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Reproductive inhibition in female common and highveld mole-rats: neuroanatomical and neuroendocrine perspectives

by

Lydia van der Walt

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Study leaders: Professor Nigel C Bennett

Professor Clive W Coen

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Abstract

The social reproductive inhibition evident in female common (*Cryptomys hottentotus hottentotus*) and highveld (*Cryptomys hottentotus pretoriae*) mole-rats was investigated, focussing on intra- and inter-subspecies differences in gonadotropin-releasing hormone (GnRH) neuroanatomy and neuroendocrinology and potential mechanisms mediating social infertility through the suppression of luteinizing hormone (LH) and/or GnRH synthesis or release.

The molecular structure of the GnRH peptide present in highveld mole-rat tissue was determined. Messenger ribonucleic acid (mRNA) coding for GnRH was isolated from hypothalamic tissue of a female highveld mole-rat. The nucleotide sequence of the transcribed complimentary deoxyribonucleic acid (cDNA) predicts amino acid sequences that indicate a potential polymorphism in the mature peptide. These sequences differ from classic 'mammalian' GnRH by either one or two amino acid positions. One form of mole-rat GnRH is identical to the unique GnRH expressed by guinea pigs, while the second form differs from guinea pig GnRH in one amino acid position. These results indicate that classic 'mammalian' GnRH is not the chief structural form of GnRH in the mole-rat.

In both the common and highveld mole-rat, GnRH structures were loosely distributed along the septo-preoptico-infundibular pathway. Highveld mole-rats consistently had more GnRH cell bodies and higher levels of the peptide than common mole-rats. The subspecies also differed with regards to the relative distribution of GnRH cell bodies in the preoptic area and the medio-basal hypothalamus.

Reproductive status and season did not influence GnRH neuroanatomy (numbers and morphology of cell bodies, distribution of GnRH structures and density of GnRH staining in the median eminence) or endogenous brain GnRH content in male or female common mole-rats. Likewise reproductive and non-reproductive male

highveld mole-rats did not differ with regards to the GnRH neuroanatomical and neuroendocrinological parameters investigated. Dominant and subordinate female highveld mole-rats had similar numbers of GnRH cell bodies, and did not differ with regards to the distribution of GnRH structures. Brain GnRH levels was however significantly lower in reproductive females compared to non-reproductive females. Similarly, GnRH staining in the median eminence was significantly less intense in reproductive compared to non-reproductive females, indicating reduced release in the latter. Excess peptide most likely accumulates in the nerve terminals and the cell bodies.

Endogenous opioid peptides (EOPs) are tentatively considered to play a role in the socially-induced infertility in non-reproductive female highveld mole-rats. Intact reproductive females and intact and ovariectmized non-reproductive females were treated with the opioid antagonist naloxone, alone and in different combinations with exogenous GnRH, to establish the role of EOPs and gonadal hormones in the reduced GnRH release evident in subordinate female highveld mole-rats. Naloxone treatment failed to alter plasma LH levels in any of the intact groups, while ovariectomized non-reproductive females showed a significant response. The pituitary response to a GnRH challenge was not influenced by either a single naloxone administration or longer term naloxone-priming regimens. In the absence of the negative feedback effects of gonadal steroids following ovariectomy, non-reproductive females showed GnRH-challenged plasma LH levels similar to that seen in intact reproductive females, both being significantly higher compared to intact non-reproductive females. Steroid-dependent mechanisms altering GnRH secretion and/or pituitary sensitivity to the endogenous peptide may therefore play a role in the social reproductive regulation displayed by the highveld mole-rat. EOPs may be indirectly involved in steroid negative feedback suppression of GnRH.

In conclusion, the GnRH neuroanatomy and neuroendocrinology of female common mole-rats provide no evidence for a physiological component to the behavioural

reproductive restraint in subordinate females due to incest avoidance. Non-reproductive female highveld mole-rats are, however, temporarily infertile due to pituitary insensitivity to GnRH coupled with suppressed hypothalamic GnRH release potentially mediated by steroid-dependent mechanisms.

Preface

'Alleen sy wat die Onsienlike sien, kan die onmoontlike doen.' - Frank Gaines

Pretoria: I trapped highveld mole-rats in the Pretoria area, and would like to thank the green keepers and janitors of the following properties: Mooikloof equestrian estate, Monumentpark golf course, University of Pretoria experimental farm and Woodhill golf estate as well as Mr and Mrs Steyn (Rietvleidam area).

Kamieskroon: Common mole-rats were trapped at Kamieskroon in Namaqualand. I lodged with Tannie Colla Swart, and trapped mole-rats on the farm Kardou. Marna Herbst, Matthew, Truida and Kieter Norval are thanked for lots of fun and support!

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I would like to thank my husband, Danie, for his endless love, support, encouragement, digging after mole-rats and all the other torturous things he had to endure. A huge thanks to my parents for their support both financially and emotionally, during my studies. And finally: thank you to all the mole-rats who, not so willingly, sacrificed their lives in the name of science.

SOLI DEO GLORIA!

'*I will lift up mine eyes unto the hills, from whence cometh my help. My help cometh from the Lord, which made heaven and earth.*' - Psalm 121: 1-2

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Chapter 1

General introduction

Animal sociality

The classic definition of the term eusocial, or truly social, was coined with species belonging to the insect orders Hymenoptera (bees, ants and wasps) and Isoptera (termites) in mind. Eusocial insect communities are characterized by: 1) reproductive division of labour (reproduction is monopolized by a few individuals with the majority of group members not breeding); 2) more than two overlapping adult generations; and 3) parental and/or alloparental care (cooperative care of young by individuals other than the parental pair; Batra, 1966; Michener, 1969; Wilson, 1971). In 1981 Jarvis discovered the first eusocial mammal in the subterranean naked mole-rat (*Heterocephalus glaber*), reminding us of Alexander's early prediction (1974) that the super-safety of the underground niche could be a precondition to eusociality in vertebrates. In naked mole-rat colonies a single female and between one and three males breed (Jarvis, 1981; Brett, 1986), there are overlapping adult generations, and cooperative care is taken of the pups (Jarvis, 1981; Jarvis, 1991; Lacey and Sherman, 1991). This vertebrate species subsequently met the requirements of classic insect eusociality. A second member of the family Bathyergidae, the Damaraland mole-rat (*Cryptomys damarensis*) is also believed to be truly social (Bennett and Jarvis, 1988; Jarvis and Bennett, 1993). Differences in colony structure, behaviour and the hormonal profiles of subordinate colony members indicate that eusociality evolved independently in the naked and Damaraland mole-rats even though the phenomenon has not been observed in any other subterranean mammal (Jarvis and Bennett, 1993).

The crucial preconditions for eusociality, namely parental care in the form of repeated food provisioning and protected nesting areas, in combination with low reproductive success of young adults or solitary pairs, seem to be similar in insects and

vertebrates (Anderson, 1984). Social insects and some social vertebrates (e.g. female marmoset monkeys, *Callithrix jacchus*) also seem to have similar neural and neuroendocrine adaptations to non-breeding or subordinate status, probably due to repeated convergent evolutionary attempts at adapting to environmental conditions unsuitable for dispersal and independent breeding (Abbott *et al.*, 1998).

Presently eusociality is not defined in a manner that is helpful to the study of the evolution of altruism in taxa other than the archetypal eusocial insects (Gadagkar, 1994). Sherman *et al.* (1995) suggest a wider use of the term with a continuum of interflowing social structures. The probability of an individual in the group ever attaining breeding status is used as a measure of the degree of sociality displayed by the group. Crespi and Yanega (1995) on the other hand, advocate stricter use of the term. These authors use the classical definition of eusociality adding a fourth requirement, namely the possession of permanent or lifelong castes. A caste is any set of individuals of a particular morphological or age cohort that perform specialized labour within the colony (Wilson, 1975). Even when applying these strict criteria, naked mole-rats still qualify as eusocial, since permanent morphological castes have been described for the species in that the lumbar vertebrae of dominant females lengthen after the commencement of reproductive activity (O'Riain *et al.*, 2000). The best working definition of eusociality probably lies somewhere between the two extremes, however in this study a continuum of social structures with eusociality at the apex, as displayed by members of the African mole-rat family Bathyergidae (Jarvis and Bennett, 1991), will be used as the context for discussions on sociality and reproductive suppression in the closely related common (*Cryptomys hottentotus hottentotus*) and highveld (*Cryptomys hottentotus pretoriae*) mole-rats.

Reproductive skew theory strives to pinpoint the major driving force(s) behind social suppression of reproduction, be it the dominant (reproductively active) or the subordinate (reproductively inhibited) group member. There are two main ideas on how reproductive quiescence is orchestrated in social species, namely the Dominant Control Model (DCM) and the Self Restraint Model (SRM). According to the DCM, the dominant female, or queen, controls reproduction in her subordinates through either chemical or behavioural interactions such as aggression and related stress effects, and pheromonal cues that block ovulation (Snowdon, 1996). However, if reproductive inhibition was not in the evolutionary best interest of subordinate individuals, natural selection should encourage helpers to develop mechanisms that allow them to ignore the chemical 'commands' from the queen (Keller and Nonacs, 1993). It thus follows that philopatry and helping behaviour must benefit the subordinate animal to a greater extent than immediate independent reproduction would. Examples of potential benefits are 1) reduced predation risk and increased foraging efficiency and territory quality as a result of increased group size; 2) the helper may increase the future probability of securing its own territory, possibly through colony fission; 3) increased probability of becoming a breeder through coalitions with other helpers or itself being selected as a breeding partner; 4) the experience and skills gained during the helping period may result in increased future fecundity; and 5) the individual may gain indirect fitness benefits by enhancing the production of non-descendent kin (Cockburn, 1998). Instead of chemical manipulators, pheromones may therefore be honest signals from the queen conveying her superior fighting ability and fecundity, to which subordinates respond by not reproducing in order to increase firstly their own, and secondarily the queen's, fitness (Keller and Nonacs, 1993). Similarly, dominance may have evolved as a reliable indicator to reduce the costs of resolving intra-group conflicts (Grafen, 1990). Under conditions where the presence of helpers greatly increases the fitness of the dominant, reproductive concessions may be made to helpers (Clutton-Brock, 1998).

Concession theory includes peace incentives (the level of subordinate reproduction necessary to prevent subordinates from challenging the dominant) and staying incentives (the level of subordinate reproduction necessary to induce subordinates to stay in the natal group; Reeve and Ratnieks, 1993). No evidence of dominant females granting reproductive concessions to subordinate animals could be found in meerkat (*Suricata suricatta*) groups (Clutton-Brock *et al.*, 2001). It is however difficult to discriminate between reproductive concessions and failures of control (Clutton-Brock, 1998).

The SRM does not involve aggression by dominant group members with the resultant suppression of reproduction in subordinates. Non-reproductive females delay independent reproduction until conditions are favourable, for example when unrelated males are present, environmental conditions are better suited, or they have gained the necessary skills to ensure successful independent reproduction (Wasser and Barash, 1983; Snowdon, 1996). The SRM acts on similar principles to that of the seasonal reproductive quiescence displayed by several species in that individuals do not breed during unfavourable environmental conditions. In most seasonal breeders photoperiod may act as the cue that terminates reproductive activity through neural signals from the retina to the pineal gland (Moore, 1978). In subordinate females the presence of a reproductive female may be similarly perceived by the central nervous system and translated into anovulation via neuroendocrine and endocrine pathways of the hypothalamo-pituitary-gonodal axis (Molteno, 1999). Behavioural and endocrine mechanisms that preclude the costs and risks associated with reproduction (courtship, mating, gestation, lactation, care of young) would be evolutionarily favoured if the offspring of subordinate individuals have sufficiently low probabilities of surviving to independence (Creel and Waser, 1997).

Reproductive suppression

According to Abbott (1988) there are three major natural inhibitors of fertility, these being a) the environment through nutritional stress and seasonal breeding; b) lactation; and c) social suppression. Social suppression occurs when an individual's reproduction is inhibited by its interaction with, and by the reproductive behaviour of other individuals (Wasser and Barash, 1983). Dominant females can enforce social infertility in helper females by 1) stress induced anovulation as a result of continual behavioural subordination; 2) interrupting social or sexual behaviour in subordinate females; and 3) direct or indirect interference leading to the early demise of subordinate offspring (Abbott, 1988).

In meerkat groups the dominant female, usually the oldest and heaviest in the group, produces about 80 % of litters and the dominant male sires more than 80 % of her offspring. Control by the dominant female and incest avoidance due to a shortage of unrelated breeding partners contribute to this enormous skew in reproductive output (Clutton-Brock *et al.*, 2001). More extreme physiological mechanisms of reproductive suppression are evident when examining the hypothalamo-pituitary-gonadal axes (HPG axis; Fig. 1.1) of some social species including the Damaraland (Bennett *et al.*, 1996) and naked mole-rats (Faulkes *et al.*, 1990; 1991) and several species of the Callitrichidae (Abbott, 1987). Non-reproductive female marmoset monkeys show several adaptations in behaviour, reproductive neuroendocrinology and non-reproductive physiology to their subordinate social status (Abbott *et al.*, 1998). Non-reproductive female marmoset monkeys show several adaptations in behaviour, reproductive neuroendocrinology and physiology to their subordinate social status (Abbott *et al.*, 1998). In this species, subordinate females display reduced pituitary sensitivity to gonadotropin-releasing hormone (GnRH) compared to dominant

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females, however, the hypothalamo-pituitary-gonadal axis (HPG axis) and ovarian function are rapidly restored when the suppressed females are removed from their social groups and housed singly (Abbott and Hearn, 1978; Abbott *et al.*, 1981; Abbott, 1988). Surprisingly, up to half of subordinate females in their intact natal colonies ovulate, especially in colonies where the mother fail to sustain pregnancies or where the father has been replaced by an unrelated male. This suggests that suppression of sexual behaviour as well as inhibition of ovulation contribute to reproductive failure in subordinate female marmoset monkeys (Saltzman *et al.*, 1997a; 1997b).

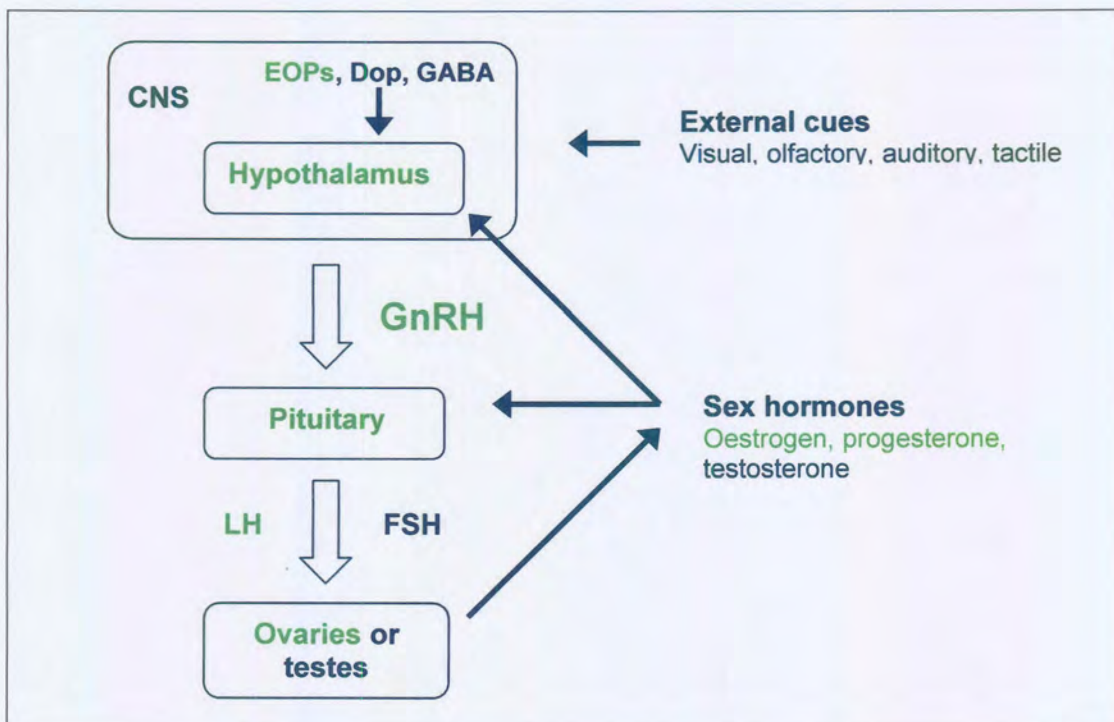

Fig. 1.1 A schematic representation of reproductive control by the hypothalamo-pituitary-gonadal axis. Green, factors investigated in this study; EOPs, endogenous opioid peptides; Dop, dopamine; GABA, γ-aminobutyric acid; CNS, central nervous system; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone (Adapted from Karsch, 1984; Molteno, 1999)

Tactile, pheromonal, visual and/or psychogenic suppressive signals may be passively or actively relayed during social interactions and translated into reproductive inhibition via GnRH-containing neurones in the hypothalamus (White *et al.*, 2002). During embryonic development populations of cells migrate from the olfactory placode to the forebrain where they express GnRH, establishing the HPG axis (Schwanzel-Fukuda *et al.*, 1985), while a second non-placodal population give rise to the posterior GnRH systems (Muske, 1993). The HPG axis revolves around GnRH, a ten-amino acid neuropeptide processed from a 92-amino acid prohormone by neurosecretory cells in the hypothalamus that have their terminals in the median eminence at the floor of the hypothalamus (Seeburg and Adelman, 1984; Eckert *et al.*, 1988). Capillaries within the median eminence converge onto a series of portal vessels that carry blood to the glandular secretory tissue of the anterior pituitary. Here the GnRH peptide interacts with receptor molecules, causing the episodic release of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The production of sex hormones (androgens, oestrogens and progesterone) is under control of LH and FSH, which target mainly the ovaries in females and testes in males (Eckert *et al.*, 1988).

There are several steps in the biochemical processing of GnRH that can influence its regulation of reproductive function, for instance the transcription of GnRH mRNA (messenger ribonucleic acid), the translation of the GnRH prohormone (proGnRH), the processing of proGnRH into the mature GnRH peptide, the transport of the peptide containing vesicles form the cell soma to the fibres, and finally the release of GnRH into the portal system (Tai *et al.*, 1997). The production, storage and release and receptor binding patterns of GnRH and its prohormone can be altered by a multitude of environmental, endocrine and behavioural parameters.

The seasonality of reproductive activity seen in some mammals is mediated by photoperiodic control of the central nervous system via a series of pathways from the retina to the pineal gland, where melatonin production is either stimulated by short day lengths or inhibited by long day lengths (Lincoln and Short, 1980; Bittman, 1984). Most seasonally breeding species such as the white-footed mouse (*Permyscus leucopus*; Glass, 1986) and the Syrian hamster (*Mesocricetus auratus*; Urbanski *et al.*, 1991) display decreased GnRH release accompanied by gonadal regression in response to the short photoperiods and increased melatonin levels of the non-breeding period. The endocrine state of the individual also influences the GnRH system and the steroid hormones oestrogen (oestradiol) and progesterone (Goodman *et al.*, 1982), neurotransmitters including dopamine, serotonin, noradrenalin, adrenalin, γ-aminobutyric acid (Kordon *et al.*, 1994) and the endogenous opioid peptides (EOPs; Brooks *et al.,* 1986; Almeida, 1993) have been implicated in both positive and negative regulation of GnRH synthesis and release. Social interactions such as courting, mating and aggressive behaviours can also influence GnRH synthesis and release (Rissman, 1996; Tai *et al.*, 1997).

It is therefore clear that GnRH is a key reproductive regulator, the complexity of its actions varying greatly between species, endocrine states and social conditions, making it an excellent starting point when looking for a mechanism of physiological reproductive suppression in the subordinate individuals of social species.

Bathyergidae

The family Bathyergidae (Greek: *bathys*, deep and *ergo*, to work) are subterranean hystricomorph rodents that are endemic to the African continent (Skinner and Smithers, 1990). The species comprising the bathyergid family display a wide array

of social structures ranging from solitary, aggressive and xenophobic (*Bathyergus, Georychus; Heliophobius*) to social (*Cryptomys*) and even eusocial (*Heterocephalus glaber, Cryptomys damarensis*; Jarvis and Bennett, 1991; Fig 1.2). The social and eusocial species of the group likewise present a fascinating admixture of strategies used to inhibit reproduction within the non-reproductive colony members (Faulkes and Bennett, 2001). Reproductive suppression in the representative species ranges from obligate incest avoidance amongst sibling colony members as in the Mashona mole-rat, *Cryptomys darlingi* (Bennett *et al.*, 1997), to strictly physiological suppression as is evident in the naked mole-rat, (Faulkes *et al.*, 1990; 1991), to social suppression that incorporates both physiological and behavioural mechanisms as in the Damaraland mole-rat (Bennett *et al.*, 1996). Herbivory and a completely subterranean life-style sets the social mole-rats apart from all other cooperatively breeding vertebrates (Bennett *et al.*, 1999) - including the 35 rodent species, representing nine families, believed to have communal lifestyles (Solomon and Getz, 1997).

The Aridity-Food-Distribution Hypothesis (AFDH; Jarvis *et al.*, 1994) has been put forward to explain the continuum of sociality seen in this remarkable group of rodent moles. According to the AFDH, increased foraging efficiency is the key driving force behind communal living and subsequent cooperative breeding in the social species of mole-rats. Among New World hystricognath rodents, group-living seems to be similarly aimed at reducing burrowing cost rather than reducing predatory risks or extending parental investment (Ebensperger and Cofré, 2001). All members of the family Bathyergidae live in extensive burrow systems excavated while foraging for the geophytes, roots and tubers that make up their diet (Jarvis and Bennett, 1991). Solitary species are confined to mesic areas with frequent and predictable rainfall, where resources are evenly distributed and the soil is easily workable for most of the year. Under such conditions a single animal will be able to find sufficient food to

sustain itself. Arid areas on the other hand are characterised by a patchy distribution of food resources and hard dry soil, restricting energetically expensive digging activity. A communally foraging group would thus be at an advantage when blindly foraging for a localized underground food source. While most solitary species do not occur in arid habitats due to the increased foraging risks (Lovegrove, 1991), social species are not precluded from mesic regions (Jarvis *et al.*, 1994; Faulkes et al., 1997). Molecular data proposes phylogenetic effects, rather than environmental constraints, as an important factor in the evolution of social behaviour in the Cavioidea (Rowe and Honeycutt, 2002). As an alternative to the AFDH, Burda *et al.*, (2000) argue that the preconditions to eusociality in the African mole-rats were a pleisiomorphic monogamous mating system with inherent cooperative broodcare and high genetic relatedness among group members, possibly negating a causal relationship between cooperative foraging for patchily distributed food sources and the evolution of (eu)sociality. However, Burland *et al.*, (2002) found that eusociality, as seen in the Damaraland mole-rat, can occur at normal familial levels of relatedness.

The cryptomids are the most species rich and widely distributed genus of African mole-rats occurring from central Africa through to the south-western tip of South Africa (Honeycutt *et al.*, 1991; Faulkes *et al.*, 1997; Bennett and Faulkes, 2000). The wide array of social structures displayed and environments inhabited by the species and subspecies belonging to the genus present us with an unparalleled opportunity to study the mechanisms driving the evolution of sociality among vertebrates. The most widely studied member of the genus is the eusocial Damaraland mole-rat. Typically, only one female and one or two males in each colony produce offspring of their own (Bennett and Jarvis, 1988). Non-reproductive female Damaraland mole-rats are rendered physiologically infertile while males of a similar social status are behaviourally restrained from breeding without any apparent physiological

manifestation of reproductive suppression (Fig. 1.2; Bennett, 1994). Anovulation in subordinate females, coupled with strict incest avoidance produce the large reproductive skew evident in colonies of the Damaraland mole-rat (Jarvis and Bennett, 1993).

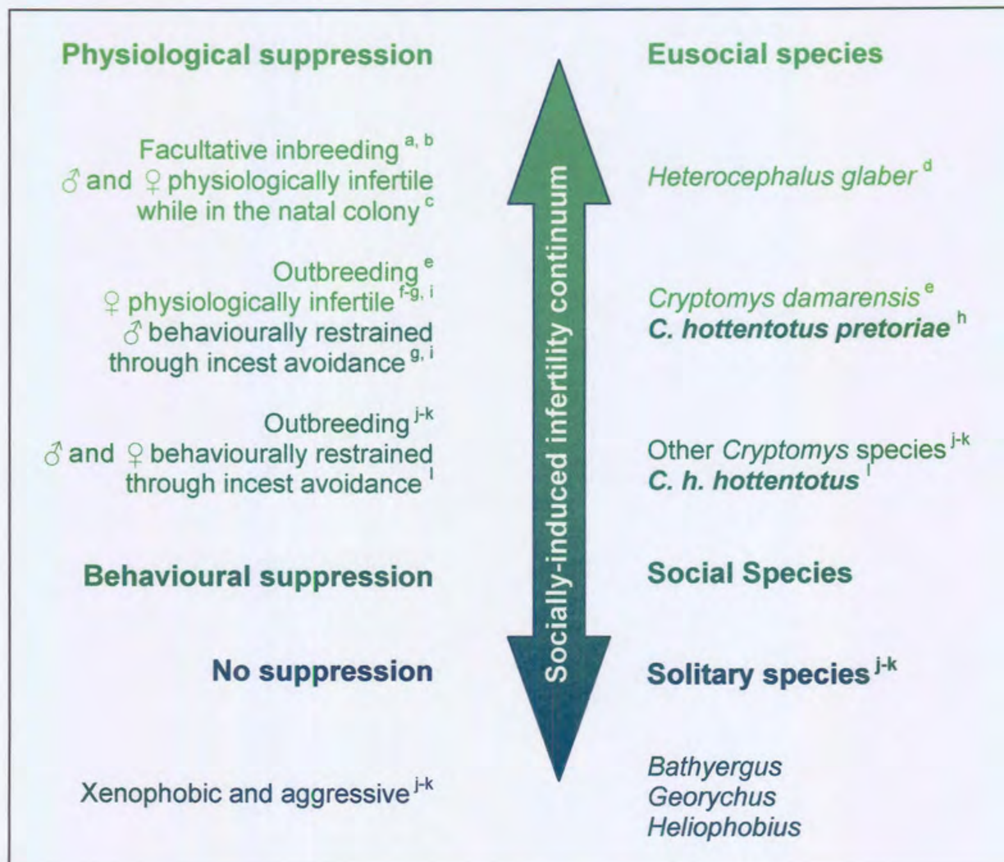

Figure 1.2 Species belonging to the family Bathyergidae grouped according to the degree of sociality displayed. [a] Reeve *et al.*, 1990; [b] Braude, 2000; [c] Faulkes *et al.*, 1990, 1991; [d] Jarvis, 1981; [e] Jarvis and Bennett, 1993; [f] Bennett *et al.*, 1996; [g] Bennett, 1994; [h] Moolman *et al.*, 1998; [i] Van der Walt *et al.*, 2001; [j] Jarvis and Bennett, 1991; [k] Bennett and Faulkes, 2000; [l] Spinks *et al.*, 1997, 1999, 2000a. ♂ male; ♀, female; *C., Cryptomys; h., hottentotus*

Common and highveld mole-rats

Common and highveld mole-rats are phylogenetically closely related subspecies (Plate 1.1; Faulkes *et al.*, 1997), and although both are unique among social bathyergids in being seasonal breeders (Bennett, 1989; Jarvis and Bennett, 1991; Moolman *et al.*, 1998; Janse van Rensburg *et al.*, 2002), the mechanisms of reproductive suppression operating within the subspecies appear to differ.

The common mole-rat has the widest habitat range of any bathyergid, occurring in both semi-arid and mesic winter rainfall regions (Skinner and Smithers, 1990). The colonies used in this study were obtained from a semi-arid habitat (Kamieskroon, Northern Cape Province, South Africa). A single breeding male and female, living in a colony made up almost entirely of their own offspring, produce a maximum of two litters in the Southern hemisphere summer (late November to January; Bennett, 1989; Spinks *et al.*, 1997, 1999). Common mole-rat colonies consist of five to fourteen animals and are transiently social, with a nearly linear dominance hierarchy and no distinctive working groups (Bennett, 1989). Subordinate males and females are not physiologically suppressed from reproducing, but behaviourally restrained as a result of the strict incest avoidance operational in all *Cryptomys* species (Spinks *et al.*, 1997, 2000a). Basal and GnRH challenged plasma LH levels are similar when compared between dominant and subordinate males and females respectively (Spinks *et al.*, 2000a). Ovulation in reproductive common mole-rat females is induced by mating (Spinks *et al.*, 1999), while the ovaries of non-reproductive females are quiescent, but 'primed for action' (Faulkes and Bennett, 2001). Although reproductive activity is seasonally restricted, male and female common mole-rats maintain physiological reproductive function out of the breeding period (Spinks *et al.*, 1997, 2000a). This pattern is unusual for seasonal mammals,

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and may facilitate intersexual recognition, pair-bond formation and ultimately outbreeding during the dispersal period of subordinate animals (Spinks *et al.*, 1997).

The highveld mole-rat occurs in the summer rainfall regions of the Northern provinces of South Africa. Highveld mole-rats are transiently social with a non-linear dominance hierarchy and no distinctive worker groups (Moolman *et al.*, 1998). Procreation in this mole-rat subspecies is restricted to the dry winter months and offspring are produced from April to July with a possible second litter in September (Janse van Rensburg *et al.*, 2002). Subordinate female highveld mole-rats show reduced pituitary sensitivity in that GnRH challenged LH levels are significantly lower in subordinate than in reproductive females. In addition, the ovaries of subordinate females are under-developed and lack the corpora lutea indicative of ovulatory activity. Non-reproductive male highveld mole-rats, on the other hand, are not physiologically suppressed from reproduction, with basal and GnRH challenged LH levels as well as gonadal anatomy similar to that of reproductive males (Van der Walt *et al.*, 2001; Janse van Rensburg *et al.*, 2003). A recent study on the genetic relatedness within highveld mole-rat colonies revealed that although all the litters in a colony are offspring of the reproductive or dominant female, paternity is shared between several males, not all of whom are present in the colony (Malherbe, 2001). The additional physiological component to social infertility in female highveld mole-rats may be in place to protect the dominant position and reproductive success of the queen in the event of an unrelated male entering the colony and overriding incest taboos (Van der Walt *et al.*, 2001).

Plate 1.1 The common mole-rat, *Cryptomys hottentotus hottentotus*

Aims of study

Common and highveld mole-rats will be used as models in the quest to elucidate the differential mechanisms of social and seasonal reproductive suppression evident in the Bathyergidae. The close phylogenetic relationship between the two subspecies, coupled with many similarities in reproductive and social biology, make them ideal candidates for this study. The fact that physiological suppression of reproduction is evident in one of the subspecies and not the other, may help us to set apart changes in the GnRH neuronal system of subordinate animals that could possibly be attributed to physiological social infertility as opposed to seasonal reproductive inactivity.

Following the exposition of the materials and methods in Chapter 2, Chapter 3 focuses on the effect of social status and season on the GnRH neuroanatomy and neuroendocrinology of reproductive and non-reproductive male and female common and highveld mole-rats. Brain GnRH levels and numbers, morphology and distribution of GnRH cell bodies will be quantified and compared between relevant groups. In Chapter 4 the role of endogenous opioid peptides and gonadal steroid hormones in the physiological reproductive suppression of subordinate highveld mole-rat females at the level of the pituitary is investigated. An EOP antagonist as well as exogenous GnRH will be used to probe the HPG axis of reproductive and non-reproductive (intact and ovariectomized) female highveld mole-rats. Chapter 5 looks at the nucleotide and amino acid sequence of mole-rat GnRH and compares it to that of classic 'mammalian' GnRH and guinea pig GnRH. Chapter 6 synthesises the findings of this study with existing knowledge on social suppression within the family Bathyergidae and proposes new lines of thought and additional fields of study

to broaden our understanding, especially of the physiological suppression evident in the subordinate female highveld mole-rat.

