 1981). In many small and often short-lived mammals from temperate regions, a continuum of photoresponsive to non-photoresponsive individuals can be found in one population which allows for a more plastic response to environmental changes and enables the non-photoresponsive individuals to breed opportunistically (Nelson, 1987; Prendergast *et al.,* 2001).

In the tropics and sub-tropics, where photoperiodic changes are less pronounced, most mammals do not use photoperiod as a proximate cue (Bernard & Hall, 1995; Nunes *et al.*, 2002) and some even appear to have abandoned any photoperiodic time-measuring systems (Bronson & Heideman, 1992). However, a few rodent species (Muteka, Chimimba & Bennett, 2006) and a shrew species (Wayne & Rissman, 1990) from sub-tropical Africa and Asia, respectively, have been found to be reproductively photoresponsive. The climate at the lower latitudes, especially in Africa, is often characterised by the occurrence of one or two rainy seasons which have a major effect on vegetation growth and therefore, rainfall with (or without) a concomitant increase in food quality and quantity has been suggested to be the main factor influencing seasonal reproduction in mammals throughout most of Africa (Neal, 1986). In the pouched mouse (*Saccostomus campestris*) and the four-striped field mouse (*Rhabdomys pumilio*) from South Africa, it has been demonstrated that food quantity affects reproduction, however, an associated influence of low ambient

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temperature on reproductive decline was also suggested (Jackson & Bernard, 2001; Tinney *et al.*, 2001).

A large number of other factors, besides photoperiod, rainfall and temperature, have been found to directly or indirectly influence reproduction in temperate as well as tropical and sub-tropical mammal species, such as social cues (Demas & Nelson, 1998), green vegetation (Reichman & van de Graaff, 1975; van de Graaff & Balda, 1973) as well as secondary plant compounds (Sanders *et al.*, 1981; Wube *et al.*, 2009). It should be noted that none of the factors mentioned above are mutually exclusive in the regulation of reproductive function and both proximate as well as ultimate factors are commonly used in combination to facilitate the best reproductive response to the environmental conditions experienced by a species, population or even a single individual.

Reproductive responsiveness to photoperiod has been studied in only a few small mammals from southern Africa, namely *S. campestris* and *R. pumilio*, but in both species, photoperiod was not the primary factor regulating reproduction (Bernard & Hall, 1995; Jackson & Bernard, 1999). However, the strongly seasonally reproducing Namaqua rock mouse (*Micaelamys namaquensis* – formerly *Aethomys namaquensis*; Skinner & Chimimba, 2005) and the Tete veld rat (*Aethomys ineptus*) from South Africa have been found to be reproductively photoresponsive (Muteka *et al.*, 2006). In order to gain further insights into the mechanisms shaping seasonality of reproduction in southern African rodents, this study aims to investigate the reproductive photoresponsiveness in the male spiny mouse (*Acomys spinosissimus*) from South Africa by comparing the development of the testes and concentrations of plasma testosterone between males subjected to either short-day (SD) or long-day (LD) photoperiods.

Acomys spinosissimus is relatively widespread in Africa, south of the equator, and occurs in Mozambique, Botswana and north-eastern South Africa in the southern African subregion (Skinner & Chimimba, 2005). The habitat of *A. spinosissimus* is characterized by one rainy season a year which lasts from about September until April in South Africa (Chapter 1). Both male and female *A. spinosissimus* have been found to reproduce seasonally (Chapter 1). The breeding season was found to

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coincide with the warm and wet spring and summer months of the southern hemisphere, while breeding was discontinued during the cold and dry autumn and winter months. It has, therefore, been suggested that rainfall, resulting in an increase in food quantity and quality, is the ultimate cause for the reproductive seasonality in *A. spinosissimus* (Chapter 1).

We hypothesized that male *A. spinosissimus* are reproductively responsive to changing photoperiods because other rodent species, which co-habit with *A. spinosissimus*, were found to be reproductively photoresponsive (Muteka *et al*., 2006). In addition, males of the golden spiny mouse (*A. russatus*) were observed to reduce spermatogenesis under SD photoperiods although the Northeast African spiny mouse (*A. cahirinus*) was found to be reproductively non-responsive to photoperiod in the same study (Wube *et al.*, 2008). We predicted that the testes of *A. spinosissimus* are regressed and the plasma testosterone concentration is lower under SD compared to LD photoperiodic conditions.

Materials and Methods

A total of 14 male *A. spinosissimus* were collected between January and March 2008, and in June 2009. The males were caught at Goro Game Reserve in the Soutpansberg region, Limpopo Province, South Africa (22°58'S, 22°57'S; 29°25'E, 29°24'E) under permits (CPM-333-00002, CPM-002-00002) from the CITES and Permit Management Office, Department of Environmental Affairs, Limpopo Province, Polokwane, South Africa. The animals were caught overnight along the rocky outcrops of the reserve with Sherman live traps (H. B. Sherman Traps, Inc. Tallahassee, Florida, U.S.A.) baited with a mixture of peanut butter, oats and fish. Male spiny mice were housed in standard polyurethane cages which were embedded with wood shavings and a paper towel was provided as shelter. They were fed daily with apples and carrots and mouse pellets and water was provided *ad libitum* during the entire experiment.

In the laboratory, animals were housed in climate controlled rooms which allowed a constant temperature of 25 °C throughout the experiment. The males were weighed in a cotton bag to the nearest 0.001 g with a digital balance (Scout Pro SPU123, Ohaus Corporation, Pine Brook, New Jersey, U.S.A.) before the onset of

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the experiments. All males were initially subjected to a photoperiod of eight hrs of light and 16 hrs of darkness (8L:16D; SD) for 40 days to attain regression of the gonads and similar reproductive status of all males before the start of the actual experimental treatments. Subsequently, male spiny mice were subjected to either a photoperiod of 8L:16D or 16 hrs of light and eight hrs of darkness (16L:8D; LD) with each experimental condition including seven individuals. After 30 days in the long or short photoperiod, respectively, male spiny mice were weighed again and subsequently euthanized with an overdose of halothane. Blood was taken from the heart of all males by exsanguination and then centrifuged at 3000 rpm for 15 mins. The blood plasma was separated from the blood cells and frozen at -35 °C until analysis for testosterone concentration. The testes were dissected out and fixed in Bouin's fluid for approximately 20 hrs after which they were rinsed and stored in 70 % ethanol. In cases where enlarged seminal vesicles were found, they were dissected out and immediately weighed to the nearest 0.001 g. All experimental procedures were approved by the animal ethics committee of the University of Pretoria (ethics clearance number: A003-07).

Histology

All excess tissue was removed from the fixed testes before they were weighed separately to the nearest 0.0001 g using a high precision scale (Ohaus Corp. Pine Brook, N.Y., U.S.A.). The testes length and width (mm) was measured with a pair of digital callipers (Sylvac Opto RS 232, Ultra Praezision Messzeuge GmbH, Germany) to the nearest 0.01 mm and then utilized to calculate testicular volume (mm^3) by using the formula for the volume of an ellipsoid: $V = 4/3\ \pi ab^2$ where a represents half the maximum length and b half the maximum width (Woodall & Skinner, 1989). The average of mass (mg) and volume was calculated for both testes per male. The tissue was dehydrated by a series of ethanol baths of increasing concentrations before it was embedded in paraffin wax. The testes were then serially sectioned in 7 µm thick sections with a rotary microtome (820 Spencer, American Optical, Scientific Instrument Division, Buffalo, N.Y., U.S.A.) and mounted on microscope slides with gelatine. After about 48 hrs drying in an oven, the sections were sequentially stained in Ehrlich's haematoxylin and eosin as described by Drury and Wallington (1967). All testes were examined for round seminiferous tubules using a light microscope (Diaplan, Ernst Leitz Wetzlar GmbH, Germany) which were then photographed at

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×10 magnification with a digital camera (Moticam 1000 1.3 M Pixel USB 2.0) attached to the microscope. The diameter of 50 seminiferous tubules (µm) per testes per animal was measured with the computer program Motic Images Plus 2.0ML (Motic China Group, LTD., Xiamen, P.R. China) and the average of all 100 diameters per individual was subsequently calculated.

Testosterone analysis

Plasma testosterone concentration was measured for all males with a coat-a-count hormone kit (Siemens Medical Solutions Diagnostics, Los Angeles, USA) according to the guidelines provided by the manufacturer. This method is based on a solid phase radioimmunoassay and requires a calibration curve for the calculation of the plasma hormone concentration (see Chapter 1 for validation). The intra-assay coefficient of variation was 3.5 % and 7.8 % and the inter-assay coefficient was 5.0 %.The minimum detectable amount of testosterone for the assay was 1.39 nmol/L.

Data analysis

A repeated measures analysis of variance (ANOVA) was performed to compare body mass before and after the experiment during which photoperiodic treatment (LD and SD) was used as an in-between factor. Analyses of Covariance (ANCOVA), with the body mass measured at the end of the experiment as a covariate, were carried out to compare testicular mass and volume, seminiferous tubule diameter and plasma testosterone concentration between the two photoperiodic treatments. The analysis of plasma testosterone concentration was done on log-transformed data. *Statistical Package for the Social Sciences* (SPSS) Statistics version 17.0 (Polar Engineering and Consulting 1993-2007) was used for all statistical analyses. All values are given as mean ± 1standard deviation and results were found to be significant at $P < 0.05$.

Results

Photoperiod affected reproductive development in male *A. spinosissimus*. Enlarged seminal vesicles were only found in males which were subjected to LD, but not in males kept under SD conditions when seminal vesicles were too small to be weighed. The mean mass of the seminal vesicles in LD males was 0.11 ± 0.06 g (range: 0.04 - 0.24 g). There was no relationship between body mass and testicular mass, diameter of seminiferous tubules and plasma testosterone concentration ($F_{1,11}$

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< 4.53; P > 0.06). Testicular volume was, however, significantly positively correlated with body mass ($F_{1,11}$ > 7.38; P < 0.02). Testicular volume and mass were significantly larger in male spiny mice subjected to LD compared to SD photoperiods ($F_{1,11}$ > 12.73; P < 0.01; Fig. 1A and 1B). The diameter of the seminiferous tubules was also significantly larger in males under LD than in males under SD ($F_{1,11}$ = 13.36; P < 0.01; Fig. 1C) and significantly more testosterone was measured in the plasma of males under LD compared to males under SD ($F_{1,11}$ = 31.20; P < 0.001; Fig. 1D).

Male spiny mice were significantly heavier at the end of the experiment than they were at the beginning ($F_{1,12}$ = 28.14; P < 0.001; Fig. 2), but body mass was not significantly different between SD and LD photoperiods ($F_{1,12}$ = 0.57; P = 0.47; Fig. 2). There was, however, no significant interaction between the photoperiodic treatments (SD and LD) and body mass measured at either the beginning or at the end of the experiment ($F_{1,12}$ = 3.02; P = 0.11; Fig. 2) indicating that changes in day length do not influence body mass in male *A. spinosissimus*.

Discussion

In a previous study, it was demonstrated that *A. spinosissimus* is a seasonal breeder which reproduces during the warm and wet spring and summer months in the southern African subregion, but the factors which lead to this reproductive pattern are unknown (Chapter 1). The present study provides the first evidence that male *A. spinosissimus* are reproductively responsive to a change in photoperiod which may be used as a proximate factor to regulate seasonal reproduction in this species. Male *A. spinosissimus* responded to SD photoperiods with a reduction in testicular mass and volume, diameter of the seminiferous tubules, mass of accessory glands (seminal vesicles) and testosterone concentrations in comparison to LD photoperiods. It is interesting to note that testes size and seminiferous tubule diameter of males subjected to LD photoperiods were similar to those found for wild caught males in July (start of breeding season in male *A. spinosissimus*) and not the size observed for males collected at the peak of the breeding season in September (e.g., testicular volume, LD: 99.6 ± 23.6 mm³, July: 100.0 ± 50.9 mm³, September: 230.6 ± 44.2 mm³; Chapter 1). This may indicate that 30 days is an insufficient time for maximum growth of the testes and it may be expected that males kept longer

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under LD conditions also show a larger increase in testes size and seminiferous tubule diameter than presently reported.

Our findings on the reproductive responsiveness of male *A. spinosissimus* are similar to those of Muteka *et al.* (2006) on *M. namaquensis* and *A. ineptus* from South Africa. Both species showed significantly higher testicular mass and volume and larger seminiferous tubule diameters under LD than under SD photoperiodic conditions, however, plasma testosterone concentration was not different between the photoperiodic conditions in *A. ineptus* although it was higher under LD than SD in *M. namaquensis.* In contrast, day length did not affect either testes size or spermatogenesis in either *S. campestris* or *R. pumilio*, both of which seem to breed opportunistically in southern Africa (Bernard & Hall, 1995; Jackson & Bernard, 1999; Jackson & Bernard, 2006). In addition, Nunes *et al*. (2002) found that photoperiod does not influence plasma testosterone concentration, testis size and seminal vesicle mass in males of the Nile grass rat (*Arvicanthis niloticus*) from an equatorial population. Other rodents, bats and shrews, which occur near the equator, have also been found to be non-responsive to photoperiod (Heideman & Bronson, 1990; O'Brien *et al.*, 1993; Rissman *et al.*, 1987). These findings suggest that opportunistically breeding small mammals from tropical and also sub-tropical regions do not use photoperiodic cues to regulate reproduction. However, sub-tropical rodents, which reproduce seasonally, seem to depend heavily on photoperiod as a proximate factor to anticipate environmental changes and shape reproduction as appears to be the case for *A. spinosissimus*, too.

Heideman and Bronson (1993) demonstrated that the Syrian hamster (*Mesocricetus auratus*) responds to photoperiodic changes at 5° and 10° latitude and, therefore, the question arises on whether populations of *A. spinosissimus* are still reproductively photoresponsive at lower latitudes or if they abandoned this photoresponsiveness because day length changes near the equator are not distinct enough. Furthermore, the day lengths chosen for the present study were much longer than the day lengths *A. spinosissimus* would experience in its natural environment. It should, therefore, be noted that the present study tested the reproductive responsiveness to photoperiod in general and not the actual responses which may be observed in the habitat of *A. spinosissimus.* Future studies should test

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if *A. spinosissimus* is also responsive to photoperiods typical of below 30° latitude. We, however, propose that male *A. spinosissimus* are able to respond to these photoperiods and, therefore, also use photoperiod to time reproduction in its natural habitat because an exclusion of any photoperiodic time-measuring systems would be expected otherwise.

Males of *A. russatus* kept under LD photoperiods were spermatogenically more active than males from SD photoperiods, but females of the same species did not show any reproductive response to photoperiod (Wube *et al.*, 2008). Both male and female *A. cahirinus* were found to be reproductively non-responsive to changing photoperiods (Wube *et al.*, 2008). In another study, however, spermatogenic activity was found to be decreased in *A. cahirinus* subjected to SD photoperiods although gonads did not regress completely (El-Bakry *et al.*, 1998). Photoperiod and other factors which influence seasonal reproduction have not been investigated in any other *Acomys* species. However, the Cape spiny mouse (*Acomys subspinosus*) from the Eastern Cape Province of South Africa might be reproductively non-photoresponsive because it is an opportunistic breeder (Fleming & Nicolson, 2002). Latitude of origin does not seem to play a role in the responsiveness of members of the genus *Acomys* to changing day length and other environmental factors may play a more important role for seasonal reproduction in the genus as a whole. Trainor *et al*. (2006) found no distinct relationship between latitude of origin and photoperiodic responsiveness in five species of *Peromyscus* from different latitudes. They, therefore, suggested that, among closely-related species, mechanisms of action of photoperiod on reproductive function may be mediated by different physiological mechanisms (Trainor *et al.*, 2006).

Body mass increased during the course of the experiment which is likely a result of captivity as the animals were provided with more protein-rich food and were probably less active in the laboratory than in their natural environment. However, there was no effect of photoperiodic treatment on body mass and the males weighed the same under SD and LD photoperiods. It appears to be fairly common in rodents that body mass is not affected by different photoperiods. El-Bakry *et al.* (1998) observed no change of body mass with varying photoperiods in four desert rodents including *A. cahirinus*. In contrast, factors which indicate body condition were affected

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by photoperiod and for example, fat pad mass and carcass lipid content increased under SD photoperiodic exposure in male *A. cahirinus* (El-Bakry *et al.*, 1998). Although photoperiod appears to be used to time reproduction, it does not seem to be of importance in regulating body mass in male *A. spinosissimus*. However, moderate photoperiodic effects on body mass may have been masked by the high food availability in the laboratory and the body condition of *A. spinosissimus* may be influenced by changing day length.

Acomys spinosissimus occurs in a highly seasonal habitat where the abundance of high quality food is mediated by seasonal rainfall. Apart from photoperiod, both food quantity and quality may regulate reproduction in the spiny mouse as described for other rodents. Food deprivation negatively affected reproductive organ growth in deer mice although not in house mice (*Mus musculus*) (Blank & Desjardins, 1984). The protein content of the diet of mammals fluctuates with seasons and it has been suggested that it may limit reproduction in tropical rodents (Field, 1975). Furthermore, different plant substances may also play a role in the seasonal reproduction of a number of rodents. Low sodium and calcium content of seeds was found to cause lower reproductive success in the California vole (Batzli, 1986). The salt content of plants increases during the dry season in xeric environments due to evaporative water loss and thus, Wube *et al.* (2009) suggested that dietary salinity may be used as a proximate factor to predict the best time for reproduction in *A. russatus*. Availability of food often interacts with photoperiod and food restriction has been found to enhance the effects of SD photoperiods (Nelson *et al*., 1992; Nelson *et al.*, 1997), whereas availability of green food may counteract the inhibitory actions of SD (Nelson *et al.*, 1983).

If the present study was compared with wild-caught animals, it appears that food quantity and possibly quality may interact with photoperiod to shape reproduction in *A. spinosissimus.* Testicular mass and volume and the diameter of the seminiferous tubules of male *A. spinosissimus* were higher in males under SD than in wild-caught individuals from the non-breeding season (e.g., testicular volume, SD: 34.0 ± 28.0 mm^3, March: 12.8 ± 8.1 mm^3; see Chapter 1) which may have been the result of the large amount of high quality food in the laboratory (see above for the effect on body mass). It is, however, possible that 70 days under SD was not long

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enough for a total regression of the testes although this is unlikely as all males were caught during the non-breeding season and the testes should have been regressed already before the start of the experiment. Moreover, fat pads around the testes appeared to be larger in individuals housed longer in captivity although time in captivity did not seem to have a significant effect on the reproductive variables (these non-significant results were not presented in the present study). We suggest that the higher food quantity and quality in the laboratory caused the difference of testicular size between laboratory and wild-caught animals. In addition, ambient temperature may also have had an effect on the size of the testes as ambient temperature was higher during the entire experiment (25 °C) than in the natural habitat during winter (< 20 °C). Nelson *et al*. (1989) demonstrated that both photoperiod and temperature affect gonadal development in male prairie voles with cold ambient temperatures further suppressing gonadal size.

Furthermore, other factors may influence reproduction in male and female *A. spinosissimus*. It is unknown if the females of *A. spinosissimus* are reproductively photoresponsive and because reproduction of females of both *A. cahirinus* and *A. russatus* have been found to be independent of photoperiodic changes (Wube *et al*., 2008), reproduction of female *A. spinosissimus* may not be influenced by photoperiod either. The onset of reproduction of female *A. spinosissimus* was previously found to coincide with the start of the rainy season (see Chapter 1). Either rainfall or water availability in general may, therefore, be proximate, but very likely ultimate factors regulating seasonal reproduction in at least female and possibly male *A. spinosissimus.* In the California mouse (*Peromyscus californicus*), for example, water availability may regulate reproduction independent of either photoperiod or the availability of food (Nelson *et al.*, 1995).

In conclusion, male *A. spinosissimus* are reproductively responsive to photoperiod with short-day length suppressing and long-day length stimulating gonadal development. As a result, photoperiod may be the proximate factor which regulates seasonal reproduction in males of this species. A number of other environmental factors are also discussed and it is likely that either rainfall, alone or by influencing plant growth and composition, or temperature affect reproduction in *A. spinosissimus* and an interaction of several factors is also likely. The present results

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show that photoperiod may possibly be an important factor in the regulation of seasonal reproduction not only in temperate species, but also in sub-tropical and perhaps even tropical rodents. Furthermore, the present study emphasizes the ecological importance for the precise and premature timing of reproductive events in seasonally breeding rodents from the sub-tropics.

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Figure legends

Fig. 1.

Standardized residual of testicular volume (mm^3) by body mass (g; A), testicular mass (mg; B), seminiferous tubule diameter (µm; C) and plasma testosterone concentration (nmol/L; D) of male spiny mice (*Acomys spinosissimus*) from South Africa subjected to either a photoperiod of 16 hrs light and 8 hrs dark (LD) or 8 hrs light and 16 hrs dark (SD). Values are presented as mean ± 1 standard deviation.

Fig. 2.

Mean body mass (g) ± 1 standard deviation of male spiny mice (*Acomys spinosissimus*) from South Africa compared between long-day (LD) and short-day (SD) photoperiodic treatments and measured at the start and end of the experiment.

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Figures

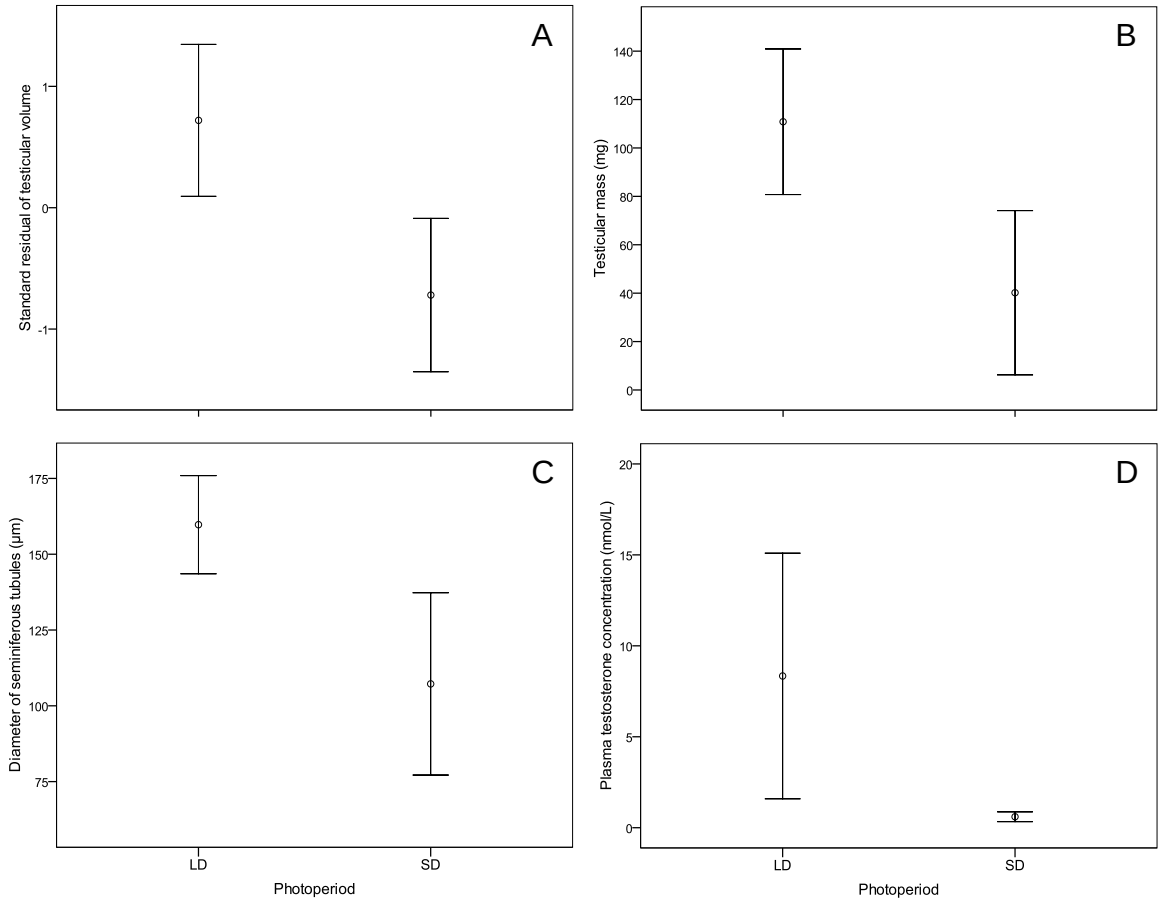

Figure 1.

