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

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).

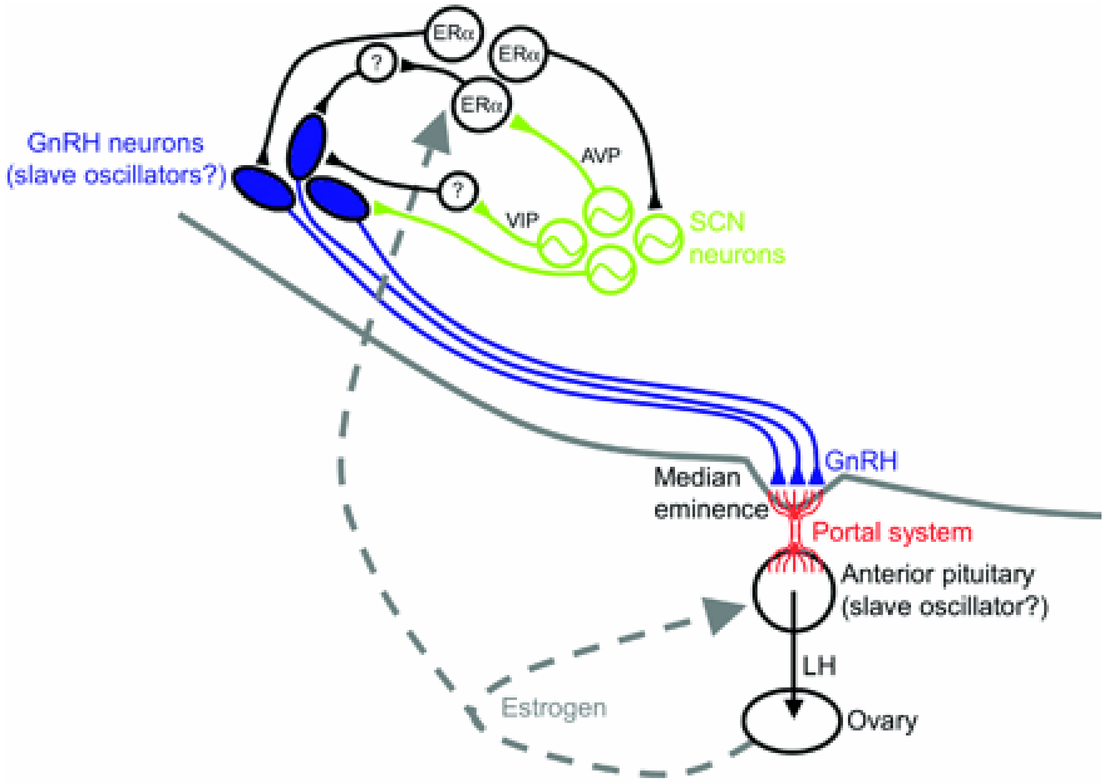

Fig. 4.1: Schematic representation of the reproductive pathway from the hypothalamus to the gonads and feedback system.

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).

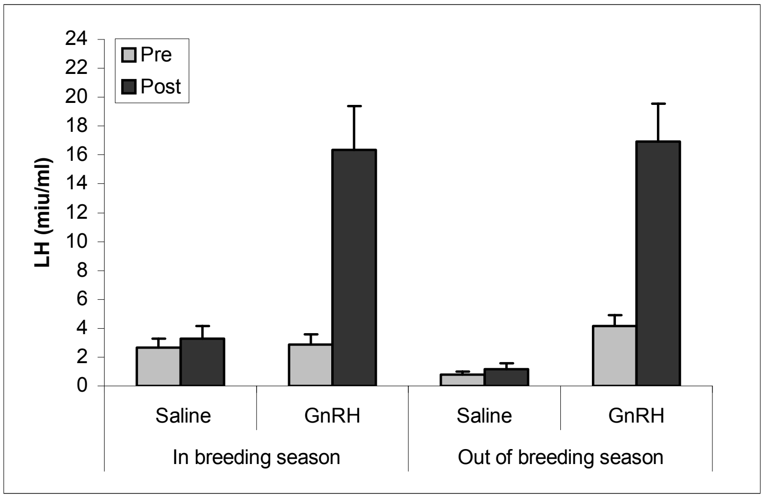

Figure 4.2: Mean basal plasma LH (Pre-GnRH) and the pituitary response (Post-GnRH) to a single 2.0μg exogenous GnRH injection, or a single injection of physiological saline control for male Cape mole-rat in and out of the breeding season.

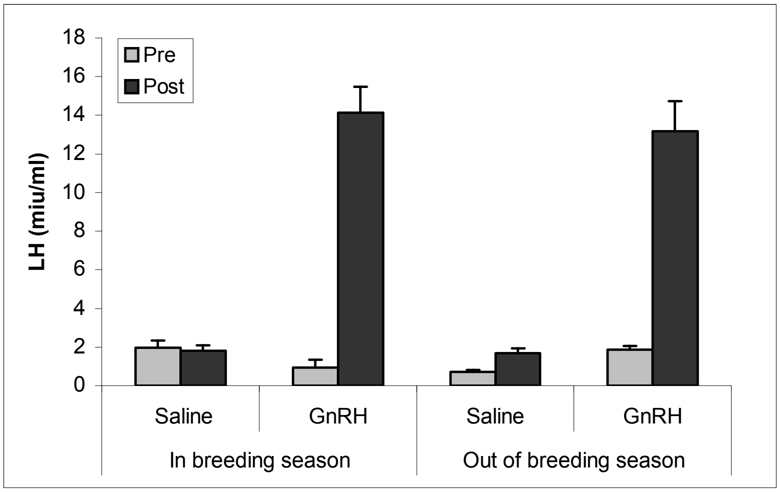

Figure 4.3: Mean basal plasma LH (Pre-GnRH) and the pituitary response (Post-GnRH) to a single 2.0μg exogenous GnRH injection, or a single injection of physiological saline control for female Cape mole-rat in and out of the breeding season.

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 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.

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).

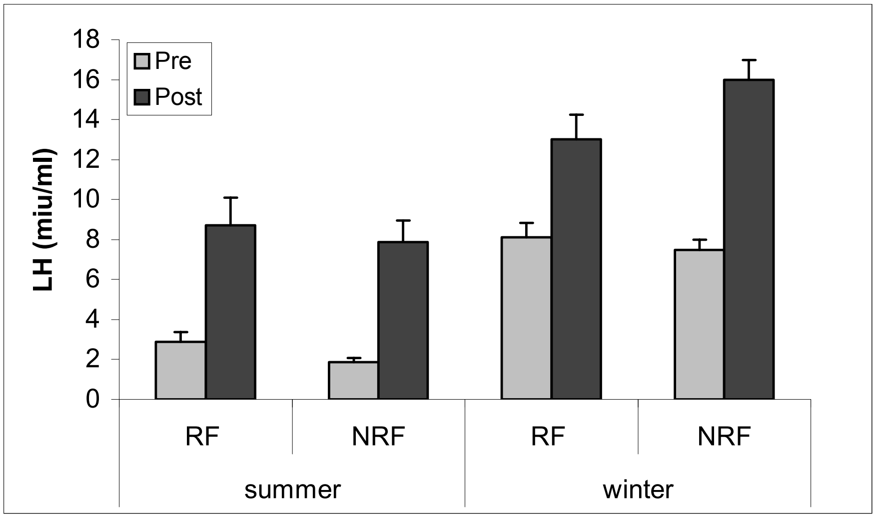

Figure 4.4: Mean basal plasma LH (Pre-GnRH) and the pituitary response (Post-GnRH) to a single 2.0μg GnRH injection, or a single injection of physiological saline for female Natal mole-rats during the summer or winter.

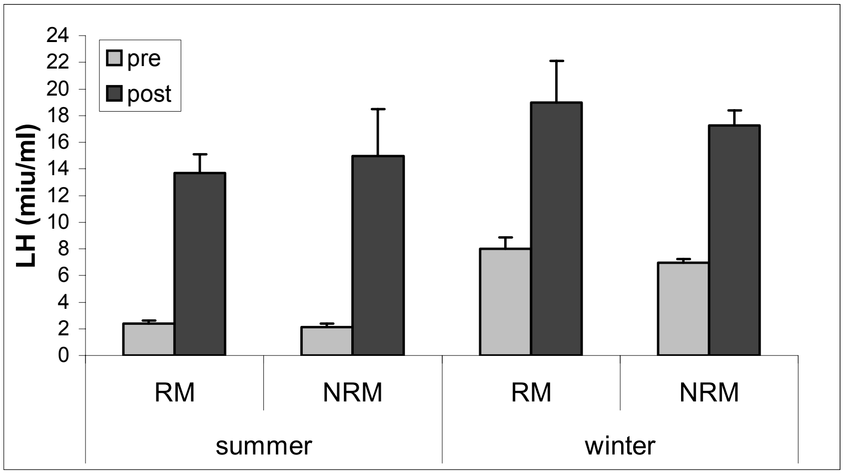

Figure 4.5: Mean basal plasma LH (Pre-GnRH) and the pituitary response (Post-GnRH) to a single 2.0μg GnRH injection, or a single injection of physiological saline for male Natal mole-rats during the summer or winter.

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 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.