GLOSSARY

AFDH	Aridity food distribution hypothesis
CNS	Central nervous system
ERα	Estrogen receptor-α
ERβ	Estrogen receptor-β
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
HPG	Hypothalamo-pituitary-gonadal
LH	Luteinising hormone
MBH	Mediobasal hypothalamus
ME	Median eminence
POA	Preoptic area

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CHAPTER 1:

GENERAL INTRODUCTION

- **Reproductive regulation in mammals**

In mammals, the hypothalamo-pituitary-gonadal (HPG) axis regulates reproduction. This axis is controlled by the central nervous system (CNS) which responds to both internal and external stimuli. Gonadotropin releasing hormone (GnRH) plays a crucial role in the regulation of reproduction. The synthesis of GnRH is stimulated by the CNS, while GnRH regulates the synthesis and secretion of pituitary gonadotropes (Batailler *et al*. 2004), thereby providing a link between the neural and endocrine systems.

GnRH involved with reproduction is produced in and secreted from a specific sub-population of GnRH neurons in the preoptic area (POA) and the mediobasal hypothalamus (MBH) of the brain (Clarke 1987). The afferents of these neurons project to the median eminence (ME) where GnRH is stored. From here, GnRH is released into the pituitary portal system to the anterior pituitary where it stimulates the release of follicle stimulating hormone (FSH) and luteinising hormone (LH) into the circulatory system (Page & Dovey-Hartman 1984). In female mammals the release of GnRH from the median eminence is related to the mode of ovulation (Milligan 1980). In spontaneously ovulating species, GnRH is released from the median eminence in a pulsatile fashion that leads to cyclical production and release of gonadal hormones to generate continuous reproductive cycles (Bakker & Baum 2000). In contrast, in mammals exhibiting induced ovulation external stimulation is required to release GnRH that produces the preovulatory LH surge. Such stimulation is typically received from coitus. The primary endocrine response to coitus is the release of LH from the anterior pituitary which results in ovulation (Bakker & Baum, 2000).

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The gonadotropes primarily control gonadal function. In females, FSH released from the pituitary is responsible for follicular development in the ovaries. The maturing follicle secretes mainly oestrogen and a small amount of progesterone. Higher levels of oestrogen in the relative absence of progesterone cause the GnRH pulse frequency to increase from the GnRH neurons while the amplitude decreases (Clarke & Pompolo 2005). The rise in GnRH release triggers a surge in LH that induces ovulation. The follicle ruptures and the oocyte is released into the fallopian tube. The remnants of the follicle remain in the ovary, and develop into a corpus luteum that secretes mostly progesterone and a small amount of oestrogen. These hormones are required to develop and maintain the endometrium for implantation and growth of the embryo. The elevated levels of oestrogen and progesterone yield GnRH secretion with a high amplitude and low frequency (Clarke 1987) that inhibits the production of FSH and LH in the pituitary. If implantation does not take place, the corpus luteum degenerates into a corpus albicans in response to the diminishing level of plasma LH, the oestrogen and progesterone levels decline and the negative feedback to the pituitary terminates (Clarke 1995) (Fig. 1.1a, Fig. 1.2).

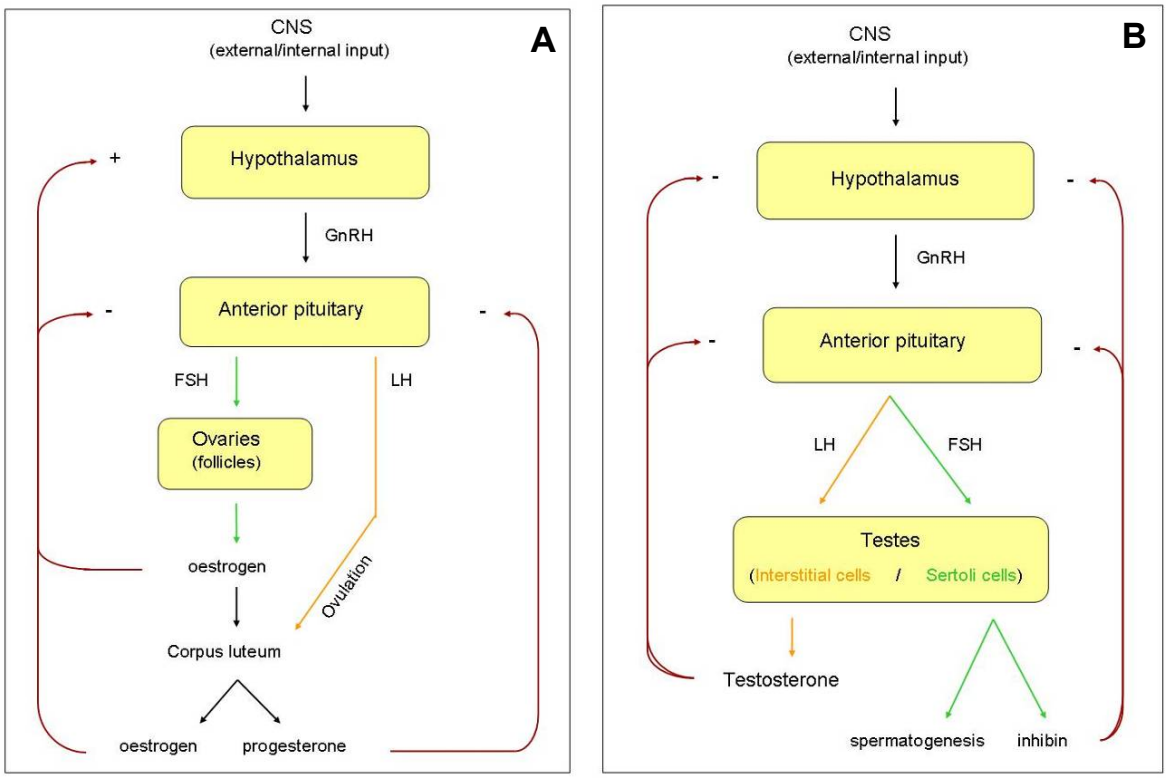

Figure 1.1: Schematic representation of the regulation of the hypothalamo-pituitary-gonadal axis in (a) females and (b) males.

In males, FSH and LH regulate spermatogenesis. FSH initiates sperm production while LH stimulates the production of testosterone in the interstitial cells (Frandson & Spurgeon 1992). Testosterone is the male hormone that maintains the male sexual characteristics. Under the influence of testosterone and other hormones, immature sperm cells develop into mature sperm called spermatozoa and are stored in the epididymus (Martan 1969). Males do not produce a LH surge due to insufficient levels of oestrogen (Parvizi 2000), or in the case of male non-human primates, rats and sheep, as a result of an inability of the hypothalamus to respond to a rapid and large LH increase in estradiol with a GnRH surge (Steiner *et al.* 1976) (Fig.1.1b).

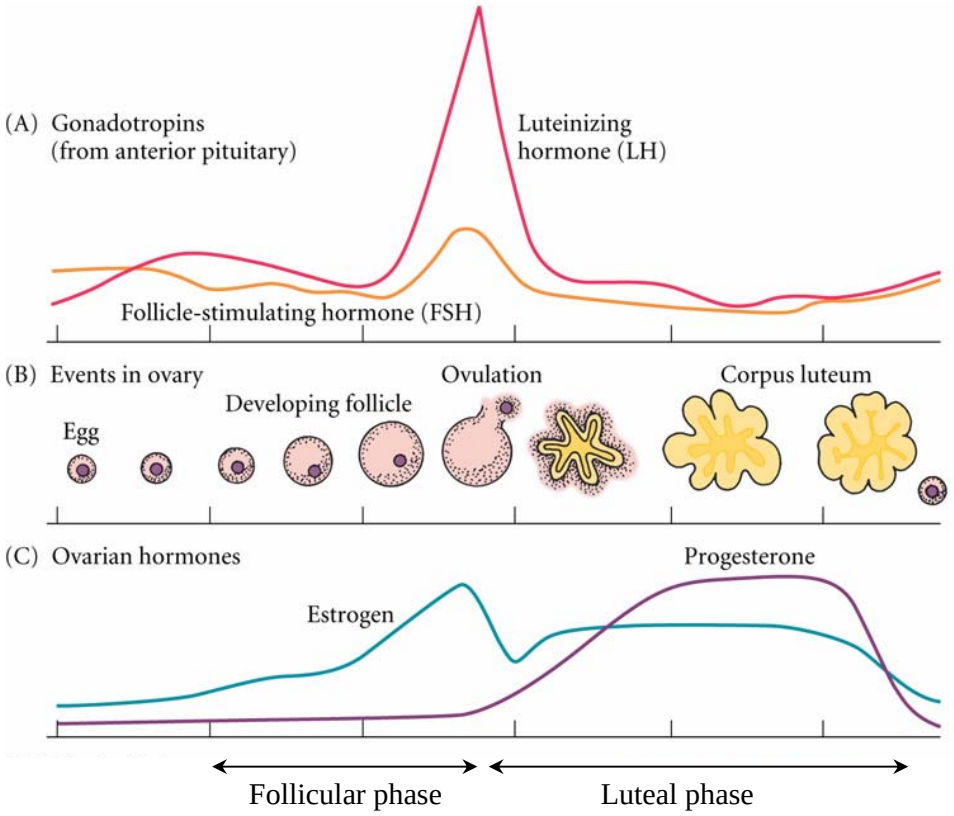

Figure 1.2: (A) Gonadotropic hormone profiles during the oestrous cycle, (B) Development of the egg into a corpus luteum, (C) Ovarian hormone profiles during the oestrous cycle.

- **Seasonal breeding**

Many species inhabiting environments with moderate to extreme seasonal fluctuations in temperature and rainfall display a reversible, annual cycle of fertility that directs their reproductive activity (Bronson & Heideman 1994, Lehman *et al*. 1997). Hence, young are usually born when environmental conditions are most favourable for optimum survival of the offspring.

Physiologically, the change in seasonal reproductive status is controlled by modifications in the activity of the HPG axis through variations in the pulsatile LH secretion. During the non-breeding season, there is a marked change in the responsiveness of the GnRH system to negative feedback of the gonadal hormones (Karsch & Moenter 1990, Lehman *et al*. 1997). In the breeding season, oestrogen inhibits the GnRH pulse amplitude while progesterone suppresses the pulse frequency. However, during the non-breeding period, the GnRH pulse frequency is inhibited by both these hormones (Goodman 1996). Outside the breeding period, there is a complete down-regulation of reproductive function in females of species such as sheep and horses (Lehman *et al.* 1997). The secretion of GnRH decreases dramatically, consequently the pituitary cannot release significant amounts of LH and FSH. Hence, gonadal steroid production diminishes (Eagle & Tortonese 2000).

In males, reproductive activity outside the breeding season is suppressed to a lesser degree than in females (Dacheux *et al.* 1981). Testicular size, testicular and plasma testosterone, sperm production and reproductive behaviour are reduced (Berndston & Desjardins 1974, Hoffmann 1979) but species differ in the degree of reproductive down-regulation. In some species like the horse, fertility is maintained at a reduced level (Guillaume 1996), whereas in some other species like hamsters (Turek *et al*. 1975), the grey squirrel (Tait & Johnson 1982) and brown bears (Tsubota *et al*. 1997), a near complete cessation of spermatogenesis occurs. In addition, intra specific variation also occurs. Some male prairie voles (*Microtus ochrogaster*) undergo complete gonadal regression during short days while others fail to respond to photoperiod and do not show any inhibition of reproduction (Kriegsfeld & Nelson 1999).

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However, 'out of season' breeding occurs in virtually all rodent populations (Moffat *et al*. 1993). The house mouse (*Mus musculus*) reproduces seasonally when in seasonal habitats, but in a constant environment, offspring is produced throughout the year (Bomford 1987, Efford *et al*. 1988). In the badger, testicular activity shows seasonal fluctuations, spermatogenesis occurs throughout the year (Audy *et al*. 1985). Thus although reproductive activity is interrupted during the non-breeding season, the hypothalamo-pituitary axis appears to remain active. This provides the possibility for opportunistic breeding throughout the year whenever conditions are favourable.

A number of environmental factors have been shown to play a role in the regulation of seasonal breeding, such as photoperiod, temperature, and humidity (Bronson & Heideman 1994). In mammals, photoperiod is the most prominent environmental cue used for timing their annual reproductive cycles. It is the single most reliable environmental cue to indicate the time of the year (Saunders 1977). The reproductive system is activated or shut down when the photoperiod reaches a certain critical day length.

Subterranean mammals spend the majority of their lives underground in burrows and therefore do not come into regular contact with environmental light cycles (Ben-Shlomo *et al*. 1995, Oelschläger *et al*. 2000, Ricco & Goldman 2000). Thus, light probably does not play a significant role in seasonal reproductive cycles in these species.

Temperature is another environmental factor that shows seasonal variation. Although not as reliable as photoperiod, some species have been shown to entrain activity rhythms to temperature cycles. Although temperature is much buffered underground, seasonal differences are detectable closer to the soil

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surface. A number of animals have been shown to entrain activity rhythms to temperature cycles (Aschoff & Tokura 1986, Gavaud 1991, Lopez-Olmeda *et al.* 2006, Rajaratnam *et al*. 1998, Tokura & Aschoff 1983, Yoshii *et al*. 2005), and in certain animals annual cycles are dependent on ambient temperature.

Around the equator, there is no annual change in photoperiod. However, rainfall tends to be strongly seasonal. Seasonal rainfall influences the availability of food and promotes seasonal breeding according to food resources (Brosset 1986).

In addition to abiotic factors, social cues can also strongly influence reproductive status of animals. Behavioural interactions between different sexes can initiate neuroendocrine events required for successful copulation, ovulation and pregnancy (Tai *et al*. 1997) In some cases sensory signals can stimulate the activation of gonadotropin releasing hormone neurons (Ferron & Gheusi 2003, Moffatt *et al*. 1995, Perret 1992, Richardson *et al*. 2004, Westberry & Meredith 2003), while in other cases coitus acts as the stimulating factor (Bakker *et al*. 2001, Dellovade *et al.* 1995, Rissman *et al.* 1997).

- **Cooperative breeding**

Cooperative breeding occurs in a number of small mammals (e.g. marmosets, suricates, mongooses, prairie voles and mole-rats) (Faulkes & Abbott 1997). The extent of cooperation varies from social groups where several males and females regularly breed to groups where reproduction is restricted to a single breeding female and one or two dominant males (Emlen 1991, Creel & Waser 1991). The non-breeding members of the colony are typically helping with

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various tasks such as foraging and tending to young (Clutton-Brock 2002, Clutton-Brock *et al*. 2001, Faulkes & Bennett 2001, Hodges 2005, Schaffner & French 1997, Solomon & Vandenbergh 1994).

Eusociality is typically associated with insects such as bees, wasps, ants and termites. An eusocial breeding strategy is considered as an evolutionarily advanced level of colonial living where groups of cooperatively breeding conspecifics have a reproductive division of labour, cooperative care of young and more than two overlapping adult generations (Wilson 1971, Wilson & Hölldobler 1995). On the eusociality continuum, social systems are rated on a scale from zero to one, such that in societies with a low skew (close to zero), all individuals have almost equal opportunities for breeding and those with a high skew (close to one) have a more despotic type of reproductive system (Sherman *et al*. 1995).

- **African mole-rats**

African mole-rats are subterreanean hystricomorph rodents endemic to sub-Saharan Africa (Skinner & Smithers 1990). The family Bathyergidae (African mole-rats) is composed of at least 16 species and five genera (Faulkes *et al*. 2004, Ingram *et al.* 2004). Of the five genera, three genera (*Georychus, Heliophobius* and *Bathyergus*) are solitary and two genera (*Cryptomys* and *Heterocephalus)* are social (Bennett & Faulkes 2000), displaying varying degrees of sociality and cooperative breeding. Social mole-rat species exhibit a reproductive division of labour; consequently reproduction in colonies is highly skewed and affects the lifetime reproductive success of subordinate animals (Faulkes & Bennett 2001).

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Two species, the Damaraland mole-rat (*Cryptomys damarensis*) and the naked mole-rat (*Heterocephalus glaber*), are considered to be truly social or eusocial (Jarvis 1981, Jarvis & Bennett 1993). Eusociality is thought to have evolved independently in these two species, it is not observed in any other subterranean mammal but further studies may include other species of mole-rat within this definition (Faulkes *et al.* 1997, Jarvis & Bennett 1993).

Comparisons between group size and the environment where the different species occur has led to the idea that the degree of sociality observed across the family Bathyergidae is correlated to the aridity of the habitat and subsequent food availability (Faulkes & Abbott 1997, Jarvis & Bennett 1991, 1993, Jarvis *et al*. 1994). The aridity food distribution hypothesis (AFDH) has been proposed to explain the subsequent costs and risks associated with foraging and dispersal in arid areas (Jarvis *et al.* 1994). Mole-rats live in extensive underground burrows that are excavated with their teeth. The primary food source of mole-rats is geophytes, roots and tubers that are encountered as they burrow (Jarvis & Bennett 1991). In areas with a predictable and frequent rainfall pattern, food resources are evenly distributed and soil is readily workable for most of the year. Solitary species are confined to these mesic areas where a single animal can easily find sufficient food to sustain itself. In arid areas mole-rats are energetically restricted by dry, hard soil, and food resources are more clumped and further apart. Since mole-rats forage blindly, a larger colony size will increase the probability of encountering a localised food patch and reduce energetic costs and tooth wear. While solitary species are confined to mesic areas, social species are not excluded from those areas. However, social species that are adapted to more arid environments tend to have larger colony sizes as the total energetic costs of foraging are reduced (Jarvis *et al.* 1994)

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Alternatively, Burda *et al*. (2000) suggests that eusociality in mole-rats results from cooperative monogamy and is reinforced by a subterranean lifestyle. In this scenario, dispersal is restricted, and it allows for continuous rather than seasonal breeding, which would lead to rapid overlap of generations. They propose that mole-rats have an ancestral tendency to be solitary. Differing rates of change between social to solitary along the different phylogenetic lines may validate any variation in the different species.

- **Reproductive skew in mole-rats**

In social mole-rat species, breeding opportunities are monopolised by dominant animals; usually a single female and one or two males that are responsible for procreation in a colony (Jarvis 1981, Jarvis & Bennett 1993). This unequal distribution of reproduction creates a reproductive skew, which in terms of lifetime reproductive success, differs significantly between species. It is not clear exactly what role the dominant animals play in maintaining this reproductive skew, and the proximate mechanisms underlying this reproductive division of labour may differ even between closely related species (Faulkes & Bennett 2001).

A number of models have been proposed to explain reproductive skew in cooperatively breeding societies (for overview see Faulkes & Bennett 2007). Two of these are particularly applicable to explain the maintenance of a reproductive skew in mole-rats (Snowdon 1996). The dominant control model suggests that the dominant animals exert some form of reproductive control over subordinate individuals (Faulkes & Bennett 2001). The breeding animal benefits from assistance provided by non-breeding colony members and avoids reproductive competition by suppressing subordinate animals

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(Snowdon 1996). Reproductive control ranges from infanticide of the offspring of a subordinate, aggression and interference with mating attempts, to suppression of the reproductive physiology of other members in the colony (Faulkes & Abbott 1997). Naked mole-rats differ from other mole-rat species in that they will spontaneously inbreed in captivity (Faulkes & Bennett 2007) although when given a choice, they do prefer outbreeding (Clarke & Faulkes 1999). There is a very high reproductive skew among members of their colonies and lifetime reproductive success is almost zero (Jarvis *et al.* 1994).

Alternatively, the self restraint model implies that incest avoidance may suffice to maintain the reproductive skew between closely related animals. Most cooperatively breeding mammals live in extended family groups, and because breeding with close relatives is often deleterious, most species have evolved mechanisms to prevent inbreeding (Cooney & Bennett 2000, Pusey & Wolf 1996). Species from the genus *Cryptomys* are obligate outbreeders, consequently a lack of unrelated breeding partners might be adequate to prevent subordinate animals from breeding (Cooney & Bennett 2000).

No individual factor can be singled out to explain delayed dispersal and group living. Non-breeding individuals may benefit from group living with respect to enhanced foraging efficiency and reduced risk of predation (Koenig *et al*. 1992). In mole-rats, ecological constraints are major factors that contribute to cooperative breeding. High energetic costs of dispersal may lead to individuals remaining in their natal colonies (Lovegrove & Wissel 1988, Lovegove 1991). Hamilton (1964) proposed that by helping closely related individuals rear their offspring, helpers gain indirect fitness benefits. Also, helpers frequently increase their own chances of becoming breeders by gaining experience (Emlen 1997). Thus, non-reproductive animals may delay their dispersal until opportunities arise, when environmental conditions are

favourable or unrelated animals are present or they have gained sufficient skills to ensure successful reproduction independently.

- **Reproductive suppression in mole-rats**

In mole-rats, the extent and type of reproductive suppression is correlated with the degree of sociality (Figure 1.3). Reproductive suppression amongst subordinate animals is either behavioural or physiological or a combination of the two. Behavioural suppression entails interference with breeding attempts of subordinate animals by dominant animals or the subordinate individuals that refrain from breeding to avoid inbreeding (Snowdon 1996). Alternatively, reproduction is physiologically interrupted and in extreme cases, reproduction can be completely suppressed by blocking ovulation (Abbott 1987, Bennett *et al*.1999).

Studies of several loosely social species with relatively small colony sizes (the Mashona mole-rat, *C.darlingi*; the common mole-rat, *C.h.hottentotus;* the giant mole-rat, *C.mechowi*; Ansell's mole-rat, *C.anseli*) have revealed that there is no difference in the pituitary function of breeding and non-breeding animals. Subordinate males and females have comparable LH levels in response to a GnRH challenge to the dominant animals (Bennett *et al.* 1997, Bennett *et al*. 2000, Burda *et al*. 1995, Spinks *et al*. 2000). Hence, it appears that the reproductive skew is maintained by inbreeding avoidance alone.

In the Damaraland mole-rat (*C.damarensis*), physiological suppression of reproduction is apparent in subordinate females. Although their ovaries possess a degree of follicular development, no Graafian follicles are present (Bennett & Jarvis 1988b). Urine and plasma progesterone concentrations as

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well as LH concentrations are measurable but lower in non-breeding females (Bennett *et al*. 1999). However the pituitary show a reduced sensitivity towards exogenous GnRH compared to that of the reproductive females (Bennett *et al*. 1993).

No physiological block is apparent in subordinate male Damaraland mole-rats. Sperm number and motility is similar to that of the breeding animals (Faulkes *et al*. 1994). Also no differences were found in urinary and plasma testosterone (Bennett 1994) plasma LH or LH responses to exogenous GnRH (Bennett *et al*. 1993).