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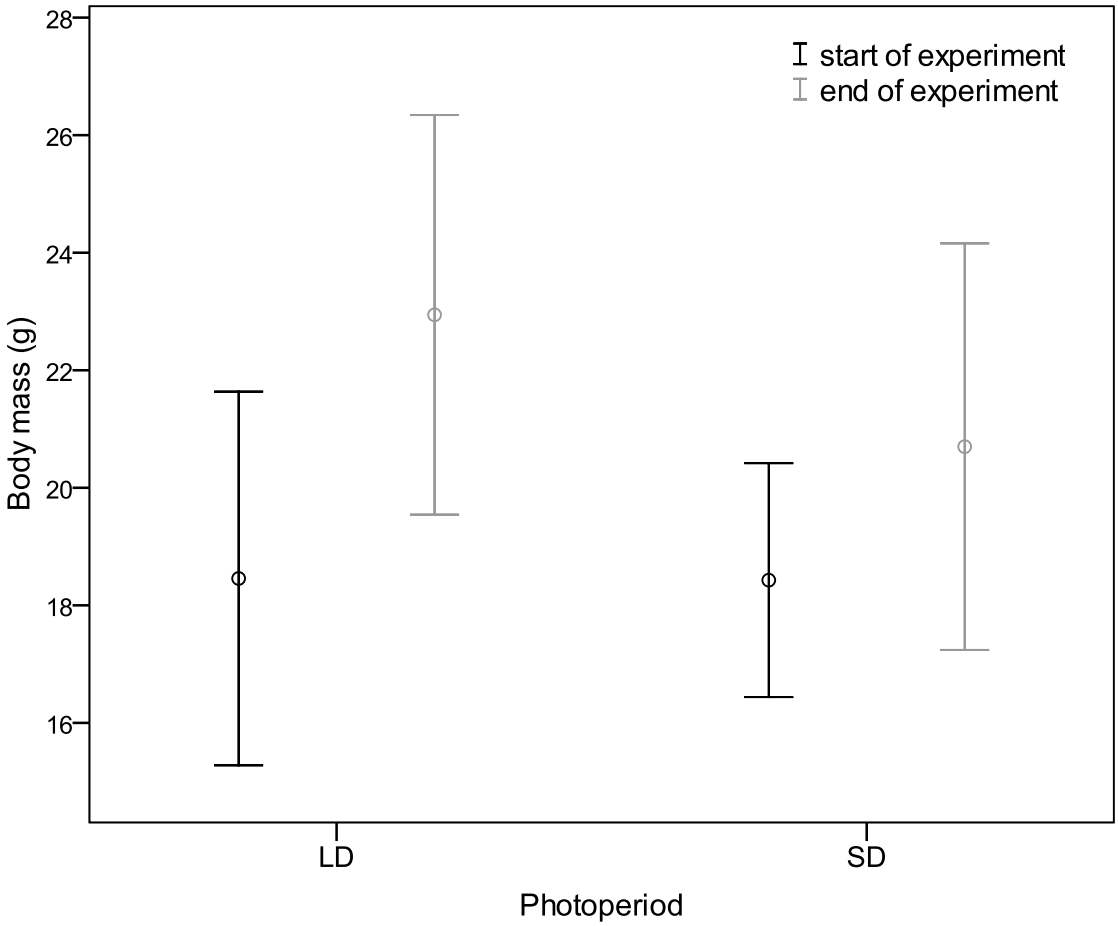

Figure 2.

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CHAPTER 4

REPRODUCTIVE RESPONSES TO PHOTOPERIOD IN MALE EASTERN ROCK ELEPHANT-SHREWS (*ELEPHANTULUS MYURUS*) FROM SOUTH AFRICA

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Abstract

Many mammals use the change in day-length to time either physiological or behavioural activities on a seasonal basis. Particularly mammals from temperate regions use photoperiod to regulate reproductive functions, however, information on small mammals from the tropics and sub-tropics are scarce. Moreover, most studies concentrated on seasonally breeding rodents, whereas the reproductive biology of most other small mammals is largely unknown. We investigated the response of the reproductive system of the male Eastern rock elephant-shrew (*Elephantulus myurus*) to photoperiods of differing length. Although a sub-tropical species, *E. myurus* breeds seasonally during the warm and wet spring and summer months of the southern hemisphere. Fourteen male *E. myurus* were subjected to either long-day (LD; 16L:8D) or short-day (SD; 8L:16D) photoperiods and the photoperiodic effect on the testes, seminiferous tubules and plasma testosterone concentration and body mass was examined. Testicular volume and mass, seminiferous tubule diameter as well as body mass were not significantly different between LD and SD conditions. However, plasma testosterone concentration was significantly lower in males on an LD compared to an SD photoperiod. Male *E. myurus* do not appear to use photoperiod as a cue to control reproductive development, but this elephant-shrew does also not seem to be devoid of a photoperiodic time-measuring system. As photoperiod is probably not the primary factor regulating seasonal reproduction in male *E. myurus*, other environmental factors such as food abundance, food compounds, or even social factors may influence the seasonal reproduction in this species. We further propose that *E. myurus* may possess an endogenous circannual clock to regulate seasonal reproduction.

Keywords: day-length, circannual rhythm, seasonal reproduction, environmental cues, Macroscelidea, southern Africa

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Introduction

In seasonal environments, resources and ultimately energy varies annually and, therefore, mammals breed during the most favourable conditions. In contrast, at times of extreme energy shortage, for example, during winter, energy may be required for other important functions such as cell maintenance and thermoregulation, and as a consequence breeding is put on hold and gonads regress (Bronson & Heideman, 1994). As a consequence, mammals need to be able to anticipate and ultimately prepare for recurring annual environmental changes in order to increase their survival and reproductive success (Bradshaw & Holzapfel, 2007). Two potential mechanisms have been identified which enable an animal to envisage environmental changes and to adapt its physiology and behaviour even before new conditions arise. The direct activation of the required changes by an environmental cue, such as photoperiod or food quantity, is the simplest of the two mechanisms (Paul *et al.*, 2008). This mechanism is mostly found in short-lived small mammals and diverse environmental factors are used by different species or even populations of the same species (Paul *et al*., 2008).

In contrast, many long-lived mammals use an endogenous circannual clock to keep track of the seasons. This clock usually operates on a cycle of approximately one year, but it may be either longer or shorter under constant environmental conditions and then, it is said to free-run (Paul *et al*., 2008). Mammals use different environmental factors to entrain this clock to the one year cycle (Paul *et al*., 2008). A change in day-length (photoperiod) is widely applied to time physiological and behavioural activities on a seasonal basis because it is most useful to predict future environmental changes (Bradshaw & Holzapfel, 2007). In particular, the reproductive biology of seasonally breeding mammals appears to depend on photoperiodic time-measuring systems in many mammal species. However, photoperiod is more reliable at higher than lower latitudes because photoperiodic changes are more marked at higher latitudes. Photoperiodic time-measuring systems are, therefore, mostly used by mammals from temperate regions, whereas other environmental factors may shape reproduction at latitudes below 30° (Bronson, 1985).

Much research on photoperiodic time-measuring systems and reproductive seasonality has been undertaken on northern hemisphere rodents (Bronson, 2009).

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Many temperate species such as species of *Peromyscus* (Lynch *et al.*, 1981; Nelson *et al.*, 1997), voles (Nelson *et al*., 1989) and lemmings (Weil *et al.*, 2006), have been found to be reproductively photoresponsive although the extent may differ between the sexes, populations and individuals. In the deer mouse (*Peromyscus maniculatus*), responsiveness to photoperiod increases with higher latitude (Dark *et al*., 1983). In contrast, responsive and non-responsive morphs have been found in a population of the northern red-backed vole (*Myodes rutilus*) (Dark *et al.*, 1983; Stevenson *et al.*, 2009). Most sub-tropical and tropical rodents breed opportunistically and use other environmental factors besides photoperiod to time reproduction. For example, the four-striped field mouse (*Rhabdomys pumilio*) has been found to be reproductively non-responsive to photoperiod, but temperature and food quantity affect reproduction of males and females of this species (Jackson & Bernard, 1999; Jackson & Bernard, 2001). In contrast, other southern African rodents have been found to regress their reproductive systems under short-day photoperiods (Muteka *et al.*, 2006; Chapter 3).

The reproductive physiology of many small African mammals is still largely unknown and although African rodents are under-represented in such research, the situation for non-murid mammals is even more depauperate. To date, we are unaware of any study which has investigated the reproductive photoresponsiveness in small mammals other than rodents in southern Africa and there are only few studies on tropical bats and shrews (Heideman & Bronson, 1994; Wayne & Rissman, 1990). The tropical musk shrew (*Suncus murinus*), for example, is reproductively photoresponsive although it is not a seasonal breeder (Rissman *et al.*, 1987). On the other hand, bats and flying foxes do not seem to use photoperiod as a cue to regulate seasonal reproduction (Heideman & Bronson, 1994; O'Brien *et al.,* 1993). The aim of the present study is, therefore, to investigate the reproductive response to differing photoperiods in the male Eastern rock elephant-shrew (*Elephantulus myurus*) from the southern hemisphere by examining gonadal development and plasma testosterone concentrations in males subjected to either long-day (LD) or short-day (SD) photoperiods.

Members of the order Macroscelidea, the elephant-shrews or sengis, are endemic to Africa (Skinner & Chimimba, 2005). *Elephantulus myurus* is insectivorous and feeds mainly on ants and termites (Churchfield, 1987). Like most elephant-

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shrews, *E. myurus* is monogamous (Ribble & Perrin, 2005) and females almost always give birth to twins which are highly precocial (van der Horst, 1946). *Elephantulus myurus* reproduces seasonally with a distinct breeding season from August until January, spanning the southern hemisphere warm and wet spring and summer months (Chapter 2; van der Horst, 1946). The gonads of both males and females regress during the cold and dry autumn and winter months (Chapter 2). To date, there are very few studies that have investigated the cues which control seasonal reproduction in either the Macroscelidea or any other African small mammal. If the regulation of reproductive function in male *E. myurus* is similar to rodents, we would predict that male *E. myurus* are reproductively photoresponsive and that the testes should be regressed with plasma testosterone concentration reduced in males subjected to SD photoperiods. This hypothesis is strengthened by the finding that rodents, inhabiting the same habitat as *E. myurus* such as the Namaqua rock mouse (*Micaelamys namaquensis* – formerly *Aethomys namaquensis*; Skinner & Chimimba, 2005) and the spiny mouse (*Acomys spinosissimus*), are reproductively photoresponsive (Muteka *et al*., 2006; Chapter 3). On the other hand, we predicted that male *E. myurus* are not reproductively photoresponsive, if the regulation of seasonal reproduction is mainly dependent on other environmental factors, besides photoperiod, as observed in longer-lived and insectivorous mammals (O'Brien *et al.*, 1993).

Materials and Methods

A total of 14 male *E. myurus* were collected at the Goro Game Reserve (22°58'S, 22°57'S; 29°25'E, 29°24'E) in the Soutpansberg region, Limpopo Province, South Africa in February, April and July 2009. The animals were trapped overnight with Sherman live traps (H. B. Sherman Traps, Inc. Tallahassee, Florida, U.S.A.) baited with a mixture of oats, peanut butter and fish. The elephant-shrews were sampled under permits issued by the CITES and Permit Management Office, Department of Environmental Affairs, Limpopo Province, South Africa (permit numbers: CPM-333-00002, CPM-002-00002) and were maintained according to the guidelines of the animal ethics committee of the University of Pretoria (ethics clearance number: EC037-08). All males were housed in large polyurethane cages (33 cm × 33 cm) with wood shavings for ground cover. In the laboratory, the animals were kept in a climate controlled room at a constant temperature of 25 °C. They were fed with canned dog

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food (Promeal Ltd., Dassenberg, South Africa), Pronutro (high protein cereal; Pioneer Foods Ltd., Bokomo Foods, Cape Town, South Africa) and grated apples and carrots. Additionally, fresh water was provided in an open dish once daily and mealworms were offered once a week.

All 14 male elephant-shrews were initially subjected to a day-night cycle of eight hrs of light and 16 hrs of darkness (8L:16D; SD) for 40 days. This was done to achieve maximum regression of the gonads and to ensure that the reproductive development of all males was comparable at the start of the experiment. Seven males sampled in February were then kept under the same light cycle for another 90 days. The seven males caught in April and July were, however, subjected to a light cycle of 16L:8D (LD) for 90 days after the initial 40 days under SD photoperiodic conditions. The animals were weighed with a digital balance (Scout Pro SPU123, Ohaus Corporation, Pine Brook, New Jersey, U.S.A.) to the nearest 0.001 g at the onset and at the end of the experiment. At the conclusion of the experiments, all males were euthanized by an overdose of halothane. Blood was taken by exsanguinations from the heart, centrifuged at 3000 rpm for 15 mins and the plasma fraction was subsequently stored at -35 °C until hormone analysis. Testes were dissected out and fixed in Bouin's fluid for about 20 hrs after which they were stored in 70 % ethanol.

Histology

All excess tissue was removed before the testes were weighed to the nearest 0.0001 g with a high precision scale (Ohaus Corp. Pine Brook, N.Y., U.S.A.). Testicular length and width (mm) were measured with a pair of digital callipers (Sylvac Opto RS 232, Ultra Praezision Messzeuge GmbH, Germany) to the nearest 0.01 mm. Testicular volume (mm^3) was then calculated by using the formula for the volume of an ellipsoid: $V = 4/3\ \pi ab^2$, where a represents half the maximum length and b half the maximum width, as described by Woodall and Skinner (1989). The average of mass (mg) and volume were calculated for both testes per male. Standard histological techniques (Chapter 2) were used to process the tissue after which the testes were cut into 7 µm thick sections with a rotary microtome (820 Spencer, American Optical, Scientific Instrument Division, Buffalo, N.Y., U.S.A.) and mounted on microscope slides. Gelatine was used as an adhesive and the slides were dried in an oven for

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about 48 hrs. The sections were then sequentially stained with Ehrlich's haematoxylin and eosin (Drury & Wallington, 1967). Fifty round seminiferous tubules per testis per animal were photographed at ×10 magnification with a digital camera (Moticam 1000 1.3 M Pixel USB 2.0) attached to a light microscope (Diaplan, Ernst Leitz Wetzlar GmbH, Germany). The diameter of the seminiferous tubules (µm) was then measured with the programme Motic Images Plus 2.0ML (Motic China Group, LTD., Xiamen, P.R. China) and the average of all diameters per individual male was calculated.

Testosterone analysis

Plasma testosterone concentration was measured with a coat-a-count hormone kit (Siemens Medical Solutions Diagnostics, Los Angeles, USA) for all 14 males. This method is based on a solid phase radioimmunoassay by which the amount of antibody bound radioactive labelled hormone is measured. The hormone concentration in the sample is subsequently calculated with a calibration curve. This assay was previously validated for *E. myurus* (see Chapter 2). The intra-assay coefficient of variation was 2.3 %, 2.7 % and 7.8 % and the inter-assay coefficient was 12.9 %.The minimum detectable amount of testosterone for the assay was 1.39 nmol/L.

Data analysis

Body mass was compared before and after the experimental treatments with a repeated measures analysis of variance (ANOVA) and photoperiodic condition (either SD or LD) used as an in-between factor. Analysis of Covariance (ANCOVA) was used to compare testicular mass, testicular volume, seminiferous tubule diameter and plasma testosterone concentration between the photoperiodic treatments during which body mass measured at the end of the experiments was utilized as a covariate to correct for any effects of body mass. The analysis of plasma testosterone concentration was performed on log-transformed data as this factor was not parametric. All statistical analyses were carried out with *Statistical Package for the Social Sciences* (SPSS) Statistics version 17.0 (Polar Engineering and Consulting 1993-2007). Data are given as mean ± 1 standard deviation and results were found to be statistically significant at $P < 0.05$.

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Results

The reproductive system of male *E. myurus* does not seem to be regulated by changing photoperiods. Testicular mass and volume and seminiferous tubule diameter were not significantly different between SD and LD photoperiods ($F_{1,11}$ < 2.49; P > 0.14; Fig. 1A-C). There was, however, a significant difference in plasma testosterone concentration between photoperiodic treatments ($F_{1,11}$ = 5.40; P = 0.04) with plasma testosterone being significantly higher in males subjected to SD than LD (Fig. 1D). Testicular mass and volume and plasma testosterone concentration was not significantly dependent on body mass ($F_{1,11}$ < 0.19; P > 0.67). There was, however, a significant negative correlation between body mass and seminiferous tubule diameter ($F_{1,11}$ = 6.01; P = 0.03).

Males were significantly heavier at the end of the experiment than at the beginning ($F_{1,12}$ = 9.79; P < 0.01; Fig. 2), but body mass was not significantly different between SD and LD photoperiods ($F_{1,12}$ = 2.23; P = 0.16; Fig. 2). There was no interaction between body mass at the beginning and end of the experiment and the photoperiodic treatments ($F_{1,12}$ = 3.13; P = 0.10; Fig. 2) indicating that body mass was not significantly influenced by photoperiod.

Discussion

Neal (1995) proposed that photoperiod may regulate seasonal reproduction in *E. myurus* because the start of the breeding season in this species is approximately a month following the winter solstice (see also Chapter 2). However, the breeding season is often described as the period when females are found to be pregnant instead of the time between recrudescence and regression of the gonads which is commonly more extended. Testicular volume and mass in *E. myurus* already increases in May, long before the first pregnant females are observed (August; Chapter 2). This also shows that the male gonads recrudesce before the winter solstice and a timing of reproduction by photoperiod in male *E. myurus* may not be as straightforward as suggested by Neal (1995). The present study suggests that photoperiod is not the primary environmental cue regulating reproduction in male *E. myurus*. Testicular mass and volume did not either regress or increase under SD or LD photoperiodic treatment, respectively, and also seminiferous tubule diameter was not significantly different between SD and LD photoperiods.

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If *E. myurus* is reproductively photoresponsive as is the case with many seasonally breeding rodent species (Nelson *et al.*, 1998), we would have expected a decrease in gonadal size and general reproductive development under SD conditions and an increase in reproductive development under LD photoperiods. In this regard, it is even more surprising that plasma testosterone concentration was increased in male *E. myurus* subjected to SD and decreased in males subjected to LD photoperiods. Effects of photoperiod on testosterone concentration have been investigated especially in rodents, but photoperiod does not affect testosterone concentration in all photoresponsive species examined. For example, in the sub-tropical Tete veld rat (*Aethomys ineptus*), testosterone was found to be similar in males under LD and SD photoperiods (Muteka *et al*., 2006). On the other hand, testosterone concentration was observed to be decreased under SD in the white-footed mouse (*Peromyscus leucopus*) (Young *et al.*, 2000) and in the Namaqua rock mouse (Muteka *et al.*, 2006). It is unclear why the opposite photoperiodic effect on plasma testosterone was observed in *E. myurus* in the present study.

The variation in seminiferous tubule diameter as well as plasma testosterone concentration was very large under SD, whereas there was only little variation in these variables in males subjected to LD. This and the difference in testosterone levels between SD and LD photoperiods suggests that photoperiod has some effect on the reproductive development of male *E. myurus* although the nature and extent are not apparent. It appears that LD photoperiods limit reproductive development in all individuals around a single instance, whereas SD photoperiods permit a wider range of reproductive responses. In many temperate rodent species, reproductively photoresponsive as well as non-responsive individuals can occur within the same population (Prendergast *et al.*, 2001). These species are often short-lived and it is, therefore, important for these to breed during any favourable opportunity during their lifespan (Bronson, 1985). Accordingly, being non-responsive to photoperiod increases reproductive success since it enables an individual to reproduce even during winter when conditions are favourable and other photoresponsive individuals are not able to reproduce (Prendergast et al., 2001).

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The large variation in responses to SD observed for some reproductive parameters of male *E. myurus* may be explained by several individuals being responsive and others non-responsive to photoperiod. The sample size is, however, too small and the reproductive responses of *E. myurus* are too variable to give a definitive answer to the question of individual photoresponsiveness. Furthermore, testicular mass and volume of *E. myurus* were not affected by photoperiodic treatment in any way. The testes of *E. myurus* are relatively small and internal (Woodall, 1995) and their size may, therefore, not be influenced by photoperiod. This is further supported by a study which did not find seasonal differences in testes size, but larger seminiferous tubules and increased spermatogenesis during the breeding season of *E. myurus* (Woodall & Skinner, 1989). It should, however, be noted that seasonal differences in testes size were established in another study (Chapter 2). In addition, these different results for seasonal variation in testes size may indicate that during some years, male *E. myurus* retain some capacity to reproduce, whereas during other times, possibly during more environmentally harsh years, males cease breeding entirely. This is also supported by the relatively small, internal testes in *E. myurus* which may require less energy than the large, external testes in other small mammals such as rodents. In summary, male *E. myurus* may exhibit a more opportunistic breeding strategy than previously reported and for which a distinctive photoperiodic regulation would be cumbersome.

Elephantulus myurus is a relatively long-lived mammal in comparison to many small rodent species. Many rodents live only for about half a year and breed only during one breeding season (Tkadlec & Zejda, 1998), whereas *E. myurus* has a life expectancy of about 12 to 13 months and may breed over two seasons (van der Horst, 1946). It is, therefore, possible that reproduction in *E. myurus* is regulated by an endogenous circannual rhythm rather than directly by an environmental cue. Endogenous circannual rhythms are frequently used by longer-lived mammals where a time-measuring system which runs for a year may be more suitable and accurate for reproductive regulation than a mechanism which is directly dependent on environmental factors (Paul *et al.*, 2008). Heideman and Bronson (1994) observed that the tropical bat (*Anoura geoffroyi*) uses a true endogenous circannual rhythm to regulate seasonal reproduction. The endogenous circannual rhythm of this bat is, however, not entrained by photoperiod and probably regulated by another

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environmental cue (Heideman & Bronson, 1994). A similar time-measuring system was also proposed to regulate seasonal reproduction in the little red flying fox (*Pteropus scapulatus*) (O'Brien *et al.*, 1993). In *E. myurus,* this rhythm either may or may not be entrained by photoperiod. The few effects of photoperiod on reproductive development, which have been discussed previously, do, however, intimate an entrainment of the endogenous circannual clock by photoperiod. The lower plasma testosterone concentration observed under LD when compared to SD may also be a consequence of an endogenous circannual rhythm regulating testosterone secretion rather than a direct effect of photoperiod. Nevertheless, the present study was too short to confirm that an endogenous circannual rhythm regulates seasonal reproduction in *E. myurus* and further studies are needed to verify this hypothesis.