Chapter 2

Materials and methods

Study animals

Trapping and maintenance

Modified Hickman live-traps baited with sweet potato, carrots or apple were used to trap individual mole-rats (Hickman, 1979). Common mole-rats (*Cryptomys hottentotus hottentotus*) were collected from residential properties in Kamieskroon (30 °S, 17 °E, Northern Cape Province, RSA) and from the farm Kardou on the outskirts of the town. Highveld mole-rats (*Cryptomys hottentotus pretoriae*) were trapped on the Monumentpark golf course, residential properties (Mooikloof equestrian estate and Woodhill golf estate) and agricultural land (University of Pretoria experimental farm and a smallholding near the Rietvleidam) in the Pretoria area (26 °S, 28 °E, Gauteng Province, RSA). For each subspecies between 15 and 18 entire colonies were trapped both during and out of the breeding seasons of 2000 to 2002. The breeding season refers to the time period when reproductive and sexual activity in the form of mating, gestation, parturition and lactation takes place. In the common mole-rat pups are born from late November to January (Spinks *et al.*, 1997, 1999), while the highveld mole-rat breeds from April to July with a possible second litter in September (Janse van Rensburg *et al.*, 2002).

Each colony was housed separately in a plastic container (1 m x 0.5 m x 0.5 m) in climate controlled rooms at the University of Pretoria (25 ± 1 °C; 50 % humidity; 12 h light: 12 h dark) for a maximum period of three weeks after capture. Untreated wood shavings were available as nesting material. The mole-rats were fed on a variety of freshly chopped fruits and vegetables on a daily basis. No free water was provided since mole-rats obtain a positive water balance from their food source.

Experimental groups

Study animals were allocated to a specific experimental group based on their sex and social status (reproductive or non-reproductive) and whether they were collected and sacrificed or experimentally treated during the breeding or non-breeding period of their species (Table 2.1).

Table 2.1 Numbers of common (*Cryptomys hottentotus hottentotus*) and highveld (*Cryptomys hottentotus pretoriae*) mole-rats collected per experimental group. Br, breeding season; N-Br, non-breeding season; RM, reproductive male; NRM, non-reproductive male; RF, reproductive female; NRF non-reproductive female

	Common mole-rat		Highveld mole-rat	
	Br	*N-Br*	*Br*	*N-Br*
RM	13	10	11	15
NRM	21	20	17	12
RF	11	9	8	19
NRF	15	20	21	36

The reproductive status of females was readily discernable by the presence of prominent axillary teats, a perforated vagina, placental scars on the uterine horns and, during the breeding season, foetuses in reproductive females. Male mole-rats were not as easy to assign reproductive status. Reproductive males were positively identified from pairs and in colonies by their involvement in copulation. Reproductive males can also be categorised by the possession of prominent abdominal testes and a stained region around the muzzle (Bennett, 1989; Spinks *et al.*, 2000a; Janse van Rensburg *et al.*, 2002).

Data, when available, were compared intra-subspecifically with regard to sex, social status and breeding season. Inter-subspecifically, animals of the same sex, social group and season were compared.

Body weight

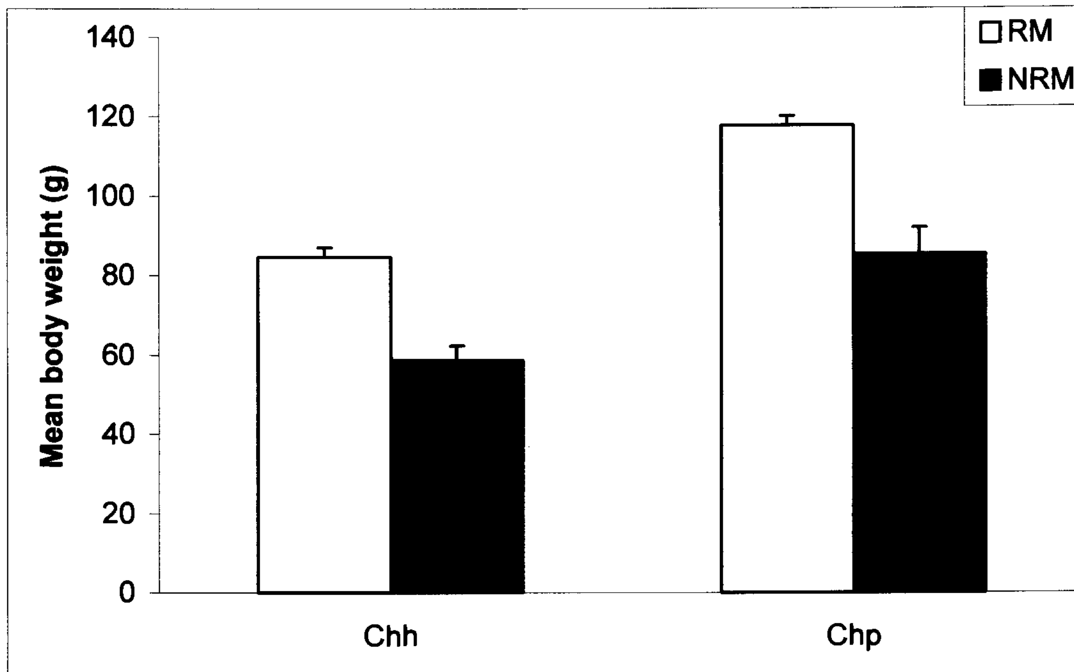

Fig. 2.1 Mean (g ± sem) body weight of reproductive and non-reproductive male common and highveld mole-rats. Chh, common mole-rat (*Cryptomys hottentotus hottentotus*); Chp, highveld mole-rat (*Cryptomys hottentotus pretoriae*); RM, reproductive male; NRM, non-reproductive male

Subspecies and reproductive status significantly influenced body weight in male common and highveld mole-rats (Kruskal Wallis analysis: $H = 74.7$, $p < 0.001$, $n = 118$). Reproductive male common mole-rats (85 ± 2 g) were significantly heavier than non-reproductive male common mole-rats (59 ± 2 g; Fig. 2.1). Similarly, reproductive male highveld mole-rats (118 ± 4 g) were significantly heavier than non-reproductive male highveld mole-rats (85 ± 7 g). Reproductive and non-reproductive

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male common mole-rats weighed significantly less than reproductive and non-reproductive male highveld mole-rats, respectively.

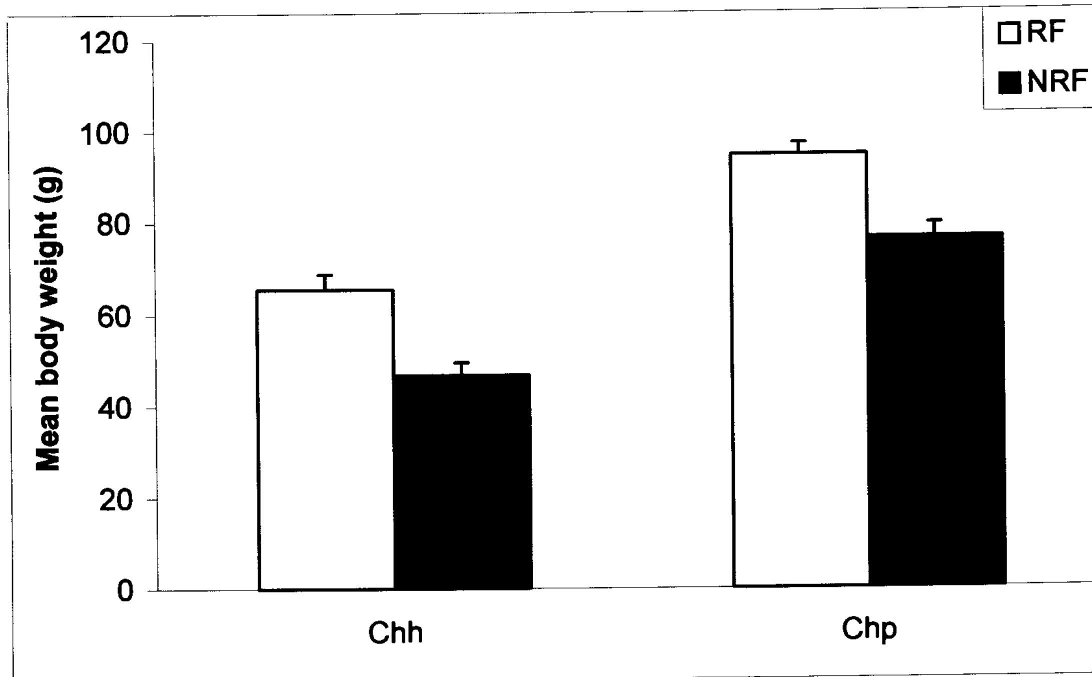

Fig. 2.2 Mean (g ± sem) body weight of reproductive and non-reproductive female common and highveld mole-rats. Chh, common mole-rat (*Cryptomys hottentotus hottentotus*); Chp, highveld mole-rat (*Cryptomys hottentotus pretoriae*); RF, reproductive female; NRF, non-reproductive female

Subspecies and reproductive status significantly influenced body weight in female common and highveld mole-rats (Kruskal Wallis analysis: $H = 72.0$, $p < 0.001$, $n = 132$). Reproductive female common mole-rats (65 ± 3 g) weighed significantly more than non-reproductive female common mole-rats (47 ± 2 g; Fig. 2.2). Similarly, reproductive female highveld mole-rats (95 ± 3 g) weighed significantly more than non-reproductive female highveld mole-rats (77 ± 3 g). Reproductive and non-reproductive female common mole-rats had a significantly lower mean body weight than reproductive and non-reproductive female highveld mole-rats.

Forebrain weight

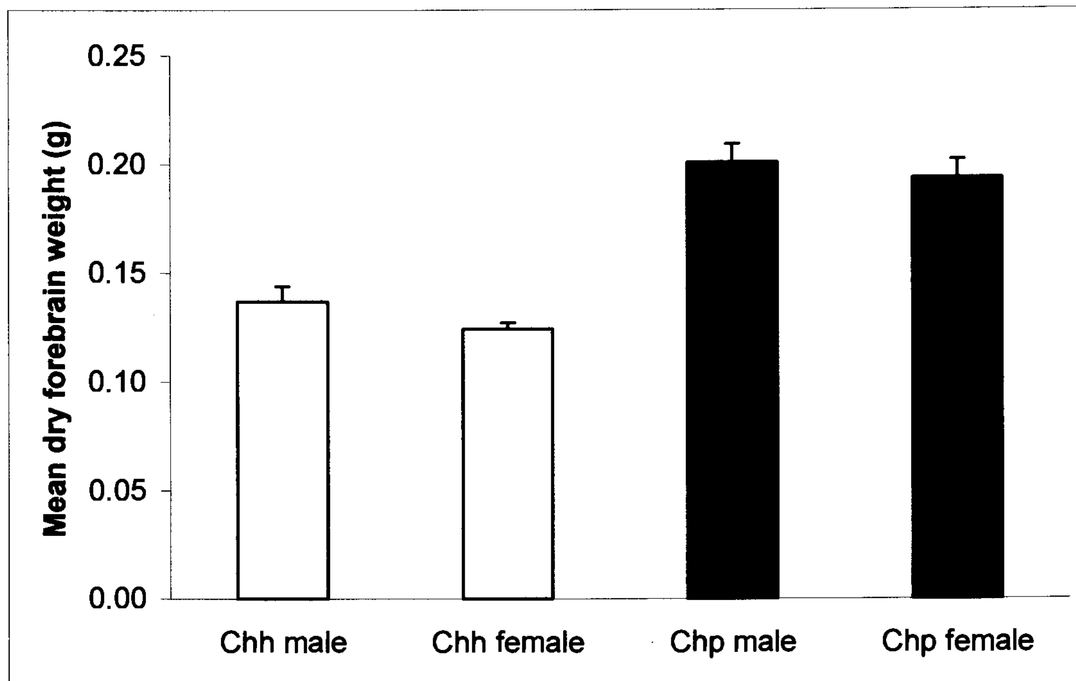

Fig. 2.3 Mean (g ± sem) dry forebrain weight of male and female common and highveld mole-rats. Chh, common mole-rat (*Cryptomys hottentotus hottentotus*); Chp, highveld mole-rat (*Cryptomys hottentotus pretoriae*)

The weight of the forebrain was determined following freeze drying and the removal of the olfactory bulbs and the cerebellum. The mean forebrain weight of male common mole-rats (0.14 ± 0.01 g) did not differ significantly compared to that of female common mole-rats (0.12 ± 0.00 g; t-test: $t = 1.882$, $p > 0.05$, $n = 6$ & 9; Fig. 2.3). Likewise, the mean forebrain weight of male highveld mole-rats (0.20 ± 0.01 g) was similar compared to that of female highveld mole-rats (0.19 ± 0.01 g; t-test: $t = 0.604$, $p > 0.05$, $n = 11$ & 12; Fig. 2.3). Common mole-rats had a significantly lower mean forebrain weight compared highveld mole-rats (t-test: $t = 8.633$, $p < 0.001$, $n = 15$ & 23).

Experimental procedures

Blood sampling

Individual animals, in small buckets with wood shavings and food, were placed in a temperature regulation chamber (38 °C for 25 minutes) causing vasodilatation, facilitating blood collection. Animals were hand restrained while between 300 μl and 400 μl whole blood was collected by venepuncture of the saphenic vein in the hind foot. Blood was collected in heparinized microhaematocrit capillary tubes, and kept at 4 °C for less than 30 minutes prior to centrifugation (eight minutes at 2 500 rpm; revolutions per minute). Plasma was stored at –70 °C until assayed.

Hormone and antagonist administration

Synthetic mammalian GnRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) was prepared on a Beckman System 990 peptide synthesizer by R Milton (Department Chemical Pathology, University of Cape Town, RSA) using conventional solid phase methodology and purified (> 96 %, at 210 nM) by preparative C-18 reverse phase high performance liquid chromatography. Both GnRH and naloxone (N-allyl-noroxymorphone-hydrochloride; Sigma Chemical Company, USA) were dissolved in sterile physiological saline and stored in aliquots at –20 °C until required. GnRH and naloxone were administered subcutaneously in 200 μl bolus injections made up with physiological saline. Basal and hormone and/or antagonist challenged luteinizing hormone (LH) levels were determined. Control animals were injected subcutaneously with sterile physiological saline (0.9 % NaCl w/v).

Hormone assays

Luteinizing hormone bioassay

LH levels were determined by an *in vitro* bioassay based on the production of testosterone by dispersed mouse Leydig cell preparations (Van Damme *et al.*, 1974). This method measures only biologically active LH and not structural LH, making it more precise than the conventional LH radioimmunoassay.

Leydig cell preparation. An incubation medium was prepared by combining 12 ml Eagle's Basal Medium (Highveld Biological, RSA), 2.1 ml $NaHCO_3$ and 2 ml foetal calf serum (Highveld Biological, RSA) in 100 ml distilled water. The incubation medium was placed on ice and slowly gassed for ten minutes with Carbogen 5 (95 % O_2: 5 % CO_2; Afrox, RSA). A six week old male mouse (NMR strain; South African Vaccine Producers, RSA) was sacrificed by cervical dislocation, the testes removed, decapsulated and minced roughly in 5 ml incubation medium. The minced cells were mixed on a magnetic stirrer for five minutes at 4 °C and filtered through nylon gauze. The cell suspension was incubated for one hour under Carbogen 5 in an agitating water bath at 34 °C. The incubated cell suspension was centrifuged for five minutes at 4 °C (2 500 rpm), decanted and resuspended in 10 ml incubation medium. The process was repeated, after which the cell suspension was slowly mixed on a magnetic stirrer for five minutes. The cells were counted on a haemocytometer (WSI, depth 0.1 mm, 1/400 mm^2) using a phase contrast microscope. The cell suspension was made up so that the number of cells counted corresponded to the final cell suspension volume in millilitres. The cell suspension was stirred slowly for five minutes. A volume of 200 µl of the cell suspension was

added to standards, controls and samples and incubated for three hours under a stream of Carbogen 5 gas and agitated in a water bath set at 34 °C.

Bioassay. LH buffer was prepared containing 0.29 g $NaH_2PO_4.2H_2O$, 2.9 g $Na_2HPO_4.12H_2O$ and 4.38 g NaCl, made up to one litre in deionised water (dH_2O) with 0.1 % bovine serum albumin (BSA, Merck, Germany). Mole-rat plasma (25 μl) was assayed at a 1: 20 dilution in LH buffer. A standard curve was prepared using serial doubling dilutions of synthetic LH (2nd international standard 1988, Code 80/552, N.I.B.S.C., UK; Storring and Gaines Das, 1993) in LH buffer over the range 360 $\mu IU.100\mu l^{-1}$ to 1.4 $\mu IU.100\mu l^{-1}$. The bioassay tubes contained 100 μl sample, standard, quality control or LH buffer (to obtain an estimate of total binding). Standards and total binding were assayed in triplicate while samples and quality controls were assayed in duplicate. Diluted cell suspension (200 μl) was added to each assay tube and incubated in Carbogen 5 for three hours in a shaking water bath at 34 °C. Testosterone production by the Leydig cells was terminated by placing the assay tubes in a water bath at 100 °C for 15 minutes, thereby killing the cells. Tubes were placed on ice and 0.3 ml phosphate buffered saline with 0.1 % gelatine was added to each tube.

Testosterone radioimmunoassay. The amount of testosterone produced by the Leydig cells in response to LH stimulation was measured by assaying 100 μl sample from each bioassay tube, in duplicate. Estimates of total counts (TC) and non-specific binding (NSB) were assayed in triplicate. The serial dilutions of LH standard and the resultant testosterone production served as the testosterone standard curve. Testosterone antiserum in phosphate buffer (100 μl) at a working dilution of 1: 800 was added to standards, reagent blanks and samples and the contents of the tubes mixed. This was followed by the addition of [1, 2, 6, 7^{3}H] testosterone TRK 402

(sp. act. 80-105 Ci/mmol; Radiochemical Centre, Amersham Biosciences, UK) in assay buffer (10 000 cpm; counts per minute). The content of each tube was mixed and incubated overnight at 4 °C. Bound and free testosterone were separated using the charcoal separation technique by adding 750 µl dextran-coated charcoal (Norit A charcoal 1 g and 0.1 g Dextran T-40 in 400 ml assay buffer) to each tube, and incubating the tubes for 12 minutes at 4 °C. The tubes were centrifuged for 15 minutes at 4 °C (2 500 rpm) and each tube was decanted into a scintillation vial with 4 ml scintillation fluid (Ultima Gold™, Analytical and Diagnostic Products, RSA). The vials were vortexed for one minute and left for two hours before counting. Each vial was counted for two minutes in a Hewlett Packard Tricarb 1500 liquid scintillation counter.

Validation of assay. The bioassay has been previously validated for the highveld mole-rat by Van der Walt *et al.* (2001) and for the common mole-rat by Spinks *et al.* (2000a). A volume of 50 µl of a known plasma pool with a high LH content was double-diluted over the range of the standard curve. When compared to the standard curve, these double-dilutions were parallel to, and not significantly different from the reference preparation (ANCOVA, $F_{2, 8} = 2.7$, $p > 0.05$). The sensitivity of the assays (determined at 95 % binding) was 10.3 µIU/tube or 2.1 mIU/ml. Intra- and interassay coefficients of variation for repeated determinations of a quality control were 3.9 % and 16.7 %, respectively.

GnRH radioimmunoassay

For the purpose of measuring the GnRH content, snap frozen cerebral tissue was used. Animals were deeply anaesthetised using Halothane (Zeneca, RSA). After the mole-rat had been weighed, the head was severed from the body, and the cranium

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opened to remove the entire brain from the skull. The brain was frozen on dry ice within five minutes from initiating anaesthesia, and stored at –70 °C until being processed. The protocol for GnRH extraction and radioimmunoassay (RIA; King and Millar, 1986) as validated for the Damaraland mole-rat by Molteno (1999) was modified and used in this study.

Iodination of GnRH. Synthetic mammalian GnRH (mGnRH; prepared as described earlier) was iodinated using the chloramine T method (Greenwood *et al.,* 1963). Five micrograms of the peptide was dissolved in 40 µl of a 0.5 M NaH_2PO_4 solution (pH 7.6). Ten microlitres ^{125}I (1mCi) was added, followed by 10 µl chloramine T (300 ng) in NaH_2PO_4. After two minutes 470 µl 0.1 M NaH_2PO_4 was added. The labelled GnRH was eluted with the use of a Sephadex column (Amersham Biosciences, UK), separating free and incorporated ^{125}I by size. As the GnRH was eluted, 2 ml fractions were collected. Fractions yielding the highest levels of radioactivity (measured as cpm) were used in the assays (fraction 24-27, Fig. 2.4). The relevant fractions were frozen and stored at –70 °C until required.

GnRH extraction. Prior to RIA, the brain tissue was lyophilised overnight. From each brain the olfactory bulbs and cerebellum were removed. The remaining tissue, inclusive of the preoptic area and hypothalamus, was weighed, minced with a sterile blade and homogenised for 30 seconds in 5 ml of a 2 N acetic acid solution with 0.001 M phenyl methane sulfonyl fluoride to block proteinase activity. The homogenate was centrifuged for 60 minutes at 4 °C (14 000 rpm). The supernatant was frozen and dried overnight. Two millilitres gel-phosphate buffered saline, or gel-PBS (31.2 g $NaH_2PO_4.2H_2O$, 45 g NaCl, 18.6 g ethylenediamine tetra-acetic acid, 0.05 g phenol red, 5 g NaN_3, made up to 5 000 ml, with 0.1 % gelatine (w/v) and 0.1

% Triton (v/v)) was added. The suspension was sonicated for 30 seconds and centrifuged for three minutes at 10 000 rpm. The supernatant was assayed.

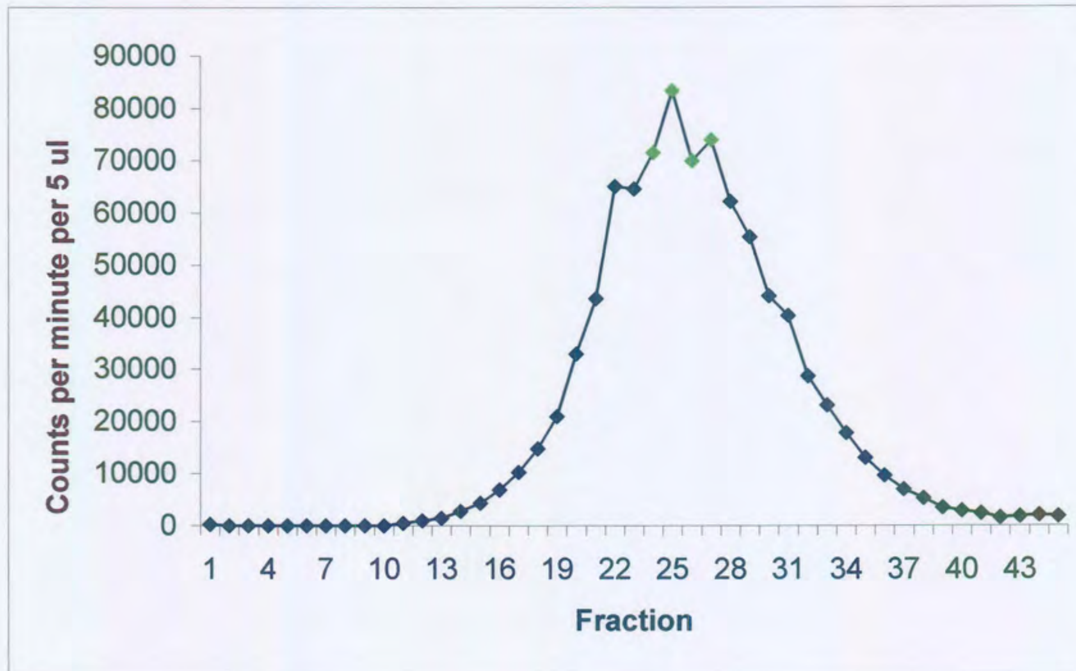

Fig. 2.4 Radioactivity (counts per minute) measured in 5 μl of each fraction of eluted iodinated mGnRH (mammalian gonadotropin releasing-hormone) separated on a Sephadex column. Fractions 24–27 (shaded green) were used in the GnRH radioimmunoassays

Radio immunoassay. GnRH antiserum 1076 against mammalian GnRH was used in preference to GnRH antiserum 678 (used by Molteno (1999) on the Damaraland mole-rat), since the former specifically binds the mammalian variant of GnRH while antiserum 678 cross-reacts with all known forms of GnRH, requiring both the NH_2 and COOH-termini for binding (King *et al.*, 1994a). Synthetic mGnRH was used as standard, and ^{125}I-mGnRH as tracer. A standard curve was generated using serial doubling dilutions of mGnRH ranging in concentration from 10 pg/μl to 0.01 pg/μl (Fig. 2.5).

The radioactive tracer was used at approximately 15 000 cpm per 100 µl in a total assay volume of 500 µl. The GnRH standard preparations were assayed in triplicate and the sample extracts in duplicate. The assay was incubated overnight at 4 °C. Bound and free peptide were separated using 1 ml activated Dextran-coated charcoal per assay tube. After 20 minutes at 4 °C, the tubes were centrifuged at 6 000 rpm for 40 minutes. The supernatant containing the bound fraction was decanted and counted for one minute on a gamma counter.

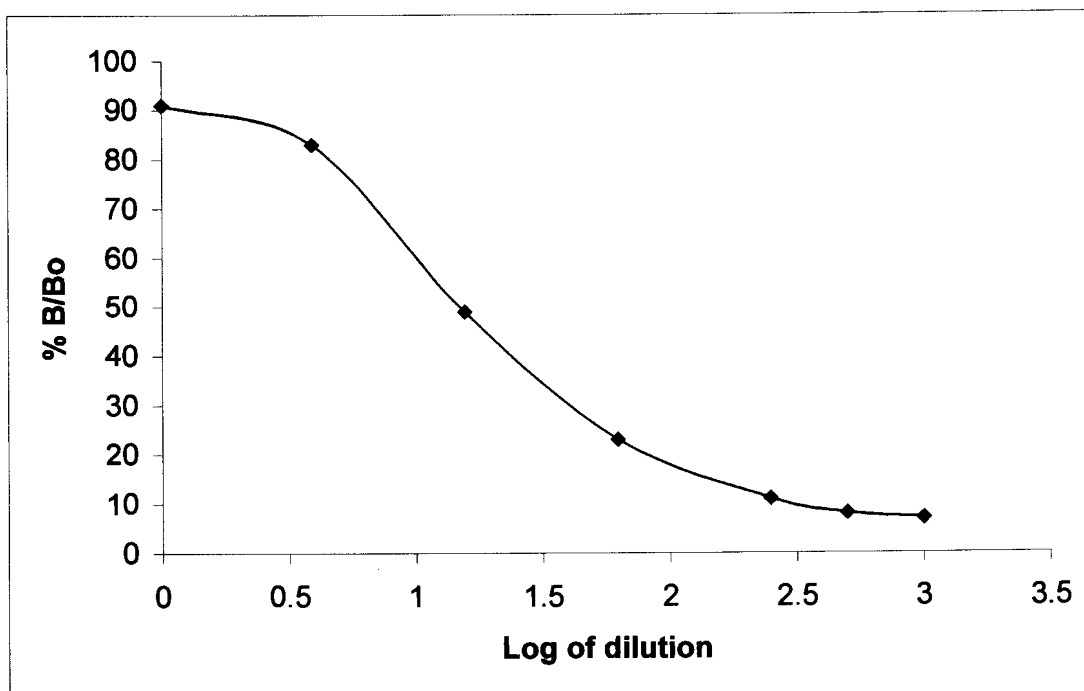

Fig. 2.5 A standard curve of synthetic mGnRH (mammalian gonadotropin releasing-hormone). Reference GnRH amounts (pg/µl) are plotted on a logarithmic scale against the logit of % B/Bo. B, binding; Bo, total binding

Protein assay. The results from the GnRH RIA were expressed as picogram GnRH per microgram protein (pg/µg). The protein concentration of each individual brain extract sample was determined with the use of the Bradford protein assay (Bradford, 1976). Zero, 2, 4, 6, 8, 10, 12, 14 and 16 µl BSA (1 mg/ml) was pipetted into

assigned wells of a 96-well microtiter plate to make up a standard curve (Fig. 2.6). The unknown samples (20 μl) were pipetted into individual wells. Bradford reagent (40 μl) was added to all standard and sample wells, and deionised water was added to all wells to bring the final volume to 200 μl. After a five minute incubation period, the absorbance was read with a 595 nm measurement filter. The sensitivity of the assays (determined at 95 % binding) was 4.4 μg/well. Intra- and interassay coefficients of variation for repeated determinations of a quality control were 5.6 % and 16.6 %, respectively.

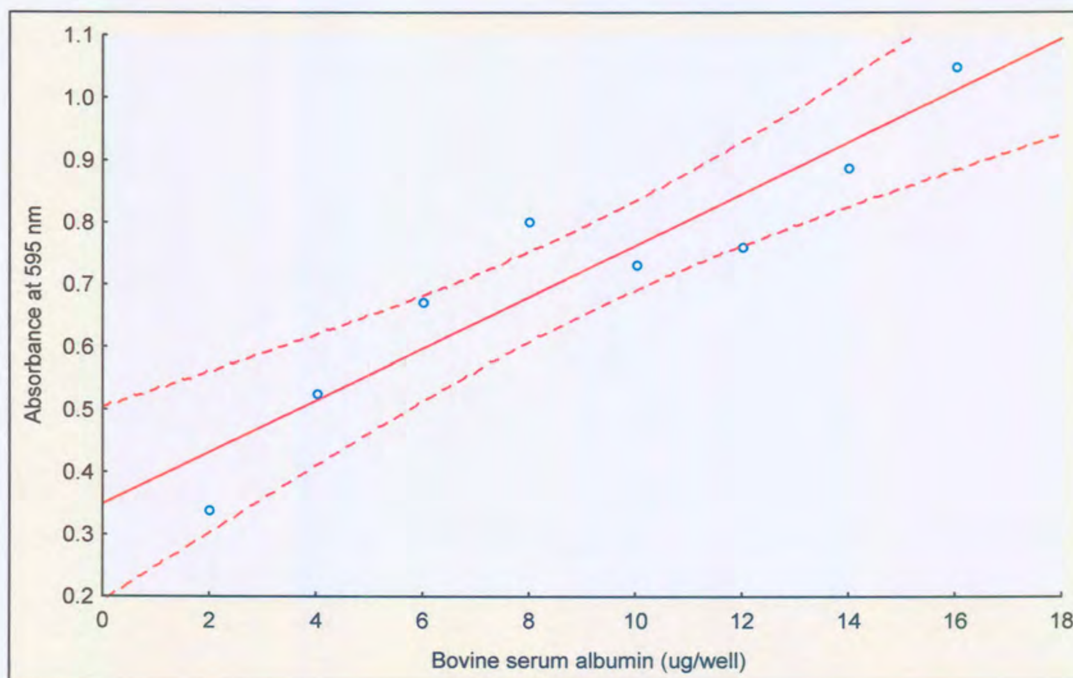

Fig. 2.6 A standard curve of protein measured (μg BSA/well) against absorbance at 595 nm. BSA, bovine serum albumin

Validation of GnRH assay. A test for parallelism between serial doubling dilutions of synthetic mGnRH standard and mole-rat brain extract was used to assess the suitability of the GnRH radioimmunoassay for use in the Damaraland mole-rat. When tested for parallelism, the slopes of the two curves were not significantly different (ANCOVA $F_{2,14} = 0.14$ $p > 0.05$; Molteno, 1999). Based on the validity of the

assay for use in the Damaraland mole-rat, the assay was assumed to be suitable for use in the closely related common and highveld mole-rats. The sensitivity of the assay (determined at 95 % binding) was 1.3 pg/tube. The intra-assay coefficient of variation for repeated determinations of a quality control was 3.7 %.