The naked mole-rat (*H.glaber*) is placed at the apex of this continuum, with the most stringent level of reproductive suppression exerted by dominant animals (Figure 1.3). A physiological block of reproduction is evident in non-breeding animals of both sexes (Faulkes *et al*. 1990a,b, Faulkes *et al*. 1991). The ovaries of subordinate naked mole-rat females have an immature appearance (Faulkes *et al.* 1990a). Urinary concentrations of oestrogen and progesterone are very low in these animals (Faulkes *et a*l. 1991, Westlin *et al*. 1994), and LH concentrations are significantly lower than that of breeding animals (Faulkes *et al*. 1990b). The LH response to exogenous GnRH suggests a reduced sensitivity of the pituitary to GnRH (Faulkes *et al*. 1990b, 1991), hence the LH production and secretion is insufficient to induce ovulation.

Subordinate male naked mole-rats have smaller testes and fewer testosterone secreting Leydig cells (Faulkes *et al.* 1994, Faulkes 1991). Although spermatogenesis takes place in subordinate males, the sperm number and motility are significantly reduced (Faulkes *et al*. 1991). All hormonal levels associated with reproduction in subordinate males are much

reduced when compared to dominant males (Faulkes & Abbott 1991, Faulkes *et al*. 1991). It appears that with higher levels of sociality, dominant animals expend more energy on suppression of reproductive function of subordinate animals.

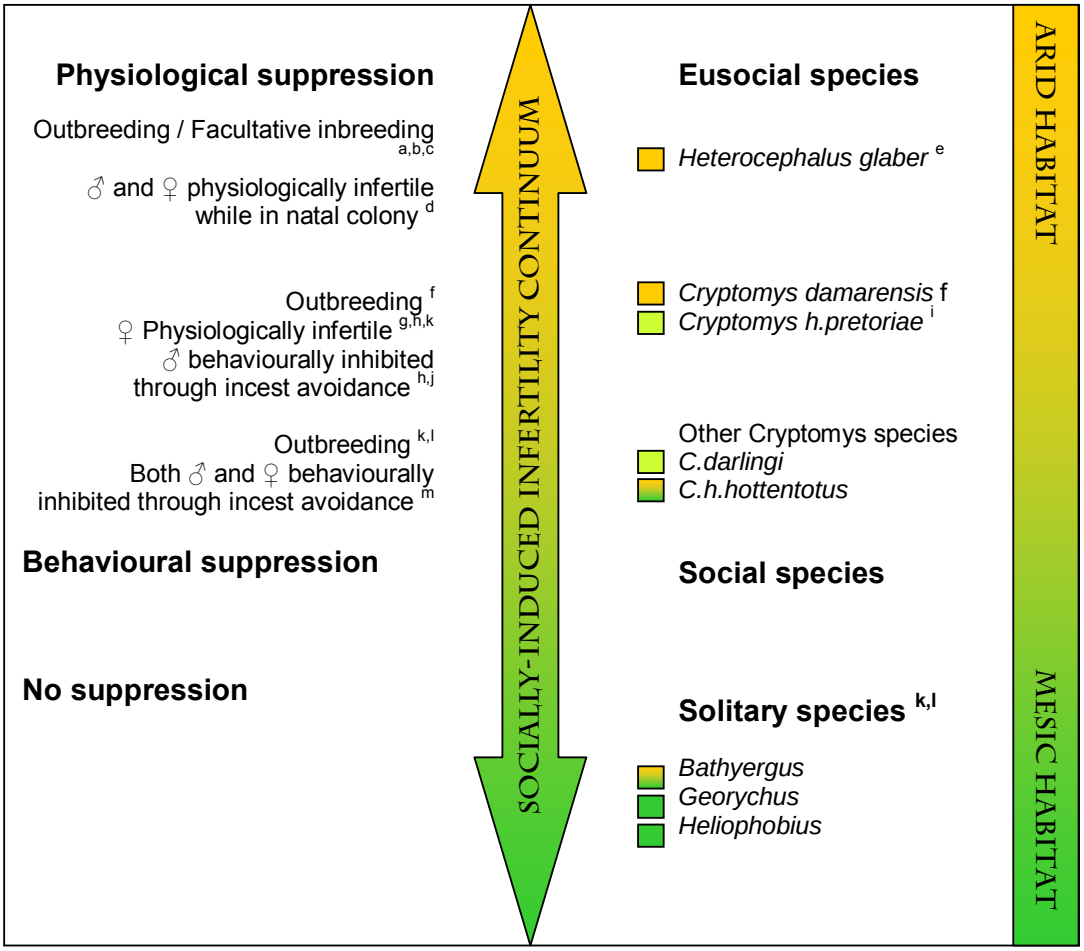

Figure 1.3: Species of the family Bathyergidae assembled according to the degree of sociality displayed and type of habitat in which they occur. (Modified from L. van der Walt 2003).

[a] Reeve *et al*. 1990; [b] Braude 2000; [c] Clarke & Faulkes 1999 [d] Faulkes *et al*. 1990a,b, 1991; [e] Jarvis 1981; [f] Jarvis & Bennett 1993; [g] Bennett *et al*. 1996; [h] Bennett 1994; [i] Moolman *et al*. 1998; [j] Van der Walt *et al*. 2001; [k] Jarvis & Bennett 1991; [l] Bennett & Faulkes 2000; [m] Spinks *et al*. 1997, 1999, 2000; ♀ - female; ♂ - male.

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Study animals

- **The Cape mole-rat (*Georychus capensis*)**

The Cape mole-rat, *Georychus capensis* is a seasonally breeding, subterranean rodent mole (Bennett & Jarvis 1988a). It primarily inhabits coastal and mountain fynbos areas of the south-western Cape regions with a distinct seasonal rainfall pattern (Skinner & Smithers 1990).

The Cape mole-rat is strictly solitary, and like other solitary mole-rats, highly aggressive and territorial (Nevo 1961, Zuri *et al.* 1998). They display extreme levels of antagonistic behaviour towards conspecifics throughout most of the year (Guttman *et al*. 1975). This xenophobic behaviour is briefly broken down during the breeding season, when other mole-rats are tolerated in their tunnels for mating and subsequently the rearing of offspring (Bennett & Jarvis 1988a). The Cape mole-rat is an induced ovulator (Van Sandwyk & Bennett 2005), and courtship and copulation is confined to the rainy, winter months (Bennett & Jarvis 1988a). The gestation period is around 45 days and young are produced from August to December. Females appear to have the reproductive potential of producing two litters per season (Bennett & Jarvis 1988a).

Neighbouring individuals communicate through the soil by drumming their hind feet on the tunnel floor (Bennett & Jarvis 1988a, Narins *et al.* 1992). This seismic signalling is used to convey information about their territorial boundaries and, during the breeding season, sex and reproductive status (Bennett & Jarvis 1988a, Narins *et al*. 1992, Rado *et al*. 1998) (Plate 1.1).

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- **The Natal mole-rat (*Cryptomys hottentotus natalensis*)**

Very little is known about the Natal mole-rat, *Cryptomys hottentotus natalensis*. Previously it has been thought to occur in groups of two or three animals (Hickman 1979a), however in the current study, colonies of up to 17 animals have been caught from a single burrow system (M. Oosthuizen, *pers.obs*). The known distributional range extends from KwaZulu-Natal to Mpumalanga in the eastern parts of South Africa (Skinner & Chimimba 2005).

The Natal mole-rat is closely related to the common mole-rat (*Cryptomys hottentotus hottentotus*) and the highveld mole-rat (*Cryptomys hottentotus pretoriae*) (Bennett & Faulkes 2000) and thus is predicted to exhibit similar reproductive characteristics. The Natal mole-rat is a cooperative breeder, with a single breeding female (M. Oosthuizen, *pers.obs*.) and 1 or possibly more breeding males, while the remaining individuals of the colony theoretically comprise the offspring of the breeding animals. To date, no information is available on the colony structure as no behavioural or genetic studies have been conducted. Information pertaining to the reproductive biology is sparse (Hickman 1982). The Natal mole-rat is an induced ovulator (Jackson & Bennett 2005), but it is unknown whether its breeding period is confined to a specific part of the year (Plate 1.2).

Plate 1.1: The Cape mole-rat (*Georychus capensis*).

Plate 1.2: The Natal mole-rat (*Cryptomys hottentotus natalensis*)

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Aims

Two mole-rat species, the solitary Cape mole-rat (*Georychus capensis*) and the social Natal mole-rat (*Cryptomys hottentotus natalensis*) were the subjects of investigation in this thesis with the explicit purpose of increasing our knowledge on their reproductive systems.

To date there is limited information on the reproductive biology of the Cape mole-rat, whereas that pertaining to the Natal mole-rat is scant (Bennett & Jarvis 1988a, Hickman 1980). The objective of this study was to obtain a comprehensive understanding of all levels of the reproductive systems of these two species. It is known that the Cape mole-rat is a seasonal breeder, therefore the focus in this species will be on seasonal differences in the reproductive system. Before commencing the study, it was unknown whether the Natal mole-rat bred seasonally or not, thus seasonal differences were investigated along with the effect of reproductive status on the reproductive system. Data are analysed according to summer (non-breeding season for the Cape mole-rat) and winter (breeding season for the Cape mole-rat).

Chapter 2

The material and methods utilised in the various chapters are presented in this chapter.

Chapter 3

The objective of this chapter was to determine whether there are any seasonal differences in gonadal hormone levels in the Cape and Natal mole-rat and whether social status has an effect upon secretion of these hormones in the latter.

My *a priori* predictions are:

Cape mole-rat:

- There would be a seasonal difference in the levels of the hormones

Natal mole-rat:

- No seasonal difference in hormonal levels
- Distinct difference between breeding and non-breeding animals

Chapter 4

This chapter focuses on the effect of season and social status on the plasma LH concentrations before and after a GnRH challenge.

My *a priori* predictions are:

The solitary Cape mole-rat:

- Both males and females would exhibit differences in the basal concentrations of LH and differential responses to an exogenous GnRH challenge in and out of the breeding season.

The social Natal mole-rat:

- There would be no seasonal differences in the basal LH concentrations and response to a GnRH challenge in either sex during any part of the year.
- The basal LH concentrations and the response to a GnRH challenge would be similar between reproductive and non-reproductive animals of either sex.

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Chapter 5

This chapter investigates the neuroanatomical and neuroendocrinological differences with regard to season in the Cape mole-rat and to social status in the Natal mole-rat. Having established that no seasonal differences are present in the Natal mole-rat, seasonal differences were not explored in this chapter. The neuroanatomy of the GnRH system in solitary and social mole-rats was compared.

My *a priori* predictions are:

Cape mole-rat:

- There would be a seasonal difference in the GnRH content of the median eminence of the female animals in and out of the breeding season.

Natal mole-rat:

- There would be a difference in the GnRH content of the median eminence in reproductive and non-reproductive animals of both sexes.

Chapter 6

This final chapter synthesizes the findings of this study and compares it in the light of the existing data of the family Bathyergidae. The findings of the study are placed into the broader context of the regulation of reproduction in the African mole-rats.

CHAPTER 2:

MATERIAL AND METHODS

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Study animals

Trapping and maintenance

Mole-rats were trapped using modified Hickman live traps (Hickman 1979b), baited with sweet potato. Cape mole-rats were captured during August 2002 (breeding season/winter) and February 2003 (non-breeding season/summer) in the Darling area, Western Cape Province (33°22'S, 15°25'E) (Plate 2.1), while Natal mole-rats were obtained from Glengarry, KwaZulu Natal (29°19'S, 29°43'E) (Plate 2.2) during six trapping expeditions spread every second month throughout one year. The mole-rats were housed in plastic containers (40 cm x 30cm x 30cm) provided with wood shavings, and were fed on sweet potato, carrots and gem squash. No free water was provided as moisture is obtained from the food. Urine and blood samples were taken no longer than two weeks after the animals were captured.

Body mass of the Cape mole-rat

The mean body mass (±SE) of male Cape mole-rats captured in the breeding season was 168±16.3g (n=6) and ranged between 121g and 222g. Out of the breeding season, the mean body mass of males was 110±17g (n=4) and ranged between 75g and 154g. In breeding season, the mean body mass of female Cape mole-rats was 142±7.8g (n=25) and ranged between 86g and 232g. The mean body mass out of the breeding season was 177±12.1g (n=17) and a range between 104g and 272g (Figure 2.1). In the population studied, male animals were encountered much less frequently than females.

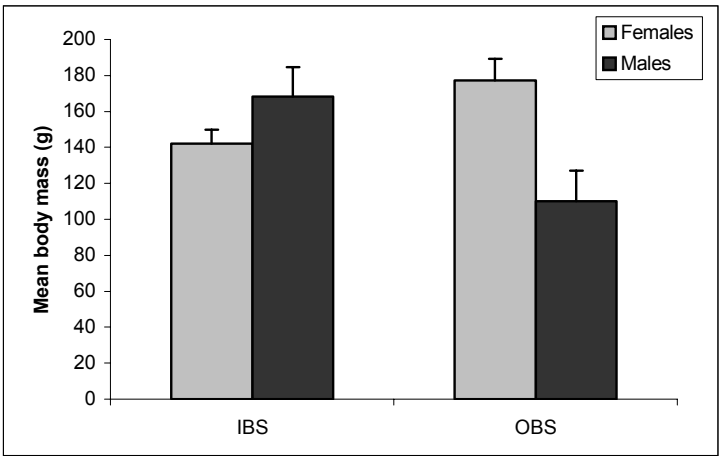

Figure 2.1: Mean (±SE) body mass (g) of the Cape mole-rat in and out of the breeding season.

Body mass of the Natal mole-rat

Breeding animals were typically heavier than non-breeding individuals in the Natal mole-rat. The mean body mass of female Natal mole-rats was 97g (n=23) for breeding animals with a range between 73g and 127g; 71g (n=72) for non-breeding animals, ranging between 47g and 111g. Breeding males had a mean body mass of 133g (n=45), ranging between 106g and 162g, and the mean body mass of the non-reproductive males was 91g (n=61) with a range between 40g and 119g (Figure 2.2).

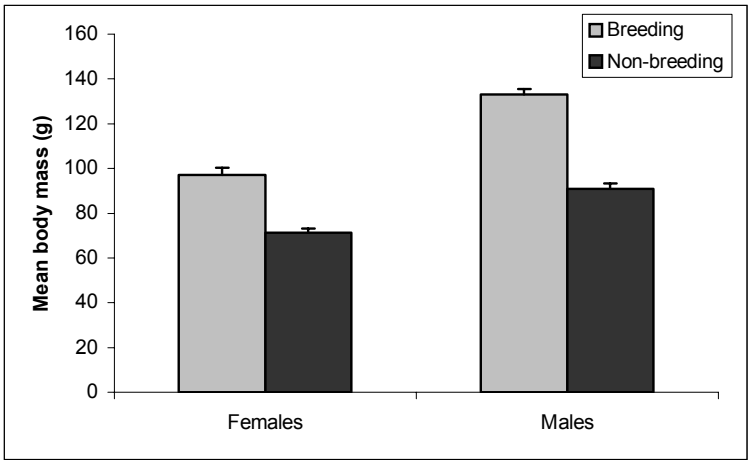

Figure 2.2: Mean (±se) body mass (g) of breeding and non-breeding/subordinate Natal mole-rats.

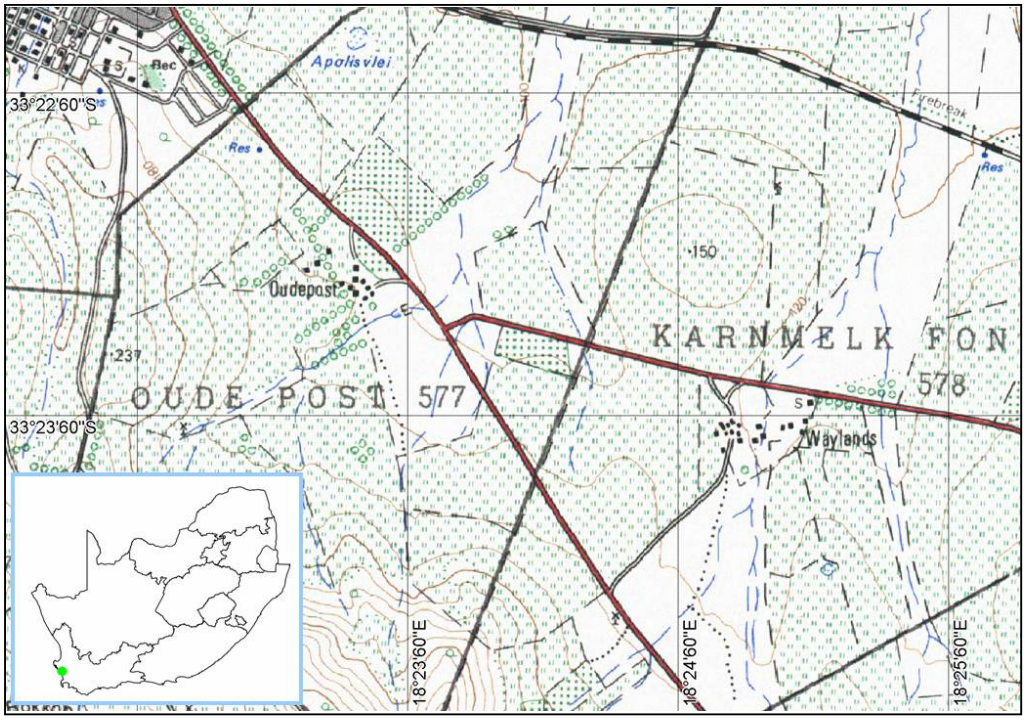

Plate 2.1: Study area near Darling, Western Cape Province, where the Cape mole-rats for this study were captured.

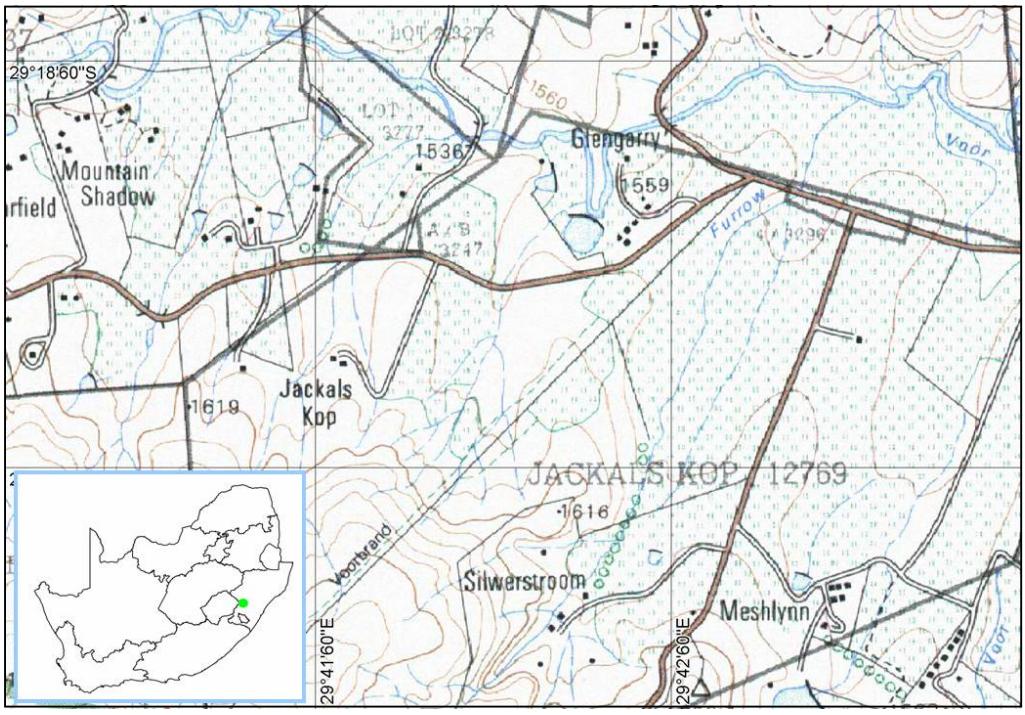

Plate 2.2: Study area near Mooiriver, KwaZulu Natal, where the Natal mole-rats for this study were captured.

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Chapter 3

Radioimmunoassays

Testosterone determination

Plasma testosterone concentrations were determined using a Coat-A-Count total testosterone kit (Diagnostic Products Corporation, Los Angeles, California, USA). Extraction or chromatography was not required for this procedure. A 50µl sample in duplicate was used for the assay. The procedure entails solid-phase radioimmunoassay based on hormone specific antibody immobilised to the wall of a polypropylene tube. ^{125}I-labeled testosterone competes for a fixed time with the specific hormone in the given sample for antibody sites. The tube is then decanted to separate bound from free and is then counted in a Cobra gamma counter.

The antiserum is highly specific for testosterone and has a low cross reactivity with other naturally occurring steroids except dihydrotestosterone, which is less than 5%.

The assay was validated by testing for parallelism using serial doubling dilutions of un-extracted plasma over the dilution range (1:1 to 1:64). The slope of the lines were compared and found not to differ significantly (ANCOVA $F_{(1,6)}$=4.3 P>0.05) following a log-logit data transformation (Chard 1987). The sensitivity of the assay (90% binding) was 2.2 nmols/l. The intra-assay coefficient of variation was 2.5% (n=6).

Oestrogen determination

Oestradiol-17β was determined in mole-rat urine using a Coat-A-Count Oestradiol-17β kit (Diagnostic Products Corporation, Los Angeles, California,

USA). A 100µl sample in duplicate was used for this assay. The method is a solid-phase radioimmunoassay that does not require purification of steroids or separation by chromatography. The antiserum is highly specific for oestradiol-17β, with a low cross reactivity with any other steroids present in the urine. The assay was validated by testing for parallelism using serial doubling dilutions of un-extracted urine over the dilution range (1:1 to 1:64) following log-logit transformation of the data (Chard 1987). The slope of the lines were compared and found not to differ significantly (ANCOVA $F_{(1,6)}$=0.09, P>0.05). The sensitivity of the assay was 2 pmols/l. The intra-assay coefficient of variation was 9% (n=8).

Progesterone determination

Urinary progesterone concentrations were determined using a Coat-A-Count progesterone radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, California, USA), as described by Bennett *et al.* (1996). A volume of 100:1 of urine in duplicate was assayed without extraction.

The antiserum is highly specific for progesterone with a low cross reactivity to all other naturally occurring steroids except 20-α- dihydroprogesterone and 11-deoxycortisol with a cross reactivity of 2% and 2.4% respectively. A pooled urine sample (one with expected high concentrations from a pregnant queen) was double diluted from 1:1 to 1:64 and assayed.