Male *E. myurus* are reproductively active during captivity independent of the photoperiodic regime under which they are placed (present study). In a previous study, it was established that the male reproductive system of *E. myurus* is quiescent during the autumn and winter months in its natural habitat (Chapter 2). It is possible that other environmental factors may, therefore, either regulate the seasonal reproduction of this species or possibly entrain its endogenous circannual clock. In southern Africa, we are not aware of any study on reproductive photoresponsiveness on small mammals other than rodents and the present study is the first in this regard on Macroscelidea which makes comparisons complicated. Many tropical and sub-tropical rodents breed seasonally and it has been suggested that seasonal rainfall and the concomitant increase in food quantity and quality are the main factors which regulate seasonal reproduction at these low latitudes (Delany, 1972; Neal, 1986). Taylor and Green (1976), for example, found that rainfall and the availability of food regulates reproduction in African rodents, particularly the Nile grass rat (*Arvicanthis niloticus*) and the Natal multimammate mouse (*Mastomys natalensis*). In contrast to most rodents, *E. myurus* is insectivorous and consumes primarily ants and termites (Churchfield, 1987).

Regrettably, there are only few studies on the influence of food on seasonal reproduction of most insectivorous mammals. Willis *et al.* (1992) observed that more termites were ingested by the aardvark (*Orycteropus afer*) in summer than in winter suggesting that termites are more readily available during the rainy season. It is likely

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that these food sources also either increase or are more accessible to the elephant-shrew after rainfall. Insect abundance would, therefore, increase during a period when the first young are born (see Chapter 2) and particularly high energetic demands have to be met by the mother during lactation (Speakman, 2008). Male *E. myurus* may adjust their reproductive development to that of the female to ensure that the young are born during this time of maximum insect abundance. It is, however, unclear what the proximate cues that regulate reproduction in *E. myurus* may be as both rainfall and increased food availability occur after the onset of reproduction in this elephant-shrew (Chapter 2). In the omnivorous Round-eared elephant-shrew (*Macroscelides proboscideus*), the increase in calcium concentration in fresh vegetation during the rainy season may result in enhanced reproduction during the same period (Bernard *et al.*, 1996). In addition, during the dry season, the salt content of plants is increased because of the high evaporative water loss which may affect reproduction. Golden spiny mice (*Acomys russatus*) fed a diet with a high salt content were found to have reduced testes mass and spermatogenesis (Wube *et al.*, 2009). In case *E. myurus* is more omnivorous than previously reported (Churchfield, 1987), this elephant-shrew may also use some plant compounds to time reproduction.

Trainor *et al*. (2006) established that the housing of a male with a female can significantly influence reproduction in some species of *Peromyscus,* but the degree of the response is dependent on the mating system of the species. In *Peromyscus polionotus*, a monogamous rodent, social housing resulted in an increase in testosterone concentration and social factors together, but also appears to be independent of photoperiod to regulate reproduction in this species (Trainor *et al*., 2006). In *Peromyscus aztecus*, which is not photoresponsive, males housed with females exhibited an increase of testes and epididymis mass and higher sperm counts and testosterone concentration compared to individually housed males (Demas & Nelson, 1998). In other small non-rodent mammals, social cues affect reproduction. In male musk shrews, for example, the presence of a female stimulated reproduction and was even able to partly reverse the inhibitory effects of SD photoperiods (Wayne & Rissman, 1990). *Elephantulus myurus* is monogamous (Ribble & Perrin, 2005) and the pair bonding may, therefore, also be important for the regulation of seasonal reproduction in this species.

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Similar to the reproductive parameters assessed in the present study, the body mass of male *E. myurus* was not significantly different between the photoperiodic treatments. An effect of captivity was, however, apparent as the elephant-shrews gained body mass during the course of the experiment probably due to a lack of activity. It should, however, be noted that although not significant, this effect was especially obvious under SD, but not under LD photoperiods indicating that day-length may affect body mass in *E. myurus*. An increase in body mass under SD photoperiod was demonstrated for collared lemmings (*Dicrostonyx groenlandicus*) which breed during the harsh winters in northern Canada and Greenland where the body mass gain is probably an adaptation to save energy by providing fat storage and increasing thermoregulatory ability (Hasler *et al.*, 1976; Weil *et al.*, 2006). In contrast, the body mass of male musk shrews from the tropics was observed to decrease under SD photoperiods (Rissman *et al.*, 1987; Wayne & Rissman, 1990). No difference in body mass was found between SD and LD photoperiods in an otherwise reproductively photoresponsive marsupial (Holloway & Geiser, 1996). In *E. myurus*, an alteration of body mass with changing photoperiods may not be advantageous. However, the comparatively larger increase in body mass in SD than LD photoperiods may indicate either a thermoregulatory or a fat storage capacity of body mass during the cold and dry winter.

In conclusion, reproductive development did not cease under SD photoperiods and, therefore, photoperiod may not be the primary cue which regulates seasonal reproduction in male *E. myurus*. However, although equivocal, there may be some evidence of photoperiodic effects that suggest that *E. myurus* may not be completely devoid of a photoperiodic time-measuring system. Other environmental factors besides photoperiod are, however, more likely to control seasonal reproduction in this elephant-shrew. Food abundance, food compounds and social factors may all be important environmental cues which regulate seasonal reproduction in *E. myurus.* In addition, we propose that male *E. myurus* may use an endogenous circannual rhythm to time reproduction that may or may not be entrained by photoperiod.

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Figure legends

Fig. 1.

Testicular mass (mg; A) and volume (mm³; B), standardized residual of seminiferous tubule diameter (µm) by body mass (g; C) and plasma testosterone concentration (nmol/L; D) of male Eastern rock elephant-shrews (*Elephantulus myurus*) from South Africa compared between long-day (LD) and short-day (SD) photoperiodic treatments. Values are mean ± 1 standard deviation.

Fig. 2.

Mean body mass (g) ± 1 standard deviation of male Eastern rock elephant-shrews (*Elephantulus myurus*) from South Africa displayed between long-day (LD) and short-day (SD) photoperiodic treatments and compared between the start and the end of the experiment.

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Figures

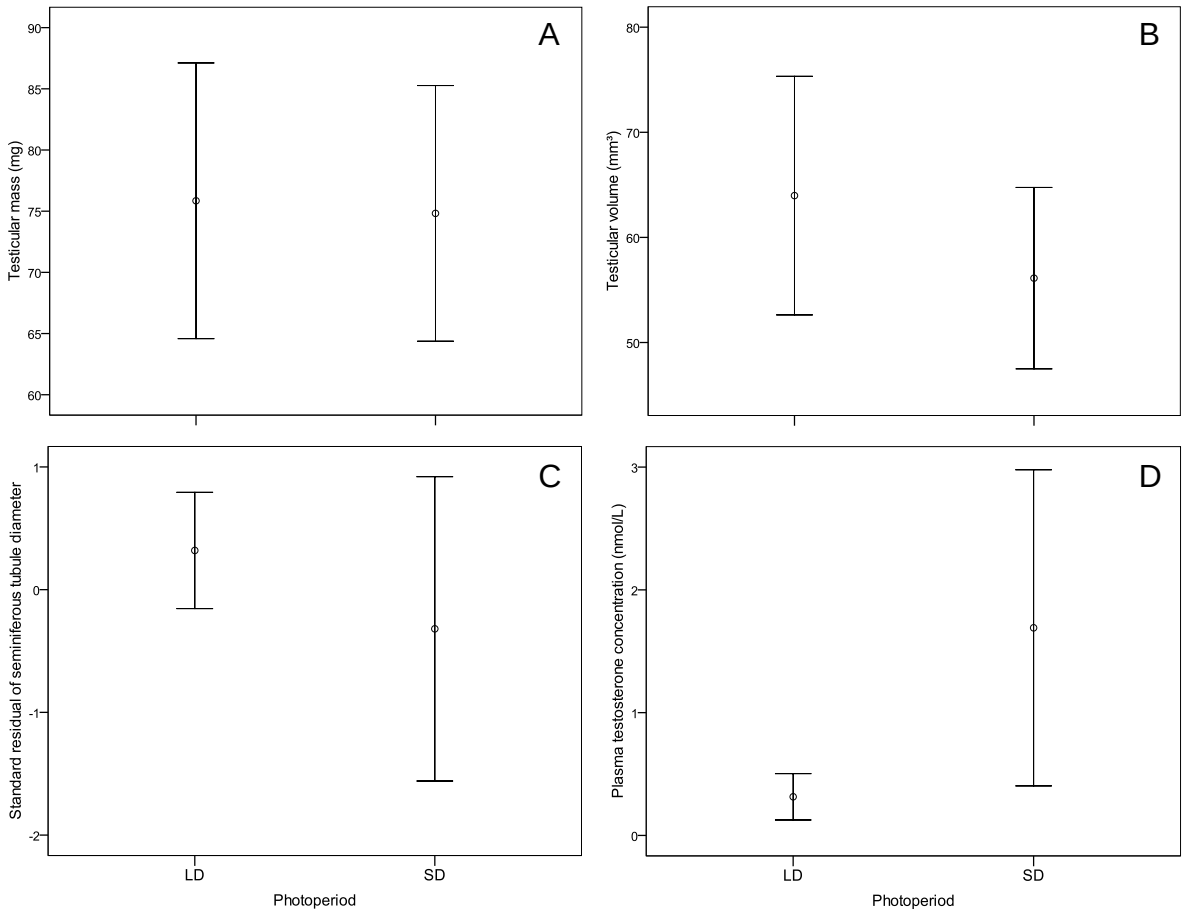

Figure 1.

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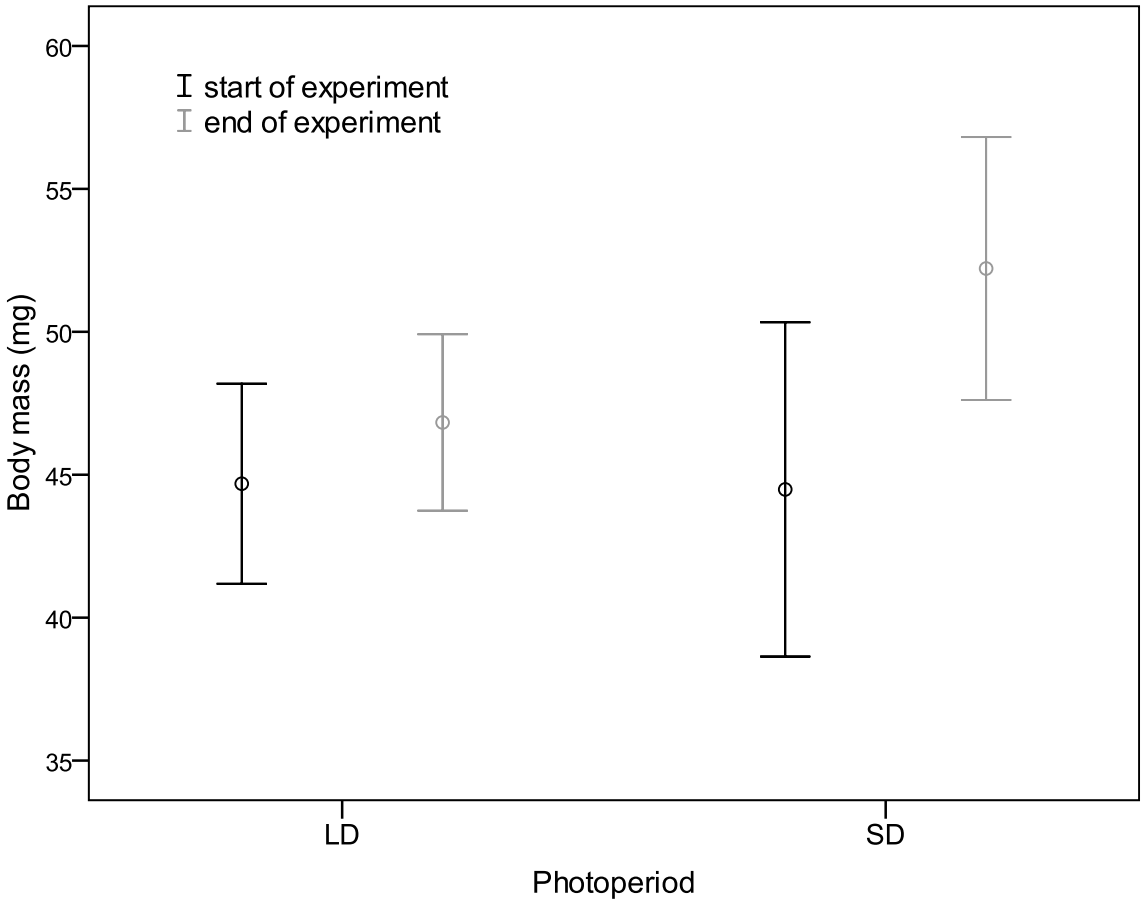

Figure 2.

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CHAPTER 5

KISSPEPTIN-IMMUNOREACTIVITY IN MALES AND FEMALES OF THE SEASONALLY BREEDING SPINY MOUSE (*ACOMYS SPINOSISSIMUS*) FROM SOUTH AFRICA: EFFECTS OF SEX AND SEASON

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Abstract

Seasonally breeding mammals use environmental factors, especially photoperiod, to coordinate reproduction and to ensure that their offspring are born during the most favourable time of the year. The mechanisms by which these factors are integrated into the regulation of reproductive function are, however, poorly understood. To elucidate the role of kisspeptin in the regulation of seasonal reproduction, it is important to study kisspeptin expression in species under semi-natural and natural conditions. The present study investigated kisspeptin-immunoreactivity in male and female spiny mice (*Acomys spinosissimus*) collected during the breeding and non-breeding seasons in their natural environment in South Africa. Kisspeptin-immunoreactive (-ir) fibres were present in the preoptic area and the arcuate nucleus (Arc) in the spiny mouse and kisspeptin-ir cell bodies were situated exclusively in the anteroventral periventricular area (AVPV) and Arc. In the AVPV, kisspeptin-ir cell bodies were found only in males caught during the breeding season intimating that kisspeptin may be involved in seasonal reproduction in males. Photoperiod may also affect kisspeptin expression in males of this reproductively photoresponsive species. In contrast, the only female spiny mouse in which kisspeptin-ir cell bodies were observed in the AVPV was the only one found to be pregnant. The absence of such cell bodies in the non-pregnant breeding females may be due to suppression of kisspeptin production during either lactation or because of an induced ovulatory pattern in this species. In the pregnant female, kisspeptin synthesis/storage may be increased because of a stimulatory effect of progesterone which is secreted by the corpora lutea. We did not observe any effects of season or reproductive state on kisspeptin-immunoreactivity in the Arc suggesting that it may not play a role in the seasonal regulation of reproduction by kisspeptin in this species. The results in the present study suggest that kisspeptin may be involved in the regulation of seasonal reproduction, although environmental factors such as photoperiod may affect kisspeptin synthesis/storage only in males, but not females of the spiny mouse.

Keywords: *Kiss1* gene, seasonal reproduction, photoperiod, lactation, pregnancy, induced ovulation

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Introduction

Most mammalian species experience marked seasonal changes during the year and, therefore, restrict breeding to a time at which conditions are most favourable and reproductive success is maximized (Bronson, 1985). Because reproduction imposes high energetic demands on an individual, breeding is frequently interrupted during periods at which energy is not readily available and needed for other bodily functions such as immune responses or thermoregulatory requirements (Nelson *et al.*, 1998). Environmental factors, such as photoperiod or food availability, are used by most seasonally breeding mammal species to assess the right time for reproduction (Bronson, 1985). These environmental signals are relayed to the neuroendocrine axis to regulate the timing of reproduction (Goldman, 2001). The hypothalamo-pituitary-gonadal (HPG) axis, which is the major pathway in the regulation of reproductive function, plays a key role in regulating seasonal reproduction in mammals (Lehman *et al*., 1997).

Kisspeptins (hereafter referred to as kisspeptin), the products of the *Kiss1* gene and endogenous ligands for the G protein coupled receptor 54 (Kiss1r) (Kotani *et al.*, 2001; Ohtaki *et al*., 2001), have recently been found to be of major importance in a wide array of mammalian reproductive functions. The major influence of kisspeptin on the reproductive system appears to be mediated through the HPG axis where it facilitates the release of gonadotrophin-releasing hormone (GnRH) from the hypothalamus and thereby the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland (Gottsch *et al*., 2004; Irwig *et al*., 2004). Kisspeptin acts directly on the GnRH neurons through its receptor Kiss1r, which has been found to be expressed by GnRH neurons (Messager *et al*., 2005) and it also induces the preovulatory GnRH/LH surge by mediating oestrogen's positive feedback to the GnRH neurons (Clarkson *et al.*, 2008; Smith *et al.*, 2006). In rodents, oestrogen positive feedback on kisspeptin neurons appears to be limited to the anteroventral periventricular nucleus (AVPV). At this site, kisspeptin neurons express oestrogen receptor α and an increase in *Kiss1* mRNA was observed at the time of the LH surge (Castellano *et al.*, 2006; Clarkson *et al.*, 2008; Smith *et al.*, 2006). In contrast, it has been speculated that kisspeptin neurons in the arcuate nucleus (Arc) are involved in the negative feedback actions of oestrogen and testosterone (D'Anglemont de Tassigny & Colledge, 2010; Kauffman, 2010a).

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Puberty onset and seasonal reproduction are regulated by the actions of kisspeptin on the HPG axis. Central administration of kisspeptin results in an advanced onset of puberty in immature rats (Navarro *et al*., 2004), whereas treatment with the kisspeptin antagonist p234 delays puberty (Pineda *et al*., 2010). In several seasonally breeding species, it has been found that kisspeptin expression varies between animals subjected to long- (LD) or short-day (SD) photoperiods, being lower under SD than LD, and these variations are possibly driven by either direct or more likely indirect actions of melatonin on kisspeptin neurons (Ansel *et al.*, 2010; Simonneaux *et al.*, 2009).

Kisspeptin expression in response to changes in photoperiod varies, however, markedly between different nuclei in the brain and between species. For example, in Syrian hamsters (*Mesocricetus auratus*), *Kiss1* expression was found to be down-regulated in both the AVPV and Arc under SD photoperiods compared to LD photoperiods (Ansel *et al*., 2010). In contrast, kisspeptin expression was higher in the AVPV, but lower in the Arc, under LD photoperiods in Siberian hamsters (*Phodopus sungorus*), while the opposite responses were observed under SD photoperiods (Mason *et al*., 2007). In addition, in ewes, an effect of season on *Kiss1* expression was found only in the Arc, with levels being reduced during the non-breeding season compared to the breeding season (Smith *et al*., 2007). Explanations for these marked differences in seasonal kisspeptin expression between species remain unknown, although they may merely reflect differences in the methods used (Greives *et al*., 2008b). However, species differences in kisspeptin expression are more likely due to marked differences in the neuroendocrine regulation of seasonal reproduction across species (Greives *et al*., 2008b). Greives *et al*. (2008b) suggested that in order to elucidate the role that kisspeptin has on seasonal reproduction, there may be a need to further ascertain its expression in a wide range of species and also under either natural or semi-natural conditions and compare these results to those of laboratory-based studies undertaken to date.

To this end, the present study attempts to determine kisspeptin-immunoreactivity in the hypothalamus of males and females of a sub-tropical rodent, the spiny mouse (*Acomys spinosissimus*) which were wild-caught in South Africa

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during both the breeding and non-breeding seasons. The spiny mouse has a wide distribution in southern Africa (Skinner & Chimimba, 2005) and has been found to breed seasonally during the warm and wet spring and summer months (September – January) of the southern hemisphere, whereas it ceases breeding during winter (February – August) (Chapter 1). Although this rodent was found to be reproductively photoresponsive (Chapter 3), its general reproductive biology remains largely unknown.

The aims of this study were three-fold, namely: 1) To map the distribution of kisspeptin-immunoreactive (-ir) cell bodies, the production site of the peptide, and fibres, which are used for peptide transport, in the hypothalamus of the spiny mouse; 2) To compare kisspeptin-immunoreactivity between the breeding and non-breeding seasons at the two hypothalamic sites previously found to be important for seasonal responses of kisspeptin, the AVPV and Arc; and 3) To compare kisspeptin-immunoreactivity between males and females at these sites because of the reported sexual dimorphism in the abundance of kisspeptin neurons in the AVPV, being more abundant in females than in males (De Vries & Södersten, 2009; Kauffman, 2010b). To the best of our knowledge, the present investigation represents the first study on kisspeptin's function in a seasonal breeder under natural conditions. Therefore, the present study has implications in our understanding of the ecological relevance of the kisspeptin system in seasonal reproduction in mammals.

Materials and Methods

Animal sampling and maintenance

Male and female *A. spinosissimus* were wild-caught over-night in 2008 and 2009 during the non-breeding season in April and during the peak of the breeding season in October. Five males and females were collected during the non-breeding season, whereas six males and five females were caught during the breeding season. The animals were trapped using Sherman live-traps (H. B. Sherman Traps, Inc. Tallahassee, Florida, U.S.A.) along rocky outcrops at Goro Game Reserve) (22°58'S, 22°57'S; 29°25'E, 29°24'E) in the Soutpansberg region, Limpopo Province, South Africa. Traps were baited with peanut butter, oats and fish. Animals were collected under permits CPM-333-00002 and CPM-002-00002 issued by the CITES and Permit Management Office, Department of Environmental Affairs, Limpopo

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Province, South Africa. Immediately after capture, all animals were weighed to the nearest 0.001 g with a digital balance (Scout Pro SPU123, Ohaus Corporation, Pine Brook, New Jersey, U.S.A.). During transportation and in the laboratory, animals were housed individually in polyurethane cages and provided with wood shavings as bedding and a paper towel for additional shelter. They were fed with mouse pellets, apples and carrots and water was provided *ad libitum*. All experimental procedures were approved by the animal ethics committee of the University of Pretoria, Pretoria, South Africa (ethics clearance number: EC037-08).

Perfusion and histology

Soon after capture, all animals were euthanized with halothane and immediately perfused transcardially with 100 ml of 0.9 % saline, followed by 100 ml of 4 % paraformaldehyde in 0.1 M phosphate buffered saline (PBS). The heads were separated from the body and the entire brain removed and stored in 4 % paraformaldehyde at 4 °C until processed. The gonads were dissected out and post-fixed in Bouin's solution for about 20 hrs after which they were stored in 70 % ethanol. Pregnancies were noted and uterine horns were examined for placental scars indicating previous pregnancies. Swollen teats were assumed to be an indication of lactation. The testes were weighed to the nearest 0.0001 g using a high precision scale (Ohaus Corp. Pine Brook, N.Y., U.S.A.). The length and width of both testes and ovaries were measured to the nearest 0.01 mm with a pair of digital callipers (Sylvac Opto RS 232, Ultra Praezision Messzeuge GmbH, Germany). Testicular and ovarian volumes (mm^3) were subsequently calculated using the formula for the volume of an ellipsoid (Woodall & Skinner, 1989): $V = 4/3\ \pi ab^2$ (in which a represents half the maximum length and b half the maximum width). The average mass and volume of both testes and ovaries were calculated for each animal. The histology of the testes and ovaries was performed as described previously (Chapter 1). Ovarian and testicular sections were stained with Ehrlich's haematoxylin and counter-stained with eosin (Drury & Wallington, 1967).