Histology

GnRH immunocytochemistry

Animals were weighed and deeply anaesthetized using Halothane (Zeneca, RSA) before the thoracic cavity was opened. Mole-rats were perfused through the aorta with approximately 100 ml 0.1 M PBS (1 g $MgCl_2$, 10.17 g NaH_2PO_4, 61 g Na_2HPO_4, 25 g NaCl, made up to 1 000 ml), followed by the same amount of 4 % paraformaldehyde (PFA; w/v) in 0.1 M PBS. Solutions were filtered and chilled to 4 °C prior to use. The heads were removed and immersion fixed in 4 % PFA. Fixed cerebral tissue was placed in a 30 % sucrose solution for 48 hours or until they sank, quick frozen on dry ice and sectioned at 25 μm in the coronal plane using a cryostat (Bright Cryostats, UK). Every sixth section was used. The sections were pre-treated with 0.5 % Triton X-100 (v/v; BDH Chemical Company, UK), followed by 0.02 % hydrogen peroxide (v/v) and then 2 % normal donkey serum (v/v) in PBS. After pre-treatment, the sections were incubated in the primary antibody, Incstar rabbit anti-LHRH (Incstar Corporation, USA), at a dilution of 1: 80 000 (4 °C, 48 hours). The secondary antibody, Biotin-SP conjugated AffiniPure donkey anti-rabbit IgG (1: 500; Jackson Immunoresearch Laboratories Inc., USA), was applied for one hour after a two hour wash in PBS. GnRH-immunoreactive (GnRH-ir) cell bodies and fibres were visualised by a blue-black reaction product formed by a 13 minute exposure to 0.05 % nickel-diaminobenzidine (w/v; 400 ml Tris buffer (Trizma 7.6, Sigma Chemical

Company, USA), 200 mg diaminobenzidine, 20 ml NH_4NiSO_4, 70µl 30 % H_2O_2 (v/v)). All the sections for a given comparative group were processed in parallel. Sections were mounted on non-coated slides, cover slipped and viewed under a light microscope.

Analysis. For each section extending from the rostral preoptic area to the posterior hypothalamus the total number of GnRH-ir cell bodies was counted. Brain tissue from common and highveld mole-rat females trapped out of the breeding season was used to map the distribution of GnRH perikarya as well as any GnRH-ir fibres on an image of each section. GnRH-ir cell bodies were classified according to the number of fibre projections from the perikaryon: bipolar cell bodies with two projections, one on each end of the spindle shaped cell body; unipolar cell bodies with one projection; and apparently nonpolar cell bodies without visible projections. Image analysis software (ImageJ version 1.30, National Institutes of Health, USA) was used to determine the size of the perikaryon of approximately 20 randomly distributed GnRH cells for each animal (Robinson *et al.*, 1997); additionally the optical density of GnRH-ir staining in the median eminence was quantified the as mean area occupied by GnRH-ir structures (Molteno *et al.*, 2004).

Oestrogen receptor immunocytochemistry

The immunocytochemical protocol to visualize oestrogen receptors (ER) is identical to the protocol for visualizing GnRH containing structures, using specific antibodies against rat oestrogen receptors. Primary antibodies against ER-α receptors (Rabbit poly-clonal Cl355, 1: 60 000, Upstate Biotechnology, USA; Zymed rabbit anti-ER-α D79, 1: 2000, Zymed Company, USA; Rabbit poly-clonal ER715, 1: 1000, National hormone and peptide programme, USA) and ER-β receptors (Zymed rabbit poly-

clonal anti-ER-β Z8P, 0.25 μg/ml, Zymed Company, USA) were applied to mole-rat tissue, and rat tissue as controls. Biotin-SP conjugated AffiniPure donkey anti-rabbit IgG (1:1000; Jackson Scientific, USA) was used as secondary antibody. Rat brain tissue stained appropriately for both oestrogen receptors-α and -β (Plate 2.1), while no staining was visible in parallel processed mole-rat tissue. It is possible that oestrogen receptors in mole-rat tissue are structurally divergent from those found in rat tissue, causing antisera against rat oestrogen receptors not to recognise the oestrogen receptors in mole-rat tissue.

***In situ* hybridisation**

Animals were weighed and deeply anaesthetized using Halothane (Zeneca, RSA) before the thoracic cavity was opened. Mole-rats were perfused, the brain removed and the cerebral tissue cut using a cryostat as described for the GnRH immunocytochemistry protocol.

Pretreatment. Sections were mounted from elvanol on gelatine-coated (0.25 % w/v) glass slides. The *in situ* hybridisation protocol as described by Nagaso *et al.* (2001) was modified and used in this study. The tissue was rehydrated by immersing the slides in 100 % ethanol, 50: 50 ethanol-xylene, 100 % ethanol, and subsequent decreasing concentrations of methanol (80 %, 50 %, 25 %; v/v). Sections were rinsed in DEPC-H_2O (0.1 % diethylpyrocarbonate; v/v) and treated with 80 % acetone (v/v) at -20 °C for ten minutes. The tissue was then treated with 0.1 % Tween-20 (polyoxyethylene sorbitan monolaureate; v/v) and 0.1 % Triton X-100 (octyl phenol ethoxylate; v/v), 4 % PFA (w/v), phosphate buffered saline and rinsed in DEPC-H_2O before being dehydrated using ascending concentrations of ethanol up to 100 %, air dried and stored until hybridisation.

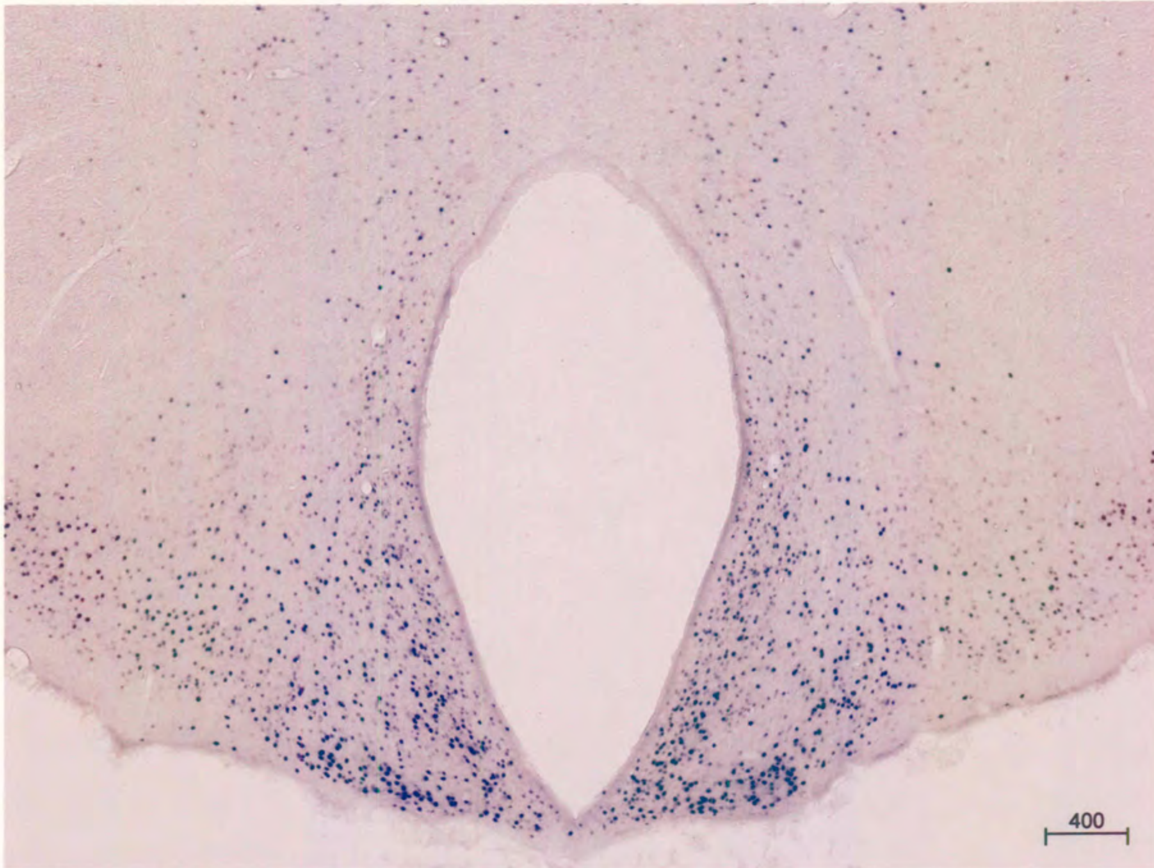

Plate 2.1 Immunocytochemical oestrogen receptor staining in control rat sections in the area of the periventricular nucleus

Hybridisation. The riboprobe (70 µl; against glutamic acid decarboxylase (GAD-65), rat GnRH mRNA or guinea pig GnRH mRNA) in hybridisation buffer (50 % deionised formamide (v/v), 20 % 20 x saline-sodium citrate buffer (SSC; v/v), 1 % Dextran sulphate (w/v), 0.3 % sodium dodecyl sulphate (SDS; v/v), 0.002 % Denhardt's solution (v/v), 0.004 % heparin (v/v), 0.005 % Herring sperm DNA (v/v), 0.005 % RNA (v/v) in DEPC-water) was applied to each section. The slides were coverslipped and the sections incubated in a humid chamber (hybridisation box with 50 % formamide (v/v) and 50 % SSC on filter paper) at 55 °C for 16 hours.

Post-hybridisation wash. The coverslips were removed exposing the hybridised tissue sections, and the slides immersed thrice for 15 minutes a time in 4 x SSC solution with 0.075 % β-mercaptoethanol (v/v), followed by 30 minutes in RNase buffer (40 µg / ml RNase, 0.5 M NaCl and 1 % Tris-ethylenediamine tetra-acetic acid at 37 °C). The tissue was then treated with 0.075 % β-mercaptoethanol (v/v) in RNase buffer for 15 minutes. The tissue was placed in a series of SSC with 0.075 % β-mercaptoethanol (v/v) ranging from 2 x SSC to 0.1 x SSC, the final concentration being applied at 60 °C. The sections were placed in 0.1 x SSC before being dehydrated respectively in 70 % and 95 % ethanol with 0.3 M NH_4Ac, and finally in absolute ethanol. The slides were left to air dry.

Development of signal. The slides were dipped in ILFORD K5 emulsion (ILFORD Ltd., UK) and exposed for up to six weeks in a light sealed box at 4 °C. The sections were then developed for ten minutes using Kodak D19 developer (80 g/l; Sigma Chemical Company, USA), washed for five minutes in dH_2O and fixed with 250 g/l $Na_2S_2O_3$ (BDH Chemical Company, UK) for ten minutes. The sections were dehydrated in increasing concentrations of ethanol from 50 % to 100 %, washed twice in xylene and coverslipped.

GnRH mRNA sequencing

Total RNA extraction. A female highveld mole-rat was deeply anaesthetized using Halothane (Zeneca, RSA) before the brain was removed and preserved in RNAlater (Qiagen, Germany) to prevent degradation of the RNA (ribonucleic acid) during transport and storage. A 4 mm^2 cube of tissue was dissected from the hypothalamic area of the brain, and total RNA extracted using TRIZOL® reagent (Life Technologies Ltd., Scotland). The tissue sample was homogenized in 800 µl TRIZOL® reagent and subsequently incubated for five minutes at 15 °C to permit the dissociation of nucleoprotein complexes. Chloroform (0.2 ml) was added and the sample was shaken by hand for 15 seconds and incubated for two minutes at 15 °C. The sample was centrifuged (12 000 rpm at 2 °C) for 15 minutes, separating the mixture into three phases with the RNA remaining exclusively in the colourless upper aqueous phase and precipitated by the addition of isopropyl alcohol. The sample was then incubated at 15 °C for ten minutes and centrifuged (12 000 rpm at 2 °C) for ten minutes. The RNA pellet was washed once with 75 % ethanol (v/v), centrifuged for five minutes (7 500 rpm, 2 °C), vacuum dried and dissolved in RNase-free water.

First-strand cDNA synthesis. The total RNA extracted from the mole-rat tissue was reverse transcribed to cDNA to serve as a template for primer construction (procedure performed by I Kalló, Department of Neurobiology, Hungarian Academy of Sciences, Hungary). The isolated RNA together with a custom-designed primer and 10 µl dH_2O was incubated at 70 °C for five minutes and chilled on ice. Four microlitres of 5 x reaction buffer, 2 µl 1.0 mM dNTP mix (containing dATP, dCTP, dGTP and dTTP, each at a final concentration of 2 mM), 20 units ribonuclease inhibitor and 20 µl nuclease free dH_2O were added and the mixture incubated at 37 °C for five minutes. Forty units of M-MuLV (Moloney Murine Leukaemia Virus)

reverse transcriptase were added, followed by a 60 minute (37 °C) incubation. The mixture was heated to 70 °C for ten minutes and chilled on ice.

Detection of mole-rat GnRH. Mole-rat GnRH cDNA was detected using PCR (polymerase chain reaction) and primers constructed from the GnRH nucleotide consensus sequences from several species (designed by E Hrabovszky, Department of Neurobiology, Hungarian Academy of Sciences, Hungary). Mole-rat GnRH cDNA was amplified with primers and cloned into the PGEMT plasmid vector (Promega). The plasmid was propagated in *E. coli* bacteria and isolated using the Plasmid Miniprep kit (Qiagen, Germany). Four ethidium bromide-stained PCR products were purified from agarose gel and sequenced using an automated sequencer. The resulting nucleotide sequences were compared to all known forms of GnRH using the Internet based program Nucleotide-Nucleotide BLAST. Sequencing was performed by P Marsh (Division of Biomolecular Sciences, King's College London, UK).

Statistical analysis

The statistical analyses for this study were done with the use of the Statistica 6 computer software package (Statsoft, USA), SigmaStat for Windows version 2.03 and SigmaPlot for Windows version 4.01. Nonparametric Mann-Whitney U tests and Kruskal Wallis Analyses of Variance (ANOVA) were used when data were not normally distributed, while parametric Two Way ANOVAs and t-tests were employed otherwise. Significance was assumed at $p < 0.05$.

Chapter 3

Neuroanatomy and -endocrinology of the gonadotropin-releasing hormone system of common and highveld mole-rats

Abstract

The gonadotropin-releasing hormone (GnRH) systems of the common and highveld mole-rats were visualised immunocytochemically and the GnRH peptide content of brain tissue inclusive of the hypothalamic area determined by radioimmunoassay.

In both subspecies GnRH neurones and fibres were loosely distributed along the septo-preoptico-infundibular pathway. Dense aggregations of GnRH fibres were present in the regions of the organum vasculosum of the lamina terminalis and the median eminence. Female common and highveld mole-rats differed with regards to the degree to which GnRH cell bodies migrated caudally: in the common mole-rat 41.4 % of cells were found in the preoptic area and 55.8% in the medio-basal hypothalamus; compared to the highveld mole-rat where 80.7 % of cell bodies were located in the preoptic area and only 13.5 % in the medio-basal hypothalamus.

Male and female common mole-rats had significantly fewer GnRH-ir (GnRH immunoreactive) cell bodies than did male and female highveld mole-rats. Similarly, the brain GnRH content of male and female common mole-rat brains was significantly lower than those of male and female highveld mole-rats. GnRH-ir staining in the median eminence of female common mole-rats was also significantly less intense compared to female highveld mole-rats.

Reproductive and non-reproductive male and female common mole-rats did not differ with regards to the numbers of GnRH-ir cell bodies or brain GnRH levels, both during and out of the breeding season. The intensity of GnRH-ir staining in the median eminence was comparable between reproductive and non-reproductive female common mole-rats.

The numbers of GnRH-ir cell bodies and brain GnRH content in male highveld mole-rats was not influenced by social status or season. Similarly, the numbers of GnRH-ir cell bodies did not differ between female highveld mole-rats with regards to social

status or season, however, reproductive females consistently had significantly reduced brain GnRH levels compared to non-reproductive animals. The mean surface area of GnRH-ir cells was significantly smaller in reproductive female highveld mole-rats compared to non-reproductive females. In addition, GnRH-ir staining in the median eminence of reproductive females was significantly less intense compared to non-reproductive female highveld mole-rats – suggesting inhibited release of the peptide in these subordinate, socially suppressed females.

Introduction

The three major natural suppressors of fertility are a) the environment through the effects of seasonal breeding and nutritional status, b) lactation and c) social dominance (Abbot, 1988). In this chapter I investigate the interplay between the effects of seasonal reproductive quiescence and socially induced infertility on selected qualitative and quantitative parameters of the gonadotropin-releasing hormone (GnRH) system in the closely related, seasonally breeding, common (*Cryptomys hottentotus hottentotus*) and highveld (*Cryptomys hottentotus pretoriae*) mole-rats. An alteration in the functioning of the GnRH system is hypothesized to be the neuroendocrine mechanism responsible for the observed anovulation and gonadal regression associated with many forms of natural infertility (Molteno, 1999). Various methods can be used to quantify this possible disruption of GnRH synthesis and/or secretion. Low concentrations of the GnRH peptide in the peripheral system (Clarke and Cummins, 1982; Ferin *et al.*, 1984) necessitate the determination of pulsatile GnRH release in the hypophysial portal blood, rendering the direct measurement of GnRH impractical. Measurement of the GnRH content in the brain through radioimmunoassay, and also by immunocytochemical visualisation of GnRH

neurones are consequently the most widely employed methods of examining GnRH production, storage and release and the possible alteration thereof (Rissman, 1996).

The effect that environmental seasonality has on the GnRH system, and therefore the reproductive functioning of the animal as a whole, varies between species. In the Syrian hamster (*Mesocricetus auratus*) short days, indicative of the non-breeding period in most rodents, lead to an increase in the size of GnRH-ir (GnRH immunoreactive) cell bodies and hypothalamic levels of the peptide, while total numbers of neurones are unaffected by season (Urbanski *et al.*, 1991). Similarly, in the Djungarian hamster (*Phodopus sungorus sungorus*), short days induce gonadal atrophy and suppress gonadotropin secretion, with numbers of GnRH-stained cells remaining unchanged (Yellon, 1989). This is contrary to the situation in the white footed mouse (*Peromyscus leucopus*) where short day lengths result in an increase in the hypothalamic GnRH content through an increase in the number and optical density of the immunological staining of GnRH-ir cell bodies and fibres, and subsequent gonadal regression (Glass, 1986; Petterborg, 1981). It therefore seems likely that the inhibition of the hypothalamo-pituitary axis in seasonally breeding species can be mediated through both decreased synthesis and/or release of the GnRH peptide.

Seasonal environmental change is but one of a multitude of factors that can influence the GnRH neuronal system. In the little brown bat (*Myotis lucifugus*), ovulation and the associated endocrine changes result in a decrease in GnRH content as well as numbers of GnRH-ir cell bodies in the brain, with the different sub-populations of GnRH neurones equally affected (Anthony *et al.*, 1989). In the rat on the other hand, spatially separate sub-populations respond differently to acute changes in the endocrine state of the animal, such as gonadectomy (King *et al.*, 1987). In the baboon, different populations of GnRH cells are postulated to have different functions

(Marshall and Goldsmith, 1980). Cells in the medial basal hypothalamus of the baboon are assumed to directly influence the anterior pituitary, while a larger population within the afferent and efferent pathways of the hypothalamus and forebrain may receive external information affecting reproduction, and neurons in the median eminence have collaterals to other brain areas - collectively suggesting that in addition to their neuroendocrine role, these cells may also have neuroregulatory functions. In the female musk shrew (*Suncus murinus*), the numbers of GnRH-ir neurones change in response to different stages in the social encounter with males (Tai *et al.*, 1997). Auditory and visual cues from males lead to the transport of GnRH out of the neurones, while copulation seems to replenish GnRH stores in the cell bodies. Similar to the rat (King *et al.*, 1987), changes are masked when considering the entire brain, while individual neurone populations in the forebrain show significant changes in the numbers of GnRH-ir cell bodies and the optical density of GnRH fibres.

A study on the Damaraland mole-rat (*Cryptomys damarensis*), an aseasonal eusocial bathyergid species, revealed that the GnRH system of non-reproductive females is compromised when compared to that of dominant females (Molteno *et al.*, 2004). Although reproductive and non-reproductive females did not differ with regard to total numbers or size of GnRH-ir cell bodies, or the distribution of GnRH-ir structures in the brain, subordinate females showed significantly elevated levels of the peptide in the brain as shown by radioimmunoassay and in the median eminence as shown by immunocytochemistry. The relatively high concentrations of GnRH displayed by subordinate females imply a reduced release of the peptide and consequently increased storage in nerve terminals in the median eminence. The GnRH system of reproductive and non-reproductive male Damaraland mole-rats did not differ significantly with regards to either GnRH content or numbers of cell bodies in the brain.

The aim of this investigation was to determine whether the socially induced infertility evident in subordinate common and highveld mole-rats may be attributed to differences in the function and morphology of the GnRH system between dominant and subordinate animals within each subspecies, and whether the mechanisms of reproductive suppression differ between the two subspecies at this level of the hypothalamo-pituitary-gonadal axis. The influence of seasonal environmental changes on reproductive function and the GnRH system was also investigated. GnRH levels in the brains of individual animals as well as numbers of GnRH-ir cell bodies were compared between both male and female, as well as dominant and subordinate individuals of each species, both during and out of the breeding season. The distribution and morphology of GnRH-ir neurones and fibre projections as well as the intensity of GnRH-ir staining in the median eminence were compared intra- and inter-subspecifically between reproductive and non-reproductive female common and highveld mole-rats out of the breeding season.

Materials and methods

Animals were trapped, housed and sacrificed as described in Chapter 2. Brain tissues for all experimental groups were either assayed radioimmunologically to determine endogenous levels of GnRH or treated immunocytochemically to visualize GnRH-ir cell bodies and fibres (see Chapter 2).

Results

Morphology of GnRH-ir cell bodies and fibres in the common and highveld mole-rat

GnRH-ir fibre projections in both the common and highveld mole-rat had a characteristically beaded appearance (Plate 3.1 a-b). In both species the GnRH-ir perikarya were spindle shaped with smooth contours and were either bipolar (two fibre projections; Plate 3.1 a) or unipolar (one fibre projection from the cell body; Plate 3.1 b). Some cell bodies appeared not to have any fibre projections (nonpolar), possibly due to the plane of the section running perpendicular to the long axis of the cell body.

Reproductive female common mole-rats had more unipolar cell bodies than non-reproductive females (Kruskal Wallis analysis: $H = 5.1$, $p < 0.05$, $n = 8$; Fig. 3.1), while numbers of bipolar cell bodies were comparable (Kruskal Wallis analysis: $H = 1.1$, $p > 0.05$, $n = 8$). Highveld mole-rat females had similar numbers of unipolar (Kruskal Wallis analysis: $H = 0.5$, $p > 0.05$, $n = 10$; Fig. 3.1) and bipolar cell bodies (Kruskal Wallis analysis: $H = 0.5$, $p > 0.05$, $n = 10$). The ratio of unipolar to bipolar cell bodies did not differ between common and highveld mole-rat females (Kruskal Wallis analysis: $H = 0.6$, $p > 0.05$, $n = 18$).

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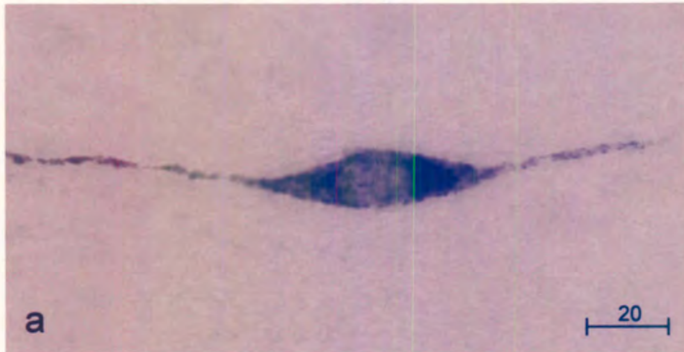

Plate 3.1 High power photomicrographs showing a) bipolar and b) unipolar GnRH (gonadotropin releasing-hormone) cells. c) GnRH-ir (GnRH immunoreactive) fibres and a GnRH perikaryon (arrow) within the subfornical organ. Scale bar units: micrometers

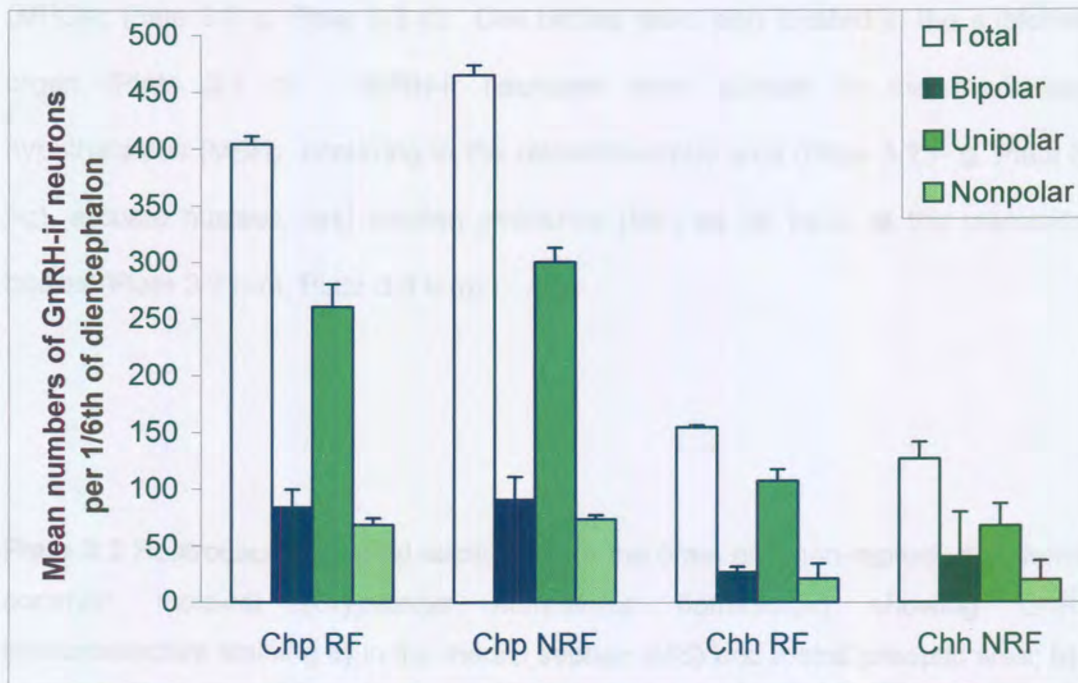

Fig. 3.1 A comparison between the mean numbers of total, bipolar, unipolar and nonpolar GnRH-ir (gonadotropin releasing-hormone immunoreactive) cell bodies per one sixth of diencephalon for reproductive and non-reproductive female common and highveld mole-rats out of the breeding season. Chh, common mole-rat (*Cryptomys hottentotus hottentotus*); Chp, highveld mole-rat (*Cryptomys hottentotus pretoriae*); RF, reproductive female; NRF non-reproductive female

Distribution of GnRH-ir cell bodies and fibres

Distribution of GnRH-ir cell bodies in the common and highveld mole-rat. In both the common and highveld mole-rat the majority of GnRH neurones formed a loose band along the septo-preoptico-infundibular tract (Plate 3.2-3.3). GnRH cell bodies were absent from the olfactory bulbs, hippocampus and central grey matter of the mesenchephalon. Rostral to the anterior commissure, GnRH cell bodies with their long axes orientated dorso-ventrally, lined the ventral median fissure (Plate 3.2 a, Plate 3.3 a). GnRH-ir perikarya were located in the horizontal and vertical diagonal bands of Broca (DBB), around the region of the organum vasculosum of the lamina terminalis (OVLT; Plate 3.2 b, Plate 3.3 b), and extending to the medial preoptic area

(MPOA; Plate 3.2 c, Plate 3.3 c). Cell bodies were also located in the subfornical organ (Plate 3.1 c). GnRH-ir neurones were present in the medio-basal hypothalamus (MBH), occurring in the retrochiasmatic area (Plate 3.2 f- g, Plate 3.3 f-g), arcuate nucleus, and median eminence (ME) as far back as the mammillary bodies (Plate 3.2 h-m, Plate 3.3 h-m).

Plate 3.2 Rostrocaudal coronal sections from the brain of a non-reproductive female common mole-rat (*Cryptomys hottentotus hottentotus*) showing GnRH-immunoreactive staining **a)** in the medial septum (MS) and rostral preoptic area; **b)** in the MS and region of the organum vasculosum of the lamina terminalis (OVLT); **c)** in the medial preoptic area and a densely immunoreactive site caudal to the OVLT and ventral to the third ventricle (3V); **d)** in the structure forming the rostral floor of the 3V in place of the optic chiasm; **e)** in the region medial to the superchiasmatic nuclei (SCN); **f-g)** in the retrochiasmatic area rostral to the median eminence (ME); **h-m)** in a dense band of fibres forming the external zone of the ME. PS, pituitary stalk; PT, pars tuberalis. Arrows (➔) indicate selected GnRH perikarya. Scale bar denotes 200 μm

Plate 3.3 Rostrocaudal coronal sections from the brain of a non-reproductive (NRF, a-m) and a reproductive female (RF, n-o) highveld mole-rat (*Cryptomys hottentotus pretoriae*) showing GnRH-immunoreactive staining **a)** in the medial septum (MS) and rostral preoptic area; **b)** in the MS and region of the organum vasculosum of the lamina terminalis (OVLT); **c)** in the medial preoptic area and a densely immunoreactive site caudal to the OVLT and ventral to the third ventricle (3V); **d)** in the membranous structure forming the rostral floor of the 3V in place of a detectable optic chiasm; **e)** in the region medial to the superchiasmatic nuclei (SCN); **f-g)** in the retrochiasmatic area rostral to the median eminence (ME); **h-m)** in a dense band of fibres forming the external zone of the ME. Fibres in the median eminence typically show stronger immunoreactive staining in l-m) non-reproductive compared to **n-o)** reproductive female highveld mole-rats. PS, pituitary stalk; PT, pars tuberalis. Arrows (➔) indicate selected GnRH perikarya. Scale bar denotes 200 μm

MS a 200
MS OVLT b
3V c
d
SCN SCN e
f
g
h
PT i
ME j
k
l
PS m

Plate 3.2

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Plate 3.3

Intra-species comparison. Reproductive and non-reproductive female common mole-rats did not differ significantly with regards to the proportion of GnRH cell bodies located in the preoptic area versus the medio-basal hypothalamus (ANOVA: $F = 3.95$, $p > 0.05$, $n = 3$ & 5; Fig. 3.2). Similarly, GnRH cell bodies were distributed in a similar fashion in reproductive and non-reproductive female highveld mole-rats (ANOVA: $F = 3.07$, $p > 0.05$, $n = 5$ & 5; Fig. 3.2).

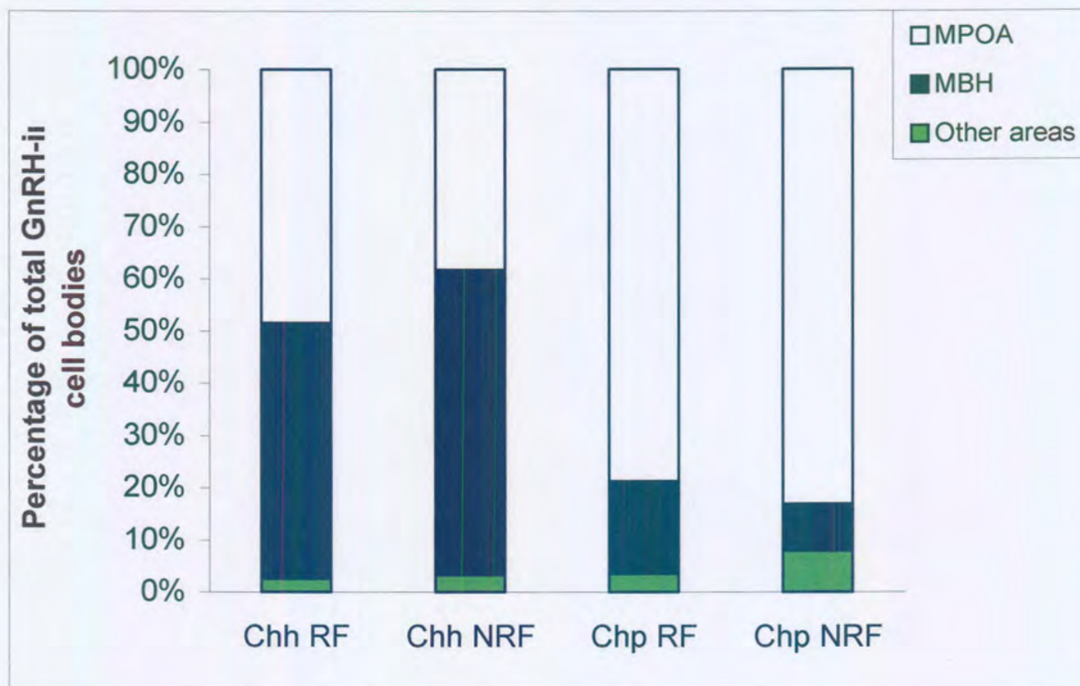

Fig. 3.2 Percentage of GnRH-ir (gonadotropin releasing-hormone immunoreactive) cell bodies in the preoptic area (MPOA) and medio-basal hypothalamus (MBH) for reproductive and non-reproductive female common and highveld mole-rats out of the breeding season. Chh, common mole-rat (*Cryptomys hottentotus hottentotus*); Chp, highveld mole-rat (*Cryptomys hottentotus pretoriae*); RF, reproductive female; NRF, non-reproductive female

Inter-species comparison. The relative distribution of GnRH-ir cell bodies differed between the species (ANOVA: $F = 188.90$, $p < 0.001$, $n = 8$ & 10; Fig. 3.2). In the common mole-rat there were fewer cell bodies in the preoptic area (41.4 %) than in the medio-basal hypothalamus (55.8 %). In the highveld mole-rat the division was

less equable, with the majority of cell bodies located in the preoptic area (80.7 %) and relatively few in the medial-basal hypothalamus (13.5 %).