In the assay were also included 6 samples at a dilution of 1:64 from a pool of low concentration progesterone. To these samples 100µl of progesterone in increasing concentrations (0.3, 1.6, 6.4, 31.8, 63.6 and 120 nmols/l) was added in duplicate. The curve was perfectly parallel to the standard curve. The assay was validated for the test species by comparing the slope of the curve produced using serial doubling dilutions of un-extracted mole-rat urine

(over the range 1:1 to 1:64) against the standard curve (ANCOVA $F_{(1,6)}$=4.9, P>0.05). The intra and inter assay coefficients of variation were 7 and 11% respectively. The sensitivity of the assay at 90% binding was 0.4nmols/l.

Creatinine determination

Urine concentration varies with fluid consumption; therefore creatinine is used to standardize samples. Creatinine is a breakdown product from tissue proteins and is excreted at a relatively constant rate (Schmidt-Nielsen 1997).
A modified Jaffe reaction was used to calculate the creatinine concentration for urine samples (Folin 1914).

The samples are assayed in duplicate. Ten microlitres of standard or sample were added to the wells of a micro plate, leaving two wells empty as a blank control. 200µl of picric reagent was added to all the wells, including the blanks. The picric reagent consists of saturated picric acid solution, alkaline triton solution (4.2 ml triton X-100, 12.5 ml 1N NaOH and 66.0 ml distilled, deionised water) and distilled deionised water in the proportion of 1:1:10. The alkaline triton can only be used once the product is homogenous. The microplate is then placed in the dark for a period of 1,5 hours, at room temperature, to allow colour development to occur. A standard curve (R^2>0.99) was used to determine all sample values.

Chapter 4

Blood sampling

Prior to sampling, the animals were placed into a temperature regulated chamber at 36°C for 20 minutes to bring about vasodilatation, thus facilitating blood collection. Mole-rats were hand restrained while blood was taken from the saphenic vein in the foot, prior to and 20 minutes after the administration

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of a saline injection or a single GnRH challenge. Heparinised capillary tubes were used to collect between 300 and 400µl of whole blood, whereafter the blood was centrifuged to separate the plasma from the cellular component of the sample. Plasma was stored at -40°C until being assayed. This method has been successfully used to investigate pituitary sensitivity and secretion in naked mole-rats (Faulkes *et al.* 1990b, 1991), Damaraland and Mashona mole-rats (Bennett *et al.* 1993, 1996, 1997) and suricates (O'Riain *et al*. 2000).

GnRH administration

A chimaeric analogue of mammalian GnRH produced in the laboratory of R.P. Millar (Chemical Pathology, University of Cape Town), was administered to the mole-rats. The hormone was synthesized using solid phase methodology and had a purity of >98% homogeneity (Millar *et al*. 1989). A dose of 2µg in 100µl of sterile physiological saline was used to challenge the pituitary. Control animals were injected with 0.2ml sterile physiological saline.

LH bioassay

LH concentrations were determined using an *in vitro* bioassay based on the production of testosterone by dispersed mouse Leydig cells (Van Damme *et al.* 1974). The incubation medium (12ml Eagle's basal medium, 2.1ml 7.5% sodium hydrogen carbonate 2ml foetal calf serum and 100ml distilled water) was placed on ice and gassed slowly under Carbogen 5 (95% O_2: 5% CO_2). A six week old male mouse was killed by cervical dislocation, the testes removed and decapsulated in 5ml incubation medium. The cell suspension was stirred on a magnetic stirrer for 5 minutes, filtered through fine nylon mesh and incubated under Carbogen 5 gas for 1 hour in a shaking water bath at 34ºC. Subsequently the incubated cell suspension was washed and centrifuged at 2500 r.p.m for 5 min at 4ºC. The supernatant was decanted and

the cells resuspended in the incubation medium. The process was repeated after which the cell suspension was slowly stirred on the magnetic stirrer for 5 minutes. A haemacytometer was used to count the number of cells. Incubation medium was added until the number of cells counted corresponded to the final cell suspension volume (in ml). The medium was stirred for 5 minutes.

The LH buffer (2.9g disodium hydrogen orthophosphate dodecahydrate, 0.29g sodium dihydrogen orthophosphate dehydrate and 4.38g sodium chloride) was made up to 1 litre in distilled water with 0.1% BSA. The mole-rat plasma samples were prepared at a 1:20 dilution in LH buffer. A standard curve was obtained by serially double diluting the mammalian LH in LH buffer, within the range of 360-1.4μIU ml/100 μl. 100μl of either plasma sample, standard, quality control or LH buffer (to obtain an estimate of total binding), was added to the bioassay tubes. Standards and total binding were assayed in triplicate while samples and quality controls were assayed in duplicate. The mammalian LH standard (2nd International standard 1988, Code 80/552, Hertfordshire, U.K.) was provided by the National Institute of Biological Standards and Controls (Storring & Gaines 1993).

After 200μl of diluted cell suspension was added to each assay tube, the tubes were incubated in a shaking water bath at 34°C for 3h under Carbogen 5 gas. Further testosterone production by the Leydig cells was inhibited by boiling the tubes containing the cell preparation in a water bath at 100°C for 15 minutes. Subsequently the tubes were placed on ice and 0.3ml phosphate buffered saline with 0.1% gelatine was added. Testosterone production during the incubation period was determined by radioimmunoassay (Bennett 1994).

Radioimmunoassay

Concentrations of testosterone were determined by radioimmunoassay of duplicate sample aliquots. Testosterone antiserum in phosphate buffer (0.1 ml) at a working dilution of 1: 800 was added to standards and reagent blanks. The contents were mixed and subsequently [1,2,6,7–^{3}H] testosterone TRK 402 (sp. Act. 80-105 Ci/mmol; Radiochemical Centre Amersham, Bucks. UK) in 0.1ml assay buffer (~10,000 cpm) was added. The contents of each tube were mixed and incubated overnight at 4^{o}C.

The tubes were cooled at 4^{o}C and separation of antibody bound and free testosterone was carried out by adding 0.5ml dextran coated charcoal (Norit A charcoal 1.0g and 0.1g Dextran T-40 in 400ml assay buffer), incubating at 4^{o}C for 12 minutes and then centrifuging at 3000 rpm for 20 minutes at 4^{o}C. The supernatant was decanted into scintillation vials and scintillation fluid (10ml) (Ready-Solve CP, Beckman Instruments (Pty) Ltd, Johannesburg, South Africa) was added to each vial. The contents of the vial were mixed, left for 1 h and finally counted for 2 min using a Tricarb Scintillation counter.

Cross-reaction with all major naturally occurring steroids was <0.1%, except for dihydrotestosterone for which it was 5.1%. The sensitivity of the assays, defined as twice the standard deviation of values obtained from the buffer blank was 0.5 miu/ml.

The inter-assay variation was 14%. Serial dilutions of plasma obtained following GnRH administration paralleled the standard curve over the dilution 1:0 to 1:64 (Cape mole-rat: ANCOVA $F_{(1,6)}$=0.883, p=0.379; Natal mole-rat: ANCOVA $F_{(1,6)}$=0.069, p=0.800).

Chapter 5

GnRH immunocytochemistry

Animals were weighed and deeply anaesthetized with an overdose of fluorothane anaesthetic (Zeneca, RSA). They were perfused intracardially with 0.9% saline at 37°C, followed by 4% paraformaldehyde (PFA) (Saarchem) in 0.1M phosphate buffer (pH 7.4) (Sigma) at 4°C. The heads were removed and the brains sectioned out. The brains were stored in 2% PFA until further treatment. Prior to sectioning, brains were placed in 30% sucrose until saturated for cryoprotection. When saturated, the brains were quick frozen with dry ice, and 25μm thick coronal sections were cut on a cryostat (Bright Cryostats, UK), and every sixth section was used.

The sections were pre-treated in 0.5%-X100 triton which increased the permeability of the cell membrane. Endogenous peroxidase was suppressed using 0.02% H_2O_2. The sections were briefly rinsed in PBS and incubated in 2% normal donkey serum for an hour after which the sections were incubated in GnRH primary antibody (manufactured in rabbits, INCSTAR) for 48 hours at 4°C (dilution 1:20 000). After a brief rinse, sections were incubated in secondary biotin-SP conjugated AffiniPure Donkey anti-rabbit IgG antibody for two hours (dilution 1:200, Jackson Immunoresearch, West Grove, PA). After rinsing in PBS the tissue was incubated in an avidin-biotin peroxidase complex (1:1000, Elite Kit, Vector Laboratories, Petersborough, United Kingdom). Following a rinse in TRIS buffer they were incubated in 0.05% diaminobenzidine (DAB) with 0.15% ammonium nickel sulphate and 0.005% H_2O_2 to visualise GnRH immunoreactivity. All the sections for a given comparative group were processed in parallel.

CHAPTER 3:

GONADAL STEROID HORMONE CONCENTRATIONS IN THE SOLITARY CAPE MOLE-RAT AND THE SOCIAL NATAL MOLE-RAT

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Abstract

Urinary gonadal steroid concentrations were measured and compared in the summer and winter for both the Cape mole-rat *(Georychus capensis*) and the Natal mole-rat (*Cryptomys hottentotus natalensis*). The Cape mole-rat breeds seasonally, with sexual activity and pregnancy recorded during the winter months in the southern hemisphere. Despite the fact that it has a distinct breeding season, the seasonal differences in the urinary hormone concentrations in both male and female Cape mole-rats were not statistically significant. This suggests that the Cape mole-rat is an opportunistic breeder and is able to make use of favourable environmental conditions when they occur during the year.

No seasonal differences in urinary sex steroid concentrations were found in either male or female Natal mole-rats. The endocrinology complements histological and post mortem data for presence of embryos in reproductive females recorded during the entire year and support the notion that the Natal mole-rat is not a seasonal breeder.

Dominant, reproductive and subordinate, non-reproductive males displayed comparable testosterone levels and the reproductive and non-reproductive females exhibit comparable oestrogen concentrations. The comparable concentrations of sex steroids in reproductive and non-reproductive animals implies that lack of reproduction in subordinate animals is likely the result of incest avoidance rather than a physiological component of suppression. Urinary progesterone concentrations of the dominant, reproductive females were found to be significantly higher than that of the subordinate, non-reproductive females, and histological data indicate that follicular development does not progress to the stage of the corpus luteum. Furthermore a resultant

LH surge to induce ovulation does not take place in subordinate animals. The reproductive physiology of the Natal mole-rat compares with loosely social mole-rat species inhabiting mesic areas.

Introduction

Gonadal hormones

Cholesterol is the common precursor for all steroid hormones. It is first converted to pregnenolone, a rate-limiting step prior to the manufacture of steroid hormones (Hsu *et al*. 2006) (Figure 3.1).

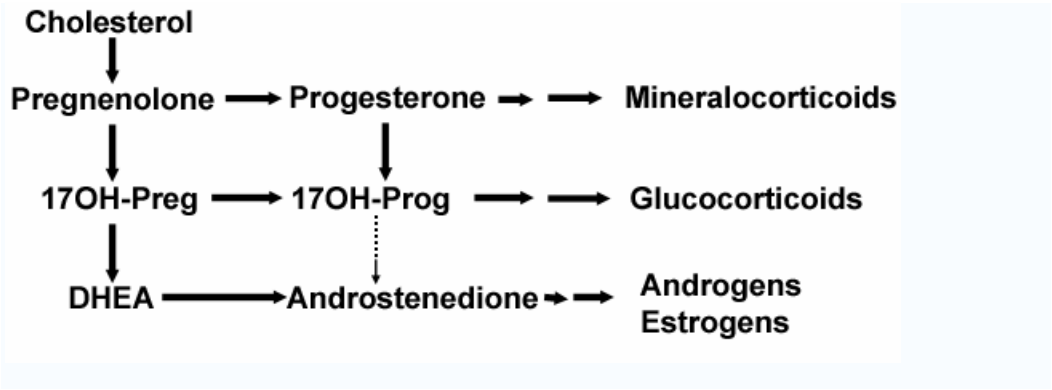

Figure 3.1: The pathway of steroid biosynthesis.

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Testosterone

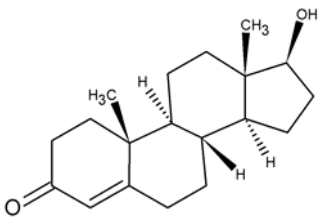

Figure 3.2: Molecular structure of testosterone.

Testosterone is secreted primarily by the testes in males, although small amounts are also secreted by the ovaries in females and the adrenal glands of both sexes. It is the principal male sex hormone, and is responsible for the development of male secondary sex characteristics (Frandson & Spurgeon 1992).

In the testes, testosterone is synthesized mainly in the interstitial Leydig cells and is regulated by luteinising hormone (LH) from the anterior pituitary. In the Sertoli cells, testosterone activates the androgen receptor to initiate and maintain spermatogenesis, and inhibit germ cell apoptosis (Dohle *et al*. 2003).

Testosterone is transported to its target organs in the blood and while being transported, it is bound to a plasma protein called sex hormone binding globulin (SHBG) (Anderson 1974). The main function of testosterone is activation of androgen receptors and it is converted to oestradiol.

Oestrogen

Oestrogens are a group of steroid compounds important in the oestrous cycle. Major oestrogens are oestradiol, oestriol and oestrone, from which oestradiol is the most prominent. Oestradiol is converted from testosterone via the

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enzyme aromatase (Balthazart *et al*. 1996). It functions as the primary female sex hormone and promotes the development of secondary sexual characteristics (Havelock *et al*. 2004). Although oestradiol is present in both males and females, the concentration in females is much higher than in males (Frandson & Spurgeon 1992).

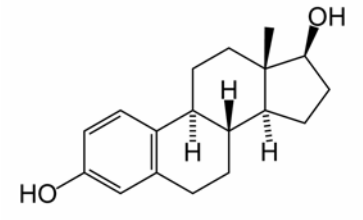

Figure 3.3: Molecular structure of oestradiol.

During the normal course of the reproductive cycle the ovarian follicles are the primary sites of oestrogen synthesis (Poutanen *et al*. 1995), however during pregnancy, oestrogen is mainly produced in the placenta. Small quantities of oestrogen are also produced in the adrenal glands and in the testes of males. Oestrogenic hormones are secreted at varying rates during the menstrual cycle throughout the period of ovarian activity (Figure 3.5).

Oestradiol acts as a growth hormone for the tissue of reproductive organs. It appears to be necessary for maintenance of oocytes in the ovary. Oestradiol produced by the growing follicles trigger hypothalamic-pituitary events that lead to the luteinising hormone surge that induces ovulation.

Progesterone

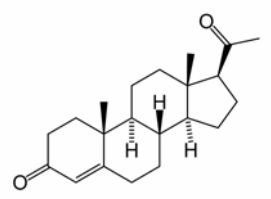

Figure 3.4: Molecular structure of progesterone.

Progesterone is primarily synthesized by the ovarian corpus luteum and the placenta. It is considered essential for the preparation and maintenance of mammalian pregnancy. Initially, the corpus luteum is responsible for the majority of progesterone production, but during the final three quarters of the pregnancy the placenta is recognised as the primary source of progesterone (Henson 1998). Small quantities of progesterone are also manufactured in the adrenal glands.

Circulating progesterone levels are characteristically low during the follicular phases of the reproductive cycle, it shows a sharp increase during the luteal phase, reaching a peak after the LH surge, after which it declines rapidly, unless pregnancy occurs (See fig. 3.4) (Clarke & Pompolo 2005).

Gonadotropins of the anterior pituitary regulate secretion of ovarian hormones, oestradiol and progesterone, hypothalamic control of pituitary gonadotropin production is in turn regulated by plasma concentrations of oestrogen and progesterone. A complex feedback system results in the cyclical phenomenon of ovulation.

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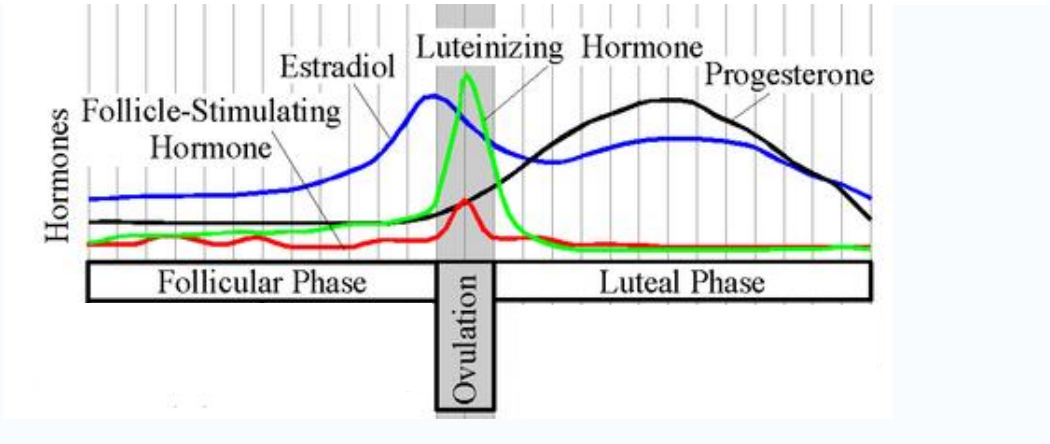

Figure 3.5: Hormonal levels during the course of the female reproductive cycle.

Chapter 3a

Cape mole-rat (*Georychus capensis*)

The Cape mole-rat (*Georychus capensis*) is a solitary rodent mole that is xenophobic and highly aggressive towards conspecifics (Nevo 1979, Bennett & Jarvis 1988a). Foot drumming is used to convey information about territorial boundaries, such that neighbouring tunnel systems may come to a metre from each other, but do not interlink (Bennett & Jarvis 1988a).

Aggressive and territorial behaviour is halted briefly for courtship and mating. This behaviour is initiated via foot drumming that, depending on the frequency, relates information about sex and reproductive state to surrounding mole-rats (Bennett & Jarvis 1988a). In the Cape mole-rat, breeding is restricted to the wet winter months. Mole-rats have a relatively long gestation time for their body size (Bennett & Faulkes 2000), such that

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young are produced towards the end of the winter and into early summer. The Cape mole-rat appears to have the potential to produce two litters per season. The Cape mole-rat is an induced ovulator, and thus does not show a constant cyclical pattern of ovulation (Van Sandwyk & Bennett 2005). The act of coitus stimulates the hypothalamus to produce gonadotropin releasing hormone (GnRH) which in turn triggers the release of follicle stimulating hormone (FSH) from the anterior pituitary. FSH promotes follicular development, from where oestrogen is secreted. Increased levels of oestrogen instigate a surge of LH that results in ovulation (Knobil 1988).

In most seasonally breeding mammals, the reproductive system undergoes some level of regression during the reproductively quiescent season. In seasonally breeding females, ovulation occurs only during the part of the year that is optimal for reproduction whereas in males testicular size is reduced and sperm production is down-regulated or terminated. Furthermore, gonadal steroid levels are secreted at much lower levels (Gerlach & Aurich 2000). The Cape mole-rat breeds seasonally, however there is no data on gonadal steroid hormone profiles for wild captured mole-rats both during the breeding season (August) and out of the breeding season (February). The aim of this study was to determine whether there is a seasonal difference in urinary steroid concentrations.

Material and methods

Gonadal steroid concentrations were measured from urine samples using Coat-a-count kits (Diagnostic Products Corporation, Los Angeles, California, USA). All hormone assays have been validated for use in mole-rats (Bennett & Jarvis 1988a, Bennett *et al*. 1994.)

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Urine concentrations of steroid hormones were standardized by measuring the creatinine content in each sample (Bonney *et al*. 1982).

Refer to Chapter 2 for detailed experimental procedures.

Statistical analysis

Due to the small sample sizes of some of the groups, non-parametric Mann-Whitney U tests were performed to determine whether there were any statistical differences between the experimental groups.

Results

- Testosterone

Urinary testosterone concentrations in male Cape mole-rats were slightly higher during the breeding season (winter) compared to out of the breeding season (summer), but the difference was not significant (Mann Whitney U-test: n_1=4, n_2=5, U=7, Z=0.735, p=0.462) (Figure 3.6).

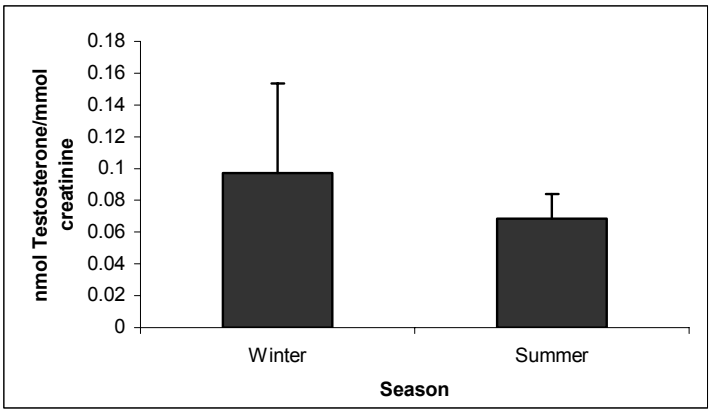

Figure 3.6: Urinary testosterone concentrations (nmol/mmol creatinine) measured in and out of the breeding season in the male Cape mole-rat.

- Oestrogen

The urinary oestrogen concentrations in Cape mole-rat females were higher during the breeding season (winter) than out of the breeding season (summer). This difference however, was not statistically significant (Mann Whitney U test: n_1=14, n_2=12, U=50, Z=1.75, p=0.08) (Figure 3.7).

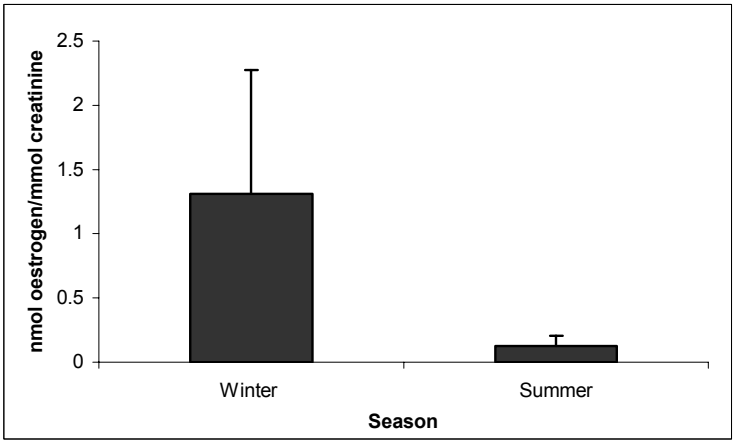

Figure 3.7: Urinary oestrogen concentrations (nmol/mmol creatinine) measured in and out of the breeding season in the female Cape mole-rat.

- Progesterone

The female Cape mole-rats have a higher concentration of progesterone during the breeding season (winter) compared to out of the breeding season (summer), however, this difference was not significant (Mann Whitney U-test: n_1=14, n_2=12, U=51, Z=1.69, p=0.089) (Figure 3.8).