Ovaries were examined for stages of follicular growth under a light microscope (Vickers Instruments, U.K.) at a magnification of ×200 and the total numbers of primary, secondary, tertiary and Graafian follicles, corpora lutea and corpora albicans were counted throughout the entire ovary (see Chapter 1). The number of primordial

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follicles has previously been found not to differ between the breeding and non-breeding seasons (Chapter 1) and primordial follicles were, therefore, not counted. Testicular sections were examined for round seminiferous tubules and photos were taken at ×10 magnification with a digital camera (Moticam 1000 1.3 M Pixel USB 2.0, Motic China Group, LTD., Xiamen, P.R. China) attached to a microscope (Diaplan, Ernst Leitz Wetzlar GmbH, Germany). Subsequently, the diameters of 100 seminiferous tubules per male were measured to the nearest 0.1 µm with the software Motic Images Plus 2.0ML (Motic China Group, LTD., Xiamen, P.R. China).

Immunohistochemistry

Brains were cryoprotected by saturation with 30 % sucrose and then quick frozen with dry ice for sectioning. Coronal sections of 25 µm were cut with a cryostat (Bright Cryostats, U.K.), beginning rostrally at the olfactory bulbs and extending caudally to the posterior end of the hypothalamus. Sections were stored in cryoprotectant at -20 °C until processed for kisspeptin-immunohistochemistry. Every sixth section was used for kisspeptin-immunohistochemistry and processed as free-floating sections. The tissue was pre-treated with 0.5 %-X100 triton and 2 % H_2O_2, to increase permeability of the tissue and to suppress endogenous peroxidase, respectively, after which the sections were incubated in 2 % normal donkey serum (Sigma-Aldrich Co., U.S.A.) for 4 hrs. Kisspeptin-ir cell bodies and fibres were labelled using rabbit anti-kisspeptin serum (Mikkelsen, J.D.) diluted at a ratio of 1:300. The sections were incubated in this primary antibody for 48 hrs at 4 °C. Subsequently, biotinylated donkey anti-rabbit IgG (1: 1000; Jackson Immunoresearch Laboratories, Inc., U.S.A.) was applied for 2 hrs. After rinses in PBS, tissue was incubated in an avidin-biotin peroxidase complex (ABC; 1:1000; Elite Kit, Vector Laboratories, Peterborough, U.K.) and kisspeptin-immunoreactivity was visualised with 0.075 % 3'-3 diaminobenzidine in 0.15 % ammonium nickel sulphate and 0.005 % H_2O_2. Sections were mounted in consecutive order (rostral to caudal) on gelatine-coated slides and cover-slipped after dehydration in increasing concentrations of ethanol and clearing in xylene.

Slides were examined for kisspeptin-ir cell bodies and fibres under bright field illumination using a microscope (Eclipse E600, Nikon Corporation, Japan) at magnifications of ×100 and ×200. By comparing the brain sections of *A.*

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spinosissimus with those in Paxinos and Watson (2007), we found that kisspeptin-immunoreactivity was particularly abundant within the preoptic area, especially the AVPV and the Arc in *A. spinosissimus*. However, kisspeptin-ir fibres in other brain regions were also observed and described (see Results). The AVPV and Arc were further examined for kisspeptin-ir cell bodies and fibres and the optical density for kisspeptin-immunoreactivity was determined. Kisspeptin-ir cell bodies in the AVPV were identified at a magnification of ×400. Only cell bodies with a clearly defined nucleus and processes were counted. In four sections containing the Arc, it was determined whether kisspeptin-ir cell bodies were visible, but the total number of cell bodies per section was not determined as it was difficult to distinguish between kisspeptin-ir cell bodies and fibres. Areas, where kisspeptin-immunoreactivity was detected, were photographed at a magnification of ×10 for further analysis of the optical density.

ImageJ, version 1.43 (National Institutes of Health, Bethesda, M.D., U.S.A) was used to analyse kisspeptin-ir density and the analysis was done according to Greives *et al.* (2008a). Optical density of kisspeptin-ir fibres was determined in two sections containing the AVPV by selecting two areas per section which showed strong staining, but areas with cell bodies were avoided. This was done because kisspeptin-ir cell bodies were found only in few animals (see results section) which made a cell-by-cell comparison difficult. In contrast, kisspeptin-immunoreactivity in the Arc was measured in areas where strong immunoreactivity was observed independent of the occurrence of cell bodies and, therefore, the optical density measured in the Arc would integrate the density of both kisspeptin-ir cell bodies and fibres. Arc optical density was measured at four levels along the rostro-caudal extent and twice in each section. The average optical density was calculated for the two measurements per section in the Arc and for the two sections of the AVPV. Optical density was analysed separately for the four sections of the Arc.

Data analysis

Body mass was compared between males and females during and outside of the breeding season with a Generalized Linear Model (GZLM). Ovarian volume, testicular mass and volume, the diameter of seminiferous tubules and numbers of primary, secondary and tertiary follicles were compared between the breeding and

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non-breeding seasons with a GZLM with body mass as a covariate. A GZLM was also used to compare the optical density in the AVPV and the Arc between the sexes and between seasons using body mass as a covariate. In addition, the optical density in the Arc was compared between the four levels with the same GZLM followed by a least significant difference (LSD) *post hoc* test. GZLMs were used for all analyses because of the small sample sizes, but only for the optical density comparisons a gamma distribution with log link was utilized because this variable was non-parametrically distributed. Chi-square (χ^2) tests were performed to compare kisspeptin-ir cell body probability in the Arc between males and females and between the breeding and non-breeding seasons. All statistical analyses were performed using the *Statistical Package for the Social Sciences* (SPSS) Statistics version 17.0 (Polar Engineering and Consulting 1993-2007). Results were assumed to be significant at $P \leq 0.05$ and are presented as mean ± 1 standard error (*SE*).

Results

Body mass and reproductive parameters

Spiny mice were significantly heavier during the breeding season than the non-breeding season (Wald $\chi^2 = 12.41$; $df = 1$; $P < 0.001$; Fig. 1). There was no significant interaction between the sexes and seasons (Wald $\chi^2 = 0.02$; $df = 1$; $P = 0.90$; Fig. 1) or a significant difference in body mass between males and females (Wald $\chi^2 = 2.94$; $df = 1$; $P = 0.09$; Fig. 1). Testicular mass and volume as well as seminiferous tubule diameter were significantly larger in breeding than non-breeding males (Wald $\chi^2 > 47.1$; $df = 1$; $P < 0.001$; Fig. 2A - C). Body mass was significantly positively related to testicular mass and seminiferous tubule diameter (Wald $\chi^2 > 6.57$; $df = 1$; $P = 0.01$), but there was no significant relationship between body mass and testicular volume (Wald $\chi^2 = 2.94$; $df = 1$; $P = 0.09$).

During the breeding season, we collected one pregnant female with three embryos and four of the five females caught, including the pregnant female, had placental scars in their uterine horns (mean = 3.7; range: 2 – 5). All females collected during the breeding season had swollen teats, but no females with swollen teats were observed during the non-breeding season. Ovarian volume was significantly larger in breeding than non-breeding females (Wald $\chi^2 = 6.86$; $df = 1$; $P < 0.01$; Fig. 2D), but was not significantly related to body mass (Wald $\chi^2 = 0.05$; $df = 1$; $P = 0.82$).

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We found significantly more primary, secondary and tertiary follicles in breeding than non-breeding females (Wald $\chi^2 > 5.86$; $df = 1$; $P < 0.02$; Table 1), but none of these follicle types was significantly related to body mass (Wald $\chi^2 < 1.72$; $df = 1$; $P > 0.19$). Graafian follicles were only found in reproductive females (Table 1). Four corpora lutea and two corpora albicans were found in the pregnant female and three corpora lutea in one non-breeding female but neither corpora lutea or corpora albicans were observed in any of the other breeding or non-breeding female spiny mice.

Kisspeptin-immunoreactivity in the preoptic area and AVPV

Kisspeptin-ir fibres of *A. spinosissimus* had a beaded appearance. They were widely distributed in the preoptic area, particularly the medial preoptic area, the medial preoptic nucleus and the AVPV. Apart from these areas, kisspeptin-ir fibres were also found in the ventromedial preoptic nucleus and periventricular nucleus. Fibres were relatively sparse and no clustering of fibres was observed in any particular area. Kisspeptin-ir cell bodies were observed only in the AVPV. Cell bodies were observed there in all breeding males and in the one pregnant female, but not in any of the other breeding females or non-breeding animals of either sex (Fig. 3). At this site, one to 18 kisspeptin-ir cell bodies (5.5 ± 0.9) were found in each section containing the AVPV in the breeding males, whereas six and 12 cell bodies (9.0 ± 3.0) were observed in the two sections obtained for the pregnant female.

Optical density of kisspeptin-ir fibres in the AVPV was not significantly different between males and females (Wald $\chi^2 = 2.05$; $df = 1$; $P = 0.15$; males: 1.2 ± 0.2, females: 1.8 ± 0.5) or between the breeding and non-breeding seasons (Wald $\chi^2 = 0.72$; $df = 1$; $P = 0.40$; breeding: 1.7 ± 0.5, non-breeding: 1.3 ± 0.2). There was no significant interaction between the sexes and the reproductive status of the spiny mice (Wald $\chi^2 = 2.29$; $df = 1$; $P = 0.13$). Body mass was not significantly related to optical fibre density in the AVPV (Wald $\chi^2 = 1.98$; $df = 1$; $P = 0.16$).

Kisspeptin-immunoreactivity in the Arc

A first clustering of kisspeptin-ir fibres was observed in the retrochiasmatic area from where they progressed caudally. At this point, a group of fibres was also observed around the periventricular hypothalamic nucleus. Kisspeptin-ir fibres and cell bodies were situated most abundantly in the Arc and fibres appeared to become denser

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further caudally (Fig. 4). Although kisspeptin-immunoreactivity was densest in the Arc, fibres were present into the dorsomedial hypothalamus and the ventromedial hypothalamic nucleus. Kisspeptin-ir fibres, but no cell bodies, were observed in the median eminence; however, their density was much less than in the Arc.

The optical density of kisspeptin-immunoreactivity measured in the Arc was significantly greater in males than in females (Wald $\chi^2 = 4.42$; $df = 1$; $P < 0.04$; Figs. 4 and 5) and was significantly different between the four rostro-caudal levels (Wald $\chi^2 = 29.63$; $df = 3$; $P < 0.001$). The optical density in the two most rostral sections was significantly less than in the two most caudal sections (LSD: $P < 0.03$; Fig. 6) and the optical density in the most rostral section was significantly less than in the second rostral section (LSD: $P < 0.05$; Fig. 6) which agrees with the general distribution observed for kisspeptin-ir fibres (see above; Fig. 4). There was no interaction between the sexes and the four levels of the Arc (Wald $\chi^2 = 0.40$; $df = 3$; $P = 0.94$). The optical density was not significantly different between the breeding and non-breeding seasons (Wald $\chi^2 = 0.23$; $df = 1$; $P = 0.63$) and there was also no significant interaction between the seasons and either the sexes (Wald $\chi^2 = 0.07$; $df = 1$; $P = 0.30$) or the four levels of the Arc (Wald $\chi^2 = 1.90$; $df = 3$; $P = 0.59$). Body mass did not significantly affect optical density in the Arc (Wald $\chi^2 = 2.05$; $df = 1$; $P = 0.15$). The probability of kisspeptin-ir cell bodies occurring in the Arc was not significantly different for males and females ($\chi^2 = 1.10$; $df = 1$; $P = 0.30$) and between the breeding and non-breeding seasons ($\chi^2 = 2.81$; $df = 1$; $P = 0.09$).

Discussion

Two recent studies carefully documented the distribution of kisspeptin-immunoreactivity in the rat and the mouse (Clarkson *et al*., 2009; Desroziers *et al*., 2010). In the female mouse, kisspeptin-ir cell bodies were exclusively observed in the periventricular continuum, the Arc and within the dorsomedial hypothalamic nucleus and posterior hypothalamus (Clarkson *et al*., 2009). Kisspeptin-ir fibres were more widely distributed throughout the mouse hypothalamus and were found in the AVPV and periventricular preoptic nuclei as well as the paraventricular, periventricular, dorsomedial and Arc among others (Clarkson *et al*., 2009). Kisspeptin-immunoreactivity in the rat contrasted to that of the mouse since kisspeptin-ir cell bodies were only found in the Arc (Desroziers *et al*., 2010). However, the distribution

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of kisspeptin-ir fibres in the rat were similar to that observed in the mouse (Desroziers *et al*., 2010).

It is interesting to note that the distribution of kisspeptin neurons observed by immunohistochemistry in *A. spinosissimus* is largely consistent to that established for the laboratory rat and mouse, but the distribution of fibres in the spiny mouse was more confined to the preoptic area and the Arc. There were, for example, no kisspeptin-ir fibres in the septohypothalamic nucleus of the spiny mouse, but such fibres are found in this region in both the mouse and the rat (Clarkson *et al*., 2009; Desroziers *et al*., 2010). Furthermore, kisspeptin-ir cell bodies were found in both the AVPV and the Arc of the spiny mouse hypothalamus, but not in any other region. Similar to the mouse and the rat (Clarkson *et al*., 2009; Desroziers *et al*., 2010), kisspeptin-ir fibres in the spiny mouse were found to be particularly dense in the Arc and surrounded the kisspeptin-ir cell bodies in this area.

A problem with kisspeptin-immunohistochemistry is the cross-reactivity of some antibodies with RFamide peptides related to kisspeptin (Oakley *et al.*, 2009). Both rat and mouse studies mentioned above took special precaution to eliminate possible non-specific binding of kisspeptin antibodies either by using two different antibodies (Desroziers *et al*., 2010) or by testing antibody specificity on *Kiss1* knockout mice (Clarkson *et al*., 2009). It should be noted that it is plausible that non-specific binding may have occurred in this study as we were not able to adjust the experimental procedure to this outcome during the course of the study. Nevertheless, we believe that the distribution of kisspeptin-immunoreactivity found in the present study is very close to the actual situation in this species because of the distinct staining observed and its concurrence with that observed in other rodents. In contrast, we suspect that some hypothalamic regions, which show kisspeptin-immunoreactivity in the spiny mouse, may have been missed because of the relatively weak kisspeptin-ir staining observed in the current study.

The AVPV and periventricular area of the third ventricle (RP3V), respectively and the Arc have been implicated as the two regions which are of major importance in reproductive functioning of kisspeptin in rodents. In ungulates and primates, however, the AVPV does not appear to be of importance (reviewed in Oakley *et al*.,

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2009). The present study demonstrated that these two areas may also play a significant role in reproduction in both male and female *A. spinosissimus*. Immunoreactivity of kisspeptin was down-regulated in the AVPV of male *A. spinosissimus* during the non-breeding season compared to the breeding season, illustrated through the presence of kisspeptin-ir cell bodies in breeding, but not in non-breeding males although the optical density of the kisspeptin-ir fibres at this site was comparable between the breeding and non-breeding seasons. These findings are consistent with the reduction in size of the testes and the seminiferous tubules of *A. spinosissimus* during the non-breeding season (this study; Chapter 1). Greives *et al*. (2008a; 2007) demonstrated a decrease in AVPV kisspeptin-ir cell body number of male Siberian hamsters which were reproductively quiescent under SD photoperiods compared to reproductively active hamsters subjected to LD photoperiods. The same photoperiodic effect on kisspeptin in the AVPV was found in male Syrian hamsters by Ansel *et al.* (2010), but not by Revel *et al*. (2006). In addition, testosterone has a positive effect on kisspeptin secretion in the AVPV as it has been shown to increase kisspeptin-ir cell body numbers and *Kiss1* mRNA expression at this site (Ansel *et al.*, 2010; Greives *et al.*, 2008a; Smith *et al.*, 2005). Male *A. spinosissimus* are reproductively photoresponsive (Chapter 3) and it is, therefore, possible that photoperiod affects kisspeptin expression or storage in the AVPV of males of this species across the seasons. This effect may be direct or indirect; thus, it may be induced by increased testosterone during the breeding season.

In contrast to the males of *A. spinosissimus*, the results obtained for the females of this species were surprising. Given the reported responses of female Siberian and Syrian hamsters exposed to SD or LD photoperiods (Ansel *et al.*, 2010; Mason *et al.*, 2007), we expected an increase in kisspeptin-immunoreactivity in breeding females of *A. spinosissimus* compared to non-breeding females; however, we found kisspeptin-ir cell bodies only in the one pregnant female. It, therefore, appears that photoperiod may not affect kisspeptin expression in females of *A. spinosissimus* as it may in males and that other factors may be responsible for the pattern observed in females. *Kiss1* expression was found to increase during pregnancy in the hypothalamus of female rats (Roa *et al*., 2006). Progesterone receptors are expressed by kisspeptin neurons in the RP3V (Clarkson *et al*., 2008), but direct evidence for a positive regulation of kisspeptin through progesterone

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seems to be lacking in mammals. Nevertheless, progesterone, produced by the corpora lutea, may stimulate kisspeptin synthesis in the AVPV of the pregnant *A. spinosissimus*. Alternatively, since LH promotes progesterone synthesis and secretion by the corpus luteum (Niswender & Nett, 1994), we may speculate that the underlying release of GnRH is driven by kisspeptin from the AVPV during pregnancy in this species.

Kisspeptin-immunoreactivity appeared to be suppressed in the non-pregnant female *A. spinosissimus* sampled during the breeding season. This is surprising since follicular development appeared to be normal in these females which had Graafian follicles present, whereas Graafian follicles were absent in females sampled during the non-breeding season. Furthermore, corpora lutea were not detected in the non-pregnant females collected during the breeding season. We suggest two possible explanations as to why kisspeptin production may have been inhibited in the non-pregnant female *A. spinosissimus* sampled during the breeding season.

Firstly, kisspeptin expression may have been suppressed because the breeding, but non-pregnant females were lactating. Yamada *et al*. (2007) demonstrated that *Kiss1* mRNA levels are lower in lactating compared to non-lactating rats; this effect was, however, observed only in the Arc, but not the AVPV. Similarly, Xu *et al.* (2009) found that *Kiss1* mRNA levels were also significantly suppressed during lactation and the level was restored by removal of the pups. Although all of the breeding female *A. spinosissimus* had swollen teats, only three of the four breeding, but non-pregnant females, had placental scars. In addition, the pregnant female possessed placental scars and corpora lutea, indicating that it gave birth shortly before it conceived again. It, therefore, appears that reproduction may not be suppressed in all lactating female *A. spinosissimus*. In addition, the presence of swollen teats is not necessarily a reliable sign of lactation (Chapter 1) and as a result it may not be a certainty that all breeding females of *A. spinosissimus* were lactating. Consequently, it may be doubtful whether lactation is a possible explanation for the observed reduction of kisspeptin-ir cell bodies in non-pregnant, but breeding females of *A. spinosissimus*.

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Secondly, in spontaneously ovulating species, ovulation is triggered by a spontaneous surge of LH from the pituitary which is induced by a positive feedback mechanism of steroids on hypothalamic GnRH neurons (Bakker & Baum, 2000). It has recently been found that the spontaneous LH surge, required for ovulation, is induced through the actions of kisspeptin/Kiss1r on GnRH neurons and the direct positive actions of oestrogen on kisspeptin neurons in the AVPV (Castellano *et al.*, 2006; Clarkson *et al.*, 2008; Smith *et al.*, 2006). In contrast, in species exhibiting induced ovulation, the preovulatory GnRH/LH surge is induced by genital stimuli during mating (Bakker & Baum, 2000). Furthermore, oestrogen appears to be the only endocrine stimulus which promotes behavioural oestrus in induced ovulators. However, its positive feedback actions on gonadotrophin-release appear to be either reduced or absent (Bakker & Baum, 2000) and, therefore, positive feedback actions of oestrogen on kisspeptin may also be lacking in such species. However, we are not aware of any study which has investigated the role of kisspeptin signalling in the hypothalamus of induced ovulators. The ovulation strategy of *A. spinosissimus* is not known, but if these animals are spontaneous ovulators, a higher level of kisspeptin-immunoreactivity might be expected in females collected during the breeding than during the non-breeding seasons. In induced ovulators, however, it is possible that kisspeptin production in the AVPV may be activated only by mating and not by the preceding effects of oestrogen. On the other hand, it may be possible that kisspeptin may not be needed for ovulation in induced ovulators and mating may stimulate the GnRH surge via a different pathway. Given that no difference in kisspeptin-immunoreativity was found between non-breeding females and breeding, but non-pregnant, females in the present study, it is possible that *A. spinosissimus* may be an induced ovulator in which either kisspeptin secretion is stimulated by mating or kisspeptin is not involved during the mating-induced preovulatory GnRH/LH surge.

In male and female Siberian hamsters, the number of kisspeptin-ir cell bodies in the Arc is higher in individuals subjected to SD (reproductively quiescent) than those under LD photoperiods (reproductively active) (Greives *et al.*, 2008a; Greives *et al.*, 2007; Mason *et al.*, 2007). Furthermore, testosterone treatment negatively affects kisspeptin-ir cell body numbers in the Arc of male Siberian hamsters, an effect which is contrary to that observed in the AVPV (Greives *et al*., 2008a). Interestingly, Syrian hamsters, however, show the opposite pattern of *Kiss1* expression in

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response to LD-SD photoperiodic treatments than that exhibited by Siberian hamsters. *Kiss1* expression in the Arc of Syrian hamsters was observed to be higher in reproductively active animals which were subjected to LD compared to animals subjected to SD photoperiods which were reproductively inactive (Ansel *et al.*, 2010; Revel *et al.*, 2006). In addition, *Kiss1* expression in the Arc has been demonstrated to be lower in anoestrous ewes than in reproductively active ewes (Clarke *et al*., 2009). Although different responses in kisspeptin expression were observed for these different species, all of these studies intimate that kisspeptin expression in the Arc is involved in the seasonal control of reproductive function (Ansel *et al.*, 2010; Clarke *et al.*, 2009; Greives *et al.*, 2008a).

The spiny mouse appears to differ in this regard because there were no differences in kisspeptin-immunoreactivity in either males or females between the breeding and non-breeding seasons. Kisspeptin production in the Arc may, therefore, be important for reproductive functions other than the regulation of seasonal reproduction. It needs to be noted, however, that the present study was conducted under natural conditions and that other environmental factors may have affected kisspeptin-immunoreactivity by influencing kisspeptin neurons to act in the opposite direction. Food restriction, for example, suppressed *Kiss1* expression in the Arc of Siberian hamsters (Paul *et al*., 2009), but not in the female rat (Kalamatianos *et al*., 2008). Apart from photoperiod, food availability may also influence seasonal reproduction in *A. spinosissimus* (Chapters 1 and 3) and may similarly affect kisspeptin expression in this species.