Distribution of GnRH-ir fibres in the common and highveld mole-rat. In both species GnRH fibres were distributed along the septo-preoptico-infundibular pathway, in a similar though slightly more widespread pattern than the GnRH cell bodies (Plate 3.2-3.3). Rostrally, a few GnRH fibres were present in the olfactory lobes, hippocampus and central grey matter of the mesenchephalon. Fibres were apparent along the midline (Plate 3.2 a, Plate 3.3 a) and the horizontal and vertical diagonal bands of Broca. GnRH fibres were also present in the subfornical organ (Plate 3.1 c). The region of the organum vasculosum of the lamina terminalis presents the first of two dense aggregations of GnRH fibres, continuing to the preoptic area along the midline beneath the third ventricle (Plate 3.2 b-c, Plate 3.3 b-c). Fibre density again increased in the retrochiasmatic area (Plate 3.2 f-g, Plate 3.3 f-g), towards the second area of intense staining, the median eminence (ME; Plate 3.2 h-m, Plate 3.3 h-m). At the level of the ME, fibres formed an arching pattern around and within the arcuate nucleus. Caudal to the infundibular stem, fibres formed a dense peripheral layer (Plate 3.2 m, Plate 3.3 m).

Density of GnRH-ir staining in the median eminence

The GnRH-ir staining in the median eminence of reproductive and non-reproductive female common mole-rats were significantly lighter (smaller area occupied by GnRH-ir structures) than that of reproductive and non-reproductive female highveld mole-rats (ANOVA: $F = 53.23$, $p < 0.001$, $n = 8$ & 10; Fig. 3.3; Table 3.1). The social status of female common mole-rats was not reflected in the intensity of GnRH staining at the level of the median eminence ($t = -1.23$, $p > 0.05$, $n = 3$ & 5; Fig. 3.3; Table 3.1). Reproductive female highveld mole-rats, however, showed significantly lighter

staining than non-reproductive female highveld mole-rats ($t = -2.61$, $p < 0.05$, $n = 5$ & 5; Fig. 3.3; Table 3.1).

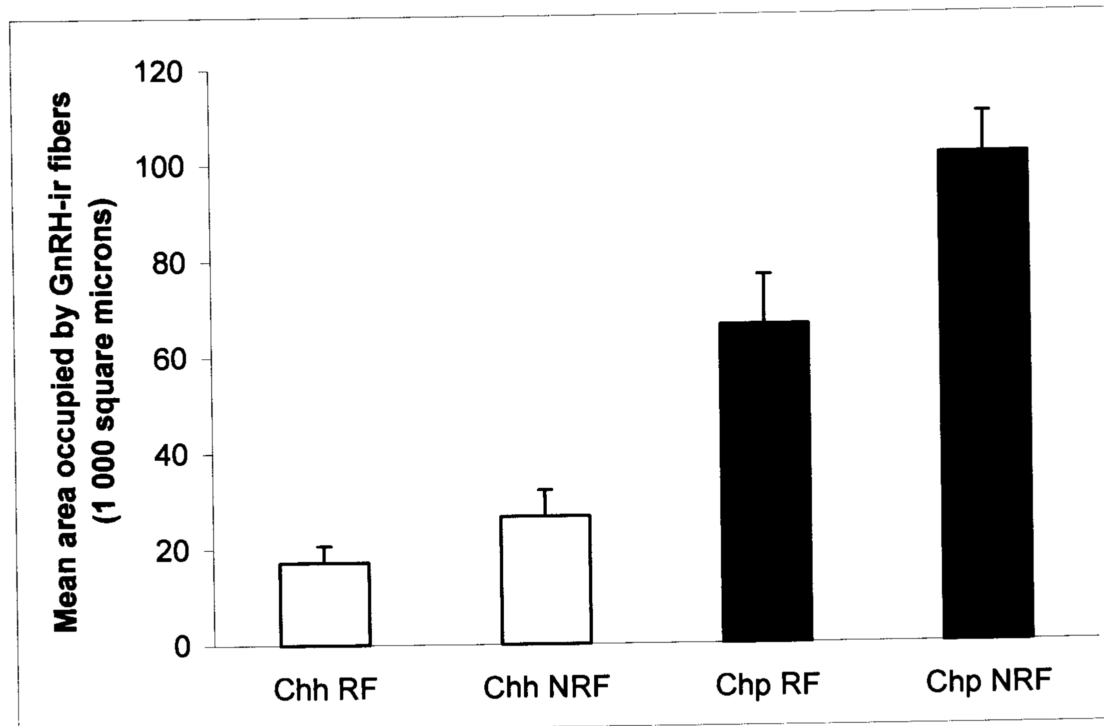

Fig. 3.3 Mean area in the median eminence occupied by GnRH-ir (gonadotropin releasing-hormone immunoreactive) fibres for reproductive and non-reproductive female common and highveld mole-rats out of the breeding season. Chh, common mole-rat (*Cryptomys hottentotus hottentotus*); Chp, highveld mole-rat (*Cryptomys hottentotus pretoriae*); RF, reproductive female; NRF, non-reproductive female

Size of GnRH-ir cell bodies

There was no significant difference in the mean surface area of individual GnRH-ir cell bodies between female common (120.1 ± 4.1 μm^2) and highveld mole-rats (127.8 ± 3.2 μm^2; ANOVA: $F = 1.22$, $p > 0.05$, $n = 8$ & 10; Fig. 3.4). Similarly reproductive (117.9 ± 8.7 μm^2) and non-reproductive (121.5 ± 4.9 μm^2) female common mole-rats did not differ significantly with regards to mean cell body size (Mann-Whitney U test: $U = 7$, $p > 0.05$, $n = 3, 5$). The GnRH-ir cell bodies of

reproductive (120.8 ± 1.6 μm^2) female highveld mole-rats, however, had a significantly smaller mean surface area than that of non-reproductive females (134.9 ± 4.3 μm^2; Mann-Whitney U test: U = 0, $p < 0.05$, n = 5, 5).

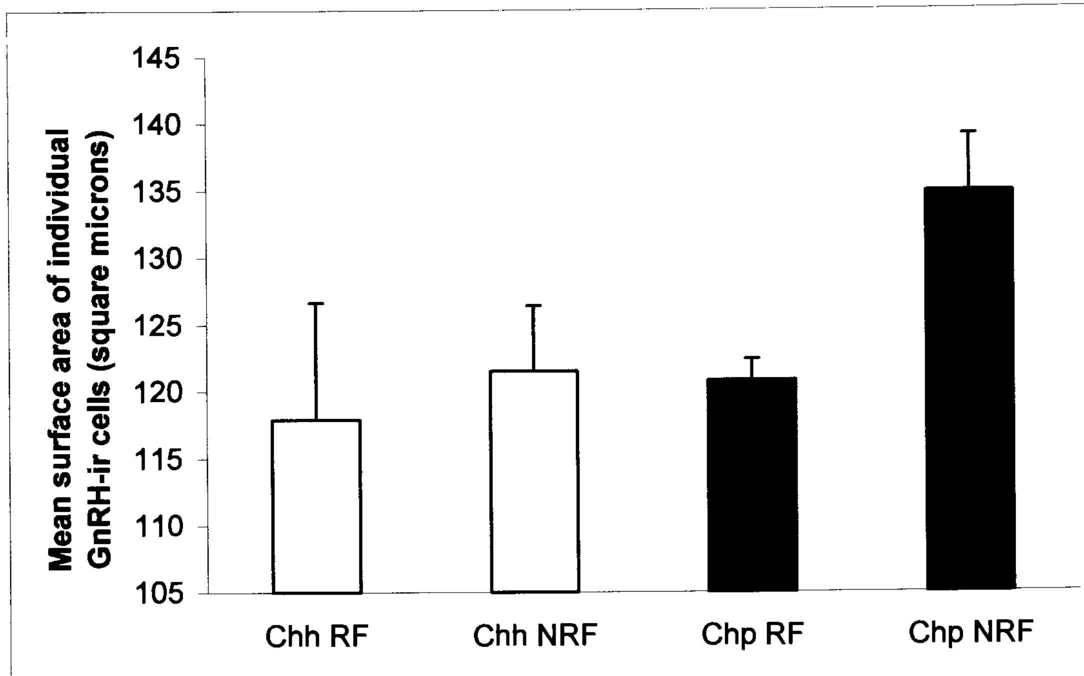

Fig. 3.4 Mean surface area of individual GnRH-ir (gonadotropin releasing-hormone immunoreactive) cells in the preoptic area of female common and highveld mole-rats out of the breeding season. Chh, common mole-rat (*Cryptomys hottentotus hottentotus*); Chp, highveld mole-rat (*Cryptomys hottentotus pretoriae*); RF, reproductive female; NRF, non-reproductive female

Number of GnRH-ir cell bodies

Influence of gender and subspecies. Male (112 ± 20) and female (101 ± 10) common mole-rats did not differ significantly in numbers of GnRH-ir cell bodies (Mann-Whitney U test: U = 127, $p > 0.05$, n = 17 & 17), similarly male (284 ± 23) and female (244 ± 29) highveld mole-rats had similar numbers of GnRH-ir cell bodies (Mann-Whitney U test: U = 111, $p > 0.05$, n = 17 & 18). Both male and female common mole-rats had significantly fewer GnRH-ir cell bodies than did male and

female highveld mole-rats (Mann Whitney U test: Males U = 23, $p < 0.001$, n = 17 & 17; Females U = 35, $p < 0.001$, n = 17 & 18).

Numbers of GnRH-ir cell bodies observed varied greatly between individuals, ranging between 23 and 354 in the common mole-rat and 36 and 524 in the highveld mole-rat (Fig. 3.5). Since every sixth tissue section was processed, these means correspond to a total of approximately 640 cells in the common mole-rat and 1650 cells in the highveld mole-rat.

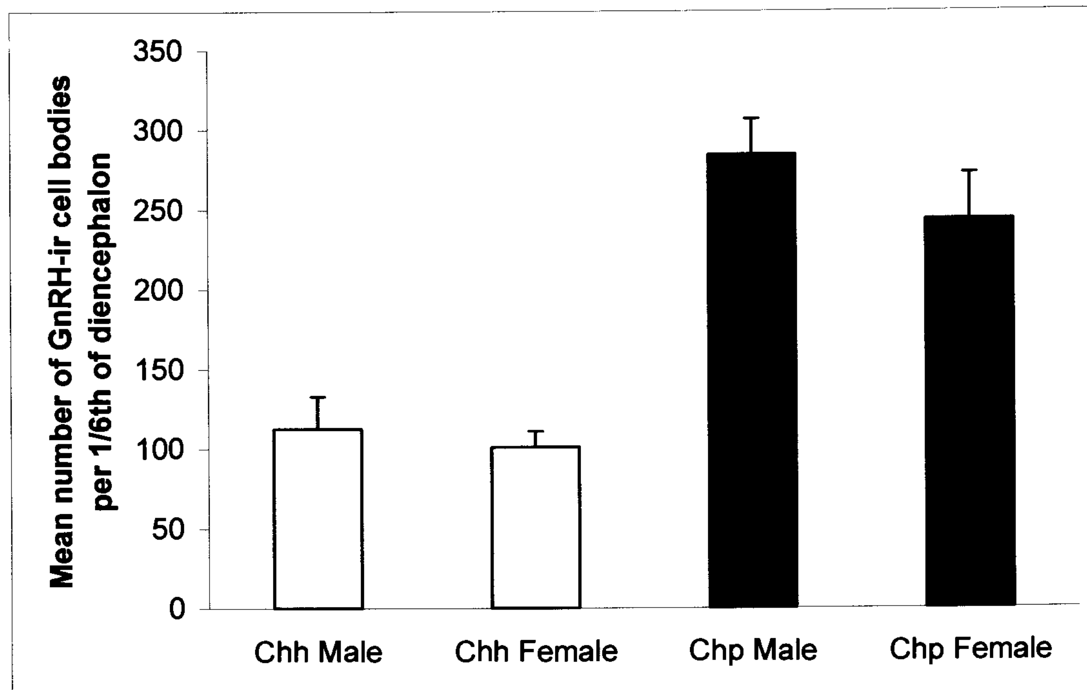

Fig. 3.5 Mean (± sem) numbers of GnRH-ir (gonadotropin releasing-hormone immunoreactive) cell bodies per one sixth of diencephalon for male and female common and highveld mole-rats. Chh, common mole-rat (*Cryptomys hottentotus hottentotus*); Chp, highveld mole-rat (*Cryptomys hottentotus pretoriae*)

Influence of social status and season in the common mole-rat. Neither differences in social status or season significantly affected numbers of GnRH-ir cell bodies in male common mole-rats (ANOVA: F = 0.00, $p > 0.05$, n = 17; Table 3.2). Similarly, there

was no difference in the numbers of GnRH-ir cell bodies between female common mole-rats with respect to social status or season (ANOVA: $F = 2.04$, $p > 0.05$, $n = 17$; Table 3.2).

Influence of social status and season in the highveld mole-rat. Numbers of GnRH-ir cell bodies in male highveld mole-rats did not vary markedly due to social status or season (ANOVA: $F = 0.00$, $p > 0.05$, $n = 18$; Table 3.2). Female highveld mole-rats also did not show significant variation in the numbers of GnRH-ir cell bodies with respect to social status or season (ANOVA: $F = 1.44$, $p > 0.05$, $n = 17$; Fig. 3.6, Table 3.2).

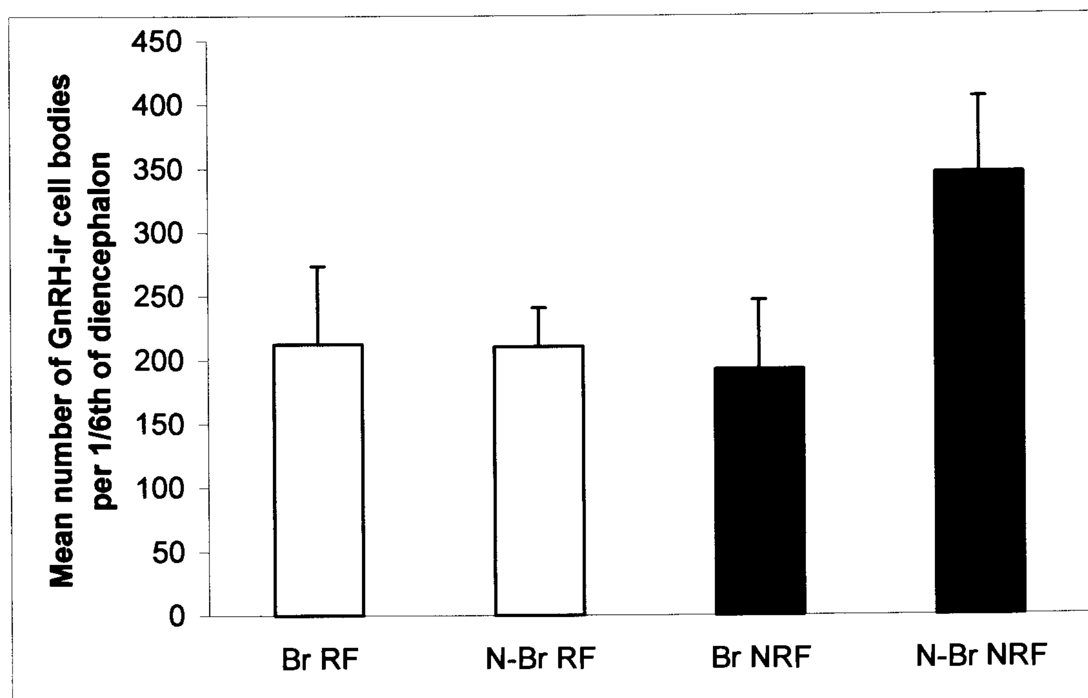

Fig. 3.6 Mean (± sem) numbers of GnRH-ir (gonadotropin releasing-hormone immunoreactive) cell bodies per one sixth of diencephalon for reproductive and non-reproductive female highveld mole-rats (*Cryptomys hottentotus pretoriae*), in and out of the breeding season. Br, breeding season; N-Br, non-breeding season; RF, reproductive female; NRF, non-reproductive female

Brain GnRH concentrations

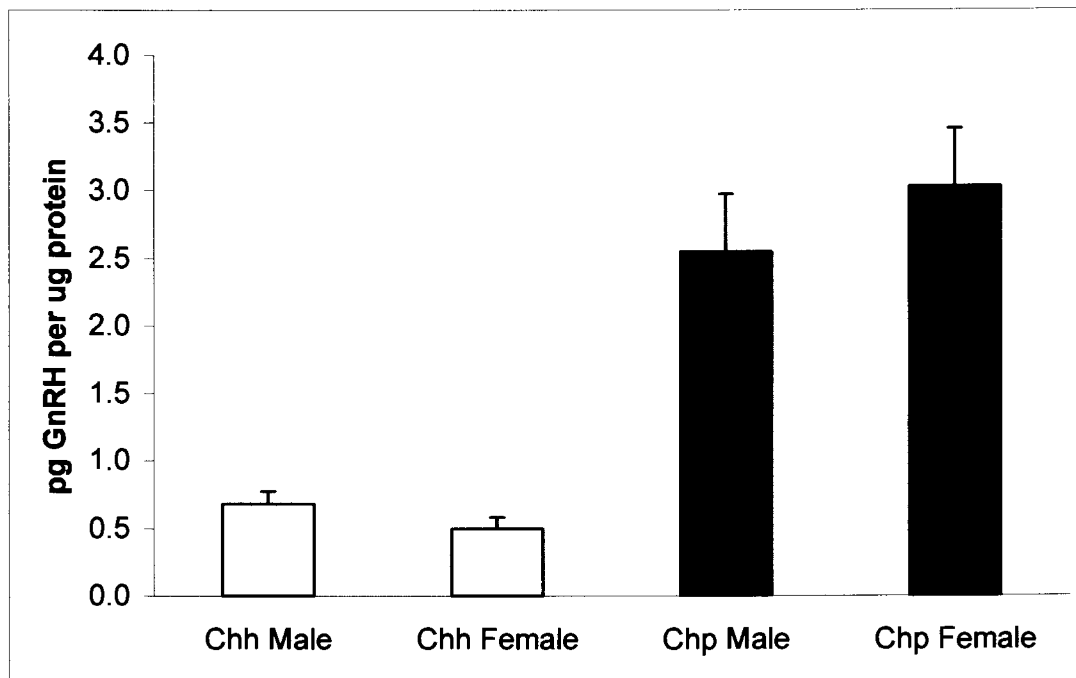

Fig. 3.7 Brain GnRH (gonadotropin releasing-hormone) levels (mean ± sem) for male and female common and highveld mole-rats. Chh, common mole-rat (*Cryptomys hottentotus hottentotus)*; Chp, highveld mole-rat (*Cryptomys hottentotus pretoriae)*

Influence of gender and subspecies. Male (0.68 ± 0.10 pg/μg) and female common mole-rats (0.50 ± 0.08 pg/μg) had similar levels of GnRH (Mann-Whitney U test: $U = 60$, $p > 0.05$, $n = 14$ & 12), similarly male (2.54 ± 0.43 pg/μg) and female highveld mole-rats (3.03 ± 0.43 pg/μg) did not significantly differ with respect to brain GnRH concentrations (Mann-Whitney U test: $U = 100$, $p > 0.05$, $n = 15$ & 18; Fig. 3.7). Male and female common mole-rats had significantly reduced GnRH levels when compared to male and female highveld mole-rats (Mann-Whitney U test: Males $U = 12$, $p < 0.001$, $n = 14$ & 15; Females $U = 7$, $p < 0.001$, $n = 12$ & 18).

Influence of social status and season in the common mole-rat. GnRH levels in brain tissue of common mole-rat males did not differ as a consequence of social status or

seasonal reproductive variation (ANOVA: $F = 1.22$, $p > 0.05$, $n = 14$; Table 3.3). Similarly, female common mole-rats had similar levels of GnRH irrespective of social status or season (ANOVA: $F = 0.06$, $p > 0.05$, $n = 14$; Table 3.3).

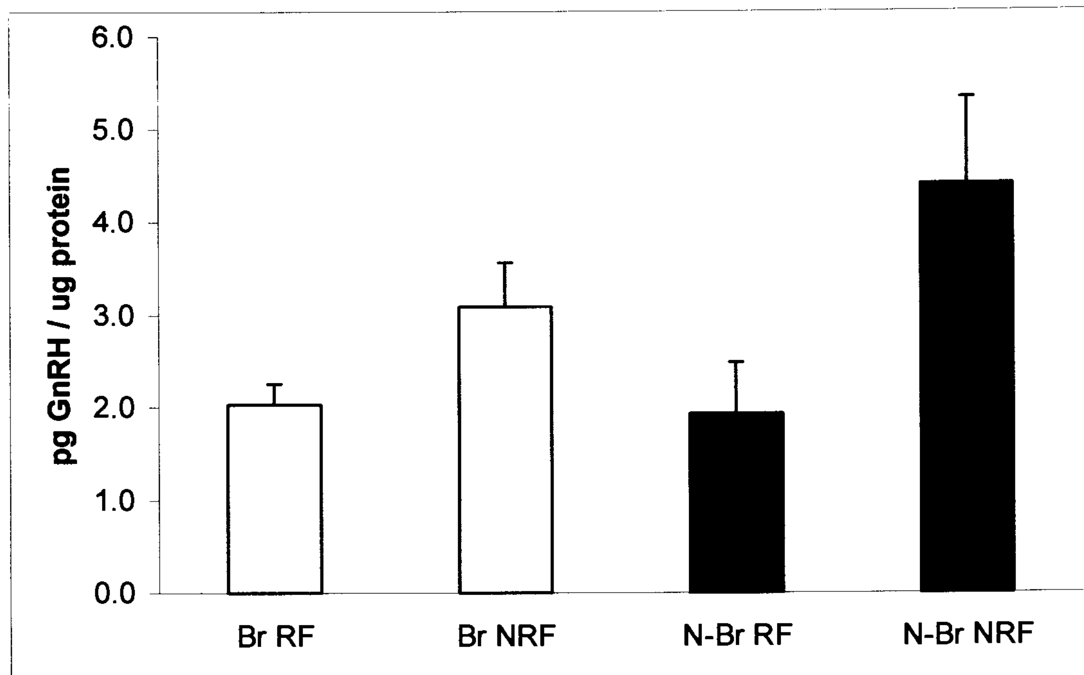

Fig. 3.8 Brain GnRH (gonadotropin releasing-hormone) levels (mean ± sem) for reproductive and non-reproductive female highveld mole-rats (*Cryptomys hottentotus pretoriae*), in and out of the breeding season. Br, breeding season; N-Br, non-breeding season; RF, reproductive female; NRF, non-reproductive female

Influence of social status and season in the highveld mole-rat. Male highveld mole-rats did not display any significant variation in brain GnRH concentrations linked to social status or season (ANOVA: $F = 3.90$, $p > 0.05$, $n = 16$; Table 3.3). Reproductive and non-reproductive female highveld mole-rats, in and out of the breeding season, differed significantly with regards to brain GnRH levels (ANOVA: $F = 5.30$, $p < 0.05$, $n = 18$; Table 3.3 and Fig. 3.8). Reproductive females had lower brain GnRH concentrations than non-reproductive females, both in and out of the breeding season. The difference was more striking out of than during the breeding

season. Non-reproductive females had increased endogenous brain GnRH levels during the breeding compared to the non-breeding season.

Discussion

This study detailed the distribution and morphology of GnRH neurones and fibres as well as endogenous brain GnRH content in common and highveld mole-rats, taking into account potential inter- and intra-species variation as a result of gender, reproductive status and breeding season. The GnRH neuronal systems of common and highveld mole-rats were found to be very similar to that of the Damaraland mole-rat as described by Molteno *et al.* (2004). GnRH fibres and cell bodies in all three species are of comparable morphology and are loosely distributed along the septo-preoptico-infundibular pathway – as is indeed typical of most mammals (Plate 3.2-3.3; Silverman *et al.*, 1994). The highveld mole-rat, however, has almost thrice the absolute number of GnRH-ir cell bodies (1652) compared to common (640) and Damaraland mole-rats (648), placing it in range with rats (Silverman *et al.*, 1994) and substantially larger species such as primates and antelopes (Silverman *et al.*, 1982; Robinson *et al.*, 1997). The numbers of GnRH cell bodies as seen in the *Cryptomys* mole-rats is in discord with the general notion that the total number of GnRH cell bodies is correlated with brain and consequently body size (Yellon and Newman, 1991), as common mole-rats have a mean adult body weight of only 77 g (Jarvis and Bennett, 1991) compared to Damaraland mole-rats and highveld mole-rats that have mean adult body weights of 104 g (Jarvis and Bennett, 1991) and 112 g (Moolman *et al.*, 1998), respectively. Differences in the number of GnRH-ir neurones between common and highveld mole-rats may simply reflect differences in body size (Fig. 2.1 and Fig. 2.2) and consequently forebrain size (Fig. 2.3), however, both male and female reproductive and non-reproductive common mole-rats differ significantly in

body weight without any marked difference in either numbers of GnRH cells or brain GnRH levels. These neuroanatomical discrepancies could mirror actual alterations in cell numbers, it is however possible that variation in the rate of peptide production and/or secretion results in different quantities of the peptide in the cell bodies altering the probability of immunocytochemical detection (Robinson *et al.*, 1997). The significance of this striking variation between such closely related (sub)species is uncertain, especially when considering that no difference in cell size was detected between the common and the highveld mole-rats.

GnRH neurones in the common and highveld mole-rat were either unipolar or bipolar, as has been found in the Damaraland mole-rat (Molteno *et al.*, 2004), Djungarian hamster (Yellon and Newman, 1991), Syrian hamster (Urbanski *et al.*, 1991) and laboratory rat (Witkin *et al.*, 1982). The importance of different cell morphologies is not fully understood, however some authors argue that more complex cells could have a greater potential to form associations with other hypothalamic elements and thereby be influenced by a greater number of neuronal inputs (Robinson *et al.*, 1997). In the male Djungarian hamster, numbers of unipolar and bipolar cell bodies change differentially during sexual maturation - probably as a result of changes in the GnRH storage and release pattern during development (Yellon and Newman, 1991).

Inter-specific variation in the degree to which GnRH neurones migrate caudally from the olfactory placode during ontogenetic development results in differences with respect to the relative presence of these neurones in the preoptic area and medio-basal hypothalamus (Silverman *et al.* 1994). In the highveld mole-rat, as in most mammals including the Damaraland mole-rat (Molteno *et al.*, 2004), very few GnRH cell bodies (13.5 %) reached the medio-basal hypothalamus. In the common mole-rat however, 55.8 % of cells migrate as far caudally as the medio-basal hypothalamus. Similarly in the mink (*Mustela vison*) 80 % of cells are found in this

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hypothalamic brain region (Ntoumi *et al.* 1994). Although social status has been shown to vary with the morphology and/or distribution of GnRH containing structures, no differences between reproductive and non-reproductive females with regards to the relative distribution of GnRH neurones were noted in either the common or highveld mole-rat.

Behavioural interactions among male African cichlid fish (*Haplochromis burtoni*) dynamically regulate reproductive behaviour and fertility through reversible changes in the size of GnRH cell bodies, depending on the social status (territorial or non-territorial) of the individual (Francis *et al.*, 1993; White *et al.*, 2002). Male springbok (*Antidorcas marsupialis*), however, do not show any relationship between social or reproductive status and either the distribution or morphology of GnRH neurones (Robinson *et al.*, 1997). In the common mole-rat the distribution of GnRH-ir structures, the number and size of GnRH-ir cell bodies, the intensity of GnRH staining and endogenous brain GnRH content are not altered by subordinate status in males and females. Similarly, neither basal or exogenously GnRH challenged LH (luteinizing hormone) levels or gonadal anatomy differs between reproductive and subordinate animals (Spinks *et al.*, 1997, 1999, 2000a). The highveld mole-rat on the other hand, seems to be more comparable to the eusocial Damaraland mole-rat. Subordinate males of both species are behaviourally restrained from reproducing whereas females are physiologically infertile (anovulatory) while in the constraints of their natal colony (Damaraland mole-rat: Bennett *et al.*, 1993, 1994; highveld mole-rat: Van der Walt *et al.*, 2001; Janse van Rensburg *et al.*, 2003). Reproductive and non-reproductive male Damaraland (Molteno *et al.*, 2004) and highveld mole-rats did not differ with regard to any aspect of the GnRH neuronal system investigated. Reproductive females on the other hand, have significantly reduced endogenous GnRH levels in brain tissue compared to their subordinate counterparts. The distribution of GnRH structures are however not altered by social status in either

species. The high levels of GnRH in socially infertile females suggest inhibition of release and subsequent accumulation of the peptide. In female Damaraland mole-rats, the similarity in soma size between the reproductive groups, and the greater intensity of GnRH immunoreactivity at the level of the median eminence and pituitary stalk in non-reproductive females, suggest that excess peptide accumulates in the nerve terminals as opposed to the cell bodies (Molteno *et al.*, 2004). In the highveld mole-rat, however, non-reproductive females have significantly larger GnRH cell soma, in addition to a greater intensity of median eminence and pituitary stalk GnRH immunoreactivity compared to reproductive females, indicating that excess peptide may accumulate in the nerve terminals as well as in the cell soma. The LH response to an exogenous GnRH challenge is also attenuated in non-reproductive female Damaraland and highveld mole-rats compared to reproductive females, suggesting pituitary desensitization (Bennett *et al.*, 1993; Van der Walt *et al.*, 2001).

Seasonal reproductive quiescence is typically manifested in the reproductive physiology of the particular animal (Clarke, 1981). The production of gametes and the subsequent unsuccessful breeding attempts during unfavourable environmental conditions are energetically wasteful, therefore the suspension of reproductive function (spermatogenesis in males and ovulation in females) during such periods is favoured from an evolutionary point of view. Seasonal mammals usually undergo gonadal atrophy coupled with an alteration in the production and/or release pattern of GnRH (Petterborg, 1981; Glass, 1986; Yellon, 1989; Urbanski *et al.*, 1991). Contrary to the norm, neither common nor highveld mole-rats suspend physiological reproductive function during the non-breeding period. Previous studies detailing the gonadal anatomy and hormone profiles of male and female common and highveld mole-rats, demonstrated continued reproductive function in males and females out of the breeding period (Spinks *et al.*, 1997; 2000a; Van der Walt *et al.*, 2001; Janse van Rensburg *et al.*, 2002). The results from this study are confirmatory in that numbers

of GnRH-ir cell bodies and GnRH brain content in common and highveld mole-rats of both sexes and social status remained constant regardless of seasonal environmental changes and the cessation of reproductive output within the colonies.