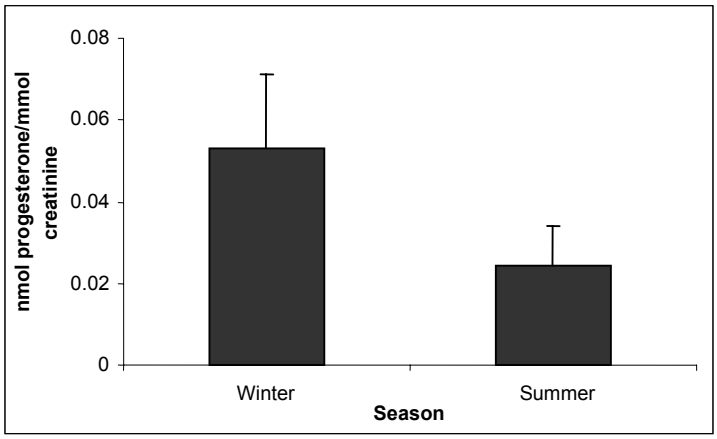

Figure 3.8: Urinary progesterone concentrations (nmol/mmol creatinine) measured in and out of the breeding season in the female Cape mole-rat.

Discussion

Seasonal breeding is common among solitary subterranean rodents (Andersen & MacMahon 1981, Bennett & Jarvis 1988a, Hansen 1960, Herbst *et al*. 2004, Jarvis 1969, Rado *et al*. 1992, Vaughan 1962). As a result, young are produced at a time of the year most favourable for their survival. Typically, the reproductive state of seasonally breeding animals is reflected in the physiology of their reproductive systems (Clarke 1981). Reproductive function is disrupted during periods when environmental conditions are unfavourable

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for breeding. In general, ovulation is suspended in females, whereas sperm production and quality is affected in males (Gerlach & Aurich 2000). GnRH plays a key role in reproductive function, and gonadal steroids both stimulate and inhibit its release. Negative feedback from gonadal hormones mainly reduces pulse frequency of GnRH release which imposes an inhibitory effect downstream on the rest of the reproductive system. Gonadal hormone concentrations that, outside the breeding season, inhibit GnRH secretion have no effect or are stimulatory during the breeding season (Karsch *et al*. 1984, 1993, Lincoln 1984). Changes in gonadal steroid feedback are at least partly responsible for seasonal changes observed in GnRH release (Ebling *et al*. 1994).

In wild captured male Cape mole-rats, urinary testosterone concentrations were not significantly higher during the breeding season (winter) than in the non-breeding season (summer). This contradicts the findings of Bennett and Jarvis (1988a), who found an increase in urinary testosterone concentrations at the onset of the breeding season. The non-significant result may be attributed to the small number of male mole-rats captured, and the samples in the two studies are likely to be collected at different times during the breeding season. Male Namaqua dune mole-rats (*Bathyergus janetta*) and Cape dune mole-rats (*Bathyergus suillus*), exhibit heightened testosterone concentrations at the onset of the breeding season (Herbst *et al*. 2004, Hart & Bennett 2006). Both of these species show two distinct peaks in testosterone levels during the breeding season, one at the beginning of the breeding period and one towards the end. The difference in testosterone concentrations between breeding and non-breeding seasons was significant in the Cape dune mole-rat but not so in the Namaqua dune mole-rat (Herbst *et al*. 2004, Hart & Bennett 2006).

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Female Cape mole-rats displayed higher circulating basal oestrogen and progesterone concentrations during the breeding season, but due to large intra-specific variation this difference was not significant for either of the steroids. High intra-specific variation could stem from the inclusion of samples taken from females that have and have not mated. Since these animals are induced ovulations, coitus will alter hormone profiles. Sharp elevations in oestrogen and progesterone levels were recorded for the Cape dune mole-rat and the Namaqua dune mole-rat (Herbst *et al*. 2004, Hart & Bennett 2006). In the Cape dune mole-rat, only the progesterone concentration was significantly higher during the breeding season than out of the breeding period (Hart & Bennett 2006), which may be as a result of the large numbers of pregnant females. The hormonal concentrations of the Namaqua dune mole-rat were not significantly different in and out of the breeding season (Herbst *et al*. 2004), which corresponds with the findings of the current study.

It is possible that the Cape mole-rat is a seasonal breeder, the lack of distinct differences in gonadal steroid concentrations in and out of the breeding season may be the result of sampling methods. Animals were captured in August, towards the end of the breeding season. Bennett and Jarvis (1988a) showed the highest testosterone levels to occur in June and July at the onset of the breeding season therefore animals sampled in the present study might well have been captured when hormonal levels were already in decline after the initial part of the breeding season. The onset of winter rainfall in the Cape is around late May hence if these animals show a similar trend to the Namaqua dune mole-rat, hormonal concentrations would have started to increase soon after the first rainfall. However, the Cape mole-rat occurs sympatric with the Cape dune mole-rat, which shows a peak in hormonal levels during August.

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At present, it is uncertain which environmental factor(s) are responsible for the seasonal control of reproduction in subterranean mole-rats. Photoperiod is the primary environmental cue used by aboveground animals to entrain their breeding patterns (Karsch *et al*. 1984). Mole-rats rarely venture above ground and therefore are not in frequent contact with photoperiodic cues. However, despite a regressed visual system, most mole-rat species are still able to entrain their daily activity rhythms to a circadian light cycle (Hart *et al.* 2004, Oosthuizen *et al.* 2003, Schöttner *et al.* 2006, Vasicek *et al*. 2005).

Temperatures in the burrow system are reasonably buffered, thus seasonal temperature changes do not fluctuate as much as aboveground (Bennett & Faulkes 2000). Yet, foraging tunnels close to the surface may relay seasonal temperature changes to mole-rats.

Rainfall influences both growth of plants, which affects food availability, and the moisture content of the soil that enables animals to excavate new tunnels (Dennis & Marsh 1997, Herbst *et al.* 2004). In addition, gonadal steroid concentrations appear to be well correlated with the rainfall profile. Breeding in the Namaqua dune mole-rat appears to be restricted to the period after the first winter rainfall (Herbst *et al*. 2004). Considering that in this study, seasonal gonadal hormone concentrations were not found to be significantly different in the Cape mole-rat, it is likely that, physiologically, this rodent mole has the capability to reproduce at any time of the year, but is restricted by unfavourable environmental conditions.

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Chapter 3b

Natal mole-rat (*Cryptomys hottentotus natalensis*)

Species in the family Bathyergidae display a broad spectrum of social organisation and mating strategies, ranging from strictly solitary to highly social (Bennett *et al*. 1999). While all solitary species currently studied appear to breed seasonally, this appears to be the exception rather than the rule among the social representatives of this family (Bennett *et al*. 1991). Reproduction in the social mole-rat species is monopolised by a single reproductive female and between one and three male consorts, with all other natal members in the colony being reproductively suppressed by the breeders (Bennett *et al*. 1993, 1994, 1996, 1997). The extent of the suppression varies along with different life history strategies displayed by the different species.

The reproductive suppression of subordinate animals in eusocial mole-rat species is much more stringent than that observed in less social species. Subordinate naked mole-rat males and females are physiologically suppressed from reproducing. Females exhibit low levels of reproductive hormones and fail to undergo follicular development and subsequent ovulation (Faulkes *et al*. 1990a). In the males, hormonal levels are also inhibited (Faulkes *et al*. 1991), and although testosterone levels are sufficient to support spermatogenesis (Faulkes *et al.* 1994), the number of spermatozoa produced is significantly less than that of the reproductive animals and most are non-motile (Faulkes *et al*. 1994, Faulkes & Abbott 1997). Female subordinates of the Damaraland and highveld mole-rat are also physiologically suppressed, whereas the subordinate males exhibit reproductive hormonal levels comparable to those of the breeding males (Bennett *et al*. 1993, Van der Walt *et al*. 2001). In transiently social species

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such as the common mole-rat and Mashona mole-rat, subordinate animals are not physiologically suppressed from reproducing. Dominant and subordinate animals of both sexes exhibit similar steroid hormone concentrations (Herbst & Bennett 2001, Bennett *et al.* 1997, Spinks *et al*. 1997).

Little is known about the reproductive biology of the Natal mole-rat, and until now it has been unknown whether this species is breeding seasonally or continuous throughout the year. Since the Natal mole-rat is phylogenetically closely related to the common mole-rat and the highveld mole-rat, both of which breed seasonally (Faulkes et *al.* 1997), it was predicted to be a seasonal breeder. Urinary gonadal steroids concentrations were measured in the Natal mole-rats collected over an entire calendar year to establish whether there is any seasonal difference in the circulating urinary steroid concentrations.

Material and methods

Steroid concentrations were measured using Coat-a-count Radioimmunoassay kits (Diagnostic Products Corporation, Los Angeles, California, USA).

Urinary oestrogen and progesterone concentrations were monitored in the female, while testosterone concentrations were measured from blood plasma samples in males. Assays were validated for use in this species and creatinine levels were determined for urine samples.

Refer to Chapter 2 for complete experimental procedures.

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Statistical analysis

As a result of small sample sizes of some of the groups, non-parametric Mann Whitney U-tests were performed on the different experimental groups to discover any significant differences between them.

Results

- Testosterone

The plasma testosterone concentrations showed no seasonal difference in either the reproductive (Mann Whitney U-test: n_1=17, n_2=16, U=128, Z=-0.29, p=0.77) or the non-reproductive (Mann Whitney U-test: n_1=27, n_2=35, U=405, Z=-0.93, p=0.352) Natal mole-rat males (Figure 3.9).

The plasma testosterone level was not different between reproductive and non-reproductive males during either the summer (Mann Whitney U-test: n_1=17, n_2=27, U=192, Z=0.90, p=0.37) or the winter (Mann Whitney U-test: n_1=16, n_2=35, U=235, Z=0.91, p=0.36) (Figure 3.9).

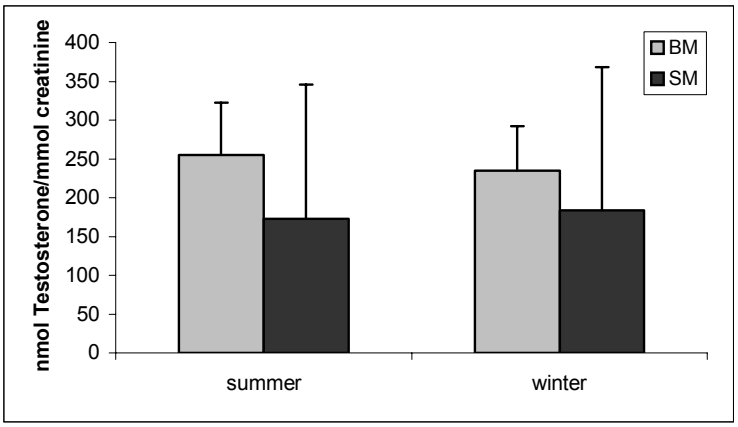

Figure 3.9: Plasma testosterone concentrations of dominant, reproductive and subordinate, non-reproductive Natal mole-rat males during the summer and winter. BM = Breeding male, SM = subordinate male

- Oestrogen

No significant difference was found in the urinary oestrogen concentrations in summer and winter for either the dominant, reproductive (n_1=7, n_2=9, U=25, Z=0.688, p=0.49) or subordinate, non-reproductive (n_1=29, n_2=25, U=270, Z=1.6, p=0.11) female Natal mole-rats (Figure 3.10).

In the summer, the non-reproductive females had a higher oestrogen concentration than the reproductive animals, although this was not significant (n_1=7, n_2=29, U=89, Z=-0.49, p=0.617). In contrast, the oestrogen concentration was higher in reproductive females during the winter, however not significantly so (n_1=9, n_2=25, U=105, Z=-0.29, p=0.769) (Figure 3.10).

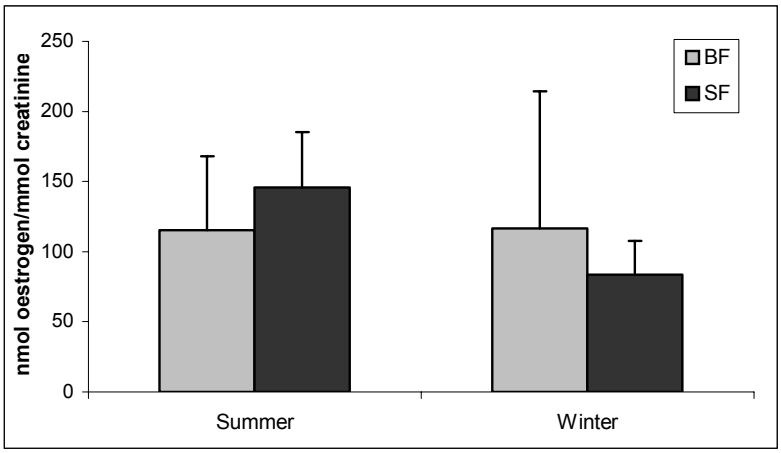

Figure 3.10: Urinary oestrogen concentrations (nmol/mmol creatinine) of dominant, reproductive and subordinate, non-reproductive female Natal mole-rats during summer and winter.

- Progesterone

There are no seasonal differences in progesterone concentrations in either the reproductive (Mann Whitney U-test: n_1=7, n_2=9, u=21, z=-1.11, p=0.266) or non-reproductive (Mann Whitney U-test: n_1=28, n_2=25, U=309, Z=-0.73, p=0.465) Natal mole-rat females, although the range of the concentrations was much broader during the winter (figure 3.11).

Reproductive females have significantly higher progesterone concentrations than the non-reproductive females during both the summer (Mann Whitney U-test: n_1=7, n_2=28, U=37, Z=2.52, p=0.011) and winter (Mann Whitney U-test: n_1=9, n_2=25, U=23, Z=3.49, p=0.0004) (figure 3.11).

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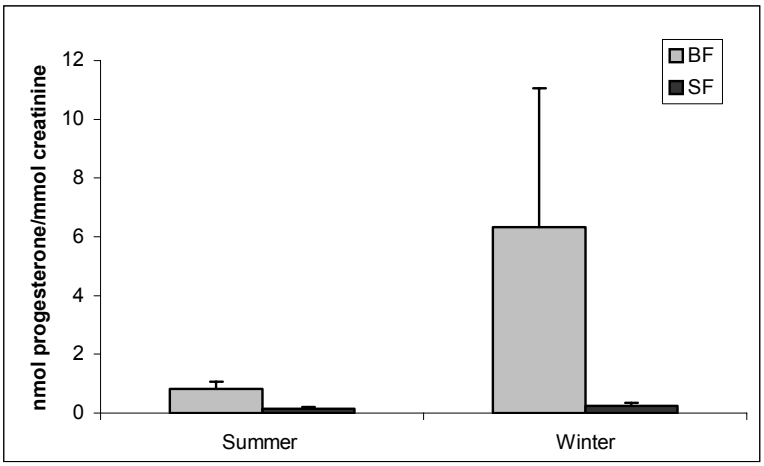

Figure 3.11: Urinary progesterone concentrations (nmol/mmol creatinine) during the summer and winter periods for reproductive and non-reproductive female Natal mole-rats.

Discussion

Breeding season

The majority of social mole-rat species do not breed seasonally (Bennett *et al*. 1999). Currently only two species of social mole-rat have been found to breed seasonally, the common mole-rat (*Cryptomys hottentotus hottentotus*) and the highveld mole-rat (*Cryptomys hottentotus pretoriae*) (Spinks *et al*. 1997, 1999, Janse v Rensburg *et al*. 2002). Despite being phylogenetically closely related to these two species, the Natal mole-rat (*Cryptomys hottentotus natalensis*) (Bennett & Faulkes 2000) does not appear to breed seasonally. No significant seasonal differences were found in any of the urinary oestrogen or plasma testosterone concentrations investigated in this study. The endocrine findings complement the post mortem findings of corpora lutea in the ovaries and embryos in the uterine horns of reproductive females during all months that mole-rats were captured (M.Oosthuizen, *pers.obs*.).

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In the seasonally breeding social highveld mole-rat, oestrogen and progesterone plasma concentrations exhibited seasonal periodicity. In female reproductive mole-rats, both oestrogen and progesterone concentrations were elevated at the onset of winter. In contrast, testosterone concentrations in male highveld mole-rat males showed no seasonality (Janse van Rensburg *et al*. 2002).

Periodicity in environmental factors plays a major role in the seasonal regulation of reproduction (Bronson & Heideman 1994). Both the common and highveld mole-rats inhabit regions with very distinct seasonal variations in most environmental parameters. The regions occupied by the Natal mole-rat also display clear seasonal differences in temperature and rainfall, however, the yearly rainfall for the region in KwaZulu Natal where the animals for this study was captured, is approximately double than that of the Cape Town area where the common mole-rat occurs, and one and a half times that of the Pretoria area that the highveld mole-rat inhabits. For most of the year, food is readily available and the soil is workable such that dispersal is easily achievable. Therefore, it seems likely that rainfall is the determining factor for seasonal breeding in social mole-rats. In the Natal mole-rat, rainfall is not a limiting factor and reproduction is possible throughout the year. This species can therefore employ an opportunistic breeding strategy and make use of favourable conditions as they arise throughout the year.

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Social status

In social cooperative breeding communities, unequal distribution of reproductive success within such a group is not uncommon (Keller & Reeve 1994). In these communities, dominant animals may inhibit reproduction of subordinate animals with behavioural interactions (Abbott 1987, Abbott *et al*. 1988). In extreme cases, reproduction can be completely suppressed by a physiological block to ovulation (Bennett & Faulkes 2000).

In social mole-rat species, reproduction is highly skewed towards a single breeding pair, and subordinate reproduction is suppressed. The degree of suppression and the mechanism by which reproduction is controlled in subordinates, vary between species (Bennett & Faulkes 2000).

In the Natal mole-rat, there was no significant difference in the plasma testosterone concentrations of reproductive and non-reproductive males. This finding supports the notion that male non-reproductive mole-rats are not physiologically suppressed while in the confines of the natal colony. In the highveld mole-rat, a similar scenario was found in the males (Janse van Rensburg *et al*. 2002), and Damaraland mole-rat where reproductive and non-reproductive males also have comparable concentrations of circulating testosterone (Bennett 1994). Interestingly, in naked mole-rat males the non-breeding animals have significantly lower urinary testosterone levels than the breeding animals, implying that they are physiologically suppressed from reproduction (Faulkes *et al*. 1991). With the exception of the naked mole-rat (Faulkes *et al.* 1990b, Reeve *et al*. 1990, O'Riain *et al*. 1996), all evidence suggests incest avoidance and outbreeding in social bathyergid species (Bennett 1994, Burda 1995, Rickard & Bennett 1997, Spinks 1998). Since mole-rat colonies typically consist of the reproductive pair and several

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generations of their offspring (Jarvis *et al*. 1994), it appears that incest avoidance alone is sufficient to inhibit reproduction in subordinate male mole-rats of the genus *Cryptomys*.

Comparable concentrations of urinary oestrogen between reproductive and non-reproductive female Natal mole-rats imply that females are not physiologically suppressed and are capable of follicular development. On the contrary, oestrogen levels were extremely low in both subordinate highveld females (Janse van Rensburg *et al*. 2002), and subordinate naked mole-rat females (Faulkes *et al*. 1991), confirming a physiological block to reproduction at the level of the ovary. The naked mole-rat is not an obligatory outbreeder, necessitating the exercise of stringent reproductive control by the breeding queen (Faulkes *et al*. 1991), whereas the Natal, highveld and Damaraland mole-rats are all outbreeding species. The difference in reproductive suppression in the latter species may result from different ecological constraints placed upon them. Regular rainfall in the Natal midlands equates to frequent dispersal opportunities for the Natal mole-rat, whereas strictly seasonal rainfall patterns in the habitats of the highveld and Damaraland mole-rats inhibit dispersal and opportunities for independent breeding are less frequent (Jarvis *et al*. 1994).

Progesterone concentrations were substantially higher in reproductive female Natal mole-rats when compared to the non-reproductive females. If one takes into account that the Natal mole-rat is an induced ovulator (Jackson & Bennett 2005) the finding is not surprising, since for ovulation to occur and subsequent corpus luteum development to take place coitus must take place. The lack of opportunities for coitus in subordinate female Natal mole-rats due to incest avoidance substantiates this finding. Progesterone is primarily secreted by the corpus luteum (Frandson & Spurgeon 1992), therefore since follicular

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development does not reach the stage of the corpus luteum in subordinate females, progesterone concentrations should remain low. Similar results have been found in both the Damaraland mole-rat and the naked mole-rat (Clarke & Faulkes 1997, Clarke *et al.* 2000, Faulkes *et al*. 1991). The breeding female exhibits the highest concentration of progesterone in the colony and the subordinate females typically have very low progesterone levels (Clarke & Faulkes 1997, Clarke *et al.* 2000). In both of these species, progesterone levels increased significantly following the removal of the breeding female. This implies that the queen has a suppressive influence over the subordinate, non-reproductive females.

Thus, it appears that reproduction in both the male and female subordinate Natal mole-rats is not through physiological suppression but rather incest avoidance. High overall rainfall ensures constant availability of food sources and softer soils provide dispersal opportunities for much of the year and as a consequence reproductive control of the subordinate, non-reproductive animals does not need to be as stringent.

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CHAPTER 4:

LUTEINISING HORMONE RESPONSES TO SINGLE DOSES OF EXOGENOUS GnRH IN THE SOLITARY CAPE MOLE-RAT (*GEORYCHUS CAPENSIS*) AND THE SOCIAL NATAL MOLE-RAT (*CRYPTOMYS HOTTENTOTUS NATALENSIS*).

Abstract

In seasonally breeding species, reproduction is usually confined to a specific period of the year. During the non-breeding season, fertility may be maintained at a reduced level or there may be a near complete cessation of reproductive function. In this chapter, the effect of breeding season on the function of the pituitary was considered in the Cape mole-rat. Basal LH concentrations were found to be significantly higher during the breeding season than out of the breeding season. In response to an exogenous GnRH injection, LH concentrations were significantly different from the basal concentrations both in and out of the breeding season. However, no difference was detected in the magnitude of the LH response either in or out of the breeding season. This supports the notion that the Cape mole-rat is an opportunistic breeder capable of breeding throughout the year should opportunity arise.

In the Natal mole-rat, no seasonal differences were seen in the LH response to an exogenous GnRH challenge, although interestingly, basal LH concentrations were significantly higher during the winter compared to the summer.

In many cooperatively breeding species, reproduction is skewed towards a few individuals while others are suppressed from reproducing. The mechanism whereby reproduction is suppressed differs among species by being either behavioural or physiological. In the Natal mole-rat, the pituitary response to a GnRH challenge was significant in both dominant and subordinate animals of either sex, implying that reproduction in the subordinate Natal mole-rats is inhibited by incest avoidance rather than a direct physiological block at the level of the pituitary and hypothalamus. This

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scenario compares well with other mole-rat species occurring in similar habitats.