The AVPV is a sexually dimorphic nucleus being larger in females than in males in mammals (De Vries & Södersten, 2009). In addition, *Kiss1* neurons are more abundant in the AVPV of adult females than in adult males (Kauffman, 2010b). This sex difference develops postnatally and during puberty arising from the positive feedback actions of oestrogen on kisspeptin neurons, which ultimately generates the preovulatory GnRH/LH surge and only occurs in females (Kauffman, 2010b). The Arc, however, is not sexually differentiated. Surprisingly, although the optical density of kisspeptin-ir fibres in the AVPV of *A. spinosissimus* did not differ between the sexes, the numbers of kisspeptin-ir cell bodies observed at this site in both sexes during the breeding season suggest that kisspeptin signalling in the AVPV is stronger

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in male than in female *A. spinosissimus.* This suggests that the AVPV may be more important in the regulation of the reproductive axis by kisspeptin in the males than in the females of this species. Whether or not this is the case, it seems possible that the AVPV may not play a major role in the oestrogen positive feedback effects culminating in the spontaneous LH surge in *A. spinosissimus,* as it does in the females of other species. This also strengthens the suggestion that *A. spinosissimus* may be an induced ovulator, in which kisspeptin production in the AVPV may not be positively regulated through oestrogen. In addition, although it may merely be a reflection of a small sample size, we observed that the optical density of kisspeptin-ir fibres and cell bodies in the Arc was greater in male than female *A. spinosissimus*. Given that this is the first time that a sex difference in the Arc has been observed in a rodent, it is difficult to ascertain reasons for this and certainly needs further investigation, although it is possible that there is a connection between the sex differences found in the AVPV and the Arc in *A. spinosissimus.*

The findings of the present study often contradict other studies and many questions either remain unanswered or have been raised for the first time. Future studies should address a number of issues raised during the course of this study such as: 1) What is the role of kisspeptin during pregnancy? 2) What is the role of progesterone in the regulation of kisspeptin expression? 3) Does kisspeptin regulate the preovulatory GnRH/LH surge in an induced ovulator? 4) Is kisspeptin expression in male and female *A. spinosissimus* regulated by photoperiod and/or other environmental factors? 5) Do these environmental factors affect kisspeptin expression in the AVPV and the Arc differently? and 6) What is the nature and extent of the sexual dimorphism of kisspeptin-immunoreactivity observed in the Arc in the present study? In addition, in order to further assess the role of kisspeptin in the regulation of reproductive functions through the neuroendocrine axis, there is a critical need to investigate kisspeptin expression in *A. spinosissimus* using other experimental methods such as *in situ* hybridisation and quantitative polymerase chain reaction (PCR) (Paul *et al.*, 2009; Revel *et al.*, 2006).

In conclusion, kisspeptin neurons were primarily found in the preoptic area, especially the AVPV, and in the Arc of *A. spinosissimus*. As has been previously observed in other mammalian species, kisspeptin appears to be important in the

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regulation of reproductive functions in *A. spinosissimus* mainly through these two regions. Kisspeptin may regulate the neuroendocrine axis in male *A. spinosissimus* according to seasonal changes in reproduction. The seasonal regulation of reproductive function in the males appears to be mediated through the AVPV by differential regulation of kisspeptin synthesis and secretion. In addition, the seasonally differing kisspeptin levels in male *A. spinosissimus* may be controlled by photoperiodic signals. In contrast to the males, kisspeptin levels were not different between the breeding and non-breeding season in females, but appeared to differ between pregnant and non-pregnant females. This implies that kisspeptin expression in the AVPV of female *A. spinosissimus* may not be regulated by photoperiod, but by other factor(s). We speculate that either lactation or the use of induced ovulation by *A. spinosissimus* suppressed kisspeptin-ir cell body numbers in non-pregnant, breeding females. In contrast, progesterone secreted by the corpora lutea may stimulate kisspeptin production in the AVPV in the pregnant females. Furthermore, kisspeptin produced in the Arc may not play a role in the regulation of seasonal reproduction in either male or female *A. spinosissimus*, a possibility which is contrary to the findings for other seasonally breeding mammalian species. It appears that kisspeptin may be of greater importance in the seasonal regulation of reproductive function through environmental factors such as photoperiod in male than in female *A. spinosissimus.*

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Tables

Table 1. Number of primary, secondary, tertiary and Graafian follicles for reproductive and non-reproductive female spiny mice (*Acomys spinosissimus*) from South Africa sampled during the breeding and non-breeding seasons. Values are mean ± 1 standard error.

	Primary follicles	Secondary follicles	Tertiary follicles	Graafian follicles
Reproductive females	38.5 ± 5.7	2.3 ± 0.4	2.6 ± 0.3	2.5
Non-reproductive females	22.5 ± 4.9	0.8 ± 0.4	0.1 ± 1.0	0

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Figure legends

Fig. 1. Mean body mass (g) ± 1 standard error of male or female spiny mice (*Acomys spinosissimus*) from South Africa during the breeding or non-breeding seasons.

Fig. 2. Standard residuals of testicular mass (mg; A) and seminiferous tubule diameter (µm; C) by body mass and testicular volume (mm^3; B), and ovarian volume (mm^3; D) of the spiny mouse (*Acomys spinosissimus*) from South Africa compared between the breeding and non-breeding seasons. Values are mean ± 1 standard error.

Fig. 3. Kisspeptin-immunoreactivity in the anteroventral periventricular nucleus of male and female spiny mice (*Acomys spinosissimus*) from South Africa sampled during the breeding and non-breeding seasons. A: reproductive male; B: pregnant female; C: non-pregnant reproductive female; D: non-reproductive male. Arrows indicate kisspeptin-immunoreactive cell bodies. The scale bar represents 200 µm.

Fig. 4. Kisspeptin-immunoreactivity at four rostro-caudal levels of the arcuate nucleus of the spiny mouse (*Acomys spinosissimus*) from South Africa compared between males and females and reproductive and non-reproductive individuals. The sections are shown from the rostral (1) to the caudal (4) end of the arcuate nucleus. A: reproductive male; B: reproductive female; C: non-reproductive male; D: non-reproductive female. The scale bar represents 200 µm.

Fig. 5. Mean optical density ± 1 standard error of kisspeptin-immunoreactivity in the arcuate nucleus of the spiny mouse (*Acomys spinosissimus*) from South Africa compared between females and males.

Fig. 6. Mean optical density ± 1 standard error of kisspeptin-immunoreactivity in the arcuate nucleus of the spiny mouse (*Acomys spinosissimus*) from South Africa compared between four rostro-caudal levels from the most rostral (1) to the most caudal (4).

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Figures

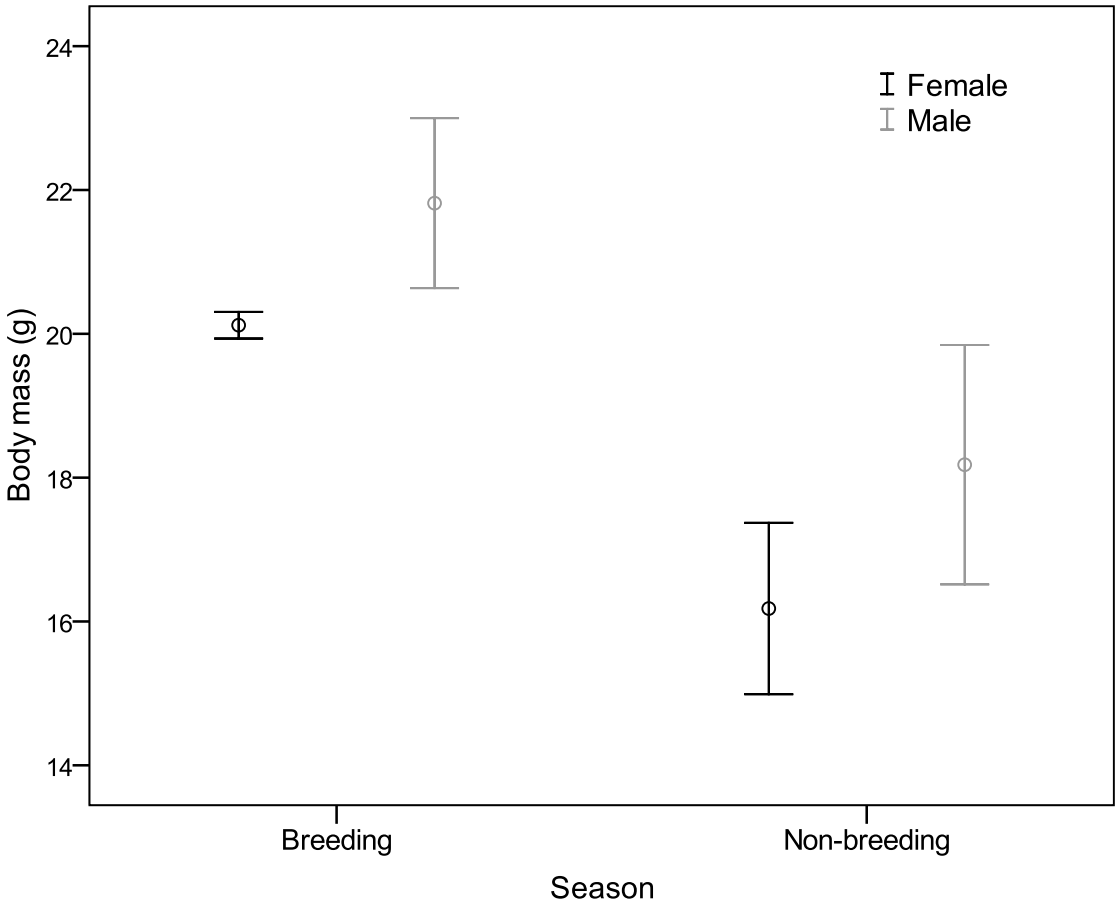

Figure 1.

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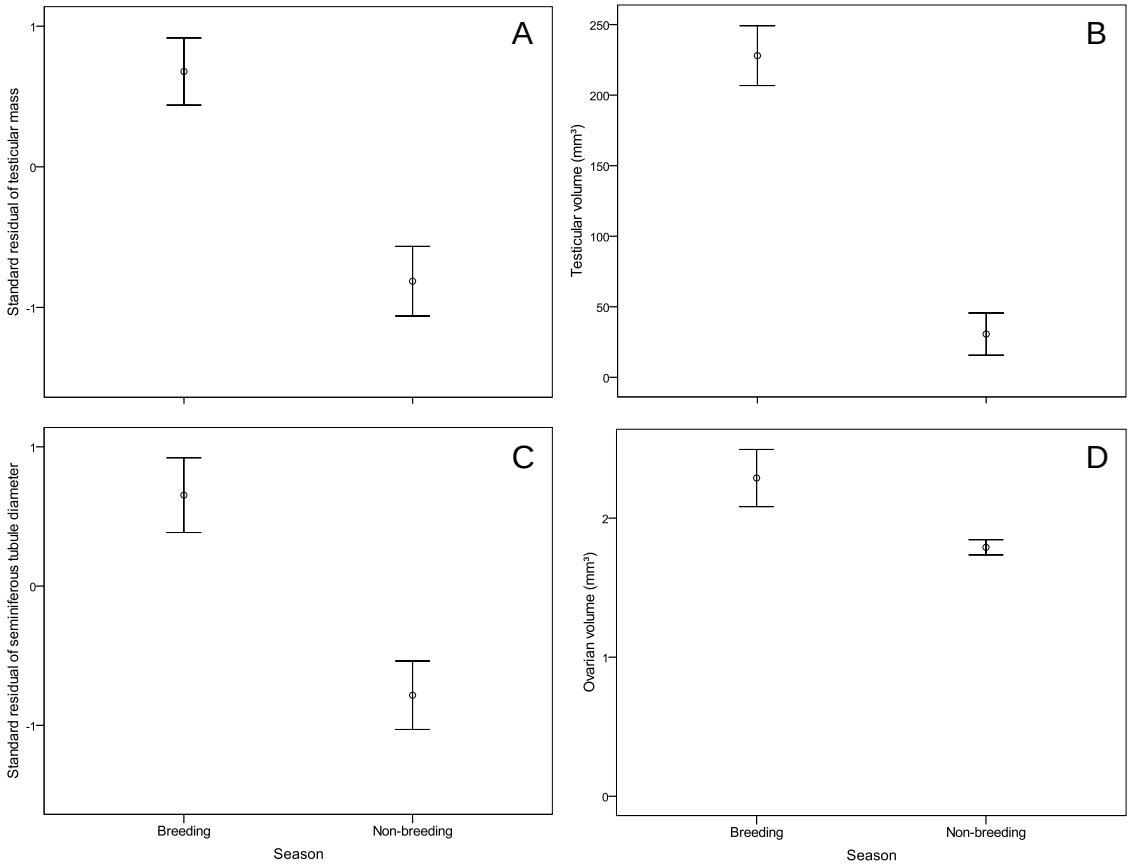

Figure 2.

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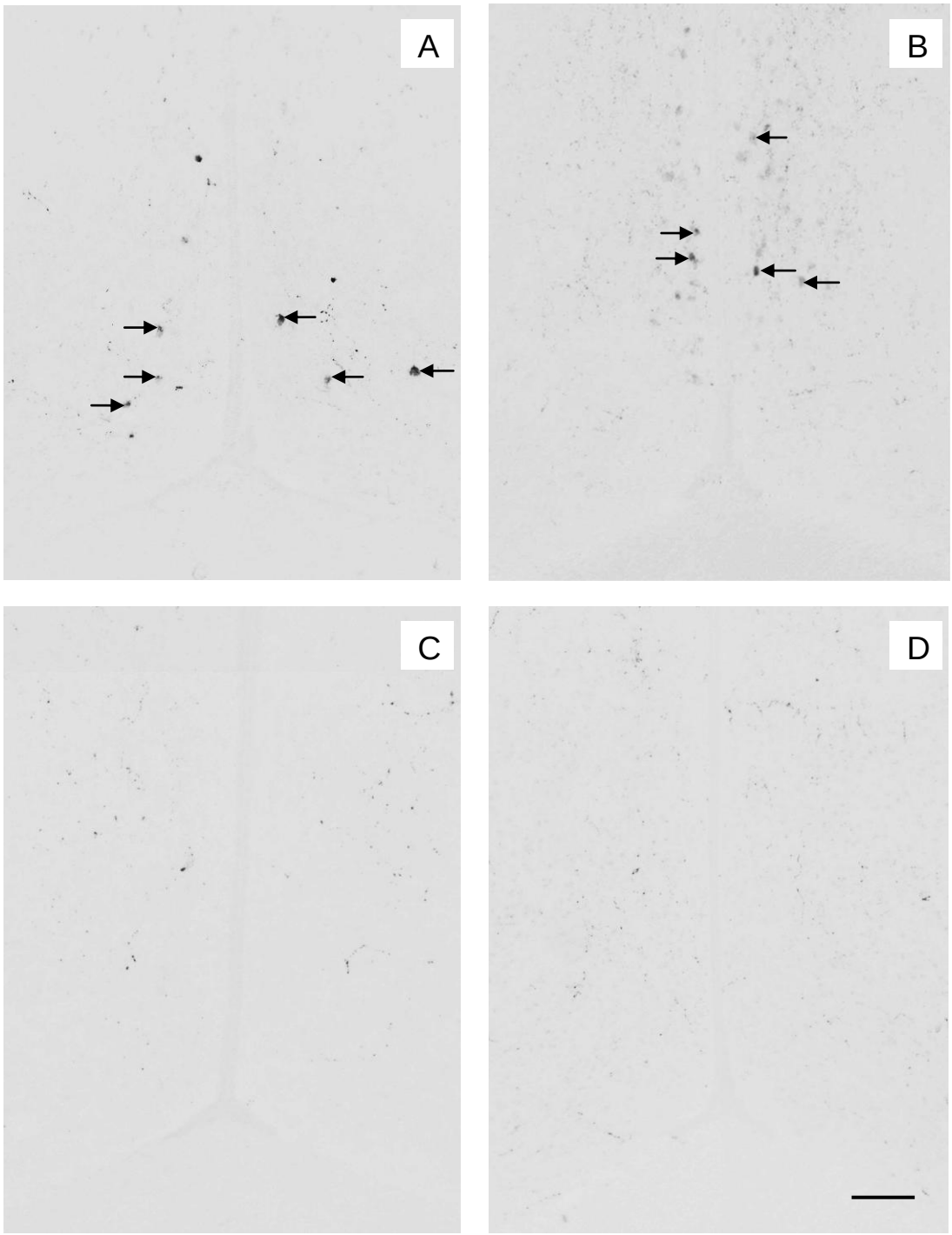

Figure 3.

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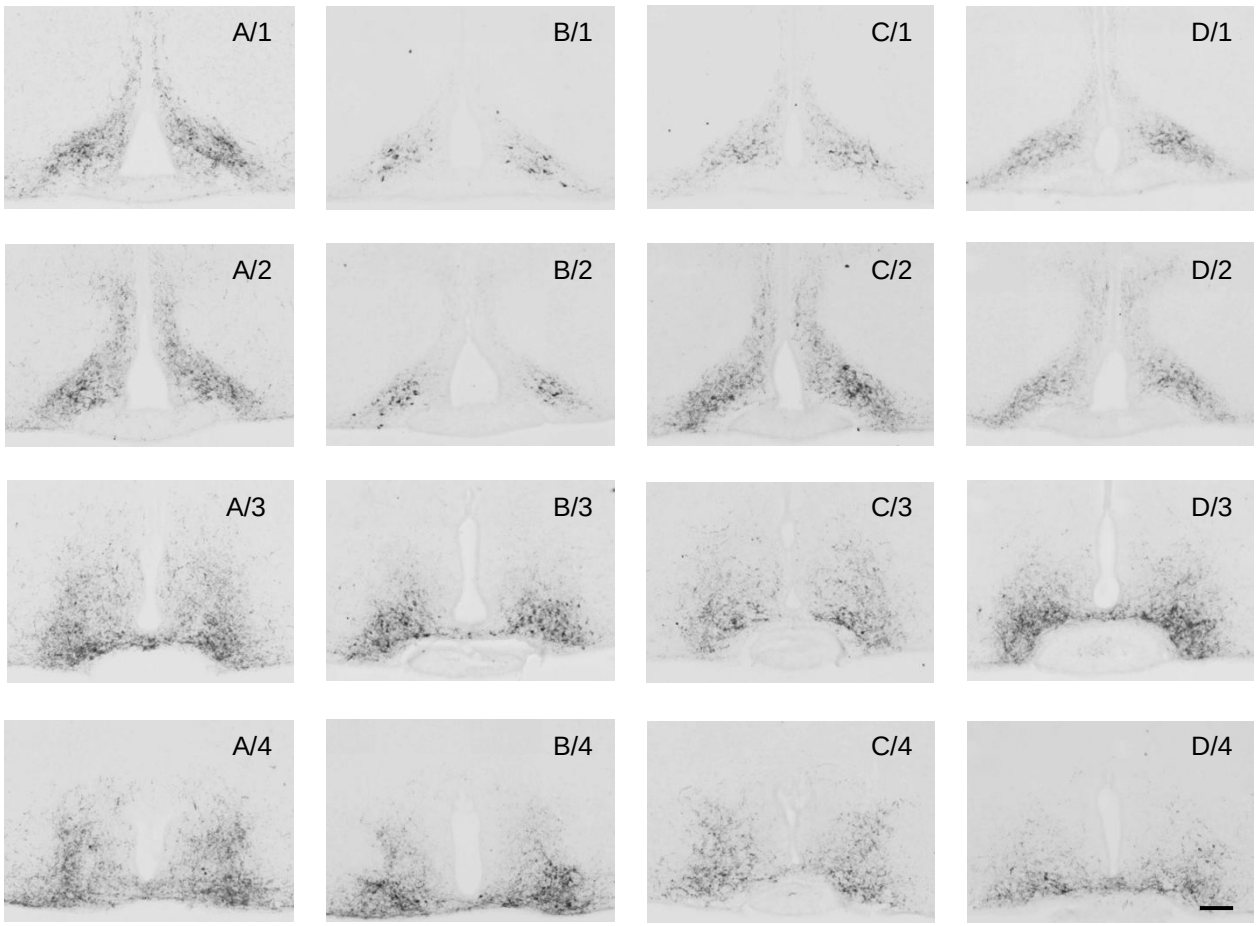

Figure 4.

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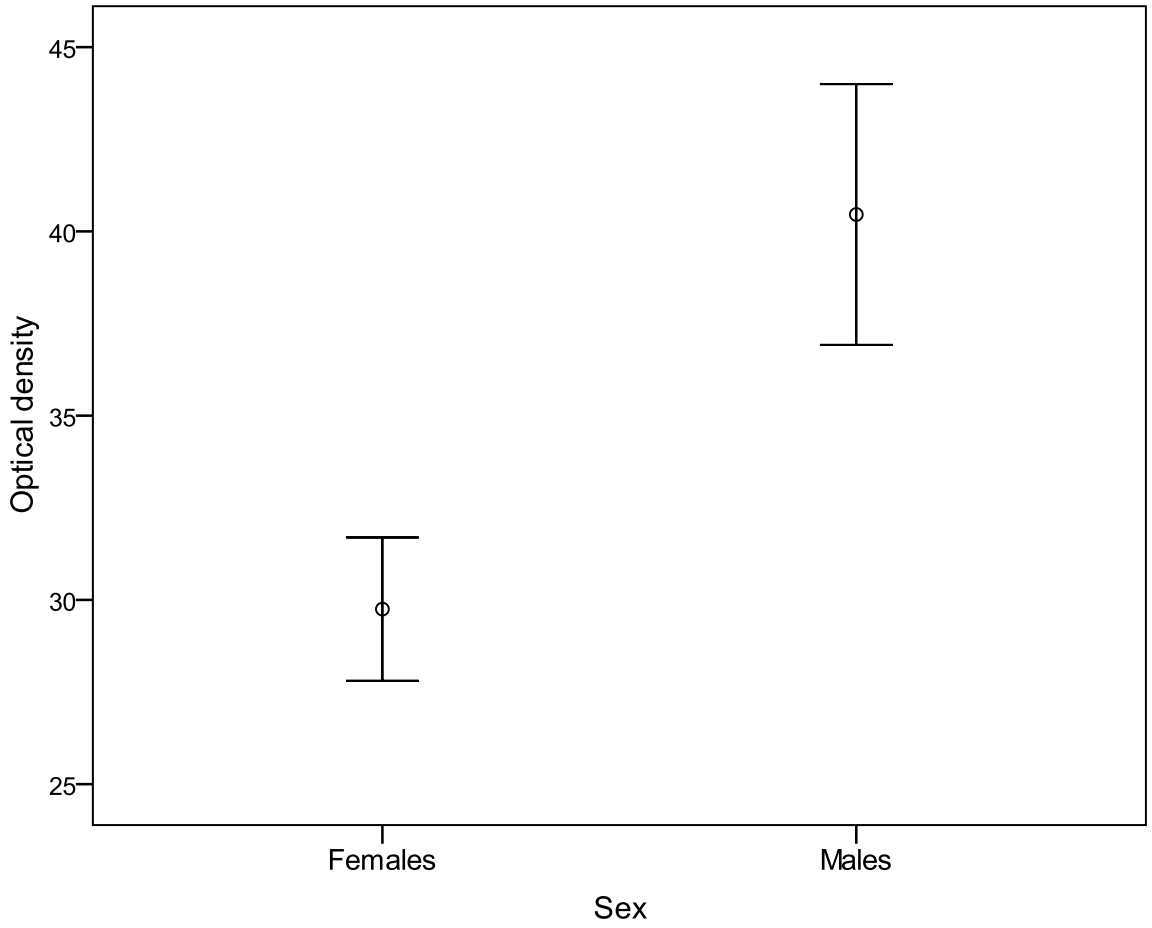

Figure 5.