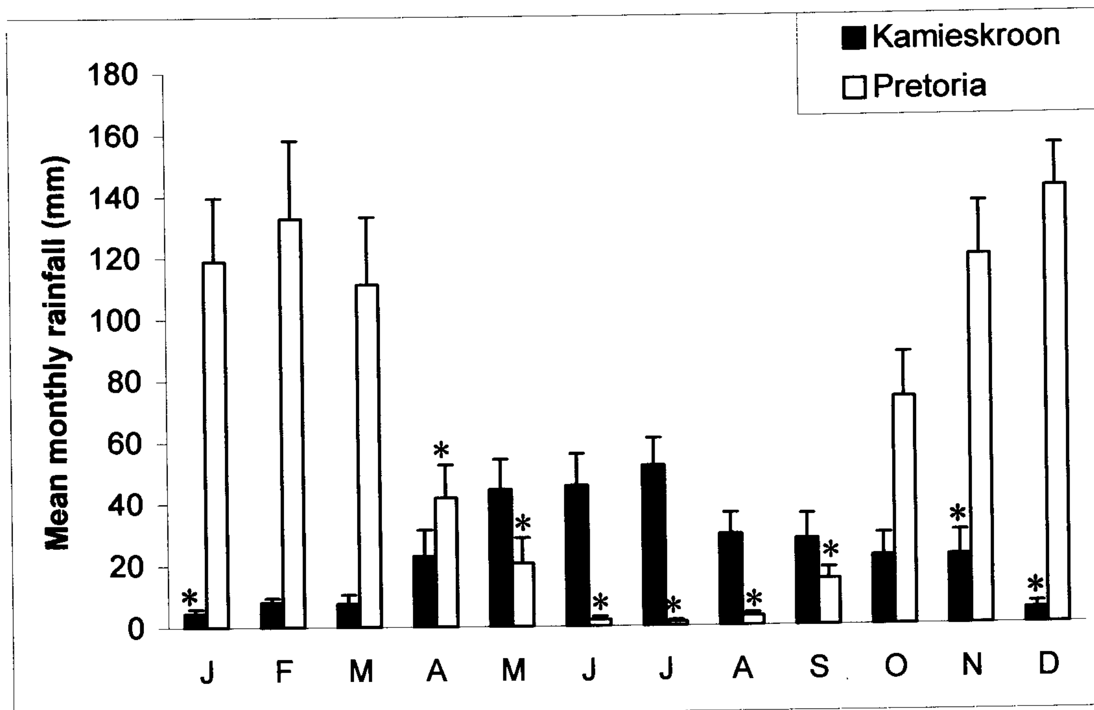

Fig. 3.9 Mean monthly rainfall for the Kamieskroon and Pretoria areas for the years 1992 to 2002 (data courtesy of the South African Weather Bureau). The breeding season of the common mole-rat is denoted by * black asterisks, and the breeding season of the highveld mole-rat by * green asterisks

Solitary mole-rats are usually distinctively cyclic with regards to reproductive characteristics (Van der Horst, 1972; Bennett and Jarvis, 1988; Herbst, 2002). Common and highveld mole-rats on the other hand, are the only social members of the family displaying seasonal breeding (Jarvis and Bennett, 1991; Spinks *et al.*, 1997; 1999; Janse van Rensburg *et al.*, 2002). This atypical life-history pattern in common and highveld mole-rats may be a consequence of the interaction between the forces of incest avoidance (Faulkes and Bennett, 2001) and limited dispersal opportunities in a seasonal environment. Subterranean dispersal is only

energetically feasible during the short post-precipitation period when the soil is soft and therefore easily workable (Jarvis and Bennett, 1991) – for both the common and highveld mole-rats this period coincides with the non-breeding season (Fig. 3.9; Spinks *et al.*, 1997; 1999; Janse van Rensburg *et al.*, 2002). Normal sexual function out of the breeding season would therefore facilitate inter-sexual recognition and pair-bond formation during the transient period when subordinate common and highveld mole-rats have the opportunity to disperse and attempt independent reproduction (Spinks *et al.*, 2000a). Interestingly, it is also towards the end of the non-breeding period when the demand for helpers in the colony is reduced due to favourable digging conditions and the presence of a new generation of workers, that suppression of the hypothalamo-pituitary-gonadal axis in non-reproductive female highveld mole-rats seems to be relaxed (Van der Walt *et al.*, 2001). In mole-rats annual changes in temperature and rainfall patterns, rather than photoperiod as is the case in most seasonal rodents (Bittman, 1984; Malpaux *et al.*, 1999), may be the ultimate cues timing reproduction (Miethe *et al.*, submitted).

The non-reproductive females of the highveld mole-rat, like the eusocial Damaraland, mole-rat exhibit natural infertility that is mediated through an inhibition of GnRH release, resulting in the suppression of reproductive function in subordinate females (this study; Molteno *et al.*, 2004). At the other end of the spectrum, in the closely related common mole-rat only behavioural suppression through incest avoidance is in place to protect the reproductive monopoly of the dominant pair (this study; Spinks *et al.*, 2000a). Which factor - ecological, behavioural, or otherwise, could result in anatomical differences in the GnRH system as well as the divergent means of social suppression operational in such closely related subspecies? In teleost fish, distinct patterns of adaptation to certain ecological environments are reflected in species-specific variation in the number and size of GnRH neurones and the reproductive tactic expressed (Foran and Bass, 1999).

Highveld mole-rat colonies are composed of a single breeding female and full siblings. Multiple paternity was however evident in three of the five colonies studied by Malherbe (2001). The paternal contributors of alleles in offspring were not all present in the studied colonies and were assumed to be foreign roaming males. A physiological component to reproductive suppression, in addition to inbreeding avoidance (Faulkes and Bennett, 2001), must therefore be in place to ensure colony cohesion as well as the dominant position and reproductive monopoly of the highveld mole-rat queen in the case of an unrelated male entering the colony circumventing incest taboos. This may seem at odds with the Aridity-Food-Distribution Hypothesis (AFDH; Jarvis *et al.*, 1994), since highveld mole-rats inhabit a summer-rainfall area with frequent predictable rainfall patterns and abundant food resources, possibly selecting for a solitary life-style as suitable soil conditions greatly improve the ability of subterranean animals to forage and thus promote dispersal and independent reproduction (Moolman *et al.*, 1999; Bennett and Faulkes, 2000). However, a trait may develop for one reason and be maintained for another: past evolutionary pressures could have promoted a high degree of sociality in this species as has been proposed for the giant Zambian mole-rat (*Cryptomys mechowi*; Bennett *et al.*, 1999).

In contrast, the common mole-rat specimens used in this study were trapped in a semi-arid habitat characterized by sporadic and unpredictable rainfall. The harsh environment, bolstered by strict incest avoidance operational in the species, may provide a sufficiently large incentive for non-reproductive animals to remain within the confines of their natal colony during the breeding season, negating the need for a physiological component to the behavioural reproductive control enforced by the dominant animals (Spinks *et al.*, 2000b). The influence of aridity on dispersal abilities and reproductive success is evident from the fact that at arid trapping sites, only

36 % of colonies had changed reproductive pairs on recapture whereas 90 % of colonies had new reproductive partners at mesic sites (Spinks *et al.*, 2000b).

The above evidence highlights the impact that environmental factors can have on the specific mechanisms of reproductive suppression evident in the social bathyergids. Restraints imposed by a harsh, arid environment and obligatory outbreeding is enough incentive to maintain a reproductive skew within common mole-rat colonies. In the highveld mole-rat, however, more favourable environmental conditions and consequently greater opportunity to disperse create the need for an added component of physiological suppression, as evidenced by reduced GnRH release in non-reproductive females, to maintain colony cohesion.

Appendix

Table 3.1 Mean area in the median eminence (μm^2) occupied by GnRH-ir (gonadotropin releasing-hormone immunoreactive) fibres for reproductive and non-reproductive female common (*Cryptomys hottentotus hottentotus*) and highveld (*Cryptomys hottentotus pretoriae*) mole-rats out of the breeding season. RF, reproductive female; NRF, non-reproductive female; N, sample size. Values indicated by * are significantly different ($p < 0.05$)

	Common mole-rat		Highveld mole-rat	
	Mean ± sem	*N*	*Mean ± sem*	*N*
RF	17 244 ± 3 376	5	66 284 ± 10 437*	3
NRF	26 700 ± 5 487	5	101 569 ± 8 595*	5

Table 3.2 Mean numbers of GnRH-ir (gonadotropin releasing-hormone immunoreactive) cell bodies per one sixth of diencephalon, for each experimental group. Br, breeding season; N-Br, non-breeding season; RM, reproductive male; NRM, non-reproductive male; RF, reproductive female; NRF non-reproductive female; sem, standard error of the mean; N, sample size

	Common mole-rat		Highveld mole-rat	
	N	*Mean ± sem*	*N*	*Mean ± sem*
Br RM	5	174 ± 63	5	209 ± 52
Br NRM	3	76 ± 4	4	278 ± 63
N-Br RM	4	93 ± 20	5	272 ± 41
N-Br NRM	5	89 ± 11	4	312 ± 52
Br RF	5	101 ± 26	4	213 ± 61
Br NRF	4	85 ± 22	5	192 ± 54
N-Br RF	3	117 ± 10	4	210 ± 30
N-Br NRF	5	103 ± 17	5	346 ± 59

Table 3.3 Mean brain GnRH (gonadotropin releasing-hormone) levels (μg GnRH per pg protein) for each experimental group. Br, breeding season; N-Br, non-breeding season; RM, reproductive male; NRM, non-reproductive male; RF, reproductive female; NRF non-reproductive female; sem, standard error of the mean; N, sample size. Values indicated by identical symbols (* ˣ) are significantly different ($p < 0.05$)

	Common mole-rat		Highveld mole-rat	
	N	*Mean ± sem*	*N*	*Mean ± sem*
Br RM	4	0.45 ± 0.09	4	1.82 ± 0.53
Br NRM	3	0.84 ± 0.38	5	2.30 ± 0.41
N-Br RM	3	0.77 ± 0.11	3	2.88 ± .79
N-Br NRM	4	0.73 ± 0.16	4	1.24 ± 0.48
Br RF	4	0.44 ± 0.10	3	2.03 ± 0.23*
Br NRF	3	0.62 ± 0.19	4	3.09 ± 0.47*
N-Br RF	3	0.37 ± 0.14	5	1.93 ± 0.55ˣ
N-Br NRF	4	0.47 ± 0.20	6	4.41 ± 0.93ˣ

Chapter 4

The effect of endogenous opioid peptides and gonadal steroids on the GnRH-system of female highveld mole-rats with special reference to non-reproductive females

Abstract

The role of gonadal steroids and endogenous opioid peptides (EOPs) in the inhibition of luteinizing hormone (LH) and/or gonadotropin-releasing hormone (GnRH) in subordinate female highveld mole-rats was investigated. Intact reproductive females and intact and ovariectomized non-reproductive females were treated according to five different protocols of exogenous GnRH and/or opioid antagonist (naloxone) administration.

Intact reproductive and ovariectomized non-reproductive females showed similar LH responses to a single exogenous GnRH challenge, significantly higher than post-challenge levels in intact non-reproductive females. Neither intact group showed a significant change in plasma LH levels after receiving a single injection of naloxone, an opioid antagonist. In ovariectomized non-reproductive females a naloxone challenge induced a significant rise in plasma LH levels, while naloxone administration followed by a single exogenous GnRH challenge resulted in plasma LH levels similar to that produced by a single exogenous GnRH challenge alone.

Short term, low dosage naloxone priming (eight hourly injections) did not significantly alter the LH response to a single exogenous GnRH challenge in either intact reproductive females or intact and ovariectomized non-reproductive females. GnRH primed LH levels were, however, significantly higher in ovariectomized non-reproductive females than in either of the intact groups. Long term, high dosage naloxone priming (eight-hourly injections over 96 hours) similarly failed to evoke an altered LH response to an exogenous GnRH challenge in any of the groups.

The results from this study suggest a tentative role for EOPs at the level of the pituitary and potentially the hypothalamic region in socially-induced infertility evident in non-reproductive female highveld mole-rats. The negative effects of gonadal steroids on the GnRH system may be mediated through EOP involvement,

particularly in subordinate females where ovariectomy induced GnRH challenged LH levels similar to that found in intact reproductive females.

Introduction

In Chapter 3 it was indicated that the socially-induced infertility evident in subordinate female highveld mole-rats is a result of reduced gonadotropin-releasing hormone (GnRH) secretion. Socially- and environmentally-induced infertility is postulated to operate through steroid-dependent and steroid-independent mechanisms suppressing gonadotropin secretion (Abbott, 1988). A study by Molteno and Bennett (2002) on the eusocial Damaraland mole-rat (*Cryptomys damarensis*) suggested that a steroid-independent mechanism is more likely to be the cause of the inhibited GnRH release and anovulation that is evident in subordinate females. The presence of a gonadal-dependent mechanism was, however, not totally precluded. Non-reproductive female Damaraland mole-rats have been described as 'pseudopregnant' with the negative feedback effects of increased progesterone levels suppressing gonadotropin secretion and consequently ovulation (Bennett *et al.*, 1996). Progesterone may inhibit the frequency of luteinizing hormone (LH) pulses by reducing the frequency of pulsatile GnRH release from the hypothalamus, while oestradiol has been shown to exert an inhibitory effect on the pituitary by lowering LH levels to such an extent that the pulsatile release of LH is abolished (Tamanini *et al.*, 1986). The negative effects of both gonadal steroids and adrenal corticosteroids (stress–induced infertility) on gonadotropin secretion may be mediated through endogenous opioid peptides (Brooks *et al.*, 1986; Almeida *et al.*, 1989).

Endogenous opioid peptides (EOPs) are naturally produced molecules with morphine-like activity that function as neuromodulators in the central nervous system,

altering the actions of other neurotransmitters by changing the electrical properties of their target neurons thereby making them more difficult to excite. Once released, opioid peptides act through receptors on their target neurons to transmit messages that mostly inhibit secondary systems (Froehlich, 1997). Opioidergic control of reproduction was first established when Barraclough and Sawyer (1955) demonstrated that the opioid agonist morphine inhibits ovulation in the rat. Endogenous opioid peptides, similar to exogenous alkaloid opiates such as morphine, can influence components of the endocrine system (Brooks *et al.*, 1986) and have been implicated in the mediation of several forms of natural infertility including seasonal reproductive quiescence (Aurich *et al.*, 1994; Roberts *et al.*, 1985, 1987), stress-induced infertility in social species (Martensz *et al.*, 1986) and infertility during lactation (Mattioli *et al.*, 1986; Sirinathsinghji and Martini, 1984) and prepubertally (reviewed by Wilkinson and Landymore, 1989). EOPs can influence the secretion of all the pituitary hormones including the gonadotropic hormones prolactin, luteinizing hormone and follicle-stimulating hormone (FSH). While prolactin secretion is markedly enhanced by EOP administration, LH secretion is inhibited or abolished through the regulation of the amplitude and frequency of pulses (Kordon *et al.*, 1994), and FSH is only weakly influenced. Both the hypothalamus and the anterior pituitary are possible sites of opioidergic action upon GnRH and/or LH secretion (Almeida, 1993). The general opioid receptor antagonist naloxone, which acts through receptor competition, has been widely used to study the intimate relationship between opioid peptides and the reproductive system (Almeida, 1993). The gonadotropin response to naloxone has been described as a functional test of reproductive pathology with a blunted response indicative of dysfunction (Kordon *et al.*, 1994).

EOPs are activated by stressful situations and can influence certain physiological effects of such stimuli (Przewlocki, 1993). In social species reproductive skew and

dominance hierarchies are often brought about by stress-induced infertility in non-breeding group members. The most likely mechanism of reproductive inhibition involves corticotrophin-releasing hormone (CRH) secreted during stress and suppressing gonadotropin secretion and sexual behaviour directly and/or indirectly through EOPs (Almeida *et al.*, 1989; Johnson *et al.*, 1992; Przewlocki, 1993). In the talapoin monkey (*Miopithecus talapoin*) chronic subordination of low-ranking males results in increased cerebrospinal fluid opioid levels and decreased reproductive activity relative to dominant males (Eberhart *et al.*, 1983; Martensz *et al.*, 1986). Naked mole-rat (*Heterocephalus glaber*) groups are similarly characterized by social stress in the form of overt aggression between dominant and subordinate females, resulting in reproductive suppression of low ranked individuals (Reeve and Sherman, 1991). A negative correlation between cortisol levels and dominance rank is evident in this species, there does however not appear to be a causal link between adrenal corticosteroid levels and reproductive suppression (Clarke and Faulkes, 1997). In the Damaraland mole-rat the lack of excessive aggression between dominant and subordinate animals (Cooney and Bennett, 2000; Clarke *et al.*, 2001) is reflected in comparable cortisol levels and naloxone-challenged LH levels between animals of different social ranks, indicating an absence of opioid involvement in social reproductive inhibition in this species (Clarke *et al.*, 2001; Molteno and Bennett, 2002).

Seasonal reproduction in mammals can be compared to recurrent puberty. In most species reproductive activity is switched *on* or *off* by changes in photoperiod (Almeida, 1993). Opioids are speculated to be involved in the transmission of photic stimuli from the retina to the hypothalamus. Additionally there is evidence that EOPs mediate the increased negative feedback effects of steroids on the hypothalamus, resulting in an increase in hypothalamic inhibition during the non-breeding period (Brooks *et al.*, 1986). Photoperiodic stimuli alter the pattern of interaction between

steroids and EOPs (Roberts *et al.*, 1987) to the extent that in the seasonally anoestrous mare the transition to ovarian cyclicity is believed to coincide with a change from continuous opioidergic suppression of the hypothalamo-pituitary-gonadal-axis (HPG axis) to intermittent inhibition of gonadotropin release activated by progesterone and resulting in the typical pulsatile pattern of GnRH secretion (Aurich *et al.*, 1994). Under short daylengths (photo-inhibition) hypothalamic β-endorphin levels in male Syrian hamsters (*Mesocricetus auratus*) are significantly elevated compared to those animals exposed to long days, additionally naloxone fails to induce an LH response in such animals (Roberts *et al.*, 1985). Mole-rats possess micropthalmic eyes and rarely if ever are exposed to light in their sealed underground burrows (Jarvis & Bennett, 1990, 1991). Seasonal changes in temperature and precipitation, instead of photoperiod, are postulated to be the ultimate cues timing reproduction in mole-rats (Miethe *et al.*, submitted). In both the common and the highveld mole-rat, behavioural reproductive quiescence during the non-breeding period does not translate into physiological shutdown, with sperm production in males and ovarian function in females being comparable to the breeding season (Spinks *et al.*, 2000a; Janse van Rensburg *et al.*, 2002; Van der Walt *et al.*, 2001). It is thus plausible that in seasonally breeding, social mole-rats EOPs do not play a definitive role in regulating the onset or termination of reproductive activity through changes in photoperiod.

This chapter aims to investigate the possible involvement of endogenous opioid peptides and ovarian hormones in the socially-induced infertility imposed on subordinate female highveld mole-rats, specifically through the suppression of GnRH and/or LH release. The opioid antagonist naloxone was administered alone, and in various combinations with exogenous GnRH, to observe the influence of EOPs on the hypothalamo-pituitary-gonadal axis of socially infertile females compared to dominant, fecund females. Non-reproductive female highveld mole-rats were treated

intact and post-ovariectomy in order to 1) sensitize the pituitary gonadotrophes through increased levels of endogenous GnRH by removing negative feedback from ovarian hormones; and 2) investigate the impact of the steroid hormones oestrogen and progesterone on basal LH secretion and the pituitary response to exogenous GnRH and naloxone administration in these socially suppressed females.

Materials and methods

Direct opioidergic action might result in the suppression of LH secretion at the level of the anterior pituitary (Cacicedo and Franco ,1986; Blank *et al.*, 1986). Since it is not known if naloxone crosses the blood-brain barrier in rodents, I investigated the response of the anterior pituitary to differing regimes of naloxone administration. Since the potential of the anterior pituitary to secrete LH is reduced in non-reproductive female highveld mole-rats, both exogenous (GnRH priming) and increased endogenous GnRH secretion (as a result of ovariectomy) were used in this study to increase pituitary sensitivity to naloxone treatment.

Mole-rats were trapped and housed as described in Chapter 2. Animals were experimentally treated during the non-breeding period of the highveld mole-rat as defined by Janse van Rensburg *et al.* (2002). Ovariectomized reproductive females died postoperatively. Non-reproductive females were allowed to recover for a minimum of four weeks, after which treatment and sampling commenced. Hormone (GnRH) and/or agonist (naloxone) administration and blood collection was performed according to five different protocols:

1. **Single GnRH challenge.** Animals were injected subcutaneously with 2 μg synthetic mammalian GnRH in 200 μg sterile physiological saline. Blood

samples were taken immediately prior to and 20 minutes after the GnRH challenge. This protocol was repeated on intact reproductive as well as intact and ovariectomized non-reproductive highveld mole-rat females to determine the baseline state of the hypothalamo-pituitary-gonadal axis.

2. **Single naloxone challenge.** In order to assess the effect of naloxone and by implication the effect of endogenous opioid peptides on LH release, intact reproductive as well as intact and ovariectomized non-reproductive highveld mole-rat females were subcutaneously injected with 250 μg naloxone in a 200 μl bolus injection. Blood samples were taken before and 20 minutes after the single naloxone challenge.

3. **Single naloxone-GnRH challenge.** A pre-treatment blood sample was obtained from the animal followed by a 250 μg naloxone injection. Twenty minutes later the animal was injected subcutaneously with 2 μg GnRH and after another 20 minute period a second blood sample was taken. These single naloxone treatments followed by GnRH challenges were administered to ovariectomized non-reproductive female highveld mole-rats to investigate the possible role of endogenous opioids in the suppression of GnRH and/or LH secretion in the absence of gonadal steroids and with the resultant increased endogenous GnRH levels and sensitised pituitaries. Plasma LH levels in response to this protocol were compared to the response following both single GnRH and single naloxone challenges.

4. **Eight hour, low dosage naloxone priming and GnRH challenges.** Animals were injected subcutaneously with 2 μg synthetic mammalian GnRH. Blood samples were taken prior to and 20 minutes after the GnRH challenge.

Thereafter animals received eight hourly injections of 100 μg naloxone. Fifteen minutes after the last naloxone treatment, a second GnRH challenge was administered to the animals, with the third blood sample being collected 20 minutes later. The aim of this experimental protocol was to attempt to sensitize the pituitaries of intact reproductive and intact and ovariectomized non-reproductive highveld mole-rat females to GnRH through priming with relatively low doses of naloxone over a short time period.

5. **96 hour, high dosage naloxone priming and GnRH challenges.** This protocol is a variation on the former, with animals receiving higher doses of naloxone over a prolonged period of time. Blood samples were collected from intact reproductive and intact and ovariectomized non-reproductive highveld mole-rat females prior to and 20 minutes after a 2 μg GnRH challenge. Animals received eight-hourly naloxone treatments (500 μg) for 96 consecutive hours. Fifteen minutes after the final naloxone treatment, a second GnRH challenge (2 μg) was administered to the animals. Blood samples were taken immediately prior to and 20 minutes after the second GnRH challenge.

Results

Controls

There was no change in circulating plasma LH levels after a subcutaneous injection of sterile physiological saline in female highveld mole-rats (pre: 2.1 ± 0.1 miu/ml; post: 2.0 ± 0.0 miu/ml; Mann-Whitney U test: $U = 11$, $p > 0.05$, $n = 5$ & 5). Saline, the

vehicle of injection for dissolved GnRH and naloxone, and the subsequent injection thus has no effect on the experimental protocol.

Single GnRH challenge

Intact reproductive females (iRF), intact non-reproductive females (iNRF) and ovariectomized non-reproductive females (oNRF) showed a significant LH response to a single GnRH challenge (Mann-Whitney U test: iRF $U = 0$, $p < 0.05$, $n = 6$ & 6; iNRF $U = 8$, $p < 0.001$, $n = 17$ & 17; oNRF $U = 0$, $p < 0.05$, $n = 8$ & 8; Fig. 4.1 and Table 4.1).

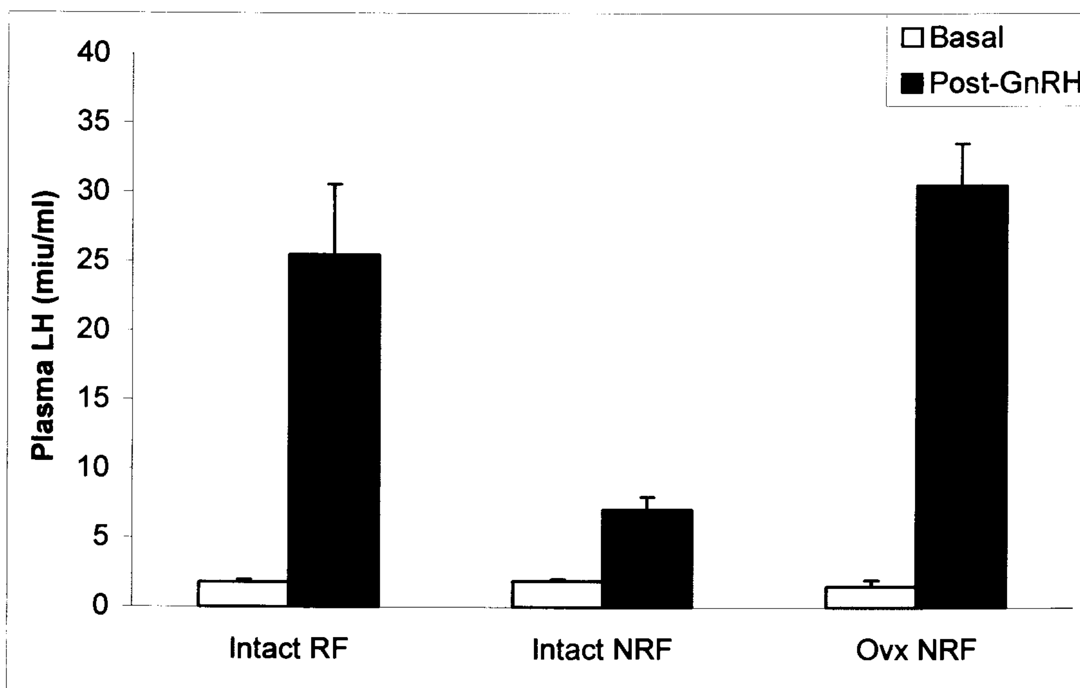

Fig. 4.1 Mean (± sem) plasma LH (luteinizing hormone) levels directly before (Basal) and 20 minutes after a single exogenously derived GnRH (gonadotropin-releasing hormone) challenge (Post-GnRH). RF, reproductive female; NRF, non-reproductive female; Ovx NRF, ovariectomized non-reproductive female

Basal plasma LH levels did not differ significantly between intact reproductive, intact non-reproductive and ovariectomized non-reproductive females (Kruskal Wallis analysis: $H = 5.8$, $p > 0.05$, $n = 31$; Fig. 4.1 and Table 4.1). GnRH challenged LH levels did however differ significantly between the three groups of females (Kruskal Wallis analysis: $H = 21.7$, $p < 0.001$, $n = 31$; Fig. 4.1 and Table 4.1). Reproductive females and ovariectomized non-reproductive females showed much higher post-challenge LH levels compared to intact non-reproductive females.

Single naloxone challenge

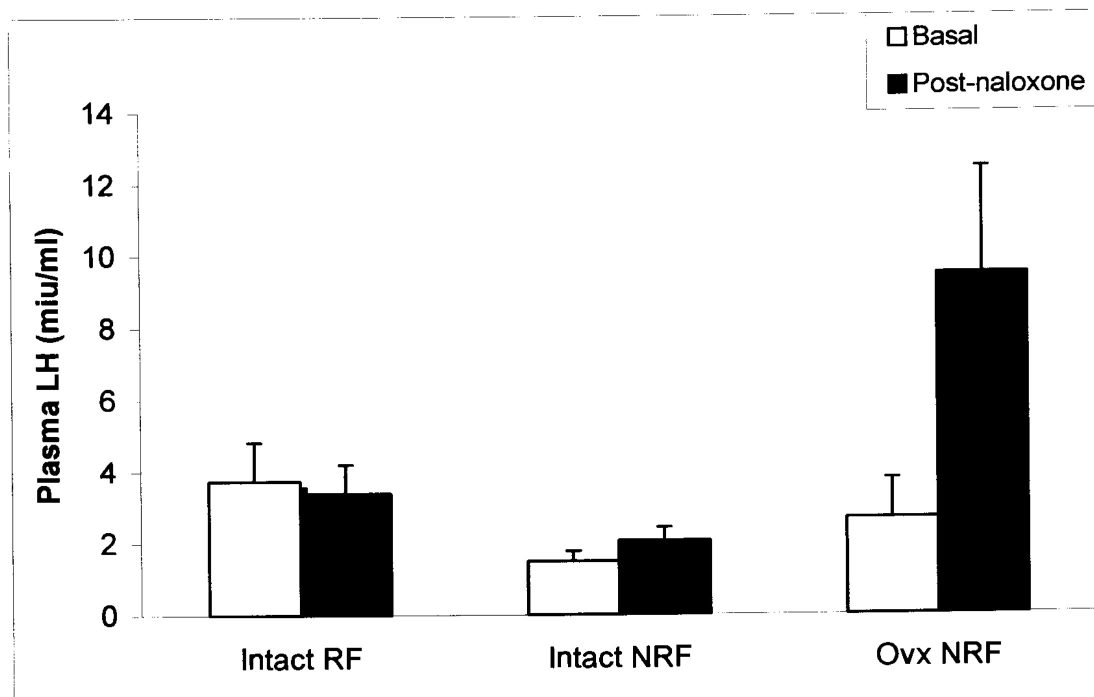

Fig. 4.2 Mean (± sem) plasma LH (luteinizing hormone) levels directly before (Basal) and 20 minutes after a single naloxone challenge (Post-naloxone). RF, reproductive female; NRF, non-reproductive female; Ovx NRF, ovariectomized non-reproductive female

Intact reproductive females and intact non-reproductive females did not show a significant LH response to a single naloxone challenge (t-test: iRF $t = 0.238$, $p > 0.05$,

n = 5 & 5; iNRF $t = -1.212$, $p > 0.05$, n = 20 & 20; Fig. 4.2 and Table 4.2). Ovariectomized non-reproductive females, however, showed a significant plasma LH response to naloxone (t-test: $t = -2.155$, $p < 0.05$, n = 8 & 8).

Basal plasma LH levels differed significantly between intact reproductive, intact non-reproductive and ovariectomized non-reproductive females (Kruskal Wallis analysis: $H = 7.3$, $p < 0.05$, $n = 33$; Fig. 4.2 and Table 4.2), with reproductive females and ovariectomized non-reproductive females having elevated levels compared to intact non-reproductive females. Naloxone challenged LH levels, although higher in ovariectomized non-reproductive females, did not differ significantly between the three groups of females (Kruskal Wallis analysis: $H = 5.5$, $p > 0.05$, $n = 33$; Fig. 4.2 and Table 4.2). However, when comparing the mean percentage change between pre-naloxone and post-naloxone values, the groups differed significantly (Kruskal Wallis analysis: $H = 5.5$, $p < 0.05$, $n = 33$; Table 4.2). Specifically, both intact groups differed significantly from ovariectomized non-reproductive females (Dunn's Pairwise Multiple Comparison: iRF vs. oNRF $Q = 3.12$, $p < 0.05$; iNRF vs. oNRF $Q = 2.61$, $p < 0.05$).

Single naloxone-GnRH challenge

There was a significant LH response to the exogenous GnRH challenge following a single naloxone administration when compared to basal plasma LH levels in ovariectomized non-reproductive female highveld mole-rats (Mann-Whitney U test: $U = 0$, $p < 0.05$, n = 7 & 7; Fig. 4.3 and Table 4.3). The LH rise in response to a single naloxone challenge was significantly lower than the response to either a single GnRH challenge (Mann-Whitney U test: $U = 4$, $p < 0.05$, n = 8 & 8) or to the naloxone followed by GnRH (Mann-Whitney U test: $U = 4$, $p < 0.05$, n = 7 & 8). There was no

difference in the plasma LH response to GnRH and to naloxone followed by an exogenous GnRH administration (Mann-Whitney U test: $U = 26$, $p > 0.05$, $n = 7$ & 8).