Introduction

Luteinising hormone (LH) is released from gonadotropic neurons in the anterior pituitary gland in response to gonadotropin releasing hormone (GnRH) stimulation. Gonadotroph cell secretion is dependent on the frequency and amplitude of GnRH pulses, therefore the plasma LH concentration shows peaks corresponding to the release of GnRH (Counis *et al*. 2005).

LH released into the systemic circulation travels to the gonads where it directs gamete production as well as gonadal hormone production (testosterone in the male and oestrogen and progesterone in the female). Depending on the phase of the ovarian cycle, ovarian oestrogen exerts either a positive or a negative feedback control over LH secretion. During the follicular phase, increasing levels of circulating oestradiol triggers a massive release of GnRH that evokes the ovulation-inducing pituitary LH surge (Herbison, *in press*). Both the positive and negative feedback of oestrogen on GnRH secretion are ERα dependent (Lindzey *et al*. 2006). GnRH neurons only express ERβ, therefore oestrogen positive feedback is likely to use an indirect pathway involving the modulation of ERα-expression neurons that project to GnRH neurons. During the negative feedback of oestroen, both Erα and Erβ are involved in inhibiting LH levels (Dorling *et al*. 2003). Since GnRH neurons express Erβ, oestrogen-mediated suppression of GnRH secretion can take place through either direct or indirect mechanisms (Roy *et al*. 1999). A change in GnRH release from the median eminence inevitably results in a

corresponding alteration of LH secretion from the pituitary gonadotrophes (Levine 1997).

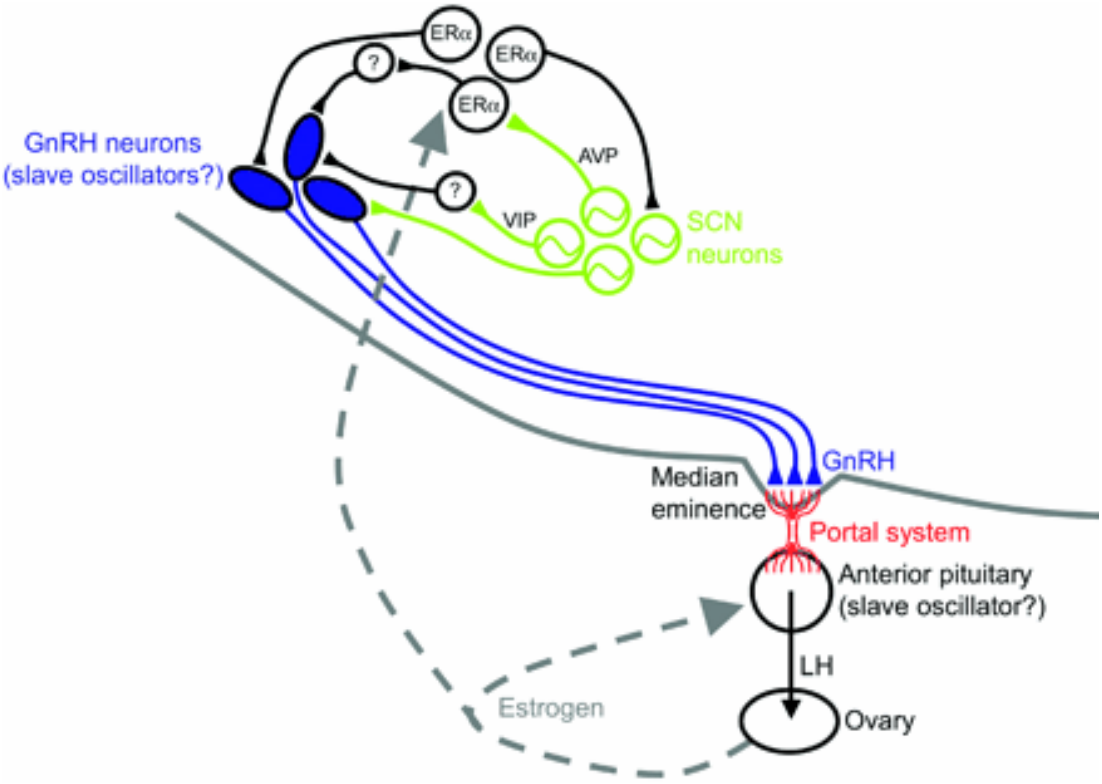

Fig. 4.1: Schematic representation of the reproductive pathway from the hypothalamus to the gonads and feedback system.

Chapter 4a

The Cape mole-rat (*Georychus capensis*)

Solitary mole-rats tend to inhabit mesic areas with relatively seasonal and predictable rainfall (Honeycutt *et al*. 1991). The Cape mole-rat occurs in

regions with a distinct winter rainfall pattern and marked seasonal differences in soil moisture and food availability. Breeding patterns restricted to a specific part of the year ensures that young are born at a time when environmental conditions are most favourable for their survival (Gerlach & Aurich 2000). During the reproductively inactive period, reproductive function and behaviour is down-regulated (Bennett & Jarvis 1988a). This, presumably, is the result of marked seasonal changes in the responsiveness to the negative feedback effects of oestradiol on the pulsatile secretion of GnRH and LH (Karsch *et al*. 1993).

The Cape mole-rat has been shown to breed during the winter or wet season (Bennett *et al*. 1988a), and gonadal steroid hormones are higher during the breeding season (See previous chapter). Following the argument that hypothalamic sensitivity towards gonadal hormones is altered during the non-breeding season, it would be predicted that LH levels should be inhibited outside of the breeding season.

Therefore, the objective of this study was firstly to determine whether basal levels of LH differ in and out of the breeding season, and secondly, an exogenous GnRH challenge was used to investigate whether the production of LH in the pituitary changes according to the season.

Methods

Blood sampling

All mole-rats were initially subjected to a saline injection prior to and after which a blood sample was taken. The saline injection acted as a control to ensure that the injection itself did not affect the LH concentration. A week

later, mole-rats were treated in a similar fashion, but received an injection of GnRH in stead of saline.

Refer to Chapter 2 for methodology of blood sampling and LH bioassays.

Statistical analyses

Non-parametric statistics were used to analyse the data since a test for homoscedacity revealed the data not to be normally distributed and small sample sizes of some of the groups. Mann-Whitney U-tests were used to determine inter group differences, and intra group differences were assessed with Wilcoxon matched pairs tests. Statistical significance was maintained at 95%.

Results

The basal LH concentrations were significantly higher during the breeding season in both the males (2.6±0.7 vs 0.8±0.2 mIU.ml) (Mann-Whitney U-test, n_1=6, n_2=4, U=0, p=0.011) (Figure 4.2), and the females (2.0±0.3 vs 0.7±0.1 mIU.ml) (Mann-Whitney U-test, n_1=25, n_2=17, U=69, p=0.001) (Figure 4.3).

In the male Cape mole-rat, there was an increase in the plasma LH concentration in response to a single GnRH challenge both in the breeding season (2.8±0.7 vs 16.4±3.0 mIU.ml) and out of the breeding season (4.2±0.8 vs 16.9±2.6 mIU.ml). This was only statistically significant in the breeding season (Wilcoxon matched pairs test, n=6, T=0, Z=2.201 p=0.027) (Figure 4.2).

The female Cape mole-rat showed significantly different levels of LH in response to a single GnRH challenge both in (0.9±0.4 vs 14.1±1.3 mIU.ml) (Wilcoxon matched pairs test, n=25, Z=4.345 p>0.0001) and out (1.9±0.2 vs 13.2±1.5 mIU.ml) (Wilcoxon matched pairs test, n=17, Z=3.574, p>0.0001) of the breeding seasons (Figure 4.3).

There was no difference in the mean magnitude of increase in concentration of LH in response to the GnRH challenge in and out of the breeding season in either the males (16.9±2.6 vs 16.4±3.0 mIU.ml) (Mann-Whitney U-test, n_1=6, n_2=4, U=11, p=0.831) (Figure 4.2) or the females (13.2±1.5 vs 14.1±1.3 mIU.ml) (Mann-Whitney U-test, n_1=25, n_2=17, U=208, p=0.908) (Figure 4.3).

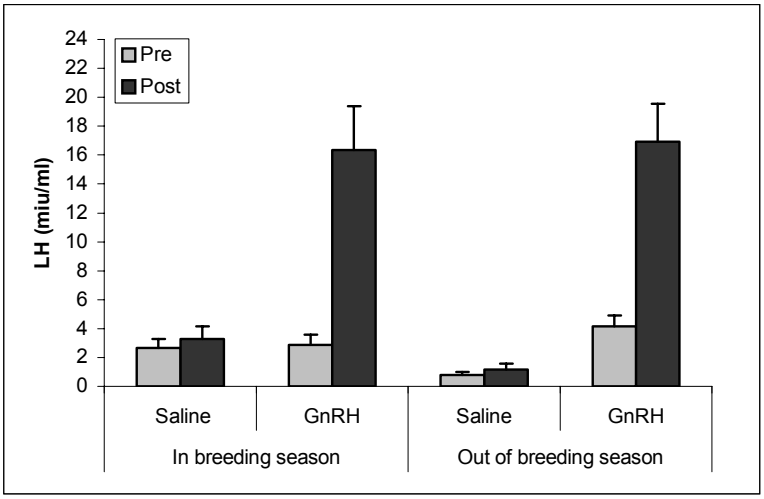

Figure 4.2: Mean basal plasma LH (Pre-GnRH) and the pituitary response (Post-GnRH) to a single 2.0μg exogenous GnRH injection, or a single injection of physiological saline control for male Cape mole-rat in and out of the breeding season.

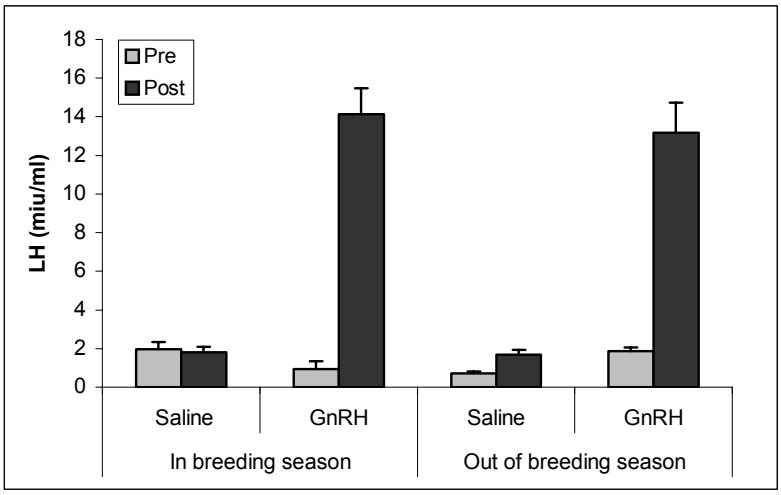

Figure 4.3: Mean basal plasma LH (Pre-GnRH) and the pituitary response (Post-GnRH) to a single 2.0μg exogenous GnRH injection, or a single injection of physiological saline control for female Cape mole-rat in and out of the breeding season.

Discussion

During the non-breeding season of many seasonally breeding mammals there is characteristically a down-regulation of gonadotropin releasing hormone production and/or secretion and the associated hormones necessary for reproductive behaviour (Gerlach & Aurich, 2000, Lincoln & Short 1980). Along with the reduction in GnRH secretion, the hypothalamo-pituitary axis becomes more sensitive to negative feedback control by the gonadal hormones, which results in a reduction in basal LH concentrations and reduced amounts of LH manufactured and subsequently stored in the pituitary (Gerlach & Aurich 2000). As a consequence, there is a reduction in follicular maturation in females and spermatogenesis in males.

Basal LH concentrations are significantly higher during the breeding period in both the male and female Cape mole-rats. A similar scenario has been reported in the Dune mole-rat (Hart & Bennett, 2006). Other seasonally breeding species such as hamsters also display a reduced basal LH concentration during the non-breeding season (Desjardins *et al*. 1971, Tsutsui *et al.* 1989).

In contrast, seasonality does not appear to affect testicular or ovarian activity in the social species of seasonally breeding mole-rats previously investigated (Spinks *et al*. 1997, Janse van Rensburg *et al*. 2002, Van der Walt *et al*. 2001), they show similar circulating plasma LH concentrations in and out of the breeding season. It has been hypothesized that it is essential to retain gonadal function outside of the breeding period in these species in order to facilitate pair bonding during times of dispersal (Spinks *et al*. 1997). The non-reproductive period is the most favourable time for dispersal consequently maintenance of reproductive activity may promote inter-sexual recognition and pair bonding, or assist in successful integration into foreign colonies (Spinks *et al*. 1997). Since solitary mole-rats are highly xenophobic towards conspecifics outside of the breeding season, and only tolerate other animals in their tunnels during a short period during the breeding season, there is no need to retain functional gonads all year round.

Female Cape mole-rats exhibited a significant release in plasma LH from the pituitary in response to an exogenous GnRH administration both in and out of the breeding season. In the males, the pituitary response to exogenous GnRH was only significant in the breeding season. Out of the breeding season, the difference in the basal and GnRH induced levels of LH was not statistically different, but this may be due to a small sample size of animals used in the

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breeding season group. It therefore appears that there is no visible seasonal difference in the LH content of the pituitary in the Cape mole-rat.

The release of pituitary LH in response to a GnRH challenge was significant in both male and female Natal mole-rats. Other solitary and social mole-rats also display comparable basal LH levels and similar LH responses when challenged with exogenous GnRH both in and out of the breeding season (Hart & Bennett 2006, Spinks *et al.* 2000, Van der Walt *et al*. 2001). Thus, it appears that none of the mole-rat species, whether seasonally or aseasonally breeding, exhibit a significant inhibition of reproductive activity at the level of the pituitary during the non-reproducing part of the year. Therefore, to date none of the mole-rat species exhibits any significant inhibition of reproductive activity, hence it can be inferred that there are no inherent physiological restrictions to prevent breeding at specific times of the year, but environmental conditions seem to be the limiting factor. Since the Cape mole-rat is a solitary species, the formation of pair bonds is not critical when individuals disperse therefore no lasting pair bonds are ever formed. The fact that the pituitary retains its function throughout the year may thus imply an opportunistic type of breeding system. Unusually aseasonal rainfall periods might allow for reproductive opportunities outside of the normal breeding season. This would favour a state of constant physiological readiness in both males and females to maximise fitness. The observation in this study that the pituitary retains its function throughout the year may represent an adaptation to maximise reproduction for an opportunistic breeding system.

In the majority of seasonally breeding mammals, the annual change in photoperiod is used as a cue for inducing alternation in active and inactive periods in reproductive processes (Lofts 1970, Reiter & Follett 1980). Since mole-rats spend almost all of their time underground, they are not exposed to

photoperiodic changes, and thus cannot use light to synchronize their reproductive activity. Consequently other external signals such as temperature and rainfall could be important cues for heralding the onset of seasonal breeding. Although temperature fluctuations underground are dampened compared to seasonal ambient changes, there is still seasonal variation in the temperatures of the burrow (Bennett, Jarvis & Davies 1988).

Chapter 4b

The Natal mole-rat (*Cryptomys hottentotus natalensis*)

The Natal mole-rat is a social subterranean rodent characterized by an extreme reproductive skew. A single female and one or two males are responsible for reproduction, while the remaining members of the colony are reproductively quiescent.

Delayed dispersal resulting in natal philopatry is thought to result in subordinate animals gaining experience in an established colony, and increasing their indirect reproductive success until conditions are favourable for dispersal. Some animals remain in their natal colonies for their entire lifetime. The lifetime reproductive success of these subordinate animals is typically very low (Jarvis *et al*. 1994).

Several hypotheses have been proposed to explain the low reproductive success of subordinate individuals. Non-reproductive animals can be reproductively suppressed by dominant animals, either behaviourally (such as aggression and interrupting reproductive behaviour) or physiologically (females anovulatory, males reduced spermatogenesis). Alternatively,

subordinate animals may refrain from reproducing as a result of incest avoidance.

If subordinate animals are physiologically suppressed by the dominant animals, LH levels are predicted to be lower in the subordinates than in the dominant animals. If suppression is behavioural or driven by incest avoidance alone, comparable plasma LH concentrations between the two reproductive catagories could be expected.

The objective of this study is to establish whether there are any differences in the circulating LH concentrations and subsequent LH levels in response to an exogenous GnRH challenge between dominant and subordinate mole-rats of either sex. In addition, to investigate whether there are any seasonal changes in either the basal or GnRH challenged LH concentrations in any of the experimental groups.

Methods

Blood sampling

All mole-rats were initially subjected to a saline injection prior to and after which a blood sample was taken. This acted as a control to ensure that the injection itself did not affect the LH concentration. A week later, mole-rats were treated in a similar fashion, but received an injection of GnRH in stead of saline.

Refer to Chapter 2 for methodology of blood sampling and LH bioassays.

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Statistical analysis

Non-parametric statistics were used to analyse the data since a test for homoscedacity revealed the data not to be normally distributed and small sample sizes of some of the groups. Mann-Whitney U-tests were used to determine inter group differences, and intra group differences were assessed with Wilcoxon matched pairs tests. Statistical significance was maintained at 95%.

Results

Basal levels of circulating LH were significantly higher in winter (dry season) than in summer (wet season) in both the reproductive (2.9±0.5 vs 8.1±0.7mIU.ml)(Mann-Whitney U-test, n_1=14, n_2=10, U=7, p<0.001) and non-reproductive females (1.9±0.2 vs 7.5±0.5mIU.ml) (Mann-Whitney U-test, n_1=42, n_2=29, U=51, p<0.001). Likewise the basal LH concentrations were higher during the winter in the reproductive (2.4±0.2 vs 8.0±0.8 mIU.ml) (Mann-Whitney U-test, n_1=16, n_2=19, U=18, p<0.001) and non-reproductive males (2.1±0.3 vs 6.9±0.3 mIU.ml) (Mann-Whitney U-test, n_1=43, n_2=34, U=37, p<0.001).

The LH concentration in response to a single GnRH challenge is higher during the winter in both the males and females. In both the reproductive (summer/winter: 8.7±1.4 vs 13.0±1.2 mIU.ml)(Mann Whitney U test, n_1=14, n_2=10, U=32, p=0.026) and non-reproductive females (pre/post 7.7±1.1 vs 15.9±0.9 mIU.ml)(Mann-Whitney U-test, n_1=42, n_2= 29, U=128, p<0.001), this difference was significant, also in the subordinate males (14.9±3.5 vs

17.3±1.1 mIU.ml)(Mann-Whitney U-test, n_1=43, n_2=34, U=427, p=0.002). Although there was a difference in the reproductive males, this was not significant (13.7±1.4 vs 18.9±3.1 mIU.ml) (Mann-Whitney U-test, n_1=16, n_2=19, U=112, p=0.185).

However, the magnitude of the difference in LH concentration in each season was only significant in the subordinate males (Mann Whitney U-test, n_1=29, n_2= 39, U=390, p=0.029).

The mean basal LH concentration was not significantly different between reproductive and non-reproductive females in either the winter (8.1±0.7 vs 7.5±0.5 mIU.ml) (Mann-Whitney U-test, n_1=14, n_2=42, U=252, p=0.427) or the summer (2.9±0.5 vs 1.9±0.2 mIU.ml) (Mann-Whitney U-test, n_1=10, n_2=29, U=88, p=0.066), neither was there a difference between the LH levels of reproductive (8.0±0.8 vs 6.9±0.3 mIU.ml) (Mann-Whitney U-test, n_1=16, n_2=43, U=264, p=0.173) and non-reproductive males (2.4±0.2 vs 2.1±0.3 mIU.ml) (Mann-Whitney U-test, n_1=19, n_2=34, U=251, p=0.182).

In both the reproductive and non-reproductive females there were a significant difference in LH concentrations in response to a single GnRH challenge during the summer and winter. Similarly, there were also significant differences in the plasma LH levels of the reproductive or non-reproductive males (Table 4.1).

Group	n	T	Z	p
Reproductive females summer	10	0.00	2.803060	0.005
Reproductive Females winter	14	3.000000	3.107436	0.002
Non-reproductive Females summer	29	3.000000	4.638177	<0.001
Non-reproductive Females winter	42	19.00000	5.407835	<0.001
Reproductive Males summer	19	0.00	3.823007	<0.001
Reproductive Males winter	16	1.000000	3.464488	<0.001
Non-reproductive Males summer	34	0.00	5.086213	<0.001
Non-reproductive Males winter	43	5.000000	5.651078	<0.001

Table 4.1: Results of a Wilcoxon matched pairs test for the comparison of LH concentrations in response to a single GnRH challenge during the summer or winter.

There were no significant differences between the LH levels of the reproductive and non-reproductive females in response to the GnRH challenge in either summer (8.7±1.4 vs 7.7±1.1 mIU.ml) (Mann-Whitney U-test, n_1=10, n_2=29, U=121, p=0.440) or winter (13.0±1.2 vs 15.9±0.9 mIU.ml) (Mann-Whitney U-test, n_1=14, n_2=42, U=219, p=0.116). Likewise, the reproductive and non-reproductive males did not have significantly different LH concentrations in the summer (13.7±1.4 vs 14.9±3.5 mIU.ml) (Mann-Whitney U-test, n_1=16, n_2=43, U=340, p=0.946) or winter (18.8±3.1 vs 17.3±1.1 mIU.ml) (Mann-Whitney U-test, n_1=19, n_2=34, U=263, p=0.266).

There was also no significant difference in the magnitude of the LH response in either the reproductive and non-reproductive females (5.3±0.1 vs 7.5±1.6 mIU.ml) (n_1=24, n_2=71, U=630, p=0.357), or the reproductive and non-reproductive males (11.1±1.7 vs 11.4±1.7 mIU.ml) (n_1=35, n_2=75, U=1304, p=0.974).

In response to a saline challenge, there was no significant difference between the pre- and post-treatment LH concentrations in either the reproductive females (5.8±1.3 vs 5.5±1.4 mIU.ml) or non-reproductive females (2.3±0.3 vs 2.6±0.7 mIU.ml). Likewise, there was no significant response in the LH concentrations in the reproductive males (1.7±0.6 vs 2.9±0.7 mIU.ml), or the non-reproductive males (2.1±0.5 vs 3.0±0.5 mIU.ml).