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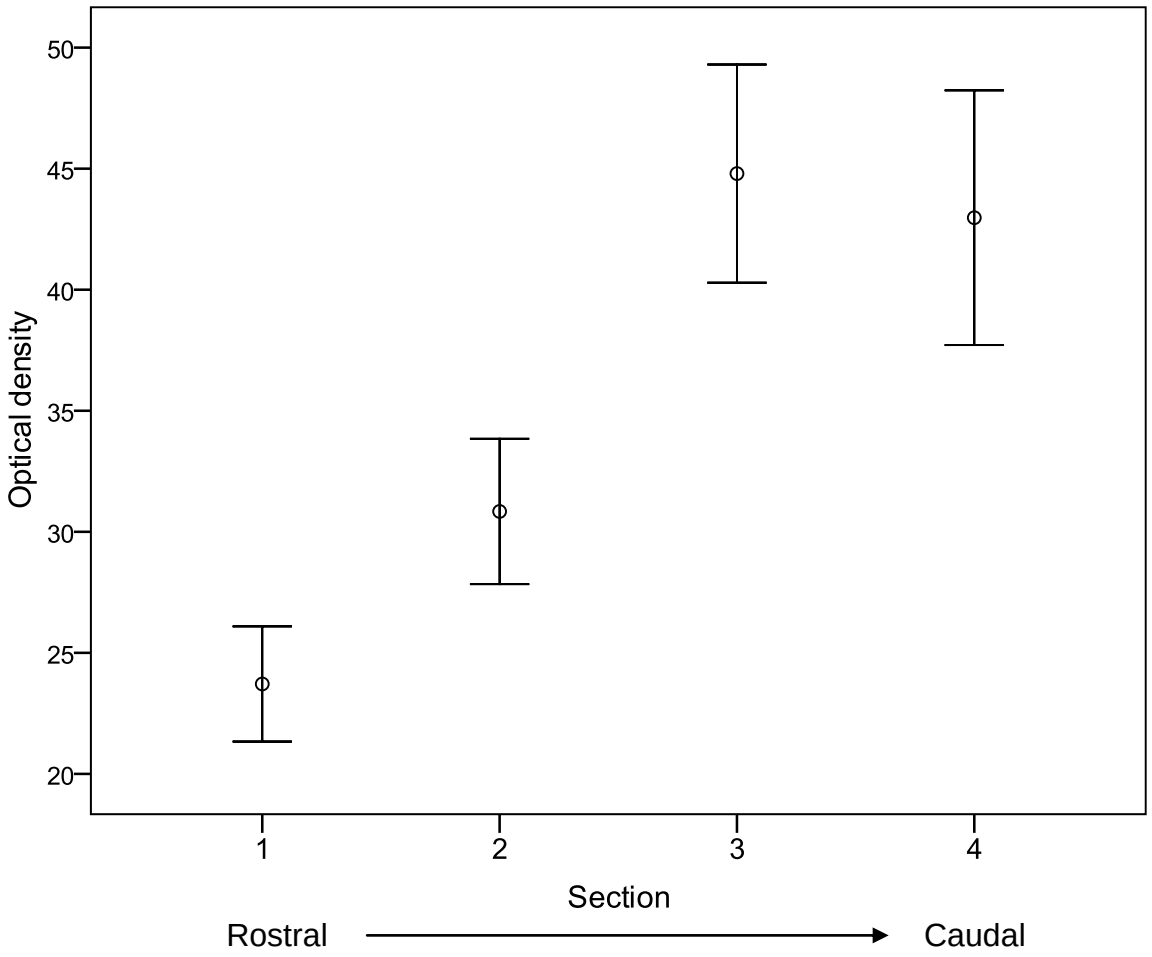

Figure 6.

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CHAPTER 6

KISSPEPTIN-IMMUNOREACTIVITY IN MALES AND FEMALES OF THE SEASONALLY BREEDING EASTERN ROCK ELEPHANT-SHREW (*ELEPHANTULUS MYURUS*) FROM SOUTH AFRICA: EFFECTS OF SEASON BUT NOT SEX

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Abstract

How kisspeptin integrates the endocrine system and environmental factors (e.g., photoperiod) to regulate reproduction in seasonally breeding mammals is uncertain. In mammals such as hamsters and sheep, it shows markedly varied expressions under different photoperiodic regimes and between breeding and non-breeding individuals. This has led to suggestions of the need for further studies on a wide range of species not only under laboratory conditions, but also in semi-natural and natural settings. The present study compared kisspeptin-immunoreactivity in wild-caught males and females of a non-rodent small mammal, the Eastern rock elephant-shrew (*Elephantulus myurus*) between the breeding (October) and non-breeding (April) seasons. Kisspeptin-immunoreactive (-ir) fibres were mostly present in the septohypothalamic nucleus (SHy), medial preoptic area, medial preoptic nucleus, anteroventral periventricular nucleus, ventromedial preoptic nucleus, retrochiasmatic area, paraventricular hypothalamic nucleus, ventromedial hypothalamic nucleus and arcuate nucleus (Arc). Kisspeptin-ir cell bodies were scarce and mostly confined to the Arc. Optical density of kisspeptin-ir fibres was compared between the breeding and non-breeding seasons in the SHy, the rostral periventricular area of the third ventricle (RP3V) and the Arc. In the RP3V and the Arc, kisspeptin-ir fibre density was significantly greater in the males and females sampled during the breeding than the non-breeding season. However, there were no differences in kisspeptin-ir fibre densities in the SHy. Kisspeptin-ir fibre densities did not differ between males and females. These results suggest that kisspeptin may contribute to the regulation of seasonal changes in reproductive functions in *E. myurus*. Given that the reproductive axis of *E. myurus* appears to be non-responsive to photoperiodic changes, other environmental factors, such as food availability, may influence seasonal kisspeptin expression in this species.

Keywords: immunohistochemistry, hypothalamus, septohypothalamic nucleus, RP3V, Arc, environmental factors

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Introduction

Kisspeptins have been the focus of numerous studies ever since it was discovered that these neuropeptides play a key role in the neuroendocrine regulation of reproductive functions. In general, kisspeptins (henceforth referred to as kisspeptin) are the products of the *Kiss1* gene and the natural ligands to the previously orphaned G protein-coupled receptor 54 (Kiss1r) (Kotani *et al*., 2001; Ohtaki *et al.*, 2001). Kisspeptin plays a vital role in regulating the mammalian hypothalamic-pituitary-gonadal (HPG) axis (Thompson *et al.*, 2004). It has been found that kisspeptin induces luteinizing hormone (LH) as well as follicle-stimulating hormone (FSH) secretion from the pituitary which is mediated through gonadotrophin-releasing hormone (GnRH) (Gottsch *et al*., 2004; Irwig *et al*., 2004; Navarro *et al*., 2005). More importantly, kisspeptin acts directly on GnRH neurons through Kiss1r which is co-localized with GnRH neurons (Clarkson *et al.*, 2008; Messager *et al.*, 2005). Furthermore, oestrogen and progesterone positive and negative feedback actions were found to differentially regulate kisspeptin expression through direct mechanisms on kisspeptin neurons (Smith *et al*., 2005a; Smith *et al*., 2005b). Consequently, kisspeptin plays an indispensable role in the hypothalamic processes underlying puberty (Funes *et al.*, 2003; Plant *et al.*, 2006), ovulation (Castellano *et a*l., 2006; Clarkson *et al*., 2008) and possibly other reproductive functions in mammals (Kalamatianos *et al.*, 2008; Roa *et al.*, 2006; Yamada *et al.*, 2007).

Although much is known about the effects of various environmental signals on the reproductive axis, the precise mechanisms by which such information is relayed to the HPG axis remains unknown (Greives *et al*., 2008b). In seasonally breeding mammals, it was found that kisspeptin is important for the transduction of environmental signals, such as photoperiodic changes, to the neuroendocrine axis (Greives *et al.*, 2008b; Revel *et al.*, 2007). In long- as well as short-day breeders, kisspeptin is important in regulating the response of the reproductive system to different photoperiodic regimes (Greives *et al*., 2007; Wagner *et al*., 2008). In addition, photoperiodic changes mediated by melatonin may be relayed to the HPG axis through kisspeptin (Ansel *et al.*, 2010; Revel *et al.*, 2006). There is evidence that the site of action of kisspeptin on the hypothalamus varies between species. Thus, although the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (Arc) seem to play a major role in the seasonal regulation of the reproductive axis

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through kisspeptin in the Siberian hamster (*Phodopus sungorus*), the Arc seems to be solely responsible for these functions in the Syrian hamster (*Mesocricetus auratus*) (Ansel *et al.*, 2010; Revel *et al.*, 2006) and the sheep (Smith *et al*., 2007; Smith *et al*., 2008). Apart from photoperiod, the effects of other environmental cues on kisspeptin expression are not well studied. However, recent studies by Kalamatianos *et al*. (2008) and Paul *et al*. (2009) suggest that food availability may also be responsible for regulating kisspeptin synthesis and secretion.

All these studies on northern hemisphere species such as hamsters and sheep (Clarke *et al.*, 2009; Simonneaux *et al.*, 2009) indicate a markedly varied kisspeptin expression under different photoperiodic regimes and between breeding and non-breeding individuals. This has led to suggestions of the need for further studies on a wide range of species not only under laboratory conditions, but also under semi-natural and natural conditions (Greives *et al.*, 2008b). Consequently, in the present study, we examined the expression of kisspeptin-immunoreactivity in wild-caught male and female Eastern rock elephant-shrews (*Elephantulus myurus*) from South Africa during the breeding (October) and the non-breeding (April) seasons.

Elephantulus myurus is reproductively active from August until January, the spring and summer months in the southern hemisphere, and is reproductively inactive during the cold and dry autumn and winter (February – July) months in South Africa (Chapter 2). Male *E. myurus* do not appear to be reproductively photoresponsive and it has been proposed that an endogenous circannual rhythm may regulate seasonal reproduction in this species (Chapter 4). The species belongs to a unique order of small mammals (the Macroscelidea) that is endemic to Africa (Skinner & Chimimba, 2005) and its general biology and particularly neuroanatomy is largely unknown (Pieters *et al*., 2010; Chapter 2). In addition, *E. myurus* is the only member of the Macroscelidea south of the equator with a marked reproductive season, but the reasons for this are uncertain (Chapter 2).

The aims of the present study were to: 1) characterize the distribution of kisspeptin-immunoreactive (-ir) cell bodies and fibres in the hypothalamus of the Eastern rock elephant-shrew; 2) compare kisspeptin-immunoreactivity between the

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breeding and non-breeding seasons under natural conditions; and 3) compare kisspeptin-ir between male and female *E. myurus*. As has been found in rodents (reviewed in Oakley *et al.*, 2009), we predicted kisspeptin-immunoreactivity in the AVPV or rostral periventricular area of the third ventricle (RP3V) and in the Arc of *E. myurus*. We further predicted that kisspeptin-immunoreactivity would be higher during the breeding than the non-breeding season. Given the observation that kisspeptin neurons are more abundant in females than in males in the sexually dimorphic AVPV (De Vries & Södersten, 2009; Kauffman, 2010), we also predicted a similar scenario for *E. myurus*. This is the first study which examined seasonal aspects of kisspeptin-immunoreactivity in a non-rodent small mammal and in a seasonally breeding, but reproductively non-photoresponsive species under natural conditions. The current study aims to contribute to the understanding of the role of kisspeptin in the seasonal regulation of reproductive functions in mammals.

Materials and Methods

Animal sampling and maintenance

Elephantulus myurus of both sexes were caught at Goro Game Reserve (22°58'S, 22°57'S; 29°25'E, 29°24'E) in the Soutpansberg region, Limpopo Province, South Africa during October (breeding season) and April (non-breeding season) in 2008 and 2009. In the breeding season, six males and five females were collected and five individuals of each sex were obtained during the non-breeding season. The animals were trapped overnight with Sherman live traps (H. B. Sherman Traps, Inc. Tallahassee, Florida, U.S.A.) baited with peanut butter, oats and fish. All *E. myurus* were weighed to the nearest 0.001 g with a digital balance (Scout Pro SPU123, Ohaus Corporation, Pine Brook, New Jersey, U.S.A.) immediately after capture. They were housed in polyurethane cages covered with wood shavings during transportation to the laboratory and fed with canned dog food (Promeal Ltd., Dassenberg, South Africa) or Pronutro, a high protein cereal (Pioneer Foods Ltd., Bokomo Foods, Cape Town, South Africa); grated apples or carrots were also provided. Fresh water was given in an open dish once daily. All animals were collected under permits from the relevant nature conservation authorities (permit numbers: CPM-333-00002, CPM-002-00002) and experimental procedures were approved by the animal ethics committee of the University of Pretoria, Pretoria, South Africa (ethics clearance number: EC037-08).

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Perfusion and histology

Shortly after capture, all animals were euthanized with halothane and immediately perfused transcardially with 100 ml of 0.9 % saline, followed by 100 ml of 4 % paraformaldehyde in 0.1 M phosphate buffered saline (PBS). The head was severed from the body and the entire brain was removed and then stored in 4 % paraformaldehyde at 4 °C until processed. It was recorded whether the females were pregnant or had placental scars in their uterine horns as an indication of prior pregnancy. Ovaries and testes were dissected out and post-fixed in Bouin's solution for about 20 hrs and then stored in 70 % ethanol. Testes and ovaries were cleaned of any fat and connective tissue, weighed to the nearest 0.0001 g with a high precision scale (Ohaus Corp. Pine Brook, N.Y., U.S.A.) and their length and width were measured to the nearest 0.01 mm with a pair of digital callipers (Sylvac Opto RS 232, Ultra Praezision Messzeuge GmbH, Germany). Subsequently, testicular and ovarian volumes (mm^3) were calculated using the formula for the volume of an ellipsoid: $V = 4/3\ \pi ab^2$ (in which a represents half the maximum length and b half the maximum width) as described by Woodall and Skinner (1989). The average of testicular and ovarian volume was calculated per male or female, respectively. Both testes and ovaries were prepared for histology as described previously (Chapter 2) and all sections were stained with Ehrlich's haematoxylin and counter-stained with eosin (Drury & Wallington, 1967).

Both ovaries per female were examined for follicular stages under a light microscope at a magnification of ×200. The total number of primary, secondary, tertiary and Graafian follicles as well as corpora lutea and corpora albicans were counted throughout the entire ovary as described previously (Chapter 2). Primordial follicles were not counted, as their number does not change between the breeding and non-breeding seasons (Chapter 2). For all males, pictures of seminiferous tubules with circular shape were taken randomly at ×10 magnification with a digital camera (Moticam 1000 1.3 M Pixel USB 2.0, Motic China Group, LTD., Xiamen, P.R. China) attached to a microscope (Diaplan, Ernst Leitz Wetzlar GmbH, Germany). From these photos, the diameters of 100 seminiferous tubules per male were measured to the nearest 0.1 µm with Motic Images Plus 2.0ML software (Motic China Group, LTD., Xiamen, P.R. China).

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Immunohistochemistry

Brains were saturated with 30 % sucrose for cryoprotection and then quick-frozen with dry ice for sectioning. Coronal sections of 25 µm were cut on a cryostat (Bright Instruments, U.K.), beginning rostrally at the olfactory bulbs and extending caudally to the posterior end of the hypothalamus. Sections were stored in cryoprotectant at -20 °C until processed for kisspeptin-immunohistochemistry. Every sixth section was used for kisspeptin-immunohistochemistry and processed as free-floating sections. To increase permeability of the tissue and suppress endogenous peroxidase, the tissue was pre-treated with 0.5%-X100 triton and 2 % H_2O_2, respectively. Afterwards, the sections were incubated in 2 % normal donkey serum (Sigma-Aldrich Co., U.S.A.) for 1 hr. Kisspeptin-ir cell bodies and fibres were labelled using rabbit anti-kisspeptin serum (Mikkelsen, J.D.) diluted at a ratio of 1:300. The sections were incubated in this primary antibody for 72 hrs at 4 °C. Subsequently, biotinylated donkey anti-rabbit lgG (1:1000; Jackson Immunoresearch Laboratories, Inc., U.S.A) was applied for 2 hrs. After rinses in PBS, tissue was incubated in an avidin-biotin peroxidase complex (ABC; 1:1000; Elite Kit, Vector Laboratories, Peterborough, U.K.) for 2 h. Kisspeptin-immunoreactivity was visualised with 0.075 % 3'-3 diaminobenzidine (DAB) in 0.15 % ammonium nickel sulphate and 0.005 % H_2O_2. Sections were mounted in consecutive order (rostral to caudal) on gelatine-coated slides and cover-slipped.

Slides were examined for kisspeptin-ir cell bodies and fibres under bright field illumination with a microscope (Eclipse E600, Nikon Corporation, Japan) at magnifications of ×100 and ×200. By comparing the brain sections of *E. myurus* with those in Paxinos and Watson (2007), we found that kisspeptin-immunoreactivity was mostly restricted to the RP3V and the Arc in *E. myurus*, but a distinct clustering of kisspeptin-ir fibres was also observed between the anterior parts of the anterior commissure, especially in the septohypothalamic nucleus (SHy) (see results section). These three hypothalamic regions were further examined and the optical density was determined for each region separately. Areas, staining positive for kisspeptin-immunoreactivity, were photographed at a magnification of ×10 with a camera (Micropublisher 5.0 RTV, QImaging, Canada) attached to the microscope for analysis of optical density. ImageJ (version 1.43; National Institutes of Health, Bethesda, M.D., U.S.A) was used to determine optical density of kisspeptin-ir fibres. The optical

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density analysis followed Greives *et al.* (2008a). Optical density of kisspeptin-ir fibres was determined for each animal in two anatomically-matched sections and in two areas per section containing the SHy and the RP3V. In the Arc, optical density was measured at four levels along its rostro-caudal extent and also twice in each section. The average optical density was determined for the two measurements per section in the Arc and for the two sections of the SHy and the RP3V. Optical density was analysed separately for the four sections of the Arc. As very few kisspeptin-ir cell bodies were observed (see results), they were presumed to be of no significance in any of the optical density measurements and, therefore, optical density in this study only evaluates the kisspeptin-ir fibre density. Kisspeptin-ir cell body distribution was determined, but their numbers were not analyzed further.

Data analysis

All analyses were performed with Generalized Linear Models (GZLM) because of the small sample sizes. All GZLM were carried out with a linear distribution and only testicular volume was log-transformed for the analysis as it was not parametric. Body mass was compared between males and females and also between the breeding and non-breeding seasons. Mass and volume of the testes and ovaries, seminiferous tubule diameter and follicle number (reproductive parameters) were compared between the breeding and non-breeding seasons, whereas kisspeptin-ir optical density in the SHy, RP3V and Arc were evaluated between the seasons as well as between males and females. For the analysis of reproductive parameters and optical densities, body mass was applied as a covariate. *Statistical Package for the Social Sciences* (SPSS) Statistics version 17.0 (Polar Engineering and Consulting 1993-2007) was used for all statistical analyses and statistical significance was accepted at $P \leq 0.05$. All results are presented as mean ± 1 standard error.

Results

Body mass and reproductive parameters

Body mass was not significantly different between male and female *E. myurus* (males: 53.6 ± 2.2 g; females: 53.3 ± 1.2 g; Wald $\chi^2 = 0.02$; $df = 1$; $P = 0.89$) or between the breeding and non-breeding seasons (breeding season: 53.8 ± 1.5 g; non-breeding season: 53.1 ± 2.0 g; Wald $\chi^2 = 0.13$; $df = 1$; $P = 0.72$). There was also no interaction between sex and season for body mass (Wald $\chi^2 = 0.42$; $df = 1$; $P =$

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0.52). Testicular volume and mass were significantly positively related to body mass (Wald $\chi^2 > 3.89$; $df = 1$; $P < 0.05$). Seminiferous tubule diameter was not significantly related to body mass (Wald $\chi^2 = 1.24$; $df = 1$; $P = 0.27$) and neither were the ovarian volume or mass or the number of the secondary follicles (Wald $\chi^2 < 1.99$; $df = 1$; $P > 0.16$). The number of primary follicles was significantly positively related to body mass (Wald $\chi^2 = 7.80$; $df = 1$; $P = 0.01$), whereas the number of tertiary follicles was significantly negatively related to body mass (Wald $\chi^2 = 14.07$; $df = 1$; $P < 0.001$). There was no significant relationship between body mass and the optical density for kisspeptin-immunoreactivity in the three hypothalamic regions analysed (Wald $\chi^2 < 3.78$; $df = 1$; $P > 0.05$). For all subsequent analyses of optical density, body mass was removed.

The reproductive parameters which were analysed, indicate that the males and females of *E. myurus* were reproductively active in October (breeding season) and reproductively inactive in April (non-breeding season). Testicular mass and volume and the diameter of the seminiferous tubules were significantly larger in males sampled during the breeding season than in males sampled during the non-breeding season (Wald $\chi^2 > 17.58$; $df = 1$; $P < 0.001$; Fig. 1). Ovarian mass and volume were significantly larger during the breeding than the non-breeding season (Wald $\chi^2 > 4.34$; $df = 1$; $P < 0.04$; Fig. 2). There were significantly more primary, secondary and tertiary follicles in the ovaries of females collected during the breeding season compared to the non-breeding season (Wald $\chi^2 > 4.12$; $df = 1$; $P < 0.05$; Table 1). Graafian follicles as well as corpora lutea and corpora albicans were observed only in female *E. myurus* sampled during the breeding season and not in any of the females sampled during the non-breeding season (Table 1). All females sampled during the breeding season were found to be pregnant and one was lactating as evident from the presence of milk in the mammary glands. None of the females sampled during the non-breeding season was pregnant, but two had placental scars in their uterine horns, indicating that they had been pregnant before.

Distribution of kisspeptin-immunoreactivity

The distribution of kisspeptin-ir fibres was similar in both males and females of *E. myurus.* Along the rostro-caudal septohypothalamic axis, the first dense continuum of kisspeptin-ir fibres was observed between the anterior parts of the anterior

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commissure, especially in the SHy, but also in the strial part of the preoptic area and the alar nucleus (Fig. 3). This half-moon-shaped distribution of fibres was later separated by the anterior commissure and fibres were then found in the SHy dorsal and ventral to the anterior commissure. Continuing caudally, kisspeptin-ir fibres were found in the medial preoptic area, medial preoptic nucleus, AVPV and ventromedial preoptic nucleus (Fig. 3). In the present study, we refer to this kisspeptin-ir fibre cluster as the RP3V (see Clarkson *et al*., 2008; Herbison, 2008).

Kisspeptin-ir fibres were sparse further caudally with a low density in the periventricular hypothalamic nucleus, but fibre density increased within the Arc (Fig. 3). In addition, kisspeptin-ir fibre density increased along the rostro-caudal extent of the Arc, as demonstrated by comparison of optical density at four anterior to posterior levels of the Arc (see below; Fig. 4). The Arc showed the highest kisspeptin-ir density compared to all other hypothalamic regions, but lower kisspeptin-ir fibre densities were also observed in the retrochiasmatic area, paraventricular hypothalamic nucleus and ventromedial hypothalamic nucleus. Due to damage of the medial eminence during removal of the brain, it was not possible to establish the presence of kisspeptin-ir fibres in this region. Kisspeptin-ir cell bodies were few in number and observed almost exclusively in the Arc, and three cell bodies were detected in the periventricular hypothalamic nucleus of only one animal (Fig. 5). In summary, the three hypothalamic regions, where kisspeptin-immunoreactivity was detected in *E. myurus*, were the SHy, the RP3V and the Arc (Fig. 3).

Optical density comparisons for kisspeptin-immunoreactivity

In the SHy, no significant difference in optical density for kisspeptin-immunoreactivity was found between the breeding and non-breeding seasons (Wald $\chi^2 = 0.89$; $df = 1$; $P = 0.35$; Table 2 and Fig. 3), and between male and female *E. myurus* (Wald $\chi^2 = 0.89$; $df = 1$; $P = 0.35$; Table 2). There was also no interaction between season and the sexes in this region (Wald $\chi^2 < 1.99$; $df = 1$; $P > 0.16$). In contrast, optical density for kisspeptin-immunoreactivity was significantly greater during the breeding than during the non-breeding seasons in the RP3V (Wald $\chi^2 = 16.47$; $df = 1$; $P < 0.001$) and in the Arc (Wald $\chi^2 = 157.28$; $df = 1$; $P < 0.001$; Table 2 and Fig. 3). There was, however, no significant difference in optical density between males and females in the RP3V (Wald $\chi^2 = 0.07$; $df = 1$; $P = 0.79$) or the Arc (Wald $\chi^2 = 2.32$; $df = 1$; $P =$

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0.13; Table 2). Similarly, there was also no significant interaction between season and the sexes in these two regions (RP3V: Wald χ^2 = 0.17; *df* = 1; *P* = 0.68; Arc: Wald χ^2 = 3.47; *df* = 1; *P* = 0.06). In the Arc, the optical density for kisspeptin-immunoreactivity differed significantly between the four levels which were assessed (Wald χ^2 = 39.02; *df* = 3; *P* < 0.001) increasing continuously from the most rostral to the most caudal level (Fig. 4). There was no significant interaction between the four levels in the Arc and either the sexes or the seasons (Wald χ^2 < 3.70; *df* = 1; *P* > 0.30).

Discussion

Kisspeptin-ir fibre distribution in the septohypothalamic continuum of *E. myurus* was consistent with that found in rodents (Clarkson *et al*., 2009; Desroziers *et al*., 2010). In the rat, for example, kisspeptin-ir fibres were found to be present in the preoptic area, SHy, AVPV, periventricular nucleus, suprachiasmatic nucleus, supraoptic nucleus, paraventricular nucleus, ventromedial nucleus and Arc (Desroziers *et al*., 2010). In contrast, kisspeptin-ir fibres were not observed in the SHy of the spiny mouse (*Acomys spinosissimus*) although the same antibody was used as in the present study (Chapter 5). There is a need for further investigation to establish whether this is due to actual differences in the function of kisspeptin or a result of different affinities of this antibody to the native peptide in the two species. However, the distribution of kisspeptin-ir fibres observed in *E. myurus* indicates a close relationship with GnRH neurons as these are also found in similar regions in the hypothalamus of most mammals (Silverman *et al.*, 1994). Kisspeptin-ir fibres have also been found to be closely associated with GnRH neurons in the rat, mouse, sheep and the horse (Clarkson & Herbison, 2006; Decourt *et al.*, 2008; Kinoshita *et al.*, 2005; Smith *et al.*, 2008). Irwig *et al*. (2004) established that about 77 % of the GnRH neurons co-express Kiss1r mRNA in the male rat. After central administration of kisspeptin, most GnRH neurons have been found to co-express cFos, a marker of neuronal activation, whereas almost none of the GnRH neurons co-expressed cFos after injections with the vehicle alone (Irwig *et al.*, 2004). These findings suggest that kisspeptin may act directly on GnRH neurons in mammals and which may also be the case in *E. myurus*. However, the possibility of direct actions of kisspeptin on GnRH neurons in *E. myurus* merits further investigation.

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Most of the previous studies on kisspeptin-immunoreactivity have focused on the distribution of immunoreactive cell bodies rather than on fibres and since we observed only very few cell bodies in *E. myurus*, further comparisons with other species regarding this parameter are difficult. As was also the case with *E. myurus*, most mammals examined to date appear to have kisspeptin-ir cell bodies in the Arc (reviewed in Oakley *et al*., 2009). In the AVPV or periventricular nucleus, kisspeptin-ir cell bodies have been observed in some species, but not in others. Similar to the results of the present study, Desroziers *et al*. (2010) did not detect kisspeptin-ir cell bodies in the AVPV/periventricular area of the rat and they speculated that less peptide may be stored in the perikaryon of rats compared to mice (Clarkson *et al*., 2009). It is, therefore, possible that in *E. myurus* kisspeptin may be rapidly transported from these cell bodies and that storage of kisspeptin may occur only under certain conditions which may explain the small numbers of kisspeptin-ir cell bodies observed. In Syrian hamsters, two different observations have been made, indicating that the method used can have major implications for the results obtained. Revel *et al*. (2006) established that there was no *Kiss1* expression in the AVPV of the Syrian hamster, whereas Ansel *et al.* (2010) observed distinct and even sexually dimorphic *Kiss1* expression in the AVPV of the very same species.

Another problem with kisspeptin-immunohistochemistry is the chance of binding of the antibody with other related peptides of the RF amide family (Oakley *et al*., 2009). Because we were not able to control for any cross-reactivity during the course of the study, it is possible that some of the kisspeptin-immunoreactivity found in *E. myurus* may be due to non-specific binding. However, the patterns observed were very distinct and agreed largely with those observed for other small mammal species (see above) such that the results obtained in the present study may represent a realistic reflection of kisspeptin expression in *E. myurus.* It should be noted, however that a comparison with more closely related species was not possible, but would have allowed a better understanding of the functions of kisspeptin in these unique mammals.

The density of kisspeptin-ir fibres in *E. myurus* was significantly different between the breeding and non-breeding seasons. In both males and females, we observed a lower density in the RP3V region and the Arc during the non-breeding

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season compared to the breeding season but there was no difference in kisspeptin-ir fibre density in the SHy between the two seasons. Males and females of *E. myurus* showed a marked down-regulation of their reproductive systems during the non-breeding season than the breeding season. The size of the testes or the ovaries was clearly reduced during the non-breeding season during which the seminiferous tubule diameter was also decreased. In addition, follicular development was suppressed during the non-breeding season, while advanced stages of follicular development and evidence of ovulation and pregnancy were observed in *E. myurus* only in the breeding season. Furthermore, it was established in Chapter 2 that plasma testosterone as well as progesterone concentrations are considerably reduced during the non-breeding season compared to the breeding season. These findings concur with the results for kisspeptin-ir fibre densities and suggest an involvement of kisspeptin in the seasonal regulation of reproductive functions in *E. myurus.*

These regulatory actions on the reproductive system may, however, be mediated primarily through the RP3V or Arc, whereas kisspeptin in the SHy may be important for other reproductive processes. It appears that a reduction in kisspeptin in the RP3V or Arc during the non-breeding season and at the end of the breeding season may, in turn, result in a simultaneous decrease of reproductive activity in both male and female *E. myurus*. In addition, the decrease in circulating testosterone and progesterone may result in reduced kisspeptin expression in the RP3V region during the non-breeding season. In rodents, it was found that testosterone, oestrogen and possibly progesterone stimulate kisspeptin expression in the RP3V/AVPV, but removal of the gonads results in reduced kisspeptin expression at this site (Greives *et al.*, 2008a; Smith *et al.*, 2005a; Smith *et al.*, 2005b; Smith *et al.*, 2006). The opposite responses have been reported for the Arc (Greives *et al.*, 2008a; Smith *et al.*, 2005a; Smith *et al.*, 2005b), such that the reduction of kisspeptin-immunoreactivity observed in the Arc of *E. myurus* during the non-breeding season may not be due to different levels of circulating sex steroids.

Ansel *et al*. (2010) established that kisspeptin expression is reduced in the AVPV and the Arc of non-reproductive male and female Siberian hamsters which concurs with our findings in the elephant-shrew. In Syrian hamsters, however, kisspeptin expression was increased in the AVPV of reproductive than non-

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reproductive males and females, while the opposite pattern was seen in the Arc (Greives *et al.*, 2008a; Greives *et al.*, 2007; Mason *et al.*, 2007). The difference found between the two hamster species is surprising although species differences in integrating either photoperiodic or melatonin signalling appear to be likely (Simonneaux *et al*., 2009). In male spiny mice, kisspeptin-ir cell bodies were found in the AVPV during the breeding season, but not during the non-breeding season, whereas kisspeptin-immunoreactivity did not vary in the Arc (Chapter 5). In female spiny mice, however, kisspeptin did not seem to be of importance in the regulation of seasonal reproduction because although kisspeptin-immunoreactivity varied between pregnant and non-pregnant females, there were no differences between the breeding and non-breeding seasons at any of the sites examined (Chapter 5). In addition to rodents, kisspeptin expression has been investigated between reproductively active and anoestrous ewes. Kisspeptin has been observed to vary between the breeding and non-breeding seasons in the Arc of ewes, where it was reduced during the non-breeding season compared to the breeding season (Smith *et al*., 2007; Smith *et al*., 2008).

Although most mammalian studies show that kisspeptin is usually reduced in non-reproductive individuals, it is difficult to compare the results of these studies with those obtained for the Eastern rock elephant-shrew. While the present study examined kisspeptin-ir fibre densities, most other mammalian studies compared kisspeptin-ir cell bodies or *Kiss1* expression. More importantly, all studies performed on seasonal breeding and kisspeptin to date were carried out on reproductively photoresponsive mammals. Melatonin has been observed to inhibit *Kiss1* expression in the Arc of the Syrian hamster, suggesting that photoperiod may mediate the effects of kisspeptin on the reproductive axis via melatonin (Ansel *et al.*, 2010; Revel *et al.*, 2006). In *E. myurus*, however, other mechanisms may be responsible for relaying environmental signals to the kisspeptin system as this species does not seem to be reproductively photoresponsive (Chapter 4).

We have previously suggested that food quantity and quality and social factors may also influence seasonal reproduction in *E. myurus* (Chapter 4). Only a few studies have examined the effect of environmental factors on kisspeptin expression and their focus has been mainly on food availability. In rats, food deprivation has

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been reported to decrease *Kiss1* expression in the hypothalamus of prepubertal rats, while central administration of kisspeptin advanced vaginal opening in under-nourished individuals (Castellano *et al*., 2005). In another study, fasting was found to suppress *Kiss1* expression in the AVPV, but not the Arc of female rats (Kalamatianos *et al*., 2008). The opposite response was observed in male Siberian hamsters, in which food deprivation reduced *Kiss1* expression in the Arc, but not the AVPV (Paul *et al*., 2009). These studies suggest a differential regulation of kisspeptin in the AVPV and Arc by environmental factors. It is, therefore, possible that certain environmental factors affect kisspeptin synthesis and/or secretion at one hypothalamic region (RP3V or Arc), while other factors may regulate kisspeptin at the other site and such a combination of effects may have resulted in the decrease in kisspeptin-ir fibre density observed in *E. myurus* during the non-breeding season. However, it is also possible that an environmentally independent regulation by sex steroids may be responsible for the regulation of kisspeptin in the AVPV (see above). Further studies should focus on kisspeptin expression independent of photoperiod to ascertain the effects of other environmental factors such as food availability and social factors on kisspeptin expression in *E. myurus*.

We did not observe any differences in kisspeptin-immunoreactivity in either the RP3V or the Arc between males and females of *E. myurus*. This was surprising since females of most rodents have been found to possess more kisspeptin neurons in the AVPV/periventricular nucleus than males (De Vries & Södersten, 2009; Kauffman, 2010). In contrast to the AVPV, the Arc of adult rodents has not been reported to show sex differences in kisspeptin expression (Kauffman *et al*., 2007). However, in sheep kisspeptin expression in the Arc is sexually differentiated (Oakley *et al*., 2009). These sex differences are believed to be related to the role of AVPV kisspeptin (or Arc kisspeptin in sheep) in initiating the oestrogen-dependent preovulatory GnRH/LH surge in female rodents (Clarkson *et al.*, 2008; Kauffman *et al.*, 2007). The kisspeptin system of *E. myurus* does not appear to be sexually differentiated, although further investigations are needed to confirm this. Whatever may be the reason for the similar kisspeptin expression between the sexes, this finding demonstrates that similar mechanisms may exist for kisspeptin's regulation of seasonal reproduction in both male and female *E. myurus*.

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In summary, high densities of kisspeptin-ir fibres were observed in the SHy, RP3V and Arc of both male and female *E. myurus.* These findings suggest a close association between kisspeptin and GnRH neurons in this species. In addition, it was found that the density of kisspeptin-ir fibres was reduced in the non-breeding compared to the breeding season of the Eastern rock elephant-shrew. This was, however, only observed in the RP3V region and the Arc of *E. myurus* and there was no difference in optical fibre density between the breeding and non-breeding season in the SHy. Kisspeptin-ir fibre densities did not differ between males and females and there was also no interaction between the sexes and the breeding and non-breeding seasons. We, therefore, suggest that kisspeptin may regulate seasonal reproduction in similar ways in male and female *E. myurus.* Alternatively, similar environmental factors may be responsible for the seasonal regulation of the neuroendocrine axis in both sexes. The environmental factors which may direct seasonal reproduction possibly via kisspeptin synthesis and secretion in *E. myurus* are unknown. Further studies are needed to identify these environmental factors and the role of kisspeptin in relaying environmental signals to the reproductive axis. Nevertheless, the present study established that kisspeptin may play an important role in the regulation of seasonal reproduction and the seasonal control of the neuroendocrine axis in both male and female *E. myurus.*

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Tables

Table 1. Numbers of primary, secondary, tertiary and Graafian follicles as well as corpora lutea and corpora albicans compared between reproductive and non-reproductive female Eastern rock elephant-shrews (*Elephantulus myurus*) from South Africa sampled during the breeding and non-breeding seasons. Values are mean ± 1 standard error.

	Primary follicles	Secondary follicles	Tertiary follicles	Graafian follicles	Corpora lutea	Corpora albicans
Reproductive females	218.1 ± 46.9	78.1 ± 23.2	37.7 ± 13.5	12.5 ± 9.9	45.3 ± 5.5	6.9 ± 2.5
Non-reproductive females	94.2 ± 22.4	30.7 ± 9.1	2.5 ± 1.3	0	0	0

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Table 2. Optical density of kisspeptin-immunoreactive fibres in the septohypothalamic nucleus (SHy), the rostral part of the third ventricle (RP3V) and the arcuate nucleus (Arc) in the Eastern rock elephant-shrew (*Elephantulus myurus*) from South Africa compared between males and females and between the breeding and non-breeding seasons. Values are mean ± 1 standard error and values which were found to be significantly different within a region are highlighted in bold.

	Optical density			
	Males	Females	Breeding season	Non-breeding season
SHy	1.2 ± 0.2	0.9 ± 0.3	1.2 ± 0.3	0.9 ± 0.3
RP3V	2.3 ± 0.6	2.3 ± 0.3	**3.2 ± 0.5**	**1.2 ± 0.1**
Arc	42.1 ± 4.4	37.8 ± 6.2	**62.6 ± 4.7**	**15.2 ± 1.8**

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Figure legends

Fig. 1. Standard residuals of testicular mass (mg) (A) and volume (mm^3) (B) by body mass (g) as well as seminiferous tubule diameter (µm) (C) in the Eastern rock elephant-shrew (*Elephantulus myurus*) from South Africa, compared between the breeding and non-breeding season. Values are mean ± 1 standard error.

Fig. 2. Mean ovarian mass (mg) and volume (mm^3) ± 1 standard error of the Eastern rock elephant-shrew (*Elephantulus myurus*) from South Africa compared between the breeding and non-breeding seasons.

Fig. 3. Kisspeptin-immunoreactivity in the septohypothalamic nucleus (SHy), the rostral area of the third ventricle (RP3V) and the arcuate nucleus (Arc) in male Eastern rock elephant-shrews (*Elephantulus myurus*) from South Africa evaluated during either the breeding or the non-breeding season. Kisspeptin-immunoreactivity was found to be similar in the females and is, therefore, not illustrated here. All scale bars represent 500 µm.

Fig. 4. Optical density of kisspeptin-immunoreactivity in the arcuate nucleus of the Eastern rock elephant-shrew (*Elephantulus myurus*) from South Africa at four rostro-caudal levels from the most rostral (1) to the most caudal (4). The scale bar represents 500 µm.

Fig. 5. Kisspeptin-immunoreactive cell bodies in the periventricular hypothalamic nucleus (Pe) and the arcuate nucleus (Arc) of the Eastern rock elephant-shrew (*Elephantulus myurus*). Cell bodies are indicated by arrows although the nature of one structure is unclear as denoted by “?”. The scale bar represents 100 µm.

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Figures

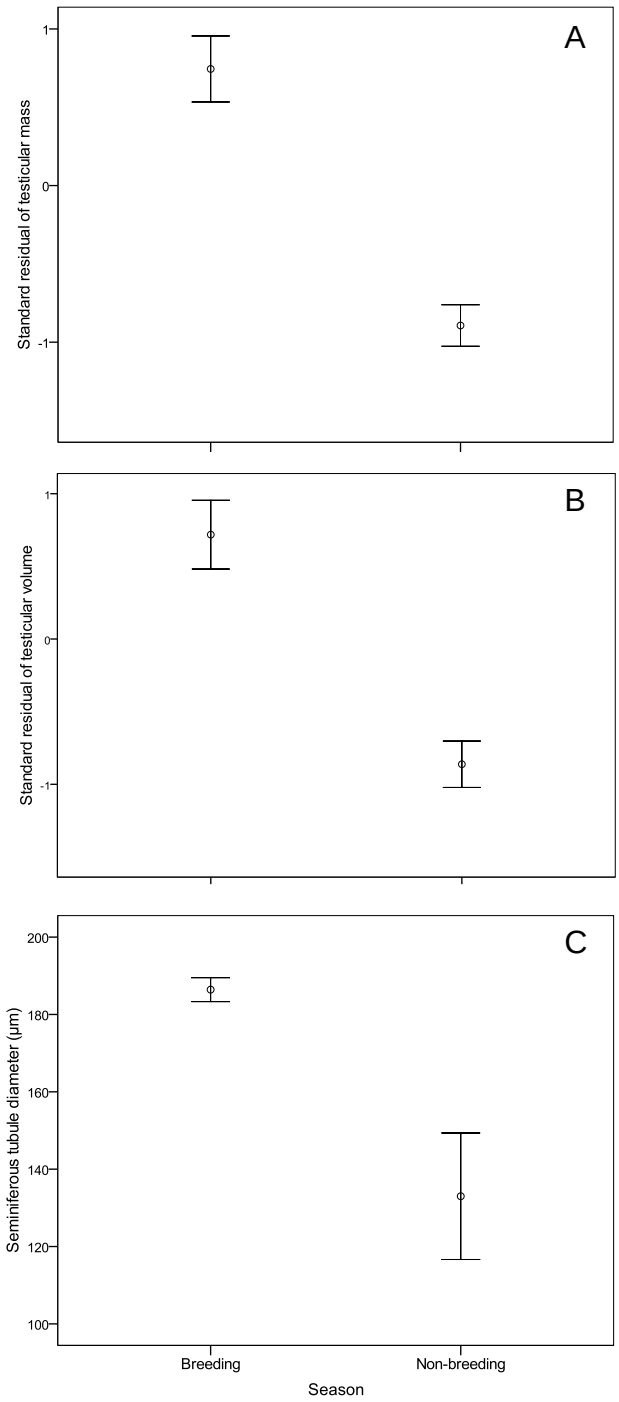

Figure 1.

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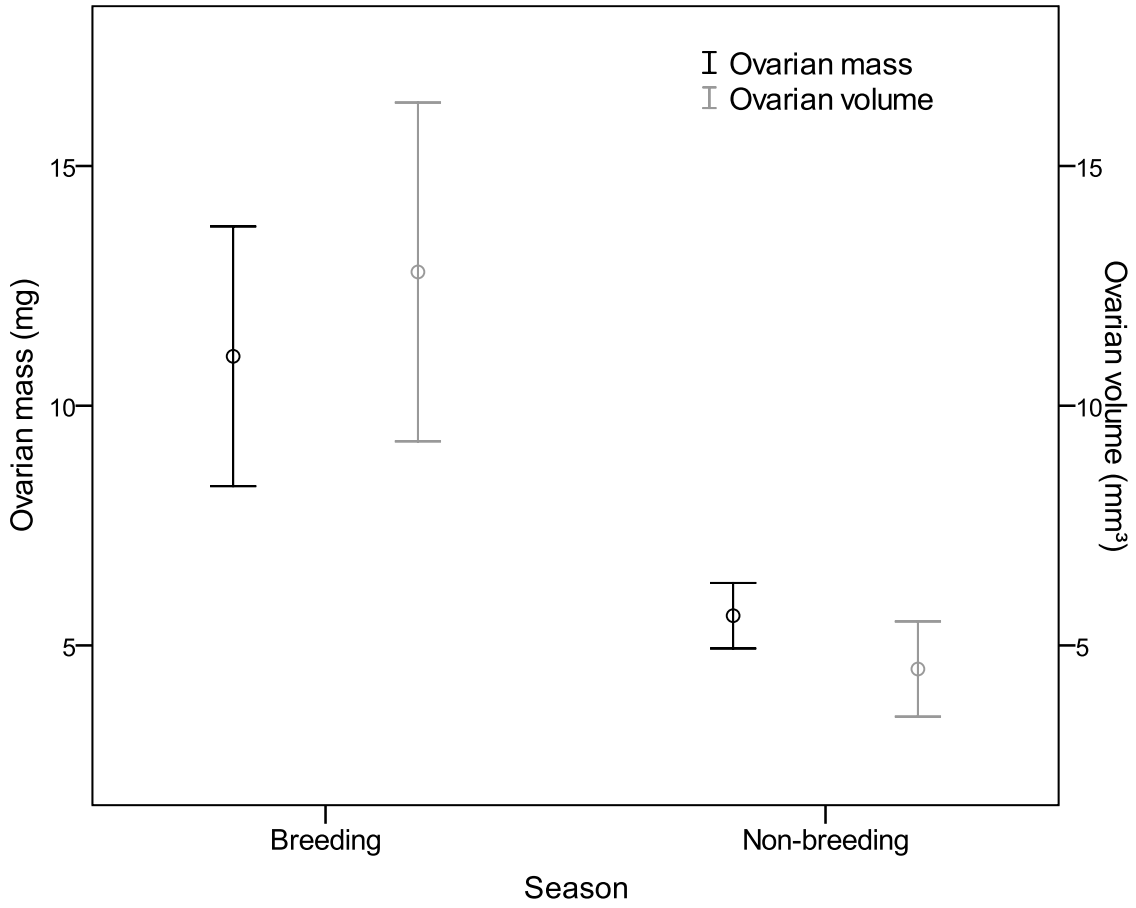

Figure 2.

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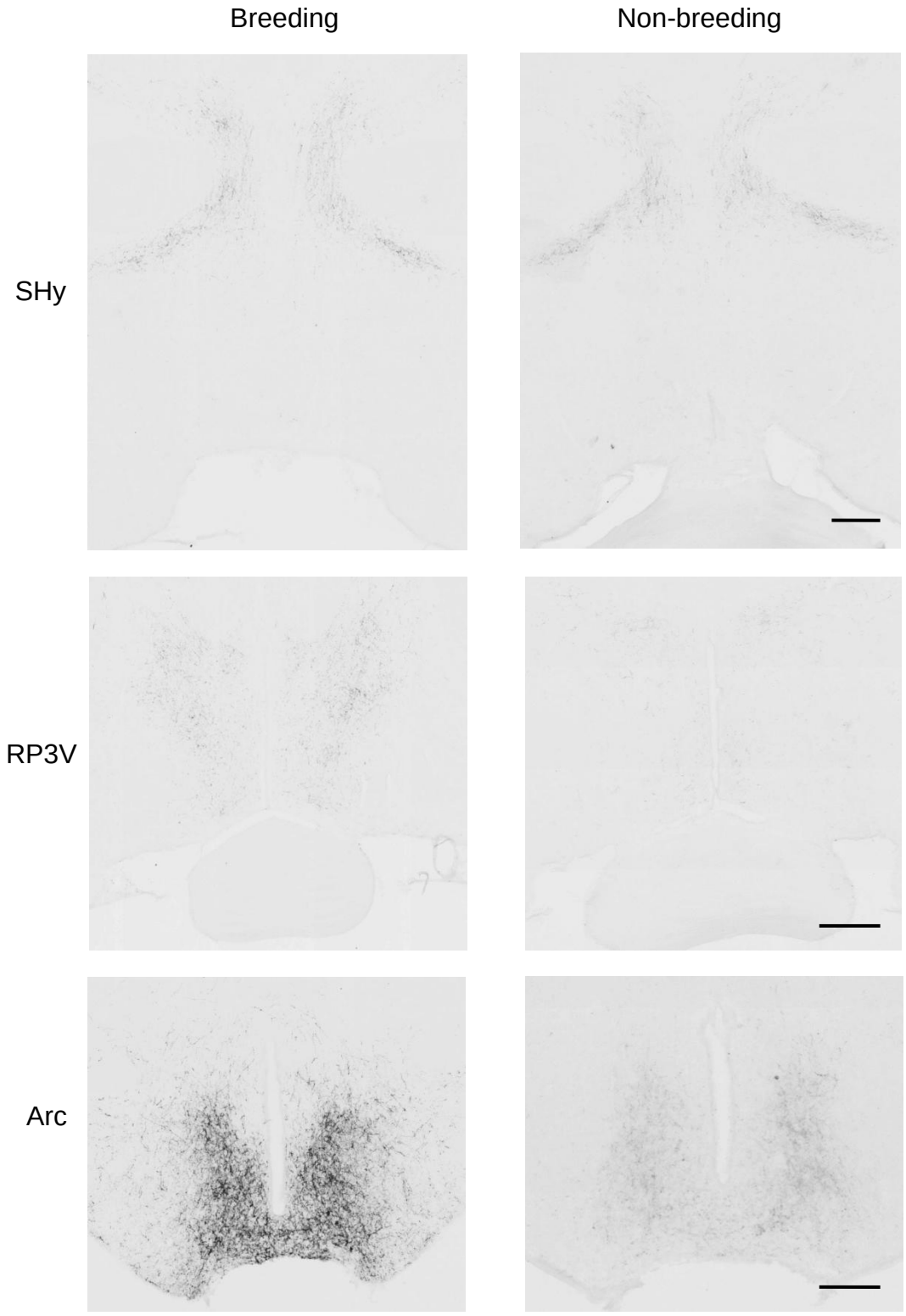

Figure 3.

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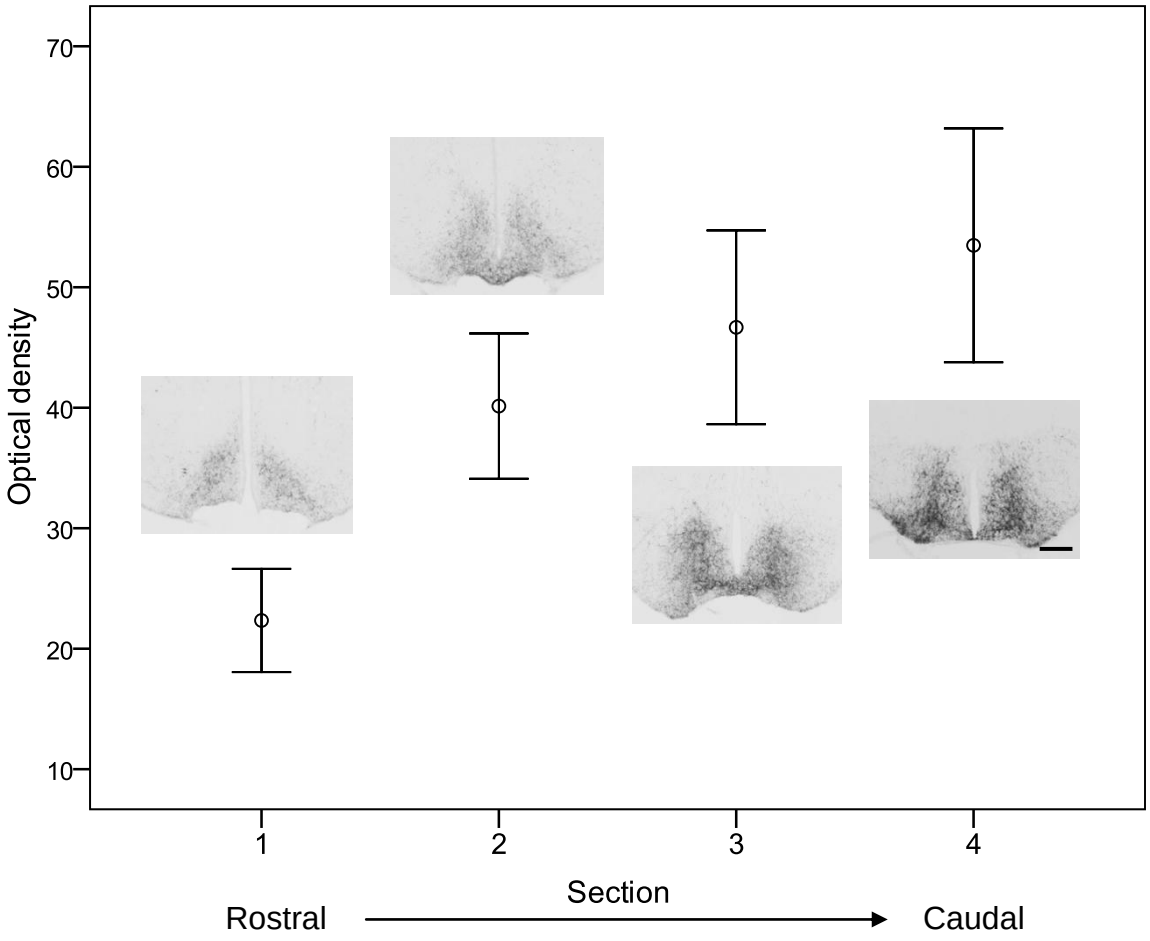

Figure 4.

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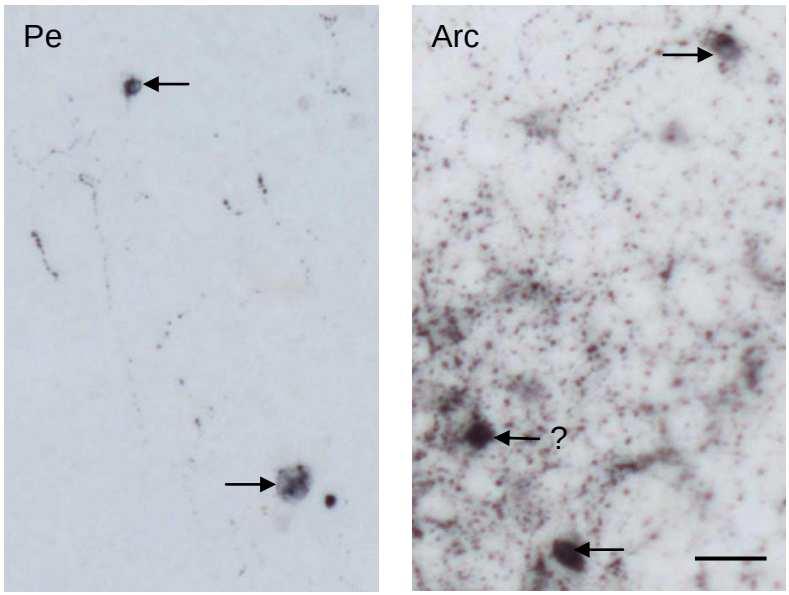

Figure 5.

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GENERAL DISCUSSION

Life history strategies

Mammals exhibit a wide range of reproductive strategies which enable them to increase their contribution to future generations and consequently, increase their fitness. Life history theory attempts to explain variations in traits such as litter size and frequency, body size at birth, age and size at maturity as well as lifespan (Stearns, 1976, 2000). The life history of an individual is shaped by both extrinsic and intrinsic factors (Stearns, 2000). This range of factors which may influence either a species' or an individuals' life history demonstrates the difficulty life history theory faces in explaining strategies utilised by organisms. Numerous life history theories and models have been developed with each focusing on a few specific traits understood to be most important (Stearns, 1976, 2000). The traits mostly associated with different life history theories include litter size, age at first reproduction and survival of juveniles and adults (Stearns, 1976; Winemiller, 1992). Two classical life history theories, "*r*- and *K*-selection" and "bet-hedging", are found in the literature which incorporate these reproductive traits and both theories are still frequently applied (McLain, 1991; Stearns, 1976, 2000). Apart from the classical approaches, new theories, for example the evolutionary game theory and adaptive dynamics, have been developed in recent years. These new theories are more explicit in their attempt to explain life history traits than the classical approaches as they also incorporate other factors such as genetics and population dynamics (Stearns, 2000). This also shows that although reproduction is vital for the fitness of an animal other factors may also be important for its life history. Phylogeny, predation, body size and energy metabolism may also affect life history strategies in mammals (Careau *et al.*, 2009; Sibly & Brown, 2009; Stearns, 1983). Careau *et al.* (2009) established that even the personality of an individual can shape its life history.

Because of their different assumptions, bet-hedging and *r*- and *K*- selection have opposing predictions. While *r*- and *K*-selection assumes constant reproductive success or juvenile and adult mortality, respectively, fluctuation in these factors is assumed by bet-hedging (Stearns, 1976). Bet-hedging is frequently associated with highly variable environments where risk avoidance is crucial and where low and

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variable fecundity but long lifespans may evolve to maximize fitness (Stearns, 1976). As indicated by the small litter size which varies only minimally in *A. spinosissimus* and *E. myurus*, reproductive success may be relatively constant in populations of these species and consequently, I will focus on the *r*- and *K*- selection rather than the bet-hedging theory. In relatively stable environments where population densities are high and it is important to be competitive and to avoid predation, all energy is directed towards maintenance and the production of a few, but precocial offspring (Pianka, 1970). Individuals facing these circumstances are selected towards the *K*-end of an *r*- and *K*-continuum and are termed *K*-selected. Other traits which are favoured by a *K*-selected strategy are slow development, delayed reproduction, several breeding periods per season, large body size and a long lifespan (Pianka, 1970). In environments with low densities and no competition, rapid population growth is facilitated resulting in small offspring, but large litters and a more *r*-selected strategy is favoured (Pianka, 1970). The *r*-selective strategy is mainly observed in unpredictable environments and it is also characterized by rapid development, early reproduction, a single reproductive event, small body size and a short lifespan (Pianka, 1970). This suggests that most small mammals follow an *r*-selected strategy, however, exceptions do exist and the key to understanding life history strategies in small mammals is to understand why these exceptions occur.

Life history strategies in *Acomys* and *Elephantulus*: a comparison

Both *A. spinosissimus* and *E. myurus* give birth to semi-precocial and precocial offspring, respectively, and have small litter sizes which is particularly restricted in *E. myurus* (Smithers, 1971; Tripp, 1972). Precocial young are often associated with *K*-selection, whereas altricial young are correlated with a more *r*-selected strategy (Perrin, 1986; Sibly & Brown, 2009). In the case of the spiny mouse and the Eastern rock elephant-shrew, precociality may be related to the lack of protective nests in these two species (Neal, 1982, 1986) which are, however, built for the protection of the young in other small mammal species. As a consequence, the increased predation risk in species which do not utilise nests is counteracted by the large body size of the juveniles and their rapid independence allowing for a greater chance to escape from predators. This general rule was supported by Sibly and Brown (2009) for mammals as a whole by establishing that mammals giving birth in the open have

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fewer, but large offspring, whereas species which use either burrows or nests have large litters of small-bodied young.

It has been postulated that precociality evolves in species that are adapted to poor quality food that is relatively constant in the environment throughout the year (Neal, 1986). The vlei rat (*Otomys irroratus*) has large precocial young, but it also builds a nest (Perrin, 1980, 1986). However, the food resource of *O. irroratus* is of poor quality and low in energy, but is available throughout the year and this combined with the small litter size, which reduces the energy needed for reproduction, enables this rodent to either extend its breeding season or even breed continually undisturbed by seasonal rainfall (Perrin, 1980, 1986). A similar combination of precociality with a stable food supply and aseasonal reproduction was also found in the wild guinea pig (*Cavia aparea*) (Trillmich, 2000). In contrast, both *A. spinosissimus* and *E. myurus* reproduce seasonally during the wet spring and summer months of the southern hemisphere (Chapters 1 and 2). *Acomys spinosissimus* is granivorous (Skinner & Chimimba, 2005) and *E. myurus* is insectivorous (Churchfield, 1987) and although fundamentally different, their food resources are likely to be more abundant during the rainy than the dry season (see also Chapters 1 to 4). Furthermore, both seeds and insects are high in energy and may, therefore, facilitate rapid reproduction and enable multiple litters during the breeding season (see also Gliwicz & Taylor, 2002). In summary, the seasonal availability of food is likely to be the primary reason for the seasonal reproduction in both small mammal species. Other factors may, however, differentially affect *A. spinosissimus* and *E. myurus*.

Acomys spinosissimus is a very small rodent and because of its unfavourable surface area to volume ratio, it is expected to have high energetic demands. These would be especially severe in the relatively cold southern African winters because heat loss may then be the main challenge this species faces. Perrin and Downs (1994) reported that *A. spinosissimus* has a basal metabolic rate lower than expected for its body size. During periods of low ambient temperatures, *A. spinosissimus* may, however, rely heavily on heat production as it is not able to increase insulation (Perrin & Downs, 1994). Consequently, it is surprising that this small rodent exhibits a life history strategy characterised by small litter sizes and semi-precocial young in a

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relatively unstable environment. These reproductive traits are even more pronounced in other larger *Acomys* species such as *A. dimidiatus* and the Crete spiny mouse (*A. minous*) (Dieterlen, 1961, 1978) which suggest that these traits are due to evolutionary rather than environmental influences. In summary, the energy needed for thermoregulation increases markedly in winter, but *A. spinosissimus* is not able to counteract this through an increase in food consumption due to shortages of its food resource. *Acomys spinosissimus*, therefore, faces a two-fold constraint which may lead it to avoid breeding during winter.

In contrast to *A. spinosissimus*, *E. myurus* is comparatively large and uses torpor to save energy (Mzilikazi & Lovegrove, 2004). Furthermore, individuals can be seen to sunbath in the mornings and may also use radiant heat from stones in the evenings (personal observation). It is, therefore, unlikely that cold ambient temperatures during winter have a severe effect on the reproduction of *E. myurus.* Nevertheless, *E. myurus* demonstrates a distinct breeding season (Chapter 2) which is surprising given that other elephant-shrew species breed throughout the year (Neal, 1995) as may be expected from a highly precocial small mammal (see above). Not surprisingly, different food preferences may be the reason for this phenomenon. *Elephantulus myurus* may depend more on insects than other elephant-shrews such as the Round-eared elephant-shrew (*Macroscelides proboscideus*) which is omnivorous (Kerley, 1995). This more restricted diet may be disadvantageous for *E. myurus* as it may not be able to switch to other food sources during times of low insect availability. This also implies that *E. myurus* may breed during winter when food is abundant suggesting a more opportunistic breeding strategy than that found in *A. spinosissimus*. Opportunism in *E. myurus* may also be demonstrated by the differences in male reproductive status found between studies on seasonal reproduction in male *E. myurus* (see Chapter 2).

Reproductive responses to photoperiod

Seasonally breeding mammals use photoperiod to anticipate environmental changes and to accurately time reproduction, whereas opportunistic species tend not to be photoresponsive (Jackson & Bernard, 1999; Prendergast *et al.*, 2001). Male *A. spinosissimus* showed strong reproductive responses to photoperiod. The male reproductive system was reduced during short-day compared to long-day

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photoperiods (Chapter 3). This may further support the notion that *A. spinosissimus* is a strongly seasonal breeder in which accurate timing of reproductive events to environmental changes is crucial for its lifetime fitness.

In contrast, male *E. myurus* demonstrated weak reproductive responses to changing light cycles and the actual significance of the observed changes remain uncertain (Chapter 4). Accurate reproductive timing may, therefore, not be as important in *E. myurus* as it is in *A. spinosissimus* and a more opportunistic breeding strategy may be further confirmed. However, it cannot be excluded that other environmental factors such as food availability and social factors, are used by *E. myurus* to time reproduction (Chapter 4).

Non-responsiveness to a given photoperiod can be the result of variations in melatonin signal processing at two main sites along the photoperiodic transduction pathway. On the one hand, photoperiod processing may already be obstructed before the pineal gland which would result in a total unresponsiveness to photoperiod (Goldman, 2001). Alternatively, photoperiodic processing can also be affected post-pineal resulting in unresponsiveness in some cases such as reproduction, but photoperiod may still regulate other types of responses (Goldman, 2001). For example, individual differences in responses to photoperiod in the white-footed mouse are most likely the result of genetic variation in the characteristics of GnRH neurons (Heideman & Pittman, 2009). Male *E. myurus* do not appear to be entirely unresponsive to photoperiod and it is, therefore, expected that genetic variation on the photoperiod system past the pineal, possibly at the GnRH neurons, may be responsible for the weak to absent responses of the reproductive system to photoperiod. The responsiveness of other elephant-shrew species to photoperiod is unknown at present. It is, however, necessary to fully understand photoperiodic responses in the Macroscelidea in general in order to gain better insights into the evolution of photoperiodic responsiveness or non-responsiveness, respectively, in *E. myurus*.

Kisspeptin and seasonality

The HPG-axis is the primary endocrine pathway responsible for the regulation of seasonal reproduction in mammals. Melatonin, which is secreted by the pineal gland

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at night, is especially important for the transduction of daylight signals to the reproductive axis of photoresponsive animals (Goldman, 2001). However, the exact target sites of melatonin on the HPG-axis are uncertain. Different neuropeptides have been implicated in the coordination and relaying of photic signals and other environmental factors to the HPG-axis. Recent research on neuroendocrine pathways in seasonal breeders has focussed on RFamide-related peptides, especially kisspeptin, but also gonadotrophin-inhibiting hormone (GnIH) (Greives *et al*., 2008). At the level of the HPG-axis, kisspeptin and GnIH have contrasting roles. Kisspeptin positively regulates GnRH release from the hypothalamus, whereas GnIH inhibits GnRH and gonadotrophin synthesis and release (Greives *et al*., 2008; Tsutsui *et al*., 2010). In contrast to kisspeptin, which acts on gonadotrophin release through GnRH, GnIH is able to directly alter the release of gonadotrophins from the pituitary (Greives *et al*., 2008; Tsutsui *et al*., 2010). Revel *et al*. (2008) demonstrated that GnIH neuron expression is altered photoperiodically and because GnIH neurons express melatonin receptors, a direct stimulation and release of GnIH through melatonin is probable (Tsutsui *et al.*, 2010). Kisspeptin and GnIH are probably interacting in the relaying of environmental signals to the HPG-axis which may enable a more precise control of reproductive timing (Greives *et al*., 2008).

In both *A. spinosissimus* and *E. myurus*, kisspeptin appears to be of importance for seasonal breeding. Kisspeptin-immunoreactivity was lower outside the breeding season than at the peak of the breeding season in males of both species and female *E. myurus* (Chapters 5 and 6). Interestingly, there was no difference in kisspeptin-immunoreactivity in female *A. spinosissimus* between the breeding and non-breeding seasons which suggests that the activation of the female reproductive system may be regulated by other factors than in the males (Chapter 5). Photoperiod most likely mediates kisspeptin expression in male *A. spinosissimus* as was established for other rodents such as the Syrian hamster (*Mesocricetus auratus*) (Ansel *et al*., 2010). In *E. myurus*, environmental factors other than photoperiod are, however, more likely to affect kisspeptin expression with food availability being the most plausible (Chapter 6). As the breeding season of both species is very distinct and the exact timing of reproductive onset is crucial for maximum reproductive success, an interaction of kisspeptin with other neuropeptides is to be expected. Besides kisspeptin, GnIH, neurokinin B and dynorphin (Greives *et al.*, 2008; Lehman

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et al., 2010) may also play a role in the regulation of reproductive functions in *A. spinosissimus* and *E. myurus*. In conclusion, kisspeptin appears to be important in the regulation of the reproductive axis to seasonal environmental changes in species as different as *A. spinosissimus* and *E. myurus.* However, the accurate mechanisms of kisspeptin regulation vary indicating different responses to environmental factors and diverse reproductive functions of kisspeptin between species.

Directions for future research

A number of new questions have arisen during the present study and the following section attempts to provide some directions for potential future studies. Many studies have intimated that food availability and especially the influx of new vegetation with the onset of the rains influences seasonal reproduction in tropical and sub-tropical mammals (Delany, 1972; Vázquez *et al.*, 2000). In a laboratory study, Jackson and Bernard (2001) observed that food restriction inhibits reproduction in both male and female four-striped field mice. In addition, several field studies tried to elucidate the effects of food supplementation on seasonal reproduction (Duquette & Millar, 1995; Jackson & Bernard, 2005; Monadjem & Perrin, 1997). Experiments, controlling the amount of food provided to animals, are crucial to our understanding of the seasonality of reproduction in both *A. spinosissimus* and *E. myurus*. Such studies should involve both laboratory and field experiments. Furthermore, studies on the effects of seasonal availability of insects for insectivorous small mammals are lacking, in particular in tropical and sub-tropical regions. It is, therefore, important to investigate how a primarily insectivorous diet affects reproduction. Besides food availability, other environmental factors such as temperature and social factors may also influence reproduction in *A. spinosissimus* and *E. myurus* (see Chapters 1 to 4). The effects of such factors should be examined to facilitate a better understanding of the evolution of seasonal reproduction in these two species as well as other tropical and sub-tropical small mammals.

The response of *E. myurus* to varying lighting regimes were not clear cut and further studies are required to tease apart the potential role of light in orchestrating reproduction. Melatonin is the primary hormone which relays photoperiodic changes to the neuroendocrine system and the reproductive axis (Goldman, 2001). It should, therefore, be investigated if *E. myurus* is responsive to changes in melatonin by, for

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example, performing pinealectomy or undertaking melatonin replacement studies. Furthermore, it would be intriguing to know if other macroscelides are reproductively photoresponsive and if physiological and/or behavioural functions other than reproduction are affected by changes in photoperiod in elephant-shrews.

In most species, females have higher energy demands during reproduction than males and are thus , more affected by changes in, for example, food availability and temperature (Speakman, 2008). As a consequence, photoperiodic regulation of reproduction may be more important for female *A. spinosissimus* and *E. myurus.* It would be interesting to investigate this and it is possible that future studies may find that females of *E. myurus* are reproductively photoresponsive although males are not. Furthermore, studies using natural photoperiods may better explain the ecological significance of photoperiodic time-measuring systems in *A. spinosissimus* and *E. myurus* and other small mammals occurring below 30° latitude. It would be interesting to repeat the work using natural summer and winter photoperiodic regimes.

The results on kisspeptin-immunoreactivity in *A. spinosissimus* have led to a range of new questions that may offer opportunities for future research (see Chapter 5). Furthermore, responses of kisspeptin to environmental changes have been investigated preliminarily in photoresponsive species and the main focus appears to be on photoperiodic effects on kisspeptin expression (Simonneaux *et al*., 2009). Future studies should also examine the effect of other factors such as food availability on kisspeptin expression and *E. myurus* may well provide a suitable non-laboratory study species for such attempts.

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