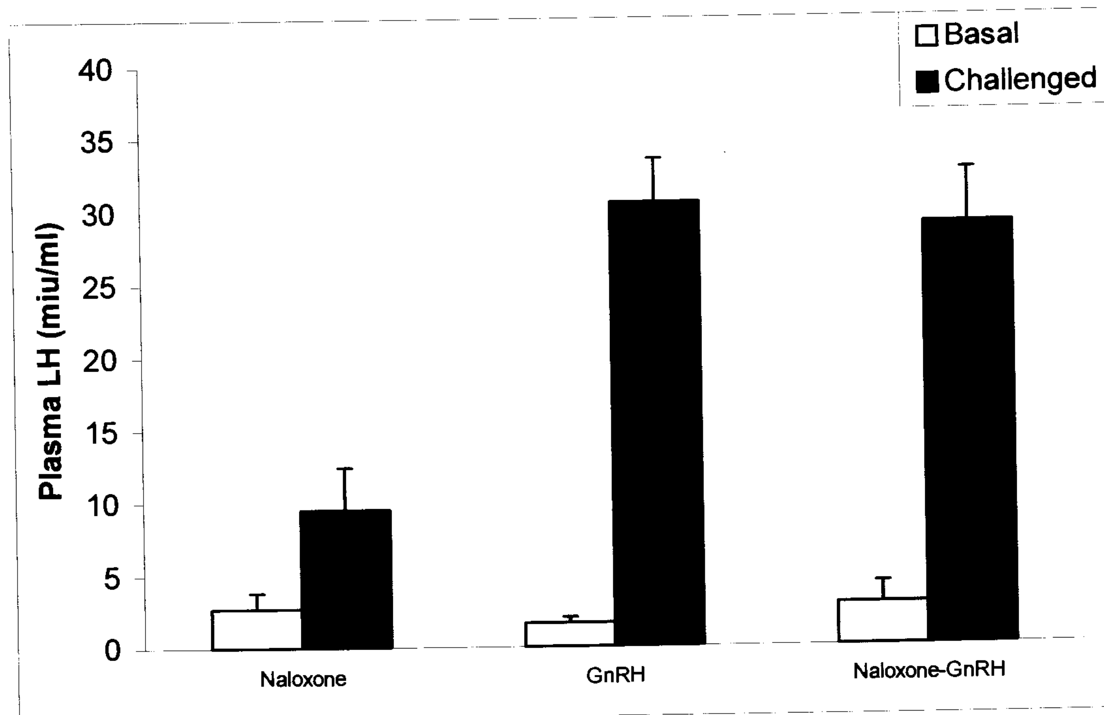

Fig. 4.3 Mean (± sem) plasma LH (luteinizing hormone) levels measured in ovariectomized non-reproductive highveld mole-rat females directly before (Basal) and 20 minutes after a single naloxone challenge, a single GnRH (gonadotropin releasing-hormone) challenge and naloxone administration followed by a GnRH challenge (Challenged)

Eight hour, low dosage naloxone priming and GnRH challenges

Plasma LH levels in response to an exogenous GnRH challenge were significantly elevated from basal concentrations both before and after the eight hours of naloxone priming in intact reproductive females (Kruskal Wallis analysis: $H = 6.9$, $p < 0.05$, $n = 10$), intact non-reproductive females (Kruskal Wallis analysis: $H = 12.0$, $p < 0.05$, $n = 59$) and ovariectomized non-reproductive females (Kruskal Wallis analysis: $H = 19.2$, $p < 0.05$, $n = 29$; Fig. 4.4 and Table 4.4). In all three groups the plasma LH

response to exogenous GnRH was similar before and after eight hours of naloxone priming (Mann-Whitney U test: iRF $U = 2$, $p > 0.05$, $n = 4$ & 2; iNRF $U = 178$, $p > 0.05$, $n = 19$ & 20; oNRF $U = 31$, $p > 0.05$, $n = 10$ & 9).

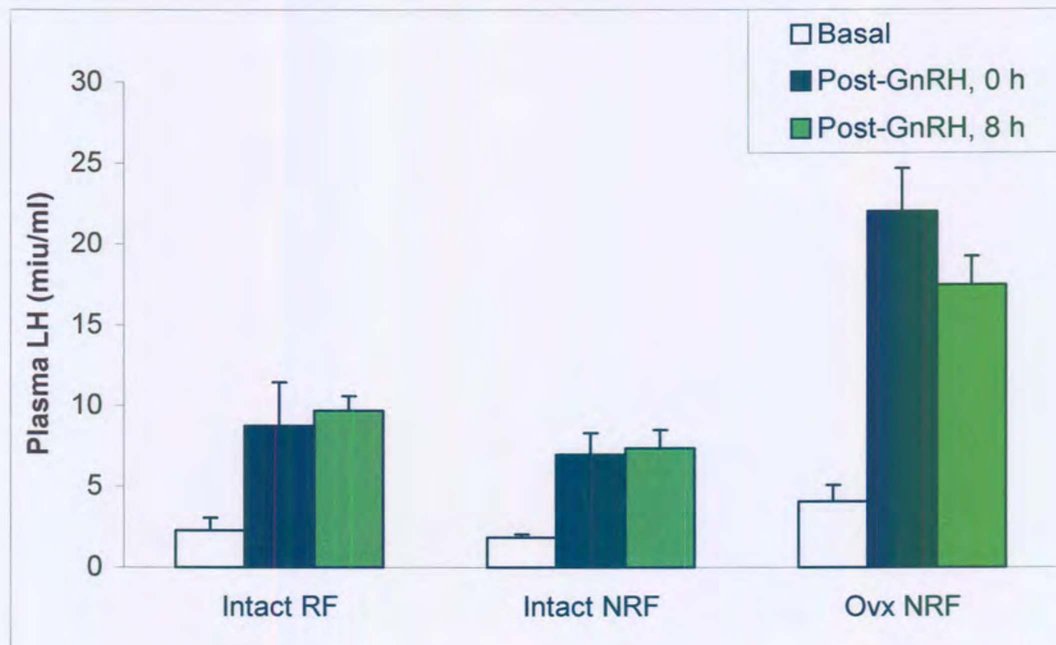

Fig. 4.4 Mean (± sem) basal plasma LH (luteinizing hormone) levels, and LH levels in response to a single exogenous GnRH (gonadotropin-releasing hormone) challenge (Post-GnRH, 0 h), and a GnRH challenge following eight hours of hourly naloxone administration (Post-GnRH, 8 h). RF, reproductive female; NRF, non-reproductive female; Ovx NRF, ovariectomized non-reproductive female

There was no difference in basal plasma LH levels between intact reproductive females, intact non-reproductive females and ovariectomized non-reproductive females (Kruskal Wallis analysis: $H = 5.6$, $p > 0.05$, $n = 34$; Fig. 4.4 and Table 4.4). Ovariectomized non-reproductive females had markedly higher plasma LH levels after a single GnRH challenge compared to intact reproductive and non-reproductive females (Kruskal Wallis analysis: $H = 16.3$, $p < 0.05$, $n = 33$). Similarly, after eight hours of hourly naloxone priming, plasma LH levels in ovariectomized females were

substantially higher than LH levels in either of the intact female groups (Kruskal Wallis analysis: $H = 13.7$, $p < 0.05$, $n = 31$).

96 hour, high dosage naloxone priming and GnRH challenges

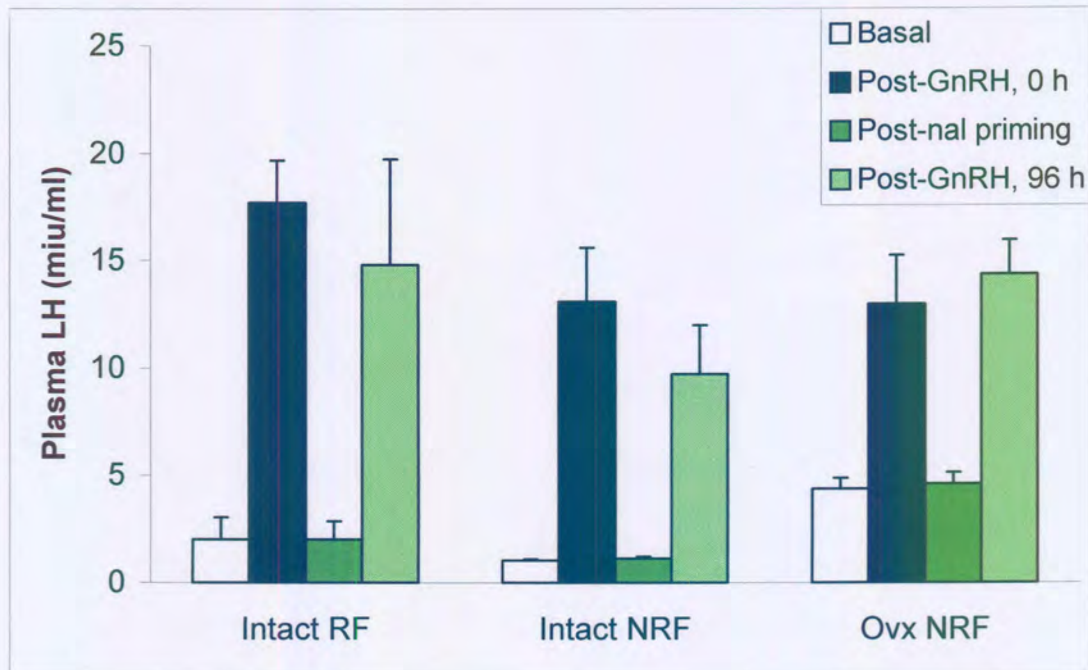

Fig. 4.5 Mean (± sem) basal plasma LH (luteinizing hormone) levels, and LH levels in response to a single GnRH (gonadotropin-releasing hormone) challenge (Post-GnRH, 0 h), plasma LH levels following 96 hours of hourly naloxone administration (Post-nal priming) and a GnRH challenge directly after the final naloxone administration (Post-GnRH, 96 h). RF, reproductive female; NRF, non-reproductive female; Ovx NRF, ovariectomized non-reproductive female

There were significant differences between basal and challenged plasma LH levels in intact reproductive females (Kruskal Wallis analysis: $H = 8.3$, $p < 0.05$, $n = 12$; Fig. 4.5 and Table 4.5), intact non-reproductive females (Kruskal Wallis analysis: $H = 24.0$, $p < 0.05$, $n = 60$) as well as ovariectomized non-reproductive females (Kruskal Wallis analysis: $H = 15.9$, $p < 0.05$, $n = 32$). Basal plasma LH levels directly after 96 hours of naloxone priming were similar to basal LH levels in all three groups

(Mann-Whitney U test: iRF $U = 4.$, $p > 0.05$, $n = 3$ & 3; iNRF $U = 100$, $p > 0.05$, $n = 15$ & 15; oNRF $U = 30$, $p > 0.05$, $n = 8$ & 8). Similarly, GnRH challenged LH levels prior to and after 96 hours of naloxone priming did not differ significantly in intact reproductive females (Mann-Whitney U test: $U = 4$, $p > 0.05$, $n = 3$ & 3), intact non-reproductive females (Mann-Whitney U test: $U = 90$, $p > 0.05$, $n = 15$ & 15) and ovariectomized non-reproductive females (Mann-Whitney U test: $U = 32$, $p > 0.05$, $n = 8$ & 8).

Basal plasma LH levels differed significantly between the three groups of females (Kruskal Wallis analysis: $H = 15.3$, $p < 0.05$, $n = 26$) with ovariectomized non-reproductive females having higher basal levels than either intact reproductive or intact non-reproductive females (Fig. 4.5 and Table 4.5). GnRH challenged LH levels prior to naloxone priming were similar in all three groups (Kruskal Wallis analysis: $H = 0.7$, $p > 0.05$, $n = 26$). Plasma LH levels directly after 96 hours of naloxone priming were again markedly higher in ovariectomized non-reproductive females than in intact reproductive females or intact non-reproductive females (Kruskal Wallis analysis: $H = 17.0$, $p < 0.05$, $n = 26$). GnRH challenged LH levels following 96 hours of naloxone priming did not differ between the three groups (Kruskal Wallis analysis: $H = 4.6$, $p > 0.05$, $n = 26$).

Discussion

In accordance with results from a previous study on the highveld mole-rat (Van der Walt *et al.*, 2001), reproductive females displayed a significantly higher LH response to an exogenous GnRH challenge compared to non-reproductive females (Fig. 4.1). Intact reproductive females and ovariectomized non-reproductive females showed similarly elevated plasma LH levels in response to a GnRH challenge. Since the

elimination of the negative feedback effects of oestrogen and progesterone through ovariectomy seems to lessen the suppression of LH and/or GnRH secretion in non-reproductive female highveld mole-rats, it is plausible that a steroid-dependent mechanism may contribute to the cause of social infertility in this species. However, in the Damaraland mole-rat ovariectomy failed to increase GnRH challenged LH levels in non-reproductive females to the same level as measured in ovariectomized reproductive females, indicating that pituitary desensitation and the resultant anovulation is not dependent on the inhibitive effects of gonadal steroids (Molteno and Bennett, 2000, 2002). Similarly, we would expect ovariectomized reproductive female highveld mole-rats to continue to show greater GnRH-stimulated LH secretion than non-reproductive females. Studies involving ovariectomized reproductive females should be conducted in order to clarify the involvement of steroid dependent versus steroid independent mechanisms in the social reproductive suppression of subordinate female highveld mole-rats.

In the anoestrous ewe, photoperiodic suppression of tonic LH secretion is mediated by steroid-dependent (catecholaminergic) and steroid-independent (serotonergic) mechanisms (Meyer and Goodman, 1986). Studies on ovariectomized ewes showed that oestradiol inhibits pituitary action by decreasing LH levels, while progesterone alters the frequency of pulsatile GnRH release (Tamanini *et al.*, 1986). However, the main site of inhibitory oestradiol action during the anestrus is ERα (oestrogen receptor alpha) containing neurons in the retrochiasmatic area, stimulating a dopaminergic pathway inhibiting LH secretion (Hardy *et al.*, 2003). Progesterone-induced suppression of pulsatile LH secretion can be reversed by naloxone during the oestradiol-independent, but not the oestradiol-dependent activation stage of GnRH surge induction (Richter *et al.*, 2001). In rats it has been shown that EOP suppression of median eminence GnRH is not dependent upon negative feedback regulation of LH secretion by physiological concentrations of gonadal steroids

(Rasmussen, 1991). However, the influence of oestrogen on EOPs through the regulation of expression, release and receptor interactions determines lordosis behaviour in female rodents (Micevych and Sinchak, 2001). The fact that opioid neurones located in the vicinity of GnRH neuronal structures bind sex hormones provide a morphological basis for the involvement of EOPs in steroid negative feedback suppression of GnRH (Morrell *et al.*, 1985).

In the highveld mole-rat, a single administration of the opioid antagonist naloxone did not elicit a significant LH response in intact reproductive or intact non-reproductive females (Fig. 4.2). Similar results were obtained for intact female Damaraland mole-rats (Molteno and Bennett, 2002), naked mole-rats (Faulkes, 1990) and marmoset monkeys (Abbott, 1988) where a transient blockade of opioid receptors failed to stimulate a significant increase in plasma LH levels. Interestingly, in ovariectomized non-reproductive females, similar to ovariectomized subordinate female marmosets (Abbott, 1993; Abbott *et al.*, 1987), the plasma LH response to naloxone was significant when compared to basal levels. However, in this experimental group, a GnRH challenge pre-empted by a single naloxone administration did not result in plasma LH levels different from that following GnRH alone (Fig. 4.3). Likewise sensitization of the pituitary through continued naloxone administration did not succeed, as neither eight hours of low dosage nor 96 hours of high dosage naloxone priming resulted in significant increases in GnRH-challenged LH levels in either intact reproductive, intact non-reproductive or ovariectomized non-reproductive females (Fig 4.4 and Fig 4.5). Data from naloxone studies have been widely used to evaluate the relationship between EOPs and the reproductive system, specifically gonadotropin secretion (for a review see Brooks *et al.*, 1986). The data obtained in this study suggests the possible involvement of endogenous opioid peptides and more explicitly β-endorphin in the socially-induced infertility that is evident in subordinate female highveld mole-rats. In male talapoin monkeys heightened levels

of aggression directed towards low-ranked individuals result in increased cerebrospinal fluid β-endorphin levels as well as lowered pituitary LH response to naloxone when compared to dominant males (Martensz *et al.*, 1986). Also, central EOP systems have also been shown to play a role in the physiology of social victory and defeat in the Mongolian gerbil (*Meriones unguiculatus*; Raab *et al.*, 1985). In female naked mole-rats ovarian hormones facilitate intrasexual aggression (Margulis *et al.*, 1995), while in the female highveld mole-rat EOPs influence LH secretion only in the absence of these hormones. Possibly an absence of overt aggression in highveld mole-rat colonies can explain the apparent low profile of opioidergic involvement in the social suppression of reproduction.

Current knowledge on the mechanisms enforcing the high reproductive skew evident in colonies of all *Cryptomys* species (Jarvis and Bennett, 1991; Faulkes *et al.*, 1997) tends to support the self-restraint model (SRM) of reproductive skew rather than dominant control (DCM) by high ranking colony members. In the common (*Cryptomys hottentotus hottentotus*) and Mashona mole-rats (*Cryptomys darlingi*), behavioural regulation of subordinate fecundity through the absence of unrelated breeding partners in the natal colony, protects the reproductive monopoly of the dominant pair (Bennett *et al.*, 1997; Spinks *et al.*, 1999, 2000a). Non-reproductive Mashona mole-rats are not behaviourally subordinated through overt aggression in the form of shoving or punishments (Gabathuler *et al.*, 1996). Likewise, in the eusocial Damaraland mole-rat, socially induced stress due to aggression does not appear to facilitate ovulatory failure in non-reproductive females (Clarke *et al.*, 2001). In the highveld mole-rat the threat of unrelated males entering the colony, thereby circumventing incest taboos, and negatively affecting colony cohesion and possibly the survival of all group members (Van der Walt *et al.*, 2001) is arguably the driving force behind the added physiological sterility seen in non-reproductive females. It therefore seems that both behavioural and physiological infertility in subordinate

social *Cryptomys* mole-rats are enforced by self-restraint on the part of non-reproductive individuals in order to avoid reproduction under sub-optimal social and/or environmental conditions. A more parsimonious explanation may simply state that subordinate females have developed a specialized neuroendocrine response to the presence of the dominant female, similar to the interaction between the external environment and the internal physiology of seasonally breeding species, inhibiting reproductive function until such a time when the dominant female becomes absent and their offspring have a lesser probability of encountering suboptimal conditions (Saltzman, 2003). An effective blend of cue control and evolved response can therefore result from social cues driving specialized responses (Abbott *et al.*, 1998; 2003).

The present results indicate that the negative feedback effects of ovarian hormones may mediate the physiological component to social infertility evident in subordinate female highveld mole-rats. The involvement of endogenous opioid peptides are tentatively supported, however, studies involving intact and ovariectomized reproductive females as controls, and intact and ovariectomized non-reproductive females housed both in their natal colonies, singly and with an unrelated male should be conducted. In this way the different influences of the suppressive and possibly stressful social environment in the colony (the presence of the dominant pair and the lack of unrelated breeding partners) could be separated. The potential influence of seasonal breeding on the opioidergic and steroid control of reproduction should also be noted.

Appendix

Table 4.1 Mean (miu/ml ± sem) basal plasma luteinizing hormone levels (Basal LH), and LH levels in response to a single gonadotropin releasing-hormone challenge (Post-GnRH). N, sample size; iRF, intact reproductive female; iNRF, intact non-reproductive female; oNRF, ovariectomized non-reproductive female. Values indicated by identical symbols (* ✚ × ✱) are significantly different ($p < 0.05$)

	Basal LH	N	Post-GnRH	N
iRF	1.8 ± 0.2*	6	25.4 ± 5.1*✱	6
iNRF	1.9 ± 0.1✚	17	7.1 ± 0.9✚✱	17
oNRF	1.6 ± 0.4×	8	30.5 ± 3.0×✱	8

Table 4.2 Mean (miu/ml ± sem) basal plasma luteinizing hormone levels (Basal LH), LH levels in response to a single naloxone challenge (Post-naloxone), and the percentage change between the values (%). N, sample size; iRF, intact reproductive female; iNRF, intact non-reproductive female; oNRF, ovariectomized non-reproductive female. Values indicated by identical symbols (* ✚ ×) are significantly different ($p < 0.05$)

	Basal LH	N	Post-naloxone	N	Percentage change
iRF	3.7 ± 1.1*	5	3.4 ± 0.8	5	-1.9 ± 9.0✚
iNRF	1.4 ± 0.3*	20	2.1 ± 0.4	20	42.1 ± 17.9×
oNRF	2.7 ± 1.1*	8	9.4 ± 3.0	8	283.31 ± 94.7✚×

Table 4.3 Mean (miu/ml ± sem) basal plasma luteinizing hormone levels (Basal LH), and LH levels in response to a single naloxone challenge, a single gonadotropin releasing-hormone (GnRH) challenge or naloxone administration followed by a GnRH challenge (LH response) in ovariectomized non-reproductive female highveld mole-rats. N, sample size. Values indicated by identical symbols (* ✚ ×) are significantly different ($p < 0.05$)

	Basal LH	N	LH response	N
Naloxone	2.7 ± 1.1	8	9.5 ± 3.0 ×	8
GnRH	1.6 ± 0.4 *	8	30.5 ± 3.0 *×	8
Naloxone-GnRH	2.9 ± 1.5 ✚	7	29.0 ± 3.7 ✚×	7

Table 4.4 Mean (miu/ml ± sem) basal plasma luteinizing hormone levels (Basal LH), and LH levels in response to a single gonadotropin releasing-hormone (GnRH) challenge (Post-GnRH, 0 h), and a GnRH challenge following eight hours of hourly naloxone administration (Post-GnRH, 8 h). N, sample size; iRF, intact reproductive female; iNRF, intact non-reproductive female; oNRF, ovariectomized non-reproductive female. Values indicated by identical symbols (* ✚ × ✱ ⋆) are significantly different ($p < 0.05$)

	Basal LH	N	Post-GnRH (0 h)	N	Post- GnRH (8 h)	N
iRF	2.3 ± 0.7 *	4	8.7 ± 2.7 *✱	4	9.7 ± 0.9 *⋆	2
iNRF	1.9 ± 0.2 ✚	20	7.0 ± 1.3 ✚✱	19	7.4 ± 1.1 ✚⋆	20
oNRF	4.1 ± 1.0 ×	10	22.0 ± 2.6 ×✱	10	17.5 ± 1.8 ×⋆	9

Table 4.5 Mean (miu/ml ± sem) basal plasma luteinizing hormone levels (Basal LH), and LH levels in response to a single gonadotropin releasing-hormone (GnRH) challenge (Post-GnRH, 0 h), plasma LH levels following 96 hours of hourly naloxone administration (Post-nal priming) and a GnRH challenge directly after the final naloxone administration (Post-GnRH, 96 h). N, sample size; iRF, intact reproductive female; iNRF, intact non-reproductive female; oNRF, ovariectomized non-reproductive female. Values indicated by identical symbols (✱ ✚ × ✸ ⋆ ✷ ✦ ❀) are significantly different ($p < 0.05$)

	Basal LH	N	Post-GnRH (0 h)	N	Post-nal priming	N	Post-GnRH (96 h)	N
iRF	2.0 ±1.0✱✦	3	17.7 ± 1.9✱	3	2.0 ± 0.9✚❀	3	14.8 ± 4.9✚	3
iNRF	1.0 ±0.1×✦	15	13.1 ± 2.5×	15	1.1 ± 0.1✸❀	15	9.7 ± 2.3✸	15
oNRF	4.4 ±0.5⋆✦	8	13.0 ± 2.3⋆	8	4.6 ± 0.5✷❀	8	14.3 ± 1.6✷	8

Chapter 5

The amino acid structure of highveld mole-rat gonadotropin-releasing hormone

Abstract

In situ hybridisation of mole-rat brain tissue with a riboprobe against the standard mammalian gonadotropin-releasing hormone (GnRH) failed to produce a signal, while hybridisation with a riboprobe against guinea pig GnRH revealed the presence of GnRH containing structures in mole-rat tissue.

Messenger ribonucleic acid (mRNA) coding for GnRH was isolated from hypothalamic tissue samples of a female highveld mole-rat (*Cryptomys hottentotus pretoriae*). The nucleotide sequence of the derived cDNA predicts an amino acid sequence for the mature peptide that differs from classic 'mammalian' GnRH by one amino acid position. The amino acid sequence of mole-rat GnRH shows a potential polymorphism in that one clone is identical to the unique form expressed by guinea pigs (*Cavia porcellus*) while a second form differs from it in position two.

These results indicate that classic 'mammalian' GnRH is not the chief structural form of GnRH in the mole-rat. Implications for the validity of previous studies on the GnRH system of African mole-rats, as well as the taxonomic placement of the Bathyergidae are discussed.

Introduction

Gonadotropin-releasing hormone (GnRH), also known as luteinizing hormone-releasing hormone (LHRH), is the chief regulator of reproductive function in all the major vertebrate classes, being responsible for the release of gonadotropins (luteinizing hormone and follicle stimulating hormone) from the anterior pituitary. GnRH-like material has also been isolated from protochordate tissue (sea squirt, *Chelyosoma productum*), suggesting that the peptide may already have been present at the evolutionary transition between invertebrates and vertebrates (Kelsall *et al.*,

1990). Although originally thought to be structurally conserved in all vertebrates, up to sixteen dissimilar forms of the GnRH peptide have been documented in fish, amphibians, reptiles, birds and mammals (King and Millar, 1979, 1995; Grove-Strawser *et al.*, 2002). In all known forms of GnRH, spanning 500 million years of evolution, the functionally important $-NH_2$ (receptor binding) and -COOH (receptor activation) terminals are conserved while amino acid positions five to eight are the most variable (Millar *et al.*, 1989).

Nearly all vertebrates studied to date express at least two forms of GnRH: almost universally chicken GnRH II thought to act as an extrahypothalamic neurotransmitter and neuromodulator (Fig. 5.1), and a second form that varies across taxa and is assumed to regulate pituitary gonadotropin release (King and Millar, 1995). The two GnRH systems have different embryonic and evolutionary origins: neurons in the embryonic olfactory placode give rise to the terminal nerve-septo-preoptic system, while cells from non-placodal precursors forms the posterior GnRH system (Muske, 1993).

	1	2	3	4	5	6	7	8	9	10
Guinea pig:	*pGlu*	Tyr	Trp	*Ser*	Tyr	Gly	Val	Arg	*Pro*	*Gly*
Mammalian:	*pGlu*	His	Trp	*Ser*	Tyr	Gly	Leu	Arg	*Pro*	*Gly*
Chicken II:	*pGlu*	His	Trp	*Ser*	**His**	Gly	**Trp**	**Tyr**	*Pro*	*Gly*

Fig. 5.1 The amino acid sequences of guinea pig GnRH, classic 'mammalian' GnRH and chicken GnRH II. **Bold**, amino acid residues that vary from classic 'mammalian' GnRH; Boxed, unique residues in the guinea pig sequence compared to all previously known forms of the GnRH peptide; *Italic,* amino acid positions that are invariant in all studied forms of GnRH (Adapted from Grove-Strawser *et al.*, 2002)

Three forms of GnRH have been purified from the brain of the Pacific herring (*Clupea harengus pallasi*): chicken GnRH II, salmon GnRH and a novel form, herring GnRH (Carolsfeld *et al.*, 2000), while four distinct GnRHs are found in the clawed toad (*Xenopus laevis*): classic 'mammalian' GnRH, hydroxyproline mammalian GnRH, chicken GnRH II and an unidentified form (King *et al.*, 1994b). In contrast to the lower vertebrates, GnRH peptide and receptor structure are highly conserved in birds and mammals (Millar *et al.*, 1989). Classic 'mammalian' GnRH and chicken GnRH II occur together in metatherian (pouched or marsupial) mammals and early evolved eutherian (placental) mammals (King *et al.*, 1988; King and Millar, 1995). A second form of GnRH, in addition to classic 'mammalian' GnRH, has only recently been documented in higher mammalian species such primates and humans (Lescheid *et al.*, 1997; White *et al.*, 1998). Almost all mammals studied to date therefore express classic 'mammalian' GnRH, with an identical amino acid sequence (*pGlu*-His-Trp-*Ser*-Tyr-Gly-Leu-Arg-*Pro*-*Gly*), but not necessarily identical nucleotide sequence. The exception to the rule is the guinea pig (*Cavia porcellus;* Jimenez-Liñan *et al.*, 1997). The major neuroendocrine form of GnRH in this animal is guinea pig GnRH (Fig. 5.1) – a novel form in more ways than one. The amino acid sequence of guinea pig GnRH indicates that it is the first peptide in the family with a substitution in position two (histidine replaced by tyrosine) thereby altering the, hitherto believed to be invariant, N-terminal sequence of the molecule. Another unique substitution relative to currently known forms of GnRH is the replacement of the leucine molecule in position seven in mammalian GnRH by valine in guinea pig GnRH (as opposed to tryptophan, phenylalanine and histidine in other variants; Jimenez-Liñan *et al.*, 1997).

The guinea pig is a singular animal in many respects. The reproductive cycle of this hystricognath rodent more closely mirrors that of a primate than that of a rat due to the presence of a true luteal phase (Jimenez-Liñan *et al.*, 1997). Similarly, the

anatomical distribution of GnRH neurons in the guinea pig resembles the distribution in primates and other non-rodent species (Silverman, 1976; King and Anthony, 1984). Furthermore, rates of amino acid replacement in several of the guinea pig's other proteins such as insulin, glucagon, vasoactive intestinal peptide and factor IX are believed to be unusually high (Jimenez-Liñan *et al.*, 1997). Several authors have proposed that the caviomorphs (guinea pig-like rodents) and the myomorphs (rat-like rodents) are not monophyletic (Graur *et al.*, 1992; Jimenez-Liñan *et al.*, 1997), it has even been debated whether the guinea pig is in actual fact a rodent (Graur *et al.*, 1991)!

The aim of this chapter is to explore the possibility that mole-rat GnRH, in a manner similar to guinea pig GnRH, is structurally divergent from the basic mammalian form of the peptide found in all other studied mammals. The notion is based on the discovery that riboprobes against rat GnRH mRNA (classic 'mammalian' GnRH) used in *in situ* hybridisation protocols, do not recognise the variant of GnRH found in mole-rat tissue (Molteno, 1999; this study). Furthermore, a fellow hystricognath, the capybara (*Hydrochaeris hydrochaeris*) synthesizes salmon GnRH in addition to the two basic variants produced by other mammals (Montaner *et al.*, 1998). The bathyergids, due to their hystricognath affiliation, may therefore be prime candidates to join the guinea pig in the ranks of the physiologically unconventional.

Materials and methods

In situ hybridisation. Animals were trapped, housed and sacrificed as described in Chapter 2. Perfused brain tissue from reproductive and non-reproductive female highveld mole-rats and laboratory rats were treated according to *in situ* hybridisation protocols (Chapter 2). Riboprobes against GAD-65 (glutamic acid decarboxylase),

classic 'mammalian' (rat) GnRH and guinea pig GnRH were applied to both highveld mole-rat and rat cerebral tissue sections.

Mole-rat GnRH sequencing. Total RNA was extracted from the cerebral tissue of one female highveld mole-rat, GnRH mRNA isolated and converted to cDNA, the nucleotide sequence determined and compared to that of classic 'mammalian' GnRH and guinea pig GnRH as described in Chapter 2.

Results

***In situ* hybridisation**

The riboprobe against GAD-65 worked on both mole-rat and rat tissue (Plate 5.1 a-b), although the signal in the mole-rat tissue was less intense compared to the signal in the rat tissue – thereby validating the specific *in situ* hybridisation technique applied (adapted from Nagaso *et al.*, 2001) for use on paraformaldehyde fixed mole-rat brain tissue. The riboprobe against classic 'mammalian' (rat) GnRH produced a signal in rat tissue but no signal in mole-rat tissue , while the probe against guinea pig GnRH did reveal the presence of GnRH-producing neurons in mole-rat neural tissue (Plate 5.2 a-c).

Mole-rat GnRH sequence

The amino acid sequence for the GnRH peptide found in mole-rat tissue (Fig. 5.2) was deduced from the nucleotide sequence of mole-rat GnRH mRNA transcripts converted to cDNA (Plate 5.3). Four individual clones revealed two different amino acid sequences for the GnRH peptide found in mole-rat tissue. Mole-rat GnRH

clones 1 and 4 differed from classic 'mammalian' GnRH only in position seven where leucine is replaced by valine. Mole-rat GnRH clones 2 and 3, however, are identical to guinea pig GnRH in that in addition to the valine in position seven, histidine in position two is substituted by tyrosine.