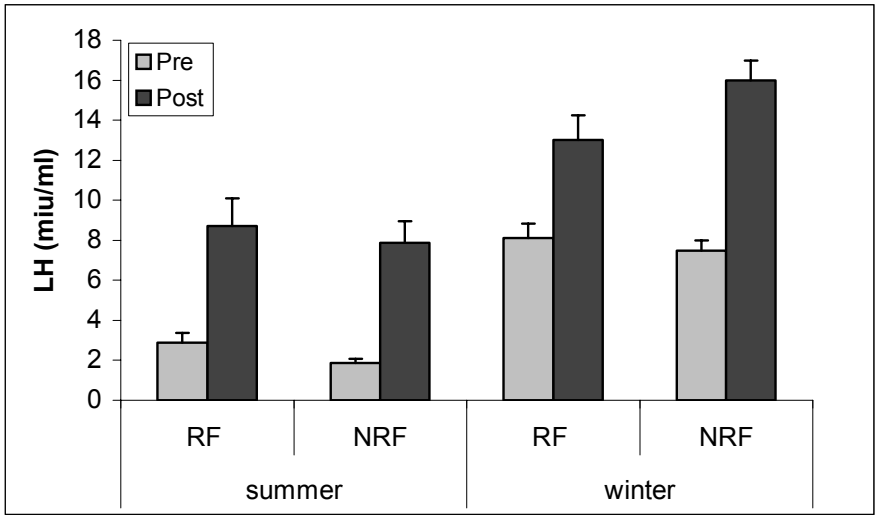

Figure 4.4: Mean basal plasma LH (Pre-GnRH) and the pituitary response (Post-GnRH) to a single 2.0μg GnRH injection, or a single injection of physiological saline for female Natal mole-rats during the summer or winter.

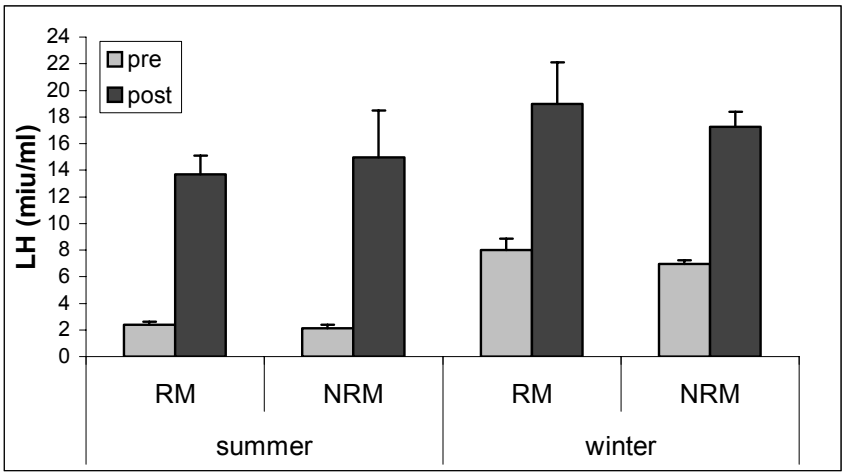

Figure 4.5: Mean basal plasma LH (Pre-GnRH) and the pituitary response (Post-GnRH) to a single 2.0μg GnRH injection, or a single injection of physiological saline for male Natal mole-rats during the summer or winter.

Discussion

Breeding season

Most social mole-rat species are not seasonally breeding. Only two species, the common mole-rat and the highveld mole-rat are known to breed seasonally, and both are phylogenetically closely related to the Natal mole-rat (Spinks *et al*. 1997, 1999, Janse van Rensburg *et al.* 2002, 2003). However, it appears that the Natal mole-rat differs from its sister species by not having a distinct breeding season. In both male and female Natal mole-rats, the response of the pituitary to a GnRH challenge was significant during both the winter and the summer periods. These results, supported by the endocrine results of the previous chapter and post mortem findings strongly suggest that the Natal mole-rat is not a seasonal breeder.

Despite the fact that the common mole-rat breeds seasonally, reproductive function is still maintained throughout the year. Spinks *et al.* (1997) suggested that normal circulating hormonal levels are required for inter-sexual recognition and pair bonding in dispersing animals and since the favourable time for dispersal is during the non-breeding season, reproductive function is retained during the year.

Interestingly, basal LH concentrations were significantly higher during the winter than the summer in both male and female Natal mole-rats. Basal LH levels may be optimal during the winter, and slightly reduced during the summer but not enough to prevent reproductive activity.

Social status

Social factors are important reproductive regulators in social animals. In social African mole-rat species a high reproductive skew is characteristic, the dominant breeding animals suppress reproduction in the subordinate individuals such that the reproductive success of subordinate animals is typically very low (See Bennett *et al.* 1999 for review). The mechanism and degree of suppression vary amongst the different mole-rat species however the type of suppression appears to be strongly correlated with the breeding strategy and the aridity of the habitat in which a particular species occur.

No physiological suppression is observed in the Natal mole-rat, reproductive and non-reproductive animals of both sexes exhibit comparable basal LH concentrations and there is no significant difference in the response to an exogenous GnRH challenge either. A similar scenario is present in the common mole-rat (*Cryptomys hottentotus hottentotus*) and the Mashona

mole-rat (*Cryptomys darlingi*) (Bennett *et al*. 1997, Spinks *et al.* 2000). In these species, reproductive inhibition is behaviourally induced rather than physiological, and anovulation of non-reproductive female animal is not associated with a reduced pituitary sensitivity to GnRH, but rather through a lack of opportunity for coitus.

Previous studies have indicated that subordinate females of the highveld mole-rat (*Cryptomys hottentotus pretoriae*) and the Damaraland mole-rat (*Cryptomys damarensis*) are both physiologically suppressed from reproducing. These mole-rats do show signs of follicular development in the ovaries but reduced pituitary activity results in failure to initiate the final stage of ovulation (Bennett 1994, Bennett *et al*. 1994, Van der Walt *et al*. 2001). In contrast, male subordinates of these two species are suppressed through an inhibition to incestuous mating. No significant difference was found in basal or GnRH challenged LH concentrations in reproductive and non-reproductive males (Bennett *et al*. 1993, Faulkes *et al.* 1994, Van der Walt *et al.* 2001).

Reproductive suppression in the eusocial naked mole-rats is the most extreme as both male and female subordinate colony members are physiologically suppressed from breeding (Faulkes *et al*. 1990a, 1991). Subordinate female animals are anovulatory and show no follicular development, and non-reproductive males have reduced levels of urinary testosterone and sperm quantity and motility is low (Faulkes *et al*. 1990b, 1991, 1994).

The various mechanisms of reproductive regulation can firstly be attributed to divergent life history tactics and mating strategies (Bennett *et al*. 1997, Spinks *et al.* 1998, 2000). Naked mole-rats are facultative inbreeders (Faulkes *et al.* 1990a) which provide a plausible explanation for the stringent reproductive

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suppression exerted by breeding animals upon subordinates. In the various species of *Cryptomys* however, all evidence points towards outbreeding and incest avoidance (Bennett 1994, Burda 1995, Bennett *et al*. 1997, Spinks *et al.* 2000, Van der Walt *et al*. 2001). Mole-rat colonies typically consist of family groups, thus close genetic relatedness would prohibit reproductive activity of the subordinate animals in a colony. When social and environmental conditions are favourable, subordinate animals may disperse and attempt to set up separate colonies.

Within the genus *Cryptomys*, aridity of the habitat appears to play a role in the stringency of reproductive suppression of the subordinate animals. Jarvis *et al.* (1994) proposed the aridity food distribution hypothesis (AFDH), to describe the subsequent costs and risks associated with foraging and dispersal in arid areas. The primary food sources of mole-rats are geophytes, roots and tubers which are encountered as they excavate their tunnels (Jarvis & Bennett 1991). In areas with regular, predictable rainfall, these plants are evenly distributed and readily obtainable. In arid areas these food sources are more clumped and further apart, and rainfall is sporadic which renders soil dry and hard for a considerable part of the year. Therefore, energetic restrictions on finding food and tooth wear are alleviated by increasing the colony size. In addition, in species inhabiting more arid areas where rainfall is less frequent, dispersal opportunities are limited, also indirectly results in an increasing colony size. Thus subordinate animals remain in their colony for extended periods of time, some never dispersing. An increased colony size increases pressure on reproductive animals to maintain their position in the colony, justifying a stricter control on reproduction of subordinate animals.

In contrast, species that occur in mesic areas with regular rainfall have frequent dispersal opportunities and food sources are available for much of

the year. The habitat of the Natal mole-rat corresponds with the latter example with ample dispersal opportunities and food. Non-reproductive animals of both sexes are not physiologically suppressed as a reduction in pituitary activity was not observed. Subordinate animals are rather inhibited from reproducing by behavioural interactions and an additional component of inbreeding avoidance.

CHAPTER 5:

NEUROANATOMY AND NEUROENDOCRINOLOGY OF THE GnRH SYSTEM OF CAPE MOLE-RATS AND THE NATAL MOLE-RATS

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Abstract

This study mapped the distribution and morphology of the GnRH neuronal systems of Cape and Natal mole-rats and the GnRH-immunoreactivity of the median eminence was quantified. A comparison was made between the winter and summer seasons in both species and also between dominant and subordinate animals in the Natal mole-rat species.

Although the Cape mole-rat is larger than the Natal mole-rat, it has a smaller number of GnRH neurons. No differences were found in the number or size of these neurons across season in either species or reproductive status in the Natal mole-rat. In both species, the size of GnRH-ir neurons was similar in the different seasons and no difference was detected according to reproductive status.

The GnRH neurons and fibres are loosely distributed along the septo-preoptico-infundibular pathway in both species. Dense clusters of fibres are visible in the area of the organum vasculosum of the lamina terminalis and the median eminence. The species differed with regard to the incidence of GnRH-ir neurons along the rostral-caudal axis of the brain. In the Cape mole-rat, almost 90% of the GnRH-ir perikarya are present in the medial septum or preoptic area, and only 10% in the mediobasal hypothalamus. In the Natal mole-rat, a much larger proportion (40%) of the total GnRH-ir neurons is located in the mediobasal hypothalamus with the remainder in the medial septum or preoptic area. In the Cape mole-rat females there is no difference in GnRH-immunoreactivity in the median eminence in and out of the breeding season. In the Natal mole-rat, dominant animals of both sexes had significantly less GnRH-immunoreactivity in the median eminence than the

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subordinate animals. This suggests that GnRH is retained in the median eminence in these subordinate, behaviourally suppressed animals.

Behaviourally, both of these species display regulated reproduction, breeding in the Cape mole-rat is restricted to a specific part of the year, while subordinate individuals of the Natal mole-rats are reproductively suppressed by dominant animals. However, neuroendocrinologically, this is only reflected in subordinate animals of the Natal mole-rat.

Introduction

Gonadotropin releasing hormone (GnRH) is a decapeptide that serves both as a hormone and a neurotransmitter in the mammalian brain. It is an essential part of the reproductive process since it is responsible for the release of luteinising hormone (LH) and follicle stimulating hormone (FSH) from the pituitary, and has been found to promote reproductive behaviour (Dellovade *et al*. 1998).

Most vertebrate species possess two or more forms of GnRH in separate cell populations. GnRH-1 was first characterised in mammals. Only two different forms of mammalian GnRH-1 have been reported; guinea-pig GnRH-1 differs by two amino acids from all other known mammalian forms (Jiminez-Liñan *et al.* 1997). Recently it has been found that the GnRH-1 gene sequence in highveld mole-rats shows similarities to that of the guinea-pig, but the translated peptide corresponds to the standard mammalian form (Kalamatianos *et al.* 2005).

GnRH-1 neurons concerned with reproductive regulation originate from the medial olfactory placode early in mammalian development, from where they migrate along the nervus terminalis to colonise the forebrain in and around the preoptic area (POA) and mediobasal hypothalamus (MBH) (Schwanzel-Fukuda & Pfaff 1989). These neurons project to the median eminence where the GnRH is stored in terminals before being released into the pituitary portal system. It is then transported to the anterior pituitary gland, where it acts upon surface receptors of gonadotropes to stimulate the production and secretion of LH and FSH.

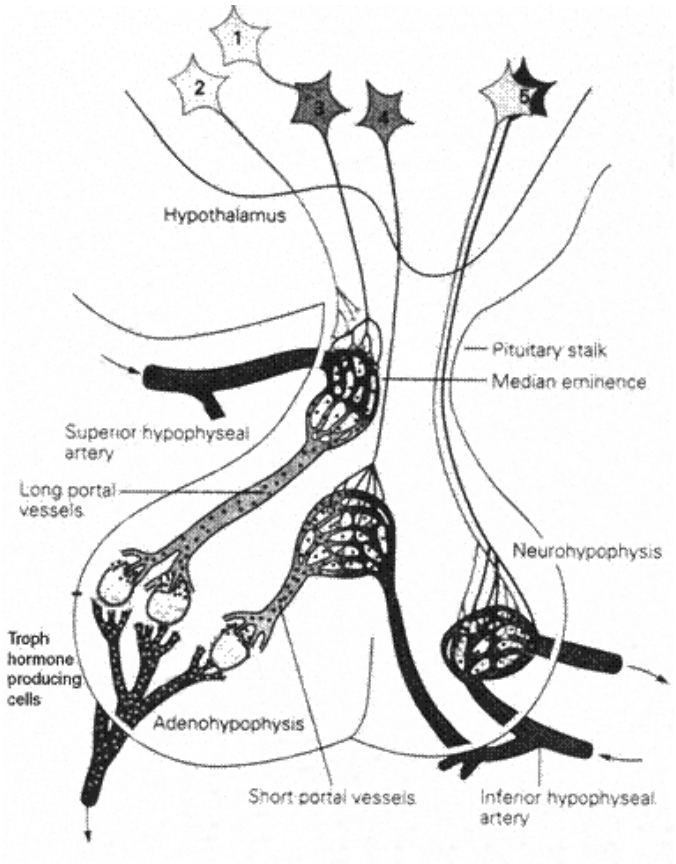

Figure 5.1: The pathway of the GnRH peptide from the perikarya to the anterior pituitary.

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In this study, we have characterised the morphology and distribution of the GnRH-1 system of the solitary Cape mole-rat and social Natal mole-rat. The research was designed to determine whether there are inter- and/or intra-species differences in the GnRH-1 systems of breeding and non-breeding members of these species.

Methods

Female Cape mole-rats (6 in the breeding season, 7 out of the breeding season) and male and female Natal mole-rats (7 dominant females, 6 subordinate females, 8 dominant males, 7 subordinate males) were used for this experiment.

Refer to chapter 2 for immunocytochemical procedure.

After the sections were mounted on gelatinised slides and cover slipped, they were analysed under a light microscope.

Analyses

The distribution of GnRH-ir processes was established and the total number of GnRH-ir cell bodies was counted in every sixth section from the confluence of the two hemispheres rostral to the posterior hypothalamus caudally. Only cell bodies with a visible nucleus were counted. The data were corrected for sampling rate of one in six sections. Image analysis software (ImageJ version 1.30, National Institutes of Health, USA) was used to determine the size of the perikaryon of 20 randomly distributed GnRH neurons for each animal.

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Additionally, the density of GnRH immunoreactivity in the median eminence was quantified according to the method of Robinson *et al*. (1997).

Results

- **Morphology and distribution of GnRH cell bodies**

Cape mole-rat

In the Cape mole-rat, GnRH-ir cell bodies are typically spindle-shaped with smooth contours (Plate 5.1 a, b). The majority of these cells are unipolar or bipolar, although a very small number of multipolar cells are present. Some cells without apparent processes are observed, most likely due to the plane of sectioning.

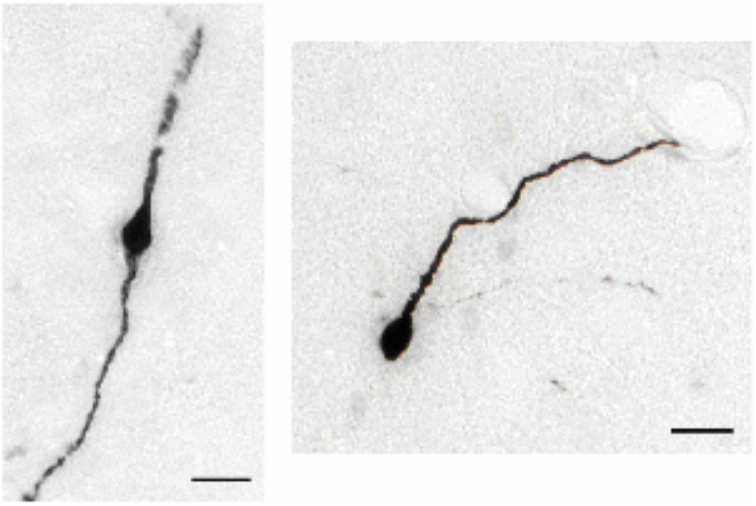

Plate 5.1(a). Bipolar cell body; **(b)**. Unipolar cell body. Scale bars = 20μm.

GnRH-ir perikarya are distributed loosely along the septo-preoptico-infundibular pathway. The majority of the GnRH-ir perikarya are located in the

medial septum (MS) and preoptic area (POA) (females BS, 87%, females OBS, 90%), and a smaller number of GnRH cell bodies are found further caudal in the mediobasal hypothalamus (MBH) (females BS, 13%, females OBS, 10%) (Figure 5.2). Very few GnRH-ir cell bodies are found around the suprachiasmatic nucleus (SCN).

No differences were observed in morphology or distribution of the GnRH cells between female Cape mole-rats in and out of the breeding season.

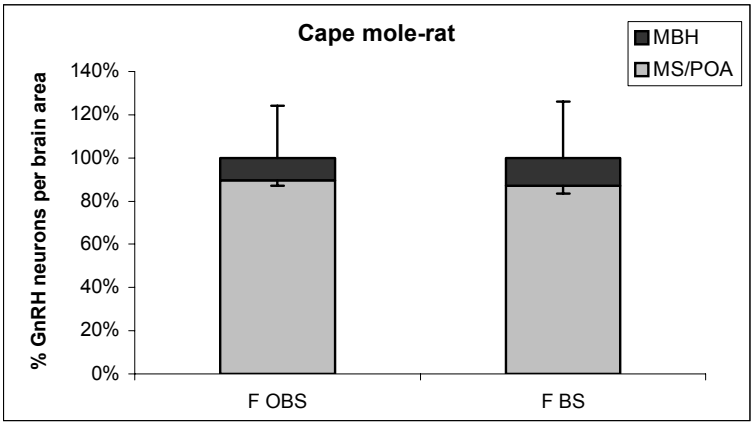

Figure 5.2: The relative distribution of GnRH-ir perikarya in the medial septum (MS)/ preoptic area (POA) and the mediobasal hypothalamus (MBH) in the brain of the Cape mole-rat. F OBS – female, out of breeding season, F BS – female breeding season.

Natal mole-rat

In terms of shape, contour and polarity, the GnRH-ir neurons in the Natal mole-rat resemble those of the Cape mole-rat. In the Natal mole-rat, the majority of GnRH-ir cell bodies are situated in the MS/POA (BF, 67%, SF, 68%, BM, 62%, SM, 60%). Nevertheless, a significant proportion of the total GnRH-ir cell bodies are present in the MBH (RF, 33%, SF, 32%, RM, 38%,

SM, 40%) (Figure 5.3). GnRH cell bodies are present around but not in the SCN. GnRH neurons are seen in the hypothalamus as far caudal as the level of the median eminence and pituitary stalk, but not in those structures.

As in the Cape-mole-rat, GnRH-ir neurons are predominantly located in the MS/POA, but in the Natal mole-rat, the proportion of GnRH-ir cells occurring in the MBH is larger than that in the Cape mole-rat.

No differences in the morphology or distribution are seen between dominant and subordinate females, or between dominant and subordinate males. Male mole-rats have a slightly higher percentage of GnRH neurons in the MBH than females, but not significantly so.

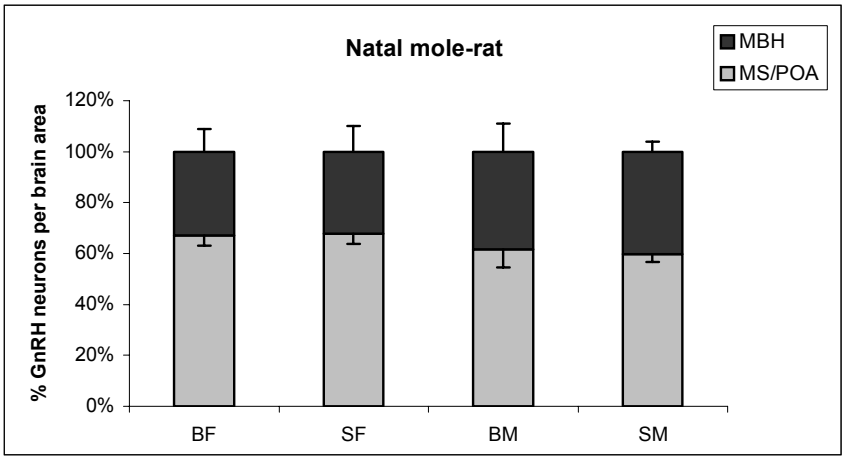

Figure 5.3: The relative distribution of GnRH perikarya in the medial septum/preoptic area and mediobasal hypothalamus in the brain of the Natal mole-rat. BF - breeding females, SF - subordinate females, BM - breeding males, SM - subordinate males. MS – medial septum, POA – preoptic area, MBH – mediobasal hypothalamus.

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- **Morphology and distribution of GnRH-ir processes**

Cape mole-rat

The GnRH-ir processes of the Cape mole-rat have a characteristic beaded appearance. They are present along the septo-preoptico-infundibular continuum from the medial septum to the median eminence. In the rostral areas of the medial septum, sparse fibres are observed in the midline, growing denser and more widespread as they proceed caudally to the anterior commissure. In this area, most fibres and cell bodies are located between the anterior commissure and the ventral limit of the brain. Some fibres are observed in the subfornical organ (SFO), but no cell bodies. A dense concentration of immunoreactive fibres is present in and around the organum vasculosum of the lamina terminalis (OVLT). These processes progress caudally around and within the vestigial optic chiasm. GnRH-ir fibres become diffuse rostral to the suprachiasmatic nuclei (SCN). Fibres are seen ventral and lateral to the SCN, none are found in the SCN. Caudal to the SCN, fibres begin forming a thin ventral aggregation as they proceed towards the median eminence. Further caudally, these fibres become denser arching towards the base of the third ventricle at the level of the median eminence. The internal part of the median eminence protrudes into the third ventricle, resulting in a bicornate recess at the base of the ventricle. GnRH-ir processes are particularly dense within the lateral margins of the median eminence.

The GnRH-ir immunoreactivity in the median eminence of the Cape mole-rat females in the breeding season is not significantly different from those out of the breeding season (Mann Whitney U-test, n1=4, n2=4, U=7, Z=-0.288, p=0.77). (Figure 5.4, Plate 5.2)

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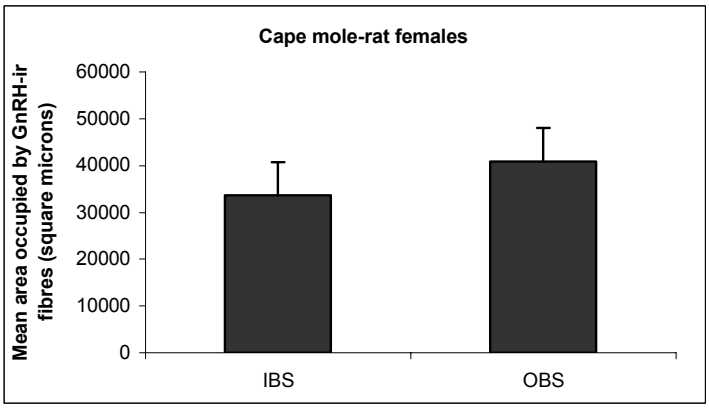

Figure 5.4: Mean area of GnRH immunoreactivity in the median eminence of female Cape mole-rats. IBS – in breeding season, OBS – out of breeding season.

Natal mole-rat

As in the Cape mole-rat, GnRH-ir fibres of the Natal mole-rat also have a beaded appearance. The general distribution of GnRH-ir fibres in the Natal mole-rat is very similar to that of the Cape mole-rat.

The Natal mole-rat appears to have a lower number of fibres in the area of the optic chiasm than the Cape mole-rat, while the area around the SCN in the Natal mole-rat has a higher density of GnRH-ir fibres than in the Cape mole-rat. The form of the median eminence differs between the two species. In the Natal mole-rat the median eminence does not appear to have the same protrusion into the third ventricle as the Cape-mole-rat.

In both the male and female Natal mole-rats, the GnRH-ir immunoreactivity of the median eminence was lower in the dominant animals compared to the subordinate animals. This difference was significant in the female animals (Mann Whitney U-test, n1=6, n2=6, U=1, p=0.006), and in the males (Mann Whitney U-test, n1=7, n2=8, U=8, p=0.02). (Figure 5.5, Plate 5.3, 5.4)

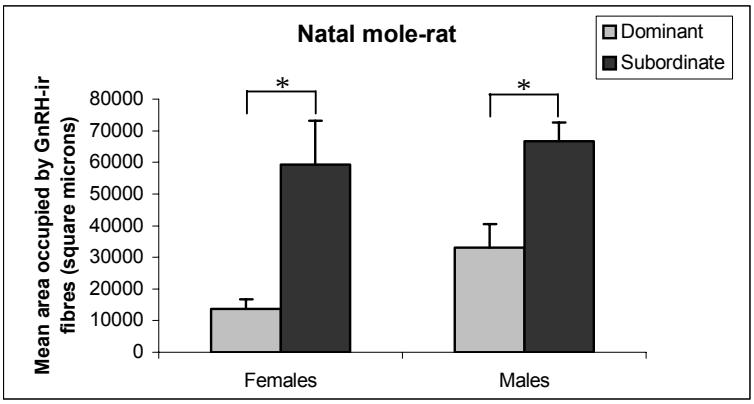

Figure 5.5: Mean area of the GnRH immunoreactivity in the median eminence of breeding and subordinate Natal mole-rats. (*=p<0.05)

- **Number and size of the GnRH cell bodies**

Cape mole-rats

The mean total number of GnRH-ir neurons observed in the Cape mole-rat brains was 423±35. There was no significant difference in number of GnRH-ir neurons between female Cape mole-rats in (394±58.6) and out (449.1±45.4) of the breeding season. (Mann-Whitney U-test, n1=6, n2=7, U=14.5, p=0.35) (Figure 5.6).

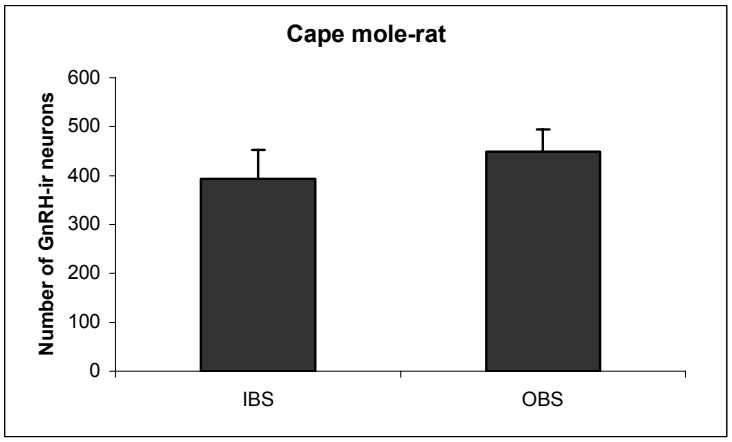

Figure 5.6: Comparison between the mean numbers of GnRH-ir neurons of female Cape mole-rats in (F BS) and out (F OBS) of breeding season.

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Plate legends:

Plate 5.2: Rostrocaudal coronal sections from the brain of female Cape mole-rat (*Georychus capensis*) showing GnRH immunoreactive staining (a) in the medial septum (MS) and rostral preoptic area, (b) in the MS and region of the organum vasculosum lamina terminalis (OVLT), (c) in the medial preoptic area and a densely innervated site caudal to the OVLT and ventral to the third ventricle (3V), (d) in the structure forming the floor of the 3V in place of the optic chiasm, (e) in the region of the suprachiasmatic nucleus (SCN), (f-g) in the mediobasal hypothalamus (MBH) caudal to the SCN, (h-i) in the rostral median eminence (ME), (j-l) in the medial and caudal ME of female mole-rats out of breeding season (OBS), (m-o) in the medial and caudal ME of female mole-rats in the breeding season (IBS). Arrows (→) indicate GnRH perikarya.

Plate 5.3: Rostrocaudal coronal sections from the brain of female Natal mole-rats *(Cryptomys hottentotus natalensis*) showing GnRH immunoreactive staining (a) in the medial septum (MS) and rostral preoptic area, (b) in the MS and region of the organum vasculosum lamina terminalis (OVLT), (c) in the medial preoptic area and a densely innervated site caudal to the OVLT and ventral to the third ventricle (3V), (d) in the structure forming the floor of the 3V in place of the optic chiasm, (e) in the region of the suprachiasmatic nucleus (SCN), (f-g) in the mediobasal hypothalamus (MBH) caudal to the SCN, (h-i) in the rostral median eminence (ME), (j-l) in the ME and pituitary stalk (PS) of non-breeding females, (m-o) in the ME and PS of breeding females. Arrows (→) indicate GnRH perikarya.

Plate 5.4: Rostrocaudal coronal sections from the brain of male Natal mole-rats *(Cryptomys hottentotus natalensis*) showing GnRH immunoreactive staining (a) in the medial septum (MS) and rostral preoptic area, (b) in the MS and region of the organum vasculosum lamina terminalis (OVLT), (c) in the medial preoptic area and a densely innervated site caudal to the OVLT and ventral to the third ventricle (3V), (d) in the structure forming the floor of the 3V in place of the optic chiasm, (e) in the region of the suprachiasmatic nucleus (SCN), (f-g) in the mediobasal hypothalamus (MBH) caudal to the SCN, (h-i) in the rostral median eminence (ME), (j-l) in the ME and pituitary stalk (PS) of non-breeding males, (m-o) in the ME and PS of breeding males. Arrows (→) indicate GnRH perikarya.

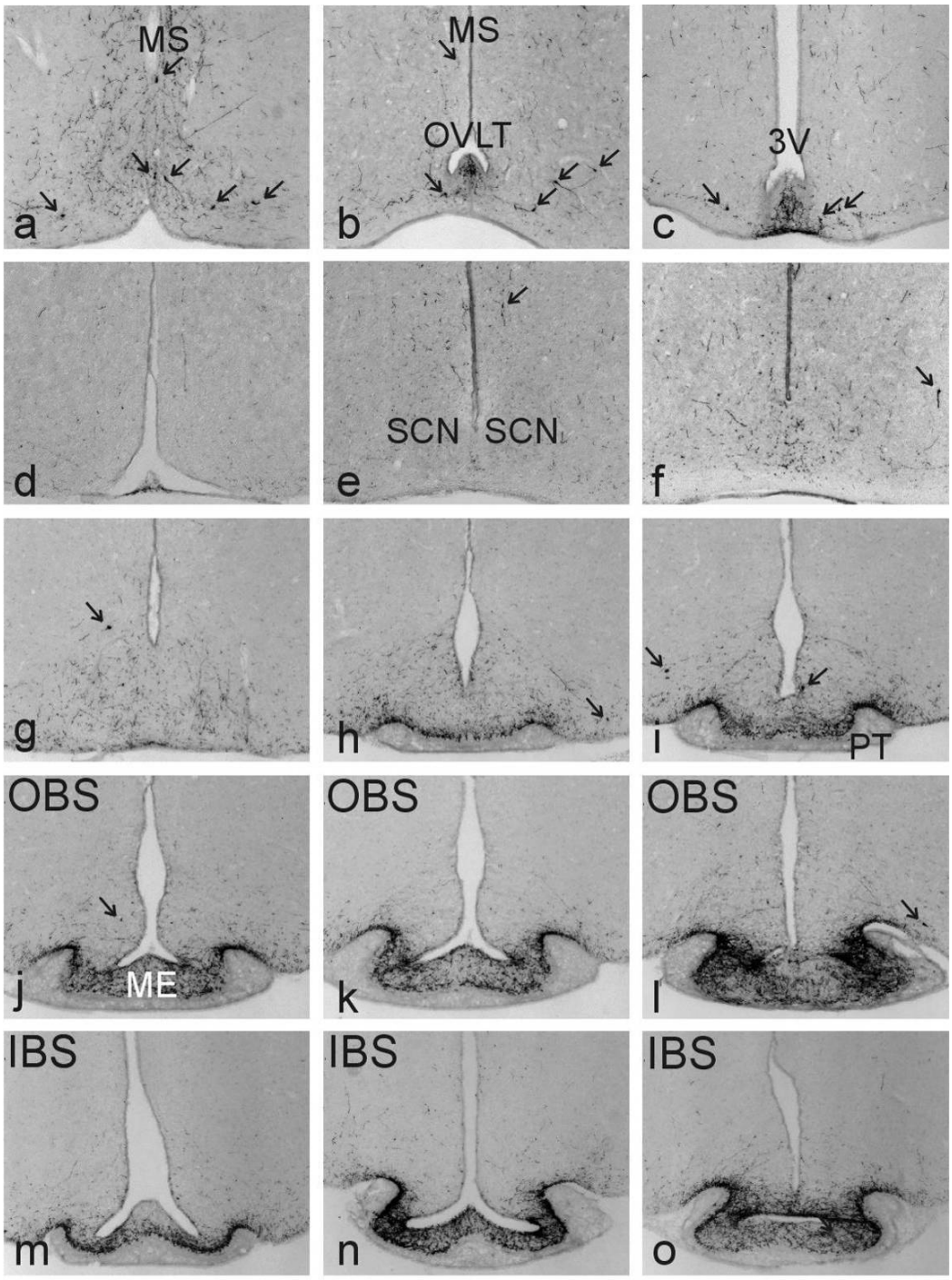
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Plate 5.2

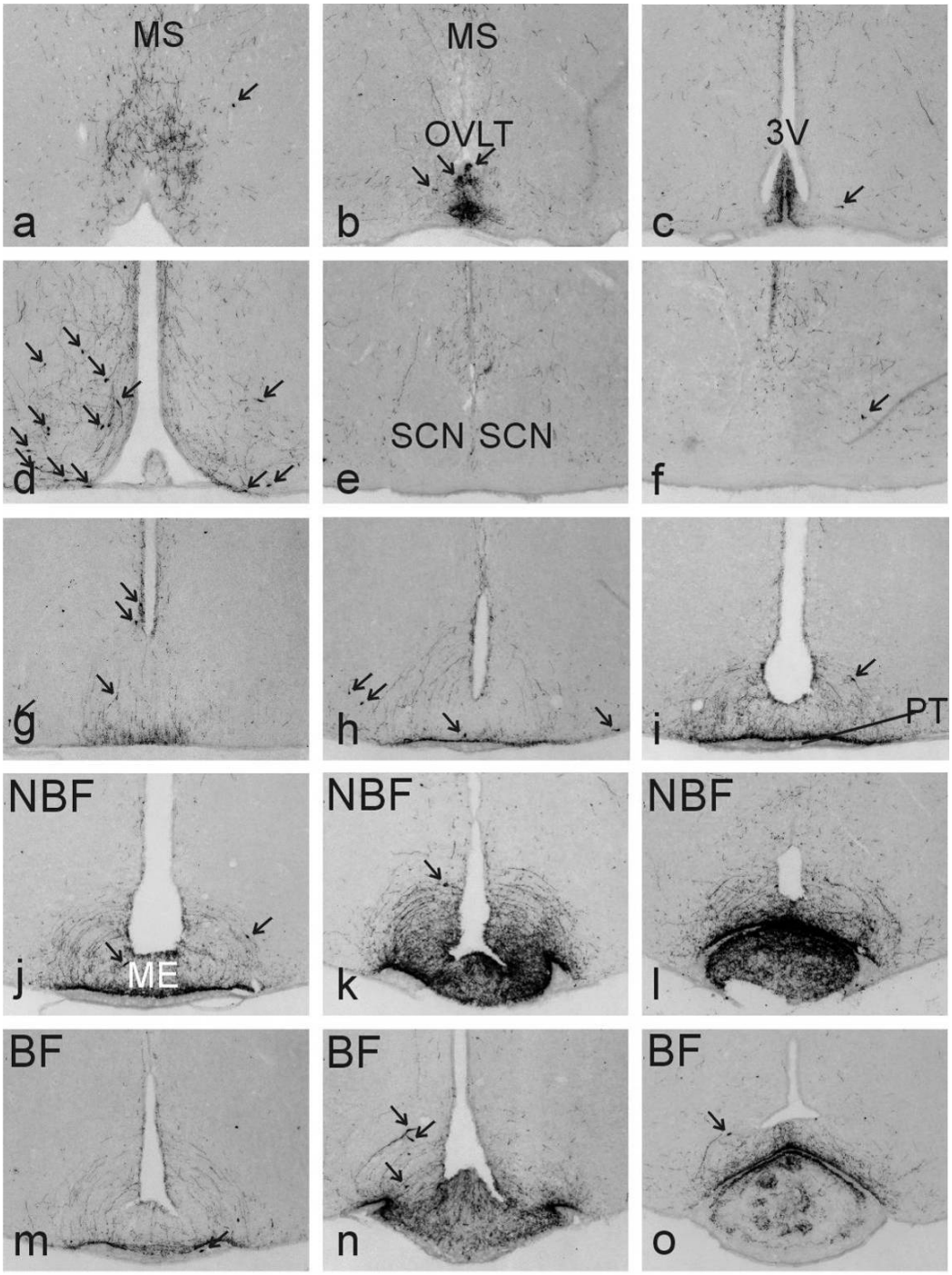
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Plate 5.3

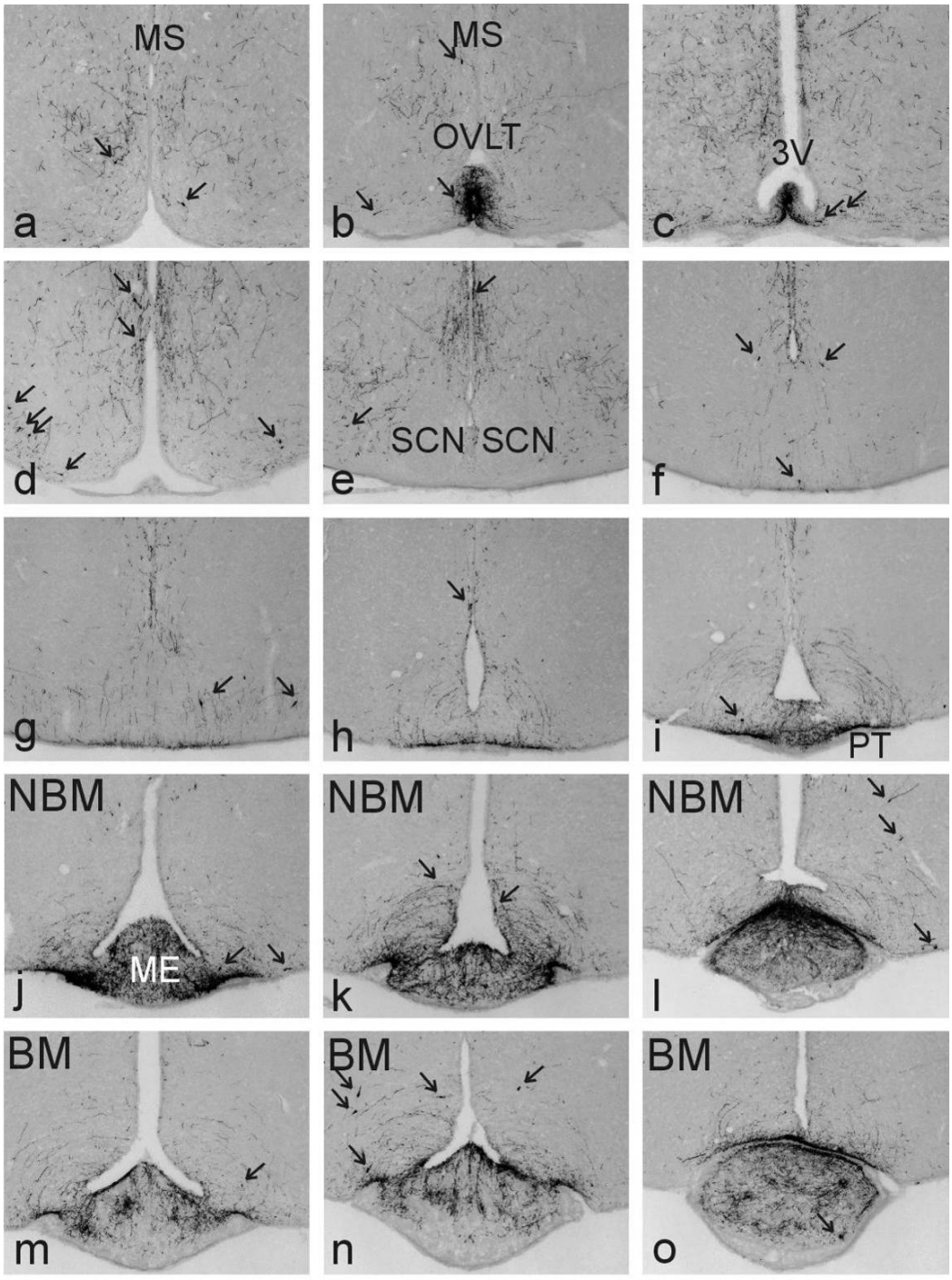
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Plate 5.4

The size of GnRH perikarya in female Cape mole-rats in the breeding season ranged from 88.77μm^2 to 113.23μm^2 (mean: 102.77±3.83μm^2); the range for the females out of the breeding season was 95.45μm^2 to 119.32μm^2 (mean: 109.93±3.11μm^2). There was no significant seasonal difference in the size of these cell bodies in the female Cape mole-rats (MWU, n1=6, n2=7, U=10, Z=1.571, p=0.116) (Figure 5.7).

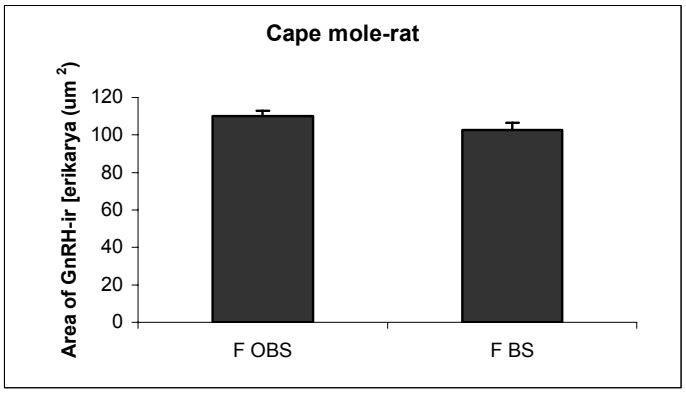

Figure 5.7: Mean size of GnRH-ir perikarya in the Cape mole-rat. F OBS – female, out of breeding season, F BS – female breeding season.

Natal mole-rat

The mean total number of GnRH cell bodies in the Natal mole rat was calculated as 721.07±41.1. No significant difference was observed in the number of cell bodies in dominant (females: 654±98.5; males: 714±72.7) and subordinate (females: 801±76.1; males: 733.7±89.2) Natal mole-rats of either sex (Females: n1=6, n2=7, U=13, Z=-1.143, p=0.253, males: n1=7, n2=8, U=27.5, Z=-0.0579, p=0.954) (Figure 5.8).

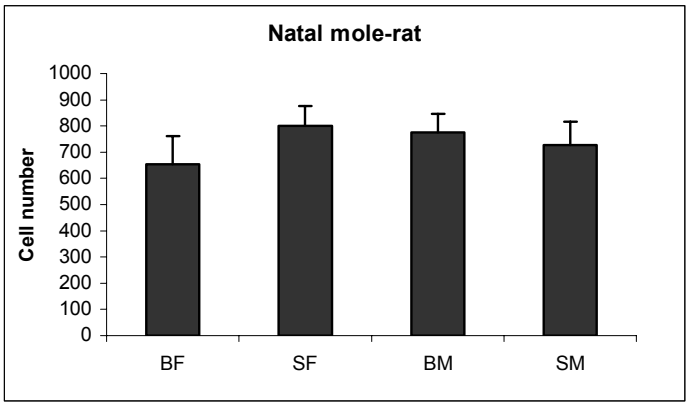

Figure 5.8: Comparison between the mean numbers of GnRH-ir neurons in the Natal mole-rat. BF – breeding females, SF – subordinate females, BM – breeding males, SM – subordinate males.

The Natal mole-rat has significantly more GnRH cell bodies than the Cape mole-rat (Mann Whitney U test, n1=18, n2=28, U=62, Z=4.276, p=0.000019).

The GnRH cell body size for breeding female Natal mole-rats ranged from 86.29 to 119.16μm^2 (mean: 100.47$\pm$5.1μm^2) and the range for the subordinate females was 86.09 to 113.77μm^2 (mean: 101.63$\pm$4.18μm^2). There was no significant difference in the neuron size between the breeding and subordinate females (MWU, n1=7, n2=6, U=21, Z=0, p=1).

The cell body size for breeding males varied from 86.29 to 113.24μm^2 (mean: 101.51$\pm$2.81μm^2) and subordinate males varied from 96.85 to 120.35μm^2 (mean: 105.52$\pm$3.63μm^2). Similarly, there was no significant difference between the breeding and subordinate males (MWU, n1=7, n2=8, U=22, Z=-0.694, p=0.479). Neither was there a significant difference between breeding

males and females (MWU, n1=7, n2=8, U=8, Z=0, p=1), or subordinate males and females (MWU, n1=7, n2=6, U=16, Z=-0.714, p=0.475) (Figure 5.9).

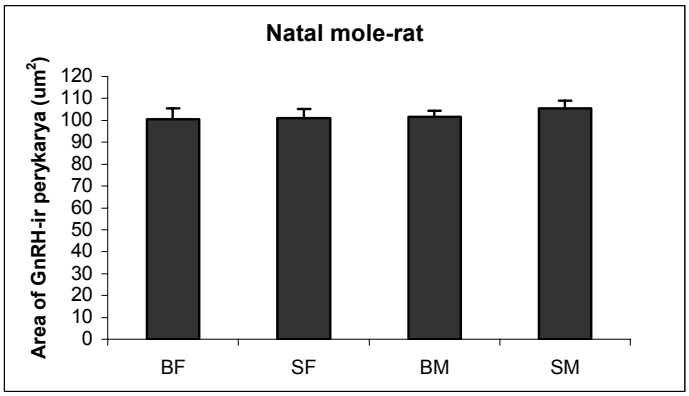

Figure 5.9: Difference in GnRH-ir perikarya size of the Natal mole-rat. BF - breeding females, SF - subordinate females, BM - breeding males, SM - subordinate males.

Discussion

The morphology and distribution of the GnRH systems of the solitary Cape mole-rat and the social Natal mole-rat is described as well as a quantification of the GnRH content in the median eminence. This is discussed and compared with regard to breeding season and reproductive status.

GnRH-ir neurons in both the Cape mole-rat and the Natal mole-rat are predominantly unipolar or bipolar. This finding is consistent with that described for the common mole-rat, the highveld mole-rat and the Damaraland mole-rat (DuToit *et al*. 2006, Molteno *et al*. 2004), as well as several other small mammal species (bat – Fernandez *et al*. 1992, white footed mouse - Glass *et al*. 1986; rat – Witkin *et al*. 1982; hamster - Yellon *et al.* 1990, Yellon & Newman 1991). While larger mammals tend to have more complex neuronal morphology (sheep - Wood *et al.* 1992, springbok -

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Robinson *et al*. 1997, pony - Melrose *et al*., 1994), the importance thereof is not fully understood. It is thought that neurons with a more complex morphology have a greater potential of association formation, hence can be influenced by a greater number of neural inputs (Robinson *et al*. 1997).

In some seasonally breeding species, there is a seasonal change in the morphology of the GnRH neurons. In sheep a larger number of dendritic processes and a higher innervation of these processes are present during the period of anoestrous (Lehman *et al*. 1986, Xiong *et al*. 1997). In contrast, neither in the Cape mole-rat nor the Natal mole-rat, were any seasonal changes in the GnRH cell body morphology observed.

A higher number of GnRH-ir cell bodies are present in the brains of the Natal mole-rat compared with those of the Cape mole-rat (721±41 vs. 423±35). It has been proposed that the total number of GnRH-ir cell bodies is related to body size (Yellon & Newman, 1991). The Cape mole-rat does not conform to this trend in that, although this mole-rat is rather larger than the other mole-rat species currently investigated (mean body sizes: Cape mole-rat 180g; common mole-rat 60-80g, highveld mole-rat 90-110g, Natal mole-rat 100g, Damaraland mole-rat 130g; Jarvis & Bennett, 1991,1993), the number of GnRH-ir cell bodies is lower than most. The opposite situation occurs in the case of the highveld mole-rat, where there is an unusually high number of GnHR-ir neurons present for its body size (Du Toit *et al*. 2006). The number of GnRH cell bodies present in the Natal mole-rat brains is roughly equivalent to the number found in the Damaraland mole-rat (648±33; Molteno *et.al*. 2004) and the common mole-rat (605±60; DuToit *et.al*. 2006), but less than half of that found in the highveld mole-rat (1489±183; DuToit *et.al.* 2006). There is thus considerable interspecies variation in the GnRH cell body numbers of mole-rats. The mean total numbers of GnRH neurons of other small mammals

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such as Djungarian hamsters (300-400, Yellon *et al*. 1990) and Syrian hamsters (650-750, Jennes & Stumpf 1980) fits well within this range, while larger mammalian species possess GnRH cell numbers of several thousands (Lehman *et al*. 1986, Robinson *et al*. 1997, Silverman *et al*. 1982).

There was no seasonal change in the size or number of GnRH-ir neurons in the Cape mole-rat or the Natal mole-rat. Similarly, the total number of GnRH-ir cell bodies in other seasonally breeding species such as the Djungarian hamster, prairie vole and Japanese wood mouse, were found to be similar in the breeding and non-breeding seasons (Yellon & Newman 1991, Kriegsfeld & Nelson 1999, Kuwahara *et al.* 2000). In contrast, the detectable GnRH-ir neuron population in the gerboa, a hibernating rodent, reduces by up to 55% in the POA in the autumn (El Quezzani *et al*. 2000).

GnRH perikarya in the Natal and Cape mole-rats were found to be of similar size. The GnRH cell body sizes of the highveld and common mole-rats were found to be slightly larger (Du Toit *et al*. 2006), whereas those of the Damaraland mole-rat had smaller perikarya (Molteno *et al*. 2004).

The GnRH cell body size and numbers did not differ between the reproductive and non-reproductive animals of either sex in the Natal mole-rat. This trend is consistent with those findings of other social mole-rat species with a reproductive division of labour (Du Toit *et al*. 2006, Molteno *et al.* 2004). Reproductive and non-reproductive springbok likewise have similar sized GnRH neurons (Robinson *et al*. 1997).

In the female Cape mole-rats, the size of the GnRH cell bodies did not differ in and out of the breeding season. Similarly, the season did not affect the GnRH-ir cell body size or number in social, seasonally breeding mole-rats (Du

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Toit *et al*. 2006). In some seasonally breeding species, however, the secretion of GnRH is suppressed during the non-breeding season; this is associated with an increase in the net GnRH in the brain and an enlargement of the GnRH neurons (Syrian hamster - Urbanski *et al*. 1991, Japanese wood mouse – Kuwahara *et al.* 2000).

Although inter-specific differences are observed, characteristically the GnRH system is distributed in a loose continuum along the septo-preoptico-infundibular pathway in mammals (El Ouezzani *et al*. 2000, Davis *et al*. 1985, Caldani *et al*. 1988, Robinson *et al*. 1997). The degree of caudal migration of GnRH neurons during ontogenetic development varies within species, resulting in the relative presence of the neurons in the preoptic area and basal hypothalamus (Silverman *et al*. 1994). In general, GnRH cell bodies are similarly distributed in all mole-rat species investigated (this study, DuToit *et al*. 2006, Molteno *et al*. 2004). In both the Cape and the Natal mole-rat, GnRH perikarya are found throughout the medial septum, ventral and horizontal limbs of the diagonal band of Brocca and preoptic area to the mediobasal hypothalamus. Although there are large inter-species differences within the Bathyergidae, the majority of GnRH-ir cell bodies are found in the preoptic area, rostral in the region of the ventral diagonal band of Broca, and further caudally below the anterior commissure (Cape mole-rat:~90%, Natal mole-rat:~65%). The remainder of the GnRH perikarya are found further caudal in the mediobasal hypothalamus scattered around the perimeter of the third ventricle and around the SCN (not within). Although fibres are observed in the subfornical organ, no cell bodies are observed in this structure in either of the species.

Reproductive status does not appear to affect the distribution or morphology of GnRH neurons in the Natal mole-rat. Similarly, the GnRH systems of the

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common mole-rat, highveld mole-rat and Damaraland mole-rat do not show alterations associated with social status in its distribution or morphology (Van der Walt 2003, Molteno *et al*. 2004).

Two main areas of dense innervation occur, namely the OVLT and the median eminence. In the Cape mole-rat, the GnRH fibres in the median eminence are not significantly more densely out of the breeding season. This suggests that the GnRH production is not affected by seasonal changes. Thus GnRH is produced and secreted during both the breeding and non-breeding seasons.

In the Natal mole-rat, the GnRH-ir fibre distribution in the median eminence was significantly higher in the non-breeding animals. This implies that the subordinate animals manufacture GnRH, but show an inhibition of its release into the portal blood capillaries to the pituitary.

Seasonally breeding species undergo a down-regulation of reproductive activity during the non-breeding season (Gerlach & Aurich 2000), and this is usually reflected in their reproductive physiology. Hypothalamic sensitivity towards steroid hormones alters, resulting in retention of GnRH in the median eminence, which inhibits the release of LH in the pituitary that in turn is responsible for the inhibition of gonadal hormones and gamete production.

Seasonally breeding mole-rats do not appear to undergo complete cessation of reproduction. Previous studies revealed that according to gonadal anatomy and hormonal responses to stimulation, the reproductive system is active during the non-breeding season (Hart & Bennett 2006, Spinks *et al*. 1997, 2000, Van der Walt *et al*. 2001, Janse van Rensburg *et al*. 2002). The hormonal profiles of the Cape mole-rat are consistent with what has been

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found in other bathyergid species (previous chapter). Hence, not surprisingly, there are no differences in the neuroanatomy (neuron numbers and size) and neuroendocrinology (GnRH-immunoreactivity in the median eminence) of the Cape mole-rat brain in and out of the breeding season. Therefore, the Cape mole-rat does not conform to the classical depiction of a seasonal breeding mammal, but it compares well with other seasonal breeding bathyergids (Spinks *et al*. 1997, 1999, Janse van Rensburg *et al*. 2002).

Subordinate members of social mole-rat colonies are reproductively quiescent, but not sterile (Bennett *et al*. 1999). Social mole-rat species display a spectrum of socially induced infertility (Bennett *et al*. 1997). The degree and mechanism of reproductive suppression varies among different species. In the highly inbred naked mole-rat, subordinate animals are physiologically suppressed from breeding. Ovulation is blocked in subordinate females, and sperm production and motility are suppressed in males (Faulkes *et al*. 1990a, 1991). The pituitary response to a GnRH challenge is significantly less than in dominant animals (Faulkes *et al*. 1990b). All other mole-rat species are outbreeding (Bennett & Faulkes 2000), and mechanisms of suppression tend more towards incest avoidance as the degree of sociality in species subsides. The Damaraland mole-rat can be classified as eusocial, female subordinates are physiologically suppressed like in the naked mole-rat, while males do not show any physiological constraints that prevent them from reproducing (Bennett *et al*. 1994, Bennett *et al*. 1996). The Natal mole-rat has two closely related sister species, the common mole-rat and the highveld mole-rat. Whereas subordinate animals from both these species exhibit a significant response to a GnRH challenge (Spinks *et al*. 2000, Van der Walt *et al*. 2001), the difference of the response to exogenous GnRH was significant between dominant and subordinate females in the highveld mole-rat (Van der Walt *et al*. 2001). Also, social status was reflected in the density of GnRH-

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immunoreactivity in the highveld mole-rat, but not the common mole-rat (Du Toit *et al*. 2006). It therefore appears that the degree of sociality-induced infertility of the Natal mole-rat lies somewhere between these two species. While there is no difference in the magnitude of the response to exogenous GnRH (previous chapter), there is an unambiguous difference in the density of the GnRH-immunoreactivity in the median eminence between dominant and subordinate animals of both sexes.

Behaviourally, both species under investigation display a form of regulated reproduction, breeding in the Cape mole-rat is restricted to a specific part of the year, while subordinate individuals of the Natal mole-rats repressed from reproduction, presumably as a result of incest taboos. However, neuroendocrinologically, reproductive regulation is only reflected in subordinate animals of the Natal mole-rat.

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CHAPTER 6

SYNTHESIS

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African mole-rats (Bathyergidae) are of particular interest as they are widely distributed across Africa and occupy a variety of different habitats and offer a number of interesting, some of which are unique, characteristics in a single family. In this family, a wide range of social behaviour is displayed, some species are strictly solitary whereas other species have highly organised social societies. Social species have a highly skewed reproductive success such that a single female and a few male consorts are responsible for reproduction. Reproduction in subordinate animals can be inhibited by the dominant individuals by various mechanisms. In extreme cases, ovulation is blocked physiologically, but more commonly, behavioural interactions and incest avoidance is sufficient to prevent subordinate animals from reproducing. This family provides the ideal model to glean insight in the mechanisms of reproductive suppression, both seasonally and socially.

This study firstly focused on the endocrine, neuroendocrine and neuroanatomical parameters of wild caught, seasonally breeding Cape mole-rats, and secondly, by using endocrine, neuroendocrine and neuroanatomical parameters, to gain insight into the seasonality of reproduction in the Natal mole-rat and the socially induced fertility of subordinate Natal mole-rats, to identify where this mole-rat fits into the spectrum of the sociality continuum.

Seasonality

All solitary African mole-rats studied to date are seasonal breeders, while the majority of social species are aseasonal (Bennett & Faulkes 2000). The Cape mole-rat has a distinct breeding season (Bennett & Jarvis 1988a) but does not appear to possess the physiological characteristics of a classical seasonal breeder. Typically, reproductive function is down-regulated during the

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reproductively quiescent period in seasonally breeding species (Gerlach & Aurich 2000). In females, ovulation occurs only during part of the year, and in males spermatogenesis is reduced or ceased.

In contrast with most seasonally breeding species, female Cape mole-rats do show follicular development during both the breeding and the non-breeding seasons (Oosthuizen & Bennett 2007). In both males and females, circulating steroid hormone concentrations, as well as the pituitary response on exogenous GnRH are not significantly different in winter compared to summer. In the brain of female Cape mole-rats, there is no difference in the number of GnRH neurons or the size of the cell bodies, moreover the amount of GnRH peptide stored in the median eminence is similar in summer and winter.

The findings of this study therefore lead us to believe that the Cape mole-rat is an opportunistic breeder with the physiological ability to produce all year round, but it is prevented from breeding during certain periods of the year by ecological constraints. During the dry season, the soil is hard and burrowing is energetically costly. Therefore the breeding season correlates well with the rainfall pattern. It is possible that unpredictable rainfall patterns due to El Nino southern ocean patterns may have selected against a strictly seasonal reproductive physiology in the Cape mole-rat.

The Natal mole-rat, although phylogenetically closely related to the common mole-rat and the highveld mole-rat (Faulkes *et al*. 1997), does not appear to have a distinct breeding season. Histological results from the gonads in both males and females show no seasonal variation in numbers of follicles or degree of follicular development (Viljoen, 2006) and post-mortem findings reveal embryos during the entire year (M.Oosthuizen *pers obs*.). No seasonal

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patterns are observed in steroid sex hormones or the pituitary response to exogenous GnRH, and GnRH neuron size and number are comparable over season. Furthermore there are no seasonal differences observed in the GnRH content of the median eminence.

The Natal mole-rats used for this study occur in a region with a much higher yearly rainfall than the habitats of the common and highveld mole-rats. For these animals, abundant food and dispersal opportunities are available throughout the year, and by utilising an opportunistic breeding strategy, they are able to reproduce when conditions are favourable. It is possible that Natal mole-rats that occur in drier areas may also exhibit seasonal reproductive quiescence if food and dispersal opportunities are restricted.

Thus it appears that a number of mole-rat species that are classified as seasonal breeders, are physiologically able to reproduce throughout the year, but are restricted from doing so by environmental factors. Rainfall seems to be an important determining factor in seasonality of mole-rats.

Sociality

All the members of the genus *Cryptomys* are social. These species occur in most habitat types in sub-Saharan Africa, and the degree of sociality is correlated both with the stringency of reproductive suppression and the aridity of the habitat where the species occur (Figure 6.1).

Neither male nor female non-reproductive Natal mole-rats appear to be physiologically inhibited from reproducing by the breeding animals. In Natal

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mole-rats, spermatogenesis takes place in reproductive and non-reproductive males, and likewise, both reproductive and non-reproductive females show follicular development (Viljoen 2006). No significant differences were seen in testosterone concentration between reproductive and non-reproductive males, neither was there a difference in the oestrogen concentrations between reproductive and non-reproductive females. Progesterone concentrations were significantly higher in reproductive females, however, the Natal mole-rat is an induced ovulator, and requires coitus to induce ovulation and follicular development to the stage of corpus luteum. No difference was seen in the response of the pituitary to an exogenous GnRH challenge between reproductive and non-reproductive animals of either sex. In the brain of the Natal mole-rat, the distribution, number and size of GnRH perikarya was similar in reproductive and non-reproductive animals of both sexes, however the GnRH innervation of the median eminence (ME) was much denser in the non-reproductive males and females compared to the reproductive animals. This implies that in non-reproductive animals, GnRH is synthesized in non-reproductive but is retained in the ME. The findings of this research suggest that the reproductive skew in the Natal mole-rat is maintained through incest avoidance and is behaviourally controlled by the dominant animals.

Jarvis *et al.* (1994) proposed the aridity food distribution hypothesis (AFDH) to explain the continuum of sociality seen in the bathyergid rodent moles. This hypothesis relates the costs and risks associated with living in an arid habitat. For mole-rats inhabiting arid areas, burrowing is a significant energetic expenditure, therefore living in large group sizes is advantageous as it reduces burrowing costs while simultaneously increasing the foraging efficiency.

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In highly social or eusocial mole-rat species (naked mole-rat and Damaraland mole-rat), reproductive control of subordinate animals is very stringent. They occur in arid areas and dispersal opportunities are infrequent. As a result, dispersal is delayed in subordinate animals and necessitate drastic measures on the part of reproductive animals to remain dominant. Non-reproductive animals in these species are frequently physiologically suppressed from reproducing (Faulkes *et al.* 1990a, Faulkes *et al.* 1991, Bennett *et al*. 1993, Bennett *et al.* 1996).

In social mole-rat species occupying more mesic habitats (Mashona mole-rat, common mole-rat, Zambian mole-rat, higveld mole-rat) reproductive control of subordinate animals tends to be enforced through behavioural mechanisms and incest avoidance. Dispersal opportunities are more predictable and regular, concequently the survival of the colony does not rely on large numbers of individuals to locate food patches. Subordinate animals have the option to leave the natal colony and set up an independent colony. The reproductive physiology of the Natal mole-rat compares well with that of representatives of this latter category (Figure 6.1). It fits in at the lower end of the continuum of socially induced fertility as proposed by Bennett *et al*. (2000) where subordinate non-reproductive males and females show a similar basal circulating LH concentration and subsequent elevation in response to a pharmacological overdose of GnRH as the dominant reproductive animals.

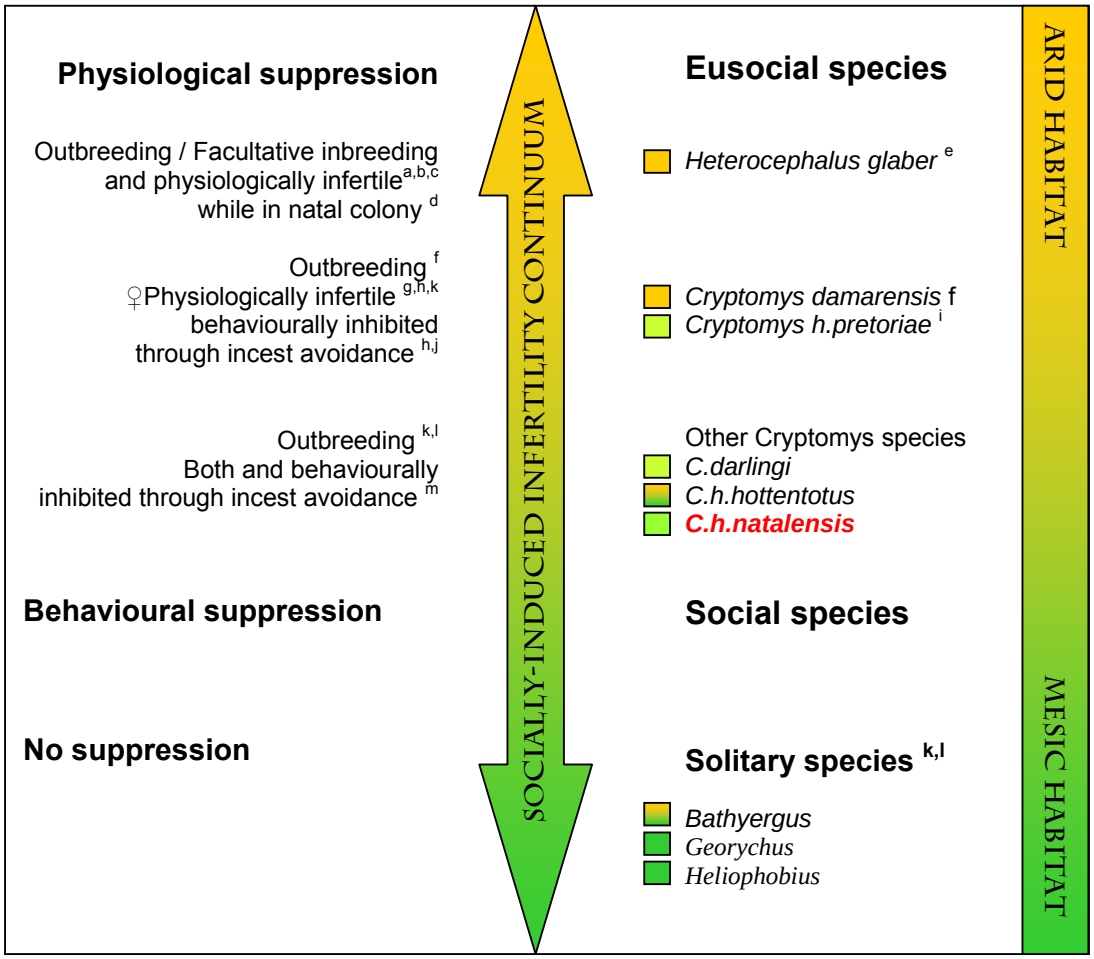

Figure 6.1: Species of the family Bathyergidae assembled according to the degree of sociality displayed and type of habitat in which they occur, with the Natal mole-rat (***C.h.natalensis***) grouped with other behaviourally suppressed species. (Modified from L. van der Walt 2003).

[a] Reeve *et al*. 1990; [b] Braude 2000; [c] Clarke & Faulkes 1999 [d] Faulkes *et al*. 1990, 1991; [e] Jarvis 1981; [f] Jarvis & Bennett 1993; [g] Bennett *et al*. 1996; [h] Bennett 1994; [i] Moolman *et al*. 1998; [j] Van der Walt *et al*. 2001; [k] Jarvis & Bennett 1991; [l] Bennett & Faulkes 2000; [m] Spinks *et al*. 1997, 1999, 2000; ♀ - female; ♂ - male.

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