	1	2	3	4	5	6	7	8	9	10
Mole-rat 1, 4:	pGlu	His	Trp	Ser	Tyr	Gly	**Val**	Arg	Pro	Gly
Mole-rat 2, 3:	pGlu	**Tyr**	Trp	Ser	Tyr	Gly	**Val**	Arg	Pro	Gly
Guinea pig:	pGlu	Tyr	Trp	Ser	Tyr	Gly	Val	Arg	Pro	Gly
Mammalian:	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly

Fig. 5.2 The amino acid sequences of mole-rat GnRH clones 1 and 4 and clone 2 and 3, guinea pig GnRH and classic 'mammalian' GnRH. **Bold**, amino acid residues in the mole-rat sequence that vary from classic 'mammalian' GnRH; boxed, amino acid residues in the mole-rat sequence that vary from the guinea pig sequence

Plate 5.1 *In situ* hybridisation signal against GAD-65 in a) highveld mole-rat (*Cryptomys hottentotus pretoriae*) tissue; and b) control rat sections in the hippocampus. Scale bar denotes 400 μm

Plate 5.2 *In situ* hybridisation signal against guinea pig GnRH mRNA in highveld mole-rat (*Cryptomys hottentotus pretoriae*) tissue. a) Low power, dark field photomicrograph of the preoptic area; High power, b) dark field and c) bright field photomicrograph of a single GnRH cell body (represented by a cluster of silver grains), corresponding to the area highlighted in the rectangle. Scale bar units in μm

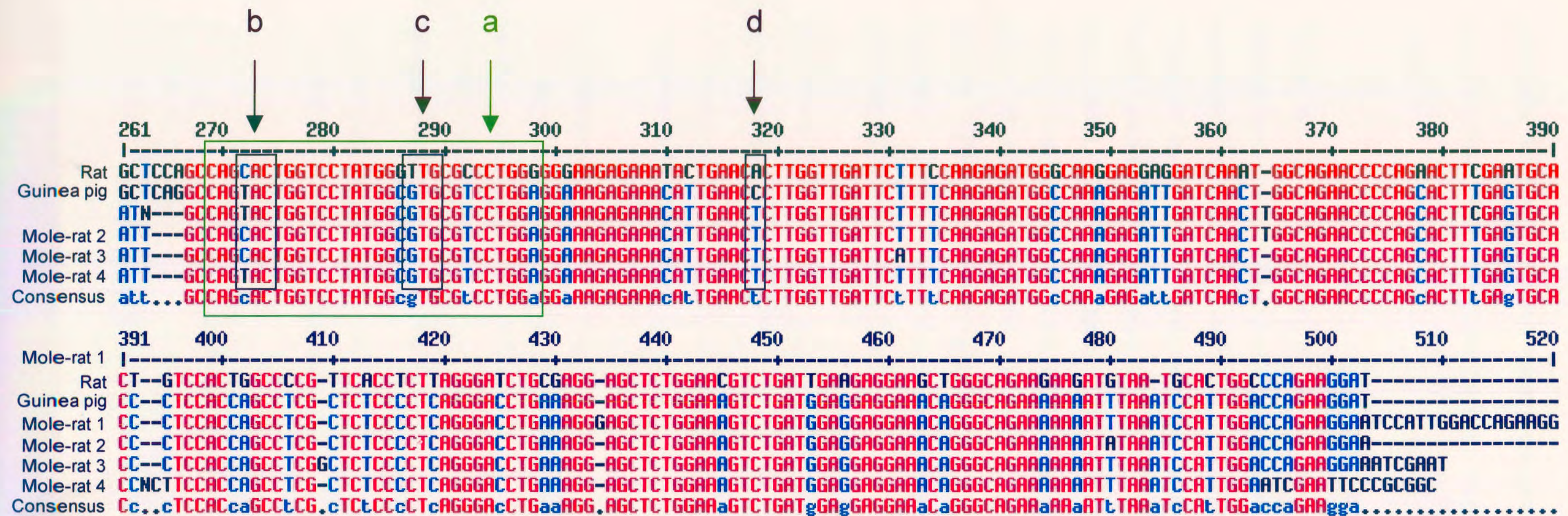

Plate 5.3 Nucleotide base sequences for classic 'mammalian' (rat) GnRH cDNA, guinea pig GnRH cDNA and mole-rat GnRH cDNA (four different clones Mole-rat 1-4) as well as the consensus sequence for the six sequences. a) The 30 nucleotide bases coding for the mature ten-amino acid GnRH peptide; b) Nucleotide bases coding for histidine (position 2) in rat GnRH and Mole-rat 2 and 3, and for tyrosine in guinea pig GnRH and Mole-rat 1 and 4; c) nucleotide bases coding for valine in rat GnRH and leucine in guinea pig GnRH and Mole-rat 1-4; d) a non-coding nucleotide base that is identical in all four mole-rat GnRH cDNA sequences (thymine) but differs from that in both rat (adenine) and guinea pig (cytosine). Sequences aligned using the Internet based program MultAlin. Red, identical in all six sequences; Blue, identical in the majority of sequences; Black, identical in the minority of sequences

Discussion

In addition to the socially induced infertility continuum displayed by the Bathyergidae (Jarvis and Bennett, 1991), the findings from this study distinguish mole-rats from the majority of other mammals in yet another way: by predicting rare and unique amino acid sequences for mole-rat GnRH. The highveld mole-rat seems to express two structural forms of the GnRH peptide, neither being classic 'mammalian' GnRH nor chicken GnRH II (the forms of GnRH expressed by most other mammals; Table 5.1). The sequence and identity of the cDNA isolated in this study have been confirmed at the level of the nucleotide and predicted amino acid sequences, by comparing mole-rat GnRH with known sequences of rat (classic 'mammalian') and guinea pig GnRH. The nucleotide sequence of mole-rat GnRH clones 1 and 4 is highly homologous to the unique structure of guinea pig GnRH – a variant of GnRH up to now only described in the guinea pig (Jimenez-Liñan *et al.*, 1997), while the sequence of mole-rat clones 2 and 3 is identical to this rare structural form of the GnRH decapeptide. Additionally, *in situ* hybridisation results indicate that a probe based on the structure of guinea pig GnRH mRNA recognises a variant of GnRH expressed in the mole-rat brain (Plate 5.2), while a probe designed for mammalian GnRH mRNA does not produce a hybridisation signal in mole-rat tissue. Mole-rat GnRH clones 1 and 4 differ from clones 2 and 3 by only one amino acid. Similar to classic 'mammalian' GnRH clones 1 and 4 have histidine in position two while clones 2 and 3 are identical to guinea pig GnRH in having tyrosine in this position and valine instead of leucine in position seven. Until now guinea pig GnRH was the only peptide in the family without histidine in position two, and with valine in position seven (Jimenez-Liñan *et al.*, 1997).

Table 5.1 Structural forms of GnRH expressed by various mammalian species (Adapted from King and Millar, 1995; Jimenez-Liñan *et al*., 1997; Lesceid *et al*., 1997; Montaner *et al*., 1998; White *et al*., 1998)

Species	GnRH structure		
Echidna (*Tachyglossus aculeatus*)			Unidentified form
Opossum (*Monodelphis domestica*)	M	CII	
Possum (*Trichosurus vulpecula*)	M	CII	
Quoll (*Dasyurus viverrinus*)	M	CII	
Bandicoot (*Isoodon macrourus*)	M	CII	
Wallaby (*Macropus eugenii*)	M		
Musk shrew (*Suncus murinus*)	M	CII	
Common tree shrew (*Tupaia glis belangeri*)	M	CII	
Mole (*Chrysochloris asiatica*)	M	CII	
Bat (*Miniopterus schreibersii*)	M		
Pig (*Sus scrofa*)	M		
Sheep (*Ovis aries*)	M		
Rat (*Rattus norvegicus*)	M		
House mouse (*Mus musculus*)	M		
Golden hamster (*Mesocricetus auratus*)	M		
Dormouse	M		
Guinea pig (*Cavia porcellus*)			Guinea pig GnRH
Capybara (*Hydrochaeris hydrochaeris*)	M	CII	Salmon GnRH
Highveld mole-rat (*Cryptomys hottentotus pretoriae*)			Guinea pig GnRH Mole-rat GnRH
Rhesus monkey (*Macaca mulatta*)	M	CII	
Stumptail monkey (*Macaca speciosa*)	M	CII	
Marmoset monkey (*Callithrix jacchus*)	M		
African green monkey (*Cercopithecus aethiops*)	M	CII	
Human (*Homo sapiens*)	M	CII	

The possibility that mole-rats express a third variant of GnRH (possibly chicken GnRH II) should, however, not be excluded at this stage, since multiple forms of GnRH have been identified in most vertebrate species studied to date (King and Millar, 1995). In the social male cichlid fish (*Haplochromis burtoni*) changes in reproductive status result in changes in the gene expression and mRNA levels of only one of three structurally divergent GnRHs expressed in distinct neuronal subpopulations (White and Fernald, 1998; White *et al.*, 2002). Separate control of the expression of multiple GnRHs with distinct locations and functions in the herring is thought to offer an evolutionary advantage in the form of refined control of reproduction, allowing the fish to spawn more than once over a prolonged time period or delay spawning to match optimal environmental conditions (Carolsfeld *et al.*, 2000). Similarly, in social mole-rat species, subordinate colony members could coordinate their reproductive behaviour and physiology with the suitability of their physical and social environment (Self Restraint Model: Wasser and Barash, 1983; Snowdon, 1996; Clarke *et al.*, 2001).

Previous studies on the GnRH system of bathyergids were based on the assumption that classic 'mammalian' GnRH is present in mole-rat tissue, and subsequently antisera and riboprobes specifically against classic 'mammalian' GnRH (in some instances chicken GnRH II) were employed - with plausible results. Antiserum 678 (JA King, Department of Chemical Pathology, University of Cape Town, RSA) and antiserum 1076 (RP Millar, Department of Chemical Pathology, University of Cape Town, RSA) were used in radioimmunoassay protocols, while Incstar rabbit anti-LHRH (Incstar Corporation, USA) was used in immunocytochemical procedures (Jastrow *et al.*, 1998; Molteno, 1999; this study). Until 1997 (Jimenez-Liñan *et al.*) the guinea pig was similarly believed to synthesize classic 'mammalian' GnRH, and antisera against this form of GnRH was routinely used to detect GnRH in guinea pig tissue (Barry and Dubois, 1974; Silverman, 1976; Kelsall *et al.*, 1990; King *et al.*,

1998). However, when antibodies raised specifically against guinea pig GnRH were used, the intensity of the immunoreactive staining and the number of cell bodies observed, appeared to be significantly greater than that described in earlier studies using conventional antibodies against classic 'mammalian' GnRH (Grove-Strawser *et al.*, 2002). It is speculated that the guinea pig GnRH receptor may have changed in response to, or concurrently with, the alteration in the GnRH peptide – importantly, the alleged changes do not eliminate the receptor's ability to react to classic 'mammalian' GnRH (Grove-Strawser *et al.*, 2002). Guinea pig GnRH and classic 'mammalian' GnRH have been shown to display conformational similarity and both can stimulate LH secretion in guinea pigs (Gao *et al.*, 2000). It therefore seems that while previous studies detailing the mole-rat gonadotropin-releasing hormone system (working under the assumption that classic 'mammalian' GnRH is virtually ubiquitous among mammals) might have quantitatively underestimated the extent of GnRH neuronal structures, they should be qualitatively correct in describing the distribution of the peptide and the influence of sociality on the hypothalamo-pituitary-gonadal axis within the social species.

The bathyergid mole-rat family is endemic to sub-Saharan Africa, with either an African or Asian origin and an exclusively African history dating to the early Miocene period (Honeycutt *et al.*, 1991; Winkler, 1994). The exact taxonomic placement of the Bathyergidae relative to other rodent families was ambiguous to such an extent that the family has at some stage been placed in each of the three rodent suborders (Hystricomorpha, Sciuromorpha and Myomorpha), as well as in a separate suborder (Bathyergomorpha; De Graaff, 1979). In evolutionary terms, the Bathyergidae form an interesting group for three reasons: 1) the family presents an early and complex rodent radiation in Africa; 2) the social structure of species in the family ranges between solitary and eusocial; and 3) the family exhibits several combinations of traits that obfuscate their placement within the order (Honeycutt *et al.*, 1991). The

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Bathyergidae do not seem to fit perfectly into any of the current rodent suborders, however, the ontogeny of the masticatory apparatus suggests a somewhat closer affinity to the Hystricognathi (Maier and Schrenk, 1987) and recently, several authors have placed the Bathyergidae unambiguously in the Hystricognathi, more specifically the Phiomorpha (Nedball *et al.*, 1994; Huchon *et al.*, 1999; Huchon and Douzery, 2001; Mouchaty *et al.*, 2001). Biogeographically hystricognath rodents are divided into two well-defined groups, the Old Word Phiomorpha (*sensu* Lavocat, 1973) to which the African mole-rats belong and the New World Caviomorpha (*sensu* Wood, 1955). Currently evidence is lacking to establish a sister-group relationship between the Bathyergidae and any other lineage within the suborder (Honeycutt *et al.*, 1991). The results from this study suggest the Bathyergidae and the guinea pig-family, Caviidae share a common ancestor – both families express highly unique forms of the GnRH peptide separating them from all other studied mammals. Interestingly, the guinea pig GnRH gene contains a second polyadenylation sequence not present in other mammals but present in some species of fish, further separating them from Old World rodents such as rats (Jimenez-Liñan *et al.*, 1997). Graur and colleagues (1992) suggest that the evolutionary lineage leading to the guinea pig may have branched off prior to the divergence among myomorphs, lagomorphs, primates, chiropterans, artiodactyls and carnivores - making the Rodentia as we currently define them 'a paraphyletic group devoid of taxonomic validity'. Another phylogenetic tree based on the mitochondrial cytochrome *b* gene suggests that the Hystricomorpha and primates form a clade, excluding the Myomorpha as an outgroup (Ma *et al.*, 1993).

Molecular phylogenies are commonly used to untangle taxonomic relationships by reconstructing the evolutionary history of living taxa (Zuckerkandl and Pauling, 1965), making the discovery of the same (mole-rat clones 2 and 3) and similar (mole-rat clones 1 and 4) anomalous variant peptides in two closely related, taxonomically

problematic groups, very valuable. However, care must be taken to use appropriate methods and adequately sized datasets before making phylogenetic inferences (Cao *et al.*, 1994), especially since the results of this study is based on data obtained from a single mole-rat.

Chapter 6

Synthesis

The primary focus of this study was to investigate the reproductive inhibition evident in female social common (*Cryptomys hottentotus hottentotus*) and highveld (*Cryptomys hottentotus pretoriae*) mole-rats, investigating 1) intra- and inter-subspecies differences in GnRH neuroanatomy and neuroendocrinology (Chapter 3); and 2) potential mechanisms of social infertility mediated by the suppression of luteinizing hormone (LH) and/or gonadotropin-releasing hormone (GnRH) synthesis or release - with special reference to the roles of endogenous opioid peptides (EOPs) and gonadal steroid hormones in the female highveld mole-rat (Chapter 4). Difficulties encountered during the study led to a probe into the molecular structure of the particular structural form of GnRH present in mole-rat brain tissue, with very exciting results (Chapter 5).

Highveld mole-rats apparently synthesize two rare variants of the GnRH peptide: one form is identical to guinea pig GnRH hitherto only described in a fellow hystricognath, the guinea pig (*Cavia porcellus*; Jimenez-Liñan *et al.*, 1997), while the second form is unique to the mole-rat, differing from both guinea pig and classic 'mammalian' GnRH in one amino acid position. This finding is made even more remarkable by the fact that all other studied mammals express two structural forms of GnRH, namely classic 'mammalian' GnRH and chicken GnRH II (King *et al.*, 1988; King and Millar, 1995). Guinea pig GnRH and/or mole-rat GnRH, as opposed to classic 'mammalian' GnRH, being the major form of this key reproductive hormone in the highveld mole-rat (and very possibly all mole-rats) may have implications for the phylogenetic placement of the Bathyergidae. The nucleotide and amino acid structure of mole-rat GnRH should be confirmed and compared to that of both New World and Old World hystricognaths, such as members of the family Hystricidae (porcupines) and Caviidae (guinea pigs). The comparative biological and immunological activity of mole-rat GnRH, guinea pig GnRH and classic 'mammalian' GnRH in the mole-rat should also be ascertained.

In the common and the highveld mole-rat, GnRH neurones and fibres were loosely distributed along the septo-preoptico-infundibular pathway. Dense fibre aggregations were present in the regions of the organum vasculosum of the lamina terminalis and the median eminence. Female common and highveld mole-rats differed with regards to the degree to which GnRH cell bodies migrated caudally to the preoptic area and medio-basal hypothalamus. Common mole-rats consistently had significantly fewer GnRH cell bodies and lower brain GnRH content than did highveld mole-rats. Similarly, GnRH-ir (GnRH immunoreactive) staining in the median eminence was significantly less intense in female common mole-rats compared to female highveld mole-rats.

The reproductive skew evident in common mole-rat colonies is primarily enforced through behavioural restraint on subordinate female reproduction as a result of obligate outbreeding (Spinks *et al.*, 1997, 2000a). The GnRH system of the common mole-rat, as described in this study, showed no evidence of a physiological component to either social or seasonal reproductive suppression in males and females. The numbers of GnRH cell bodies as well as the morphology, distribution and intensity of immunoreactive staining of cell bodies and fibres were similar in dominant and subordinate males and females, regardless of season. Similarly, brain endogenously produced GnRH content did not differ between any of the groups. These results confirm the current knowledge on pituitary sensitivity to exogenous GnRH and ovarian and testicular function in the common mole-rat (Spinks *et al.*, 1999, 2000a). The harsh semi-arid environment our study colonies were collected from, in combination with incest taboos, may provide sufficient incentive for subordinate animals to remain in their natal colonies during the breeding season; a physiological component to reproductive suppression would therefore be unneccesary in this species (Spinks *et al.*, 2000b).

Male highveld mole-rats, similar to the common mole-rat, are behaviourally restrained from reproducing while in the confines of their natal colonies (Van der Walt *et al.*, 2001; Janse van Rensburg *et al.*, 2003). As expected, the GnRH system of reproductive and non-reproductive males did not differ with regards to the neuroanatomical or -endocrinological parameters measured in this study. Reproductive suppression in female highveld mole-rats, on the other hand, is much more pronounced. Dominant and subordinate females had similar numbers of GnRH cell bodies, likewise the morphology and distribution of GnRH structures did not differ between the two groups. Reproductive female highveld mole-rats did, however, have significantly smaller GnRH-ir cell bodies and significantly less intense GnRH-ir staining in the median eminence compared to non-reproductive females. Similarly, endogenously produced brain GnRH content was significantly lower in reproductive females compared to non-reproductive females, indicating an inhibition of GnRH release in the latter group. Excess peptide most likely accumulates in the nerve terminals and the cell bodies, and not only the terminals as has been speculated for non-reproductive female Damaraland mole-rats (Molteno *et al.*, 2004).

Intact reproductive females and intact and ovariectomized non-reproductive female highveld mole-rats were treated with the opioid antagonist naloxone, alone and in different combinations with exogenous GnRH, to establish the role of EOPs and gonadal hormones in the reduced GnRH release evident in subordinate female highveld mole-rats. Naloxone treatment failed to alter plasma LH levels in any of the intact groups, while ovariectomized non-reproductive females showed a significant response. The pituitary response to a GnRH challenge was not influenced by either a single naloxone administration or longer-term naloxone-priming regimes. EOPs are tentatively considered to play a role in the socially-induced infertility experienced by subordinate female highveld mole-rats. In the absence of the negative feedback effects of gonadal steroids (post-ovariectomy) non-reproductive females showed

GnRH-challenged plasma LH levels similar to that seen in intact reproductive females, both being significantly higher compared to intact non-reproductive females. Steroid-dependent mechanisms altering GnRH secretion and/or pituitary sensitivity to the endogenous peptide may therefore partly play a role in the social reproductive regulation displayed by the highveld mole-rat. EOPs may be involved in steroid negative feedback suppression of GnRH as opioid-producing neurones in the region of GnRH neuronal structures have been found to accumulate sex hormones (Morrell *et al.*, 1985).

The highveld mole-rat shows several parallelisms to the eusocial Damaraland mole-rat (*Cryptomys damarensis*; Table 6.1). In both species subordinate males are behaviourally restrained from reproducing while females of the same social status have an added component of physiological sterility (Bennett *et al.*, 1996; Van der Walt *et al.*, 2001). The pituitaries of non-reproductive female highveld and Damaraland mole-rats are less sensitive to stimulation by exogenous GnRH compared to reproductive females (Bennett *et al.*, 1994; Van der Walt *et al.*, 2001; Molteno and Bennett, 2002), likewise the ovaries of subordinate females lack the corpora lutea indicative of ovulation (Bennett *et al.*, 1994; Van der Walt *et al.*, 2001; Janse van Rensburg *et al.*, 2002). The GnRH neuroanatomy and –endocrinology of non-reproductive female highveld and Damaraland mole-rats are also similar - in both species release of the peptide is apparently suppressed in subordinate females (Molteno *et al.*, 2004). Previously the level of social organization displayed by the giant Zambian mole-rat (*Cryptomys mechowi*) was thought to mirror that of the Damaraland mole-rat more closely than many of the other social cryptomids. Similar to the highveld mole-rat, this species occurs in mesic regions where a solitary lifestyle would be predicted (Wallace and Bennett, 1998). Past evolutionary and environmental pressures may be responsible for the high degree of sociality displayed by both the highveld and giant Zambian mole-rats (Bennett *et al.*, 1999).

Alternatively, overlapping generations due to phylogenetically constrained slow postnatal development have been proposed as a causal factor of eusociality in the Bathyergidae, using the giant Zambian mole-rat as a model (Burda and Kawalika, 1993).

Table 6.1 A comparison of the socially induced infertility imposed on non-reproductive female Damaraland, highveld and common mole-rats while in the constraints of their natal colonies. [a] Bennett, 1988; [b] Jarvis and Bennett, 1993; [c] Bennett *et al.*, 1993; [d] Molteno *et al.*, 2004; [e] Molteno and Bennett, 2002; [f] Skinner and Smithers, 1990; [g] Bennett and Jarvis, 1988; [h] Faulkes *et al.*, 1997; [i] Van der Walt *et al.*, 2001; [j] Janse van Rensburg *et al.*, 2002; [k] Moolman *et al.*, 1998; [l] Spinks *et al.*, 1999; [m] Faulkes and Bennett, 2001; [n] Spinks *et al.*, 2000a; [o] Spinks *et al.*, 2000b, 1997; [p] Bennett, 1989. **Bold**, parameters investigated in this study; RF, reproductive female; NRF, non-reproductive female; 2°, secondary; 3°, tertiary; LH, luteinizing hormone; GnRH, gonadotropin-releasing hormone; EOPs, endogenous opioid peptides; NR, non-reproductives

	Damaraland mole-rat *Cryptomys damarensis*	**Highveld mole-rat** *C hottentotus pretoriae*	**Common mole-rat** *C h hottentotus*
Ovarian development	NRF anovulatory 2° + 3° follicles luteinize [a]	NRF anovulatory no corpora lutea [i]	NRF, quiescent but primed for action [l, m]
Oestrogen, progesterone	RF ≥ NRF [b]	RF ≥ NRF [j]	RF = NRF [a]
Basal LH	RF ≥ NRF [c]	**RF = NRF** [i]	RF = NRF [n]
GnRH-challenged LH	RF ≥ NRF [c]	**RF ≥ NRF** [i]	RF = NRF [n]
Brain GnRH levels	RF ≤ NRF [d]	**RF ≤ NRF**	**RF = NRF**
GnRH cell body numbers	RF = NRF [d]	**RF = NRF**	**RF = NRF**
GnRH cell body size	RF = NRF [d]	**RF ≤ NRF**	**RF = NRF**
Distribution of GnRH cell bodies	RF = NRF [d]	**RF = NRF**	**RF = NRF**
GnRH staining intensity	RF ≤ NRF [d]	**RF ≤ NRF**	**RF = NRF**
EOPs and LH/GnRH	No apparent role [e]	**Tentative role**	Not known
Habitat	Semi-arid [f]	Mesic [k]	Semi-arid and mesic [f]
Dispersal	> 90 % of NR never disperse [b]	Not known	More dispersals in mesic than arid habitat [o]
Reproductive seasonality	Aseasonal [b]	Seasonal [j]	Seasonal [o]
Social hierarchy	Strongly linear [g]	Almost linear [k]	Non linear [p]
Mean months of rainfall > 25 mm	4.29 ± 0.55 [h]	6.43 ± 0.98 [h]	7.08 ± 0.86 [h]

Burda *et al.* (2000) classifies all *Cryptomys* mole-rats as eusocial, adding permanent (as opposed to transient) philopatry as an additional criterion to the traditional entomological definition of eusociality. The authors also discount the Aridity-Food-Distribution Hypothesis (AFDH) as a plausible explanation for the socially-induced infertility continuum displayed by the Bathyergidae. However, it has been shown in common mole-rats that natal philopatry is greater in harsher, semi-arid than in mesic habitats (Spinks *et al.*, 2000b). The Damaraland mole-rat inhabits an arid environment where opportunities for dispersal and independent reproduction are severely limited, and consequently lifelong philopatry is very likely (Jarvis and Bennett, 1993). The highveld mole-rat, on the other hand, occurs in a mesic habitat where solitary animals or newly formed pairs should be able to survive independently (Moolman *et al.*, 1998). The major difference between the Damaraland and highveld mole-rats lies in the potential life-time reproductive success of individual animals in the wild: the probability of non-reproductive highveld mole-rats successfully dispersing from their natal colonies and becoming breeders should therefore be markedly higher compared to non-reproductive Damaraland mole-rats where less than 8 % of individuals ever achieve direct reproductive success (Jarvis *et al.*, 1994). Similarly, subordinate naked mole-rats (*Heterocephalus glaber*) have an even lower chance (less than 1 %) of ever attaining reproductive status (Jarvis and Bennett, 1993; Jarvis *et al.*, 1994). Sherman *et al.* (1995) proposed the likelihood of an individual in the group ever breeding independently as a measure of the degree of sociality displayed by the group, distinguishing the social (highveld mole-rat) from the *eu*social (Damaraland mole-rat).

Finally, two closely related hormones, oxytocin and vasopressin, have recently been implicated in the central neural mediation of complex social behaviours such as pair bond formation in monogamous mammals (Insel, 1997). Oxytocin and vasopressin are produced in the hypothalamus and released through the axon terminals in the

posterior pituitary and also centrally within the brain (Gainer and Wray, 1994). Prairie voles (*Microtus ochrogaster*) are monogamous rodents living in multigenerational family groups, and similar to the social mole-rats their offspring remain sexually suppressed while in the parental group (Getz *et al.*, 1981; Getz and Hofman, 1986). The closely related montane vole (*Microtus montanus*) is the social opposite of the prairie vole, being solitary and breeding promiscuously (Jannett, 1980, 1982). Species-specific patterns of oxytocin and vasopressin receptor distribution and gene expression seem to underlie the social differences between the two species (Young *et al.*, 1998). In future studies it could be rewarding to investigate potential differences in oxytocin and vasopressin neurobiology, comparing solitary and social bathyergid mole-rats - possibly even comparing social species at different positions along the socially-induced infertility continuum.

List of references

ABBOTT DH (1987) Behaviourally mediated suppression of reproduction in female primates *Journal of Zoology, London* 213: 455-470

ABBOTT DH (1988) Natural suppression of fertility *Symposium of the Zoological Society of London* 60: 7-28

ABBOTT DH (1993) Social conflict and reproductive suppression in marmoset and tamarin monkeys In: *Primate social conflict* pp 331-372 Eds. Mason WA & Mendosa SP State University of New York Press, New York

ABBOTT DH AND HEARN JP (1978) Physical, hormonal and behavioural aspects of sexual development in the marmoset monkey, *Callithrix jacchus* *Journal of Reproduction and Fertility* 53: 155-166

ABBOTT DH, KEVERNE EB, BERCOVITCH FB, SHIVELY CA, MENDOSA SP, SALTZMAN W, SNOWDON CT, ZIEGLER TE, BANJEVIC M, GARLAND T Jr AND SAPOLSKY RM (2003) Are subordinates always stressed? A comparative analysis of rank differences in cortisol levels among primates *Hormones and Behavior* 43: 67-82

ABBOTT DH, MCNEILLY AS, LUNN SF, HULME MJ AND BURDEN FJ (1981) Inhibition of ovarian function in subordinate female marmoset monkeys (*Callithrix jacchus*) *Journal of Reproduction and Fertility* 63: 335-345

ABBOTT DH, O'BYRNE KT, SHEFFIELD JW, LUNN SF AND GEORGE LM (1987) Neuroendocrine suppression of LH secretion in subordinate female marmoset monkeys (*Callithrix jacchus*) In: *Comparative reproduction in mammals and man, Proceedings of the NCRR Conference Nairobi* Ed. Eley RM National Museums of Kenya, Nairobi

ABBOTT DH, SALTZMAN W, SCHULTZ-DARKEN NJ AND TANNENBAUM PL (1998) Adaptations to subordinate status in female marmoset monkeys *Comparative Biochemistry and Physiology C (Pharmacology, Toxicology and Endocrinology)* 119: 261-274

ALEXANDER RD (1974) The evolution of social behaviour *Annual Review in Ecology and Systematics* 5: 325-383

ALMEIDA OFX (1993) Opioids and the neuroendocrine control of reproduction In: *Opioids II* pp 497-524 Ed. Herz A Springer-Verlag, Berlin

ALMEIDA OFX, NIKOLARAKIS KE, SIRINATHSINGHJI DJS AND HERZ A (1989) Opioid-mediated inhibition of sexual behaviour and luteinizing hormone secretion by corticotrophin-releasing hormone In: *Brain opioid systems* pp 149-164 Eds. Dyer RG & Bicknell RJ Oxford University Press, Oxford

ANDERSON M (1984) The evolution of eusociality *Annual Review in Ecology and Systematics* 15: 165-189

ANTHONY ELP, WESTON PJ, MONTVILO JA, BRUHN TO, NEEL K AND KING JC (1989) Dynamic aspects of the LHRH system associated with ovulation in the little brown bat (*Myotis lucifugus*) *Journal of Reproduction and Fertility* 87: 671-686

AURICH C, SCHLOTE S, HOPPEN H-O, KLUG E, HOPPE H AND AURICH JE (1994) Effects of the opioid antagonist naloxone on release of luteinizing hormone in mares during the anovulatory season *Journal of Endocrinology* 142: 127-138

BARRACLOUGH CA & AND SAWYER CH (1955) Inhibition of the release of pituitary ovulatory hormone in the rat by morphine *Endocrinology* 57: 329-337

BARRY J AND DUBOIS MP (1974) Immunofluorescence study of the preoptico-infundibular LH-RH neurosecretory pathway of the guinea pig during the estrous cycle *Neuroendocrinology* 15: 200-208

BATRA SWT (1966) Nests and social behavior of halicitine bees of India *Indian Journal of Entomology* 28: 375–393

BENNETT NC (1988) The trend towards sociality in three species of southern African mole-rats (Bathyergidae): cause and consequences Unpublished PdD dissertation, University of Cape Town

BENNETT NC (1989) The social structure and reproductive biology of the common mole-rat, *Cryptomys hottentotus hottentotus* and remarks on the trends in reproduction and sociality in the family Bathyergidae *Journal of Zoology, London* 219: 45-59

BENNETT NC (1994) Reproductive suppression in social *Cryptomys damarensis* colonies – a lifetime of socially-induced sterility in males and females (Rodentia: Bathyergidae) *Journal of Zoology, London* 234: 25-39

BENNETT NC AND FAULKES CG (2000) *African mole-rats: ecology and eusociality* Cambridge University Press, Cambridge

BENNETT NC, FAULKES CG AND JARVIS JUM (1999) Socially induced infertility, incest avoidance and the monopoly of reproduction in cooperatively breeding African mole-rats, family Bathyergidae *Advances in the Study of Behavior* 28: 75-113

BENNETT NC, FAULKES CG AND MOLTENO AJ (1996) Reproductive suppression in subordinate, non-breeding female Damaraland mole-rats: two components to a lifetime of socially induced infertility *Proceedings of the Royal Society of London B* 263: 1599-1603

BENNETT NC, FAULKES CG AND SPINKS AC (1997) LH responses to single doses of exogenous GnRH by social Mashona mole-rats: a continuum of socially induced infertility in the family Bathyergidae *Proceedings of the Royal Society London B* 264: 1001-1006

BENNETT NC AND JARVIS JUM (1988) The social structure and reproductive biology of colonies of the mole-rat, *Cryptomys damarensis* (Rodentia, Bathyergidae) *Journal of Mammology* 69: 293-302

BENNETT NC, JARVIS JUM, FAULKES CG AND MILLAR RP (1993) LH responses to single doses of exogenous GnRH by freshly captured Damaraland mole-rats, *Cryptomys damarensis* *Journal of Reproduction and Fertility* 99: 81-86

BENNETT NC, JARVIS JUM, MILLAR RP, SASANO H AND NTSHINGA KV (1994) Reproductive suppression in eusocial *Cryptomys damarensis* colonies: socially-induced infertility in females *Journal of Zoology, London* 233: 617-630

BITTMAN EL (1984) Melatonin and photoperiodic time measurement: evidence from rodents and ruminants In: *The pineal gland* pp 155-192 Ed. Reiter RJ Raven Press, New York

BLANK MS, FABBRI A, CATT JK AND DUFAU ML (1987) Inhibition of luteinizing hormone release by morphine and endogenous opiates in cultured pituitary cells *Endocrinology* 118: 2097-2101.

BRADFORD MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding *Analytical Biochemistry* 72: 248-254

BRAUDE S (2000) Dispersal and new colony formation in wild naked mole-rats: evidence against inbreeding as the system of mating *Behavioral Ecology* 11: 7-12

BRETT RA (1986) The ecology and behaviour of the naked mole-rat (*Heterocephalus glaber* Rüppell) (Rodentia: Bathyergidae) Unpublished PhD thesis, University of London

BROOKS AN, LAMMING GE AND HAYNES NB (1986) Endogenous opioid peptides and the control of gonadotrophin secretion *Research in Veterinary Science* 41: 285-299

BURDA H, HONEYCUTT RL, BEGALL S, LOCKER-GRÜTJEN O AND SCHARFF A (2000) Are naked and common mole-rats eusocial and if so, why? *Behavioral Ecology and Sociobiology* 47: 293-303

BURDA H AND KAWALIKA M (1993) Evolution of eusociality in the Bathyergidae. The case of the giant mole rats (*Cryptomys mechowi*) *Naturwissenschaften* 80: 235-237

CACICEDO L AND FRANCO SF (1986) Direct action of opioid peptides and naloxone on gonadotropin secretion by cultured rat anterior pituitary cells *Life Sciences* 38: 617-625

CAO Y, ADACHI J, YANO T-A AND HASEGAWA M (1994) Phylogenetic place of guinea pigs: no support of the rodent-polyphyly hypothesis from maximum-likelihood analyses of multiple protein sequences *Molecular Biology and Evolution* 11: 593-604

CAROLSFELD J, POWELL JFF, PARK M, FISCHER WH, CRAIG AG, CHANG JP, RIVIER JE AND SHERWOOD NM (2000) Primary structure and function of three gonadotropin-releasing hormones, including a novel form, form an ancient teleost, herring *Endocrinology* 141: 505-512

CLARKE FM AND FAULKES CG (1997) Dominance and queen succession in captive colonies of the eusocial naked mole-rat, *Heterocephalus glaber* *Proceedings of the Royal Society London B* 264: 993-1000

CLARKE FM, MIETHE GH AND BENNETT NC (2001) Reproductive suppression in female Damaraland mole-rats *Cryptomys damarensis*: dominant control or self-restraint? *Proceedings of the Royal Society London B* 268: 899-909

CLARKE JR (1981) Physiological problems of seasonal breeding in eutherian mammals *Oxford Reviews of Reproductive Biology* 3: 244-312

CLUTTON-BROCK TH (1998) Reproductive skew, concessions and limited control *Trends in Ecology and Evolution* 13: 288-292

CLUTTON-BROCK TH, BROTHERTON PNM, RUSSELL AF, O'RIAIN MJ, GAYNOR D, KANSKY R, GRIFFIN A, MANSER M, SHARPE L, MCILRATH GM, SMALL T, MOSS A & AND MONFORT S (2001) Cooperation, control, and concession in meerkat groups *Science* 291: 478-481

COCKBURN A (1998) Evolution of helping behavior in cooperatively breeding birds *Annual Review in Ecology and Systematics* 29: 141-177

COONEY RM AND BENNETT NC (2000) Incest avoidance and reproductive skew in a cooperatively breeding mammal *Proceedings of the Royal Society London B* 267: 801-806

CREEL SR AND WASER PM (1997) Variation in reproductive suppression among dwarf mongooses: interplay between mechanisms and evolution In: *Cooperative breeding in mammals* pp 150-170 Eds. Solomon N and French J Cambridge University Press, Cambridge

CRESPI BJ AND YANEGA D (1995) The definition of eusociality *Behavioural Ecology* 6: 109-115

DE GRAAFF G (1979) Molerats (Bathyergidae, Rodentia) in South African national parks: notes on their taxonomic 'isolation' and hystricomorph affinities of the family *Koedoe* 22: 89-107

EBENSPERGER LA AND COFRÉ H (2001) On the evolution of group-living in the New World cursorial hystricognath rodents *Behavioral Ecology* 12: 227-236

EBERHART JA, KEVERNE EB AND MELLER RE (1983) Social influences on the circulating levels of cortisol and prolactin in male talapoin monkeys *Physiology of Behaviour* 30: 361-369

ECKERT R, RANDALL D AND AUGUSTINE G (1988) Chemical messengers and regulators In: *Animal physiology – mechanisms and adaptations 3rd ed.* pp 266-328 W.H. Freeman and Company, New York

FAULKES CG (1990) Social suppression of reproduction in the naked mole-rat, *H. glaber* Unpublished *PhD dissertation*, University of London

FAULKES CG, ABBOTT DH AND JARVIS JUM (1990) Social suppression of ovarian cyclicity in captive and wild colonies of naked mole-rats, *Heterocephalus glaber* *Journal of Reproduction and Fertility* 88: 559-568

FAULKES CG, ABBOTT DH AND JARVIS JUM (1991) Social suppression of reproduction in naked mole-rats, *Heterocephalus glaber* *Journal of Reproduction and Fertility* 91: 593-604

FAULKES CG AND BENNETT NC (2001) Family values: group dynamics and social control of reproduction in African mole-rats *Trends in Ecology and Evolution* 16: 184-190

FAULKES CG, BENNETT NC, BRUFORD MW, O'BRIEN HP, AGUILAR GH AND JARVIS JUM (1997) Ecological constraints drive social evolution in the African mole-rats *Proceedings of the Royal Society London B* 264: 1619-1627

FERIN M, VAN VUGT D AND WARDLAW S (1984) The hypothalamic control of the menstrual cycle and the role of endogenous opioid peptides *Recent Progress in Hormone Research* 40: 441-485

FORAN CM AND BASS AH (1999) Preoptic GnRH and AVT: axes for sexual plasticity in teleost fish *General Comparative Endocrinology* 116: 141-152

FRANCIS RC, SOMA K AND FERNALD RD (1993) Social regulation of the brain pituitary-gonadal axis *Proceeding of the National Academy of Science, USA* 90: 7794-7798

FROEHLICH JC (1997) Opioid peptides *Neurotransmitter Reviews* 21: 132-136

GABATHULER U, BENNETT NC AND JARVIS JUM (1996) The social structure and dominance hierarchy of the Mashona mole-rat, *Cryptomys darlingi* (Rodentia: Bathyergidae) from Zimbabwe *Journal of Zoology* 240: 221-231

GADAGKAR R (1994) Why the definition of eusociality is not helpful to understand its evolution and what should we do about it *Oikos* 70: 485-488

GAINER H AND WRAY S (1994) Cellular and molecular biology of oxytocin and vasopressin In: *The physiology of reproduction, 2nd Ed.* pp 1099-1130 Eds. Knobil E and Neill JD Raven Press, New York

GAO CQ, VAN DEN SAFFELE J, GIRI M AND KAUFMAN JM (2000) Guinea-pig gonadotropin releasing hormone: immunoreactivity and biological activity *Journal of Neuroendocrinology* 12: 355-359

GETZ LL CARTER CS AND GAVISH L (1981) The mating system of the prairie vole *Microtus ochrogaster*: field and laboratory evidence for pair bonding *Behavioral Ecology and Sociobiology* 8: 189-194

GETZ LL AND HOFMAN JE (1986) Social organization in free living prairie voles, *Microtus ochrogaster* *Behavioral Ecology and Sociobiology* 18: 275-282

GLASS JD (1986) Short photoperiod-induced gonadal regression: effects on the gonadotropin-releasing hormone (GnRH) neuronal system of the white-footed mouse, *Permyscus leucopus* *Biology of Reproduction* 35: 733-743

GOODMAN RL, BITTMAN EL, FOSTER DL AND KARSCH FL (1982) Alterations in the control of luteinizing hormone pulse frequency underlie the seasonal variation in estradiol negative feedback in the ewe *Biology of Reproduction* 27: 580-589

GRAFEN A (1990) Biological signals as handicaps *Journal of Theoretical Biology* 144: 517-546

GRAUR D, HIDE WA AND LI WH (1991) Is the guinea-pig a rodent? *Nature* 351: 649-652

GRAUR D, HIDE WA, ZHARKIKH A AND LI WH (1992) The biochemical phylogeny of guinea-pigs and gundis, and the paraphyly of the order rodentia *Comparative Biochemistry and Physiology* 101: 495-498

GREENWOOD FC, HUNTER WM AND GLOVER JS (1963) The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity *Journal of Biochemistry* 89: 114-120

GROVE-STRAWSER D, SOWER SA, RONSHEIM PM, CONNOLLY JB, BOURN CG AND RUBIN BS (2002) Guinea pig GnRH: localisation and physiological activity reveal that it, not mammalian GnRH, is the major neuroendocrine form in guinea pigs *Endocrinology* 143: 1602-1612

HARDY SL, ANDERSON GM, VALENT M, CONNORS JM AND GOODMAN RL (2003) Evidence that estrogen receptor alpha, but not beta, mediates seasonal changes in the response of the ovine retrochiasmatic area to estradiol *Biology of Reproduction* 68: 846-852

HERBST M (2002) The biology and population ecology of the Namaqua dune mole-rat, *Bathyergus janetta* from the Northern Cape Province, South Africa Unpublished *MSc thesis*, Department of Zoology and Entomology, University of Pretoria

HICKMAN CG (1979) A live-trap and trapping technique for fossorial mammals *South African Journal of Zoology* 14: 9-12

HONEYCUTT RL, ALLARD MW, EDWARDS SV AND SCHLITTER DA (1991) Systematics and evolution of the family Bathyergidae In: *Biology of the naked mole-rat* pp 45-65 Eds. Sherman PW, Jarvis JUM and Alexander RD Princeton University Press, Princeton

HUCHON D, CATZEFLIS FM AND DOUZERY EJ (1999) Molecular evolution of the nuclear von Willebrand factor gene in mammals and the phylogeny of rodents *Molecular Biology and Evolution* 16: 577-589

HUCHON D AND DOUZERY EJ (2001) From the Old World to the New World: a molecular chronicle of the phylogeny and biogeography of Hystricognath rodents *Molecular Phylogenetics and Evolution* 20: 238-251

INSEL TR (1997) A neurobiological basis of social attachment *American Journal of Psychiatry* 154: 726-735

JANNETT FJ (1980) Social dynamics in the montane vole *Microtus montanus* as a paradigm *Biologist* 62: 3-19

JANNETT FJ (1982) The nesting patterns of adult voles (*Microtus montanus*) in field populations *Journal of Mammalogy* 63: 495-498

JANSE VAN RENSBURG L, BENNETT NC, VAN DER MERWE M AND SCHOEMAN AS (2002) Seasonal reproduction in the highveld mole-rat, *Cryptomys hottentotus pretoriae* (Rodentia: Bathyergidae) *Canadian Journal of Zoology* 80: 810-820

JANSE VAN RENSBURG L, BENNETT NC, VAN DER MERWE M, SCHOEMAN AS AND BRINDERS J (2003) Are non-reproductive male highveld mole-rats, *Cryptomys hottentotus pretoriae* physiologically suppressed while in the confines of the natal colony? *Journal of Zoology, London* 260: 73-38

JARVIS JUM (1981) Eusociality in a mammal: Cooperative breeding in naked mole-rat colonies *Science* 212: 571-573

JARVIS JUM (1991) Reproduction of naked mole-rats In: *Biology of the naked mole-rat* pp 384-425 Eds. Sherman PW, Jarvis JUM and Alexander RD Princeton University Press, Princeton

JARVIS JUM AND BENNETT NC (1990) The evolutionary history, population biology and social structure of African mole-rats: Family Bathyergidae In: *Evolution of subterranean mammals at the organismal and molecular levels* pp 97-128 Eds. Nevo E and Reig OA Alan R Liss, New York

JARVIS JUM AND BENNETT NC (1991) Ecology and behavior of the family Bathyergidae In: *Biology of the naked mole-rat* pp 66-96 Eds. Sherman PW, Jarvis JUM and Alexander RD Princeton University Press, Princeton

JARVIS JUM AND BENNETT NC (1993) Eusociality has evolved independently in two genera of bathyergid mole-rats – but occurs in no other subterranean mammal *Behavioral Ecology and Sociobiology* 33: 253-260

JARVIS JUM, O'RIAIN MJ, BENNETT NC AND SHERMAN PW (1994) Mammalian eusociality: a family affair *Trends in Ecology and Evolution* 9: 47-51

JASTROW H, BURDA H AND OELSCHLÄGER HHA (1998) Unilateral absence of the terminal nerve and distribution of gonadotropin-releasing hormone immunoreactive neurons in the brain of the common mole-rat (*Cryptomys*, Rodentia) *Brain Research* 813: 229-240

JIMENEZ-LIÑAN M, RUBIN BS AND KING JC (1997) Examination of guinea pig luteinizing hormone-releasing hormone gene reveals a unique decapeptide and existence of two transcripts in the brain *Endocrinology* 138: 4123-4130

JOHNSON EO, KAMILARIS TC, CHROUSOS GP AND GOLD PW (1992) Mechanisms of stress: a dynamic overview of hormonal and behavioral homeostasis *Neuroscience and Behavioral Reviews* 16: 115-130

KARSCH FJ (1984) The hypothalamus and the anterior pituitary gland In: *Hormonal control of reproduction* Eds. Austin CR and Short RV Cambridge University Press, Cambridge

KELLER L AND NONACS P (1993) The role of queen pheromones in social insects: queen control or queen signal? *Animal Behaviour* 45: 787-794

KELSALL R, COE IR AND SHERWOOD NM (1990) Phylogeny and ontogeny of gonadotropin-releasing hormone: comparison of guinea pig, rat, and a protochordate *General Comparative Endocrinology* 78: 479-494

KING JA, HASSAN MF, MEHL AE AND MILLAR RP (1988) Gonadotropin-releasing hormone molecular forms in mammalian hypothalamus *Endocrinology* 122: 2742-2752

KING JA AND MILLAR RP (1979) Heterogeneity of vertebrate luteinizing hormone-releasing hormone *Science* 206: 67-69

KING JA AND MILLAR RP (1986) Identification of His^5, Trp^7, Tyr^8 –GnRH (chicken GnRH II) in amphibian brain *Peptides* 7: 827-834

KING JA AND MILLAR RP (1995) Evolutionary aspects of gonadotropin-releasing hormone and its receptor *Cellular and Molecular Neurobiology* 15: 5-23

KING JA, STENEVELD AA, CURLEWIS JD, RISSMAN EF AND MILLAR RP (1994a) Identification of chicken GnRH II in brains of metatherian and early-evolved eutherian species of mammals *Regulatory Peptides* 54: 467-477

KING JA, STENEVELD AA AND MILLAR RP (1994b) Differential regional distribution of gonadotropin-releasing hormones in amphibian (clawed toad, *Xenopus laevis*) brain *Regulatory Peptides* 50: 277-289

KING JC AND ANTHONY EL (1984) LHRH neurons and their projections in humans and other mammals: species comparisons *Peptides* 5: 195-207

KING JC, KUGEL G, ZAHNISER D, WOOLEDGE K, DAMASSA DA AND ALEXSAVICH B (1987) Changes in populations of LHRH-immunopositive cell bodies following gonadectomy *Peptides* 8: 721-735

KING JC, LIU E, RONSHEIM P, SLONIMSKI M AND RUBIN BS (1998) Expression of fos within luteinizing hormone-releasing hormone neurons, in relation to the steroid-induced luteinizing hormone surge in guinea pigs *Biology of Reproduction* 58: 316-322

KORDON C, DROUVA SV, DE LA ESCALERA GM AND WEINER RI (1994) Role of classic and peptide neuromediators in the neuroendocrine regulation of luteinizing hormone and prolactin In: *The physiology of reproduction* pp 1621-1681 Eds. Knobil E and Neill JD Raven Press, New York

LAVOCAT R (1973) Les rongeurs du Miocène d'Afrique Orientale. I. Miocène inférieur *Mém. Trav. Inst. Montpellier Ecole Pratique Hautes Etudes* 1: 1-284

LACEY EA AND SHERMAN PW (1991) Social organization of naked mole-rat colonies: evidence for division of labour In: *Biology of the naked mole-rat* pp 275-336 Eds. Sherman PW, Jarvis JUM and Alexander RD Princeton University Press, Princeton

LESCHEID DW, TERASAWA E, ABLER LA, URBANSKI HF, WARBY CM AND MILLAR RP (1997) A second form of gonadotropin-releasing hormone (GnRH) with characteristics of chicken GnRH II is present in the primate brain *Endocrinology* 138: 5618-5629

LINCOLN GA AND SHORT RV (1980) Seasonal breeding: nature's contraceptive *Recent Progress in Hormone Research* 36: 1-52

LOVEGROVE BG (1991) The evolution of eusociality in molerats (Bathyergidae): a question of risks, numbers and costs *Behavioral Ecology and Sociobiology* 28: 37-45

MA D-P, ZHARKIKH A, GRAUR D, VANDEBERG JL AND LI W-H (1993) Structure and evolution of opossum, guinea pig, and porcupine cytochrome *b* genes *Journal of Molecular Evolution* 36: 327-224

MAIER W AND SCHRENK F (1987) The hystricomorphy of the Bathyergidae, as determined from ontogenetic evidence *Sonderdruck aus Z. f. Saügertierkunde* 52: 156-164

MALHERBE GP (2001) Aspects of the sociality, ecology, reproductive biology and genetic relatedness of colonies of the highveld mole-rat, *Cryptomys hottentotus pretoriae* Unpublished MSc thesis, Department of Zoology and Entomology, University of Pretoria

MALPAUX B, THIERY JC AND CHEMINEAU P (1999) Melatonin and the seasonal control of reproduction *Reproduction and Nutritional Development* 39: 355-366

MARGULIS SW, SALTZMAN W AND ABBOTT DH (1995) Behavioral and hormonal changes in female naked mole-rats (*Heterocephalus glaber*) following removal of the breeding female from a colony *Hormones and Behavior* 29: 227-247

MARSHALL PE AND GOLDSMITH PC (1980) Neuroregulatory and neuroendocrine GnRH pathways in the hypothalamus and forebrain of the baboon *Brain Research* 193: 353-372

MARTENSZ ND, VELLUCCI SV, KEVERNE EB AND HERBERT J (1986) β-endorphin levels in the cerebrospinal fluid of male talapoin monkeys in social groups related to dominance status and the luteinizing hormone response to naloxone *Neuroscience* 18: 651-658

MATTIOLI M, CONTE F, GALEATI G AND SEREN E (1986) Effect of naloxone on plasma concentrations of prolactin and LH in lactating sows *Journal of Reproduction and Fertility* 76: 167-173

MEYER SL AND GOODMAN RL (1986) Separate neural systems mediate the steroid-dependent and steroid-independent suppression of tonic luteinizing hormone secretion in the anestrous ewe *Biology of Reproduction* 35: 562-571

MICEVYCH P AND SINCHAK K (2001) Estrogen and endogenous opioids regulate CCK in reproductive circuits *Peptides* 22: 1235-1244

MICHENER CD (1969) Comparative social behaviour of bees *Annual Review in Entomology* 14: 277–342

MIETHE GH, JANSE VAN RENSBURG L, MALPAUX B, RICHTER TA AND BENNETT NC (Submitted) Can the highveld mole-rat (*Cryptomys hottentotus pretoriae*), regulate the plasma levels of melatonin to reflect changes in daylength?

MILLAR RP, FLANAGAN CA, MILTON RC DEL AND KING JA (1989) Chimeric analogues of vertebrate gonadotropin-releasing hormones comprising substitutions of the variant amino acids in positions 5, 7, and 8 *Journal of Biological Chemistry* 264: 21007-21013

MOLTENO AJ (1999) Reproductive regulation in female Damaraland mole-rats, *Cryptomys damarensis*: physiological and neuroendocrine mechanisms Unpublished *MSc thesis*, Department of Zoology and Entomology, University of Pretoria

MOLTENO AJ AND BENNETT NC (2000) Anovulation in nonreproductive female Damaraland mole-rats (*Cryptomys damarensis*) *Journal of Reproduction and Fertility* 119: 35-41

MOLTENO AJ AND BENNETT NC (2002) Social suppression in nonreproductive female Damaraland mole-rats, *Cryptomys damarensis*: no apparent role for endogenous opioid peptides *Hormones and Behavior* 41: 115-125

MOLTENO AJ, KALLÓ I, BENNETT NC, KING JA AND COEN CW (2004) A neuroanatomical and neuroendocrinological study into the relation between social status and the GnRH system in cooperatively breeding female Damaraland mole-rats *Cryptomys damarensis* *Reproduction*

MONTANER AD, SOMOZA GM, KING JA, BIANCHINI JJ, BOLIS CG AND AFFANNI JM (1998) Chromatographic and immunological identification of GnRH (gonadotropin-releasing hormone) variants. Occurrence of mammalian an a salmon-like GnRH in the forebrain of an eutherian mammal: *Hydrochaeris hydrochaeris* (Mammalia, Rodentia) *Regulatory Peptides* 73: 197-204

MOOLMAN M, BENNETT NC AND SCHOEMAN AS (1998) The social structure and dominance hierarchy of the highveld mole-rat *Cryptomys hottentotus pretoriae* (Rodentia: Bathyergidae) *Journal of Zoology, London* 246: 193-210

MOORE RY (1978) The innervation of the mammalian pineal gland In: *Progress in reproductive biology, Vol. 4* pp 1-29 Ed. Reiter RJ Basel, Karger

MORRELL JL, MCGINTY JF AND PFAFF DW (1985) A subset of β-endorphin- or dynorphin-containing neurons in the medial basal hypothalamus accumulates estradiol *Neuroendocrinology* 41: 417-426

MOUCHATY SK, CATZEFLIS F, JANKE A AND AMASON U (2001) Molecular evidence of an African Phiomorpha – South American Caviomorpha clade and support for Hystricognathi based on the complete mitochondrial genome of the cane rat (*Thryonomys swinderianus*) *Molecular Phylogenetics and Evolution* 18: 127-135

MUSKE LE (1993) Evolution of gonadotropin-releasing hormone (GnRH) neuronal systems *Brain and Behavioural Evolution* 42: 215-230

NAGASO H, MURATA T, DAY N AND YOKOYAMA KK (2001) Simultaneous detection of RNA and protein by *in situ* hybridisation and immunological staining *Journal of Histochemistry and Cytochemistry* 49: 1177-1182

NEDBALL MA, ALLARD MW AND HONEYCUTT RL (1994) Molecular systematics of hystricognath rodents: evidence form the mitochondrial 12S rRNA gene *Molecular Phylogenetics and Evolution* 3: 206-220

NTOUMI F, MARTINET L AND MONDAIMONVAL M (1994) Effects of melatonin treatment on the gonadotropin releasing-hormone neuronal system and on gonadotropin secretion in male mink, *Mustela vison*, in the presence and absence of testosterone feedback *Journal of Pineal Research* 16: 18-25

O'RIAIN MJ, JARVIS JUM, ALEXANDER RD, BUFFENSTEIN R AND PETERS C (2000) Morphological castes in a vertebrate *Proceedings of the National Academy of Science, USA* 97: 13194-13197

PETTERBORG LJ (1981) The reproductive consequences of pineal manipulation and melatonin administration in the white-footed mouse, *Peromyscus leucopus* Unpublished *PhD dissertation,* Health Science Centre at San Antonio, University of Texas

PRZEWLOCKI R (1993) Opioid systems and stress In: *Opioids P* pp 293 Ed. Herz A Springer-Verlag, Berlin

RAAB A, SEIZINGER BR AND HERZ A (1985) Continuous social defeat induces an increase of endogenous opioids in discrete brain areas of the mongolian gerbil *Peptides* 6: 387-391

RASMUSSEN DD (1991) High postovariectomy LH levels are not due to decreased opioid inhibition of GnRH *Brain Research Bulletin* 26: 663-666

REEVE HK AND RATNIEKS FLW (1993) Queen-queen conflicts in polygynous societies: mutual tolerance and reproductive skew In: *Queen number and sociality in insects* pp 45-85 Ed. Keller L Oxford University Press, Oxford

REEVE HK AND SHERMAN PW (1991) Intracolonial aggression and nepotism by the breeding female naked mole-rat In: *Biology of the naked mole-rat* pp 337-357 Eds. Sherman PW, Jarvis JUM and Alexander RD Princeton University Press, Princeton

REEVE HK, WESTNEAT DF, NOON WA, SHERMAN PW AND AQUADRO CF (1990) DNA 'fingerprinting' reveals high levels of inbreeding in colonies of the eusocial naked mole-rat *Proceedings of the National Academy of Science, USA* 87: 2496-2500

RICHTER TA, SPACKMAN DS, ROBINSON JE, DYE S, HARRIS TG, SKINNER DC AND EVANS NP (2001) Role of endogenous opioid peptides in mediation of progesterone-induced disruption of the activation and transmission stages of the GnRH surge induction process *Endocrinology* 142: 5212-5219

RISSMAN EF (1996) Behavioural regulation of gonadotropin-releasing hormone *Biology of Reproduction* 54: 413-419

ROBERTS AC, MARTENSZ ND, HASTINGS MH AND HERBERT J (1985) Changes in photoperiod alter the daily rhythms of pineal melatonin content and hypothalamic β-endorphin content and the lutheinizing hormone response to naloxone in the male Syrian hamster *Endocrinology* 117: 141-148

ROBERTS AC, MARTENSZ ND, HASTINGS MH AND HERBERT J (1987) The effects of castration, testosterone replacement and photoperiod upon hypothalamic β-endorphin levels in the male Syrian hamster *Neuroscience* 23: 1075-1082

ROBINSON JE, SKINNER DC, SKINNER JD AND HAUPT MA (1997) Distribution and morphology of luteinizing hormone-releasing hormone neurons in a species of wild antelope, the springbok (*Antidorcas marsupialis*) *Journal of Comparative Neurology* 389: 444-452

ROWE DL AND HONEYCUTT RL (2002) Phylogenetic relationships, ecological correlates, and molecular evolution within the Cavioidea (Mammalia, Rodentia) *Molecular Biology and Evolution* 19: 263-277

SALTZMAN W (2003) Reproductive competition among female common marmosets (*Callithrix jacchus*): proximate and ultimate cues In: *Sexual selection and reproductive competition in primates: new perspectives and directions* pp 197-229 Eds. Jones CB and Norman OK American Society of Primatologists

SALTZMAN W, SCHULTZ-DARKEN JN AND ABBOTT DJ (1997a) Familial influences on ovulatory function in common marmosets (*Callithrix jacchus*) *American Journal of Primatology* 41: 159-177

SALTZMAN W, SEVERIN JM, SCHULTZ-DARKEN JN AND ABBOTT DJ (1997b) Behavioral and social correlates of escape from suppression of ovulation in female common marmosets housed with the natal family *American Journal of Primatology* 41: 1-21

SCHWANZEL-FUKUDA M, MORELL, JI AND PFAFF DW (1985) Ontogenesis of neurons producing luteinizing hormone-releasing hormone (LHRH) in the nervus terminalis of the rat *Journal of Comparative Neurology* 238: 348-364

SEEBURG PH & ADELMAN JP (1984) Characterization of cDNA for precursor of human luteinizing hormone-releasing hormone *Nature* 311: 666-668

SHERMAN PW, LACEY EA, REEVE HK AND KELLER L (1995) The eusociality continuum *Behavioural Ecology* 6: 102-108

SILVERMAN AJ (1976) Distribution of luteinizing hormone-releasing hormone (LHRH) in the guinea pig brain *Endocrinology* 99: 30-41

SILVERMAN AJ, ANTUNES JL, ABRAMS GM, NILAVER G, THAU R, ROBINSON JA, FERIN J AND KREY LC (1982) The lutheinizing hormone pathways in Rhesus (*Macaca mulatta*) and pigtailed (*Macaca nemestrina*) monkeys: new observations on thick unembedded sections *Journal of Comparative Neurology* 211: 309-317

SILVERMAN AJ, LIVNE I AND WITKIN JW (1994) The gonadotropin-releasing hormone (GnRH) neuronal systems: immunocytochemistry and *in situ* hybridisation In: *The physiology of reproduction, 2nd Ed.* pp 1683-1709 Eds. Knobil E and Neill JD Raven Press, New York

SIRINATHSINGHJI DJS AND MARTINI L (1984) Effects of bromocriptine and naloxone an plasma levels of prolactin, LH and FSH during suckling in the female rat: responses to gonadotropin releasing hormone *Journal of Endocrinology* 100: 175-182

SKINNER JD AND SMITHERS RHN (1990) The mammals of the southern African subregion pp 186-197 University of Pretoria, Pretoria

SNOWDON CT (1996) Infant care in cooperatively breeding species *Advances in the Study of Behaviour* 25: 643-689

SOLOMON NG AND GETZ LL (1997) Examination of alternative hypotheses for cooperative breeding in rodents In: *Cooperative breeding in mammals* pp199-230 Eds. Solomon NG and French JA Cambridge University Press, New York

SPINKS AC, BENNETT NC, FAULKES CG AND JARVIS JUM (2000a) Circulating LH levels and the response to exogenous GnRH in the common mole-rat: Implications for reproductive regulation in this social, seasonal breeding species *Hormones and Behaviour* 37: 221-228

SPINKS AC, BENNETT NC AND JARVIS JUM (1999) Regulation of reproduction in female common mole-rats, *Cryptomys hottentotus hottentotus*: the effects of breeding season and reproductive status *Journal of Zoology London* 248: 161-168

SPINKS AC, JARVIS JUM AND BENNETT NC (2000b) Comparative patterns of philopatry and dispersal in two common mole-rat populations: implications for the evolution of mole-rat sociality *Journal of Animal Ecology* 69: 224-234

SPINKS AC, VAN DER HORST G AND BENNETT NC (1997) Influence of breeding season and reproductive status on male reproductive characteristics in the common mole-rat, *Cryptomys hottentotus hottentotus* *Journal of Reproduction and Fertility* 109: 79-86

STORRING PL AND GAINES DAS RE (1993) The second international standard for human pituitary LH; its collaborative study by bioassay and immunoassay *Journal of Endocrinology* 138: 345-359

TAI VC, SCHIML PA, LI X AND RISSMAN EF (1997) Behavioural interactions have rapid effects on immunoreactivity of prohormone and gonadotropin-releasing hormone peptide *Brain Research* 772: 87-94

TAMANINI C, CROWDER ME AND NETT TM (1986) Effects of oestradiol and progesterone on pulsatile secretion of luteinizing hormone in ovariectomized ewes *Acta Endocrinologica* 111: 172-178

URBANSKI HF, DOAN A AND PIERCE M (1991) Immunocytochemical investigation of luteinizing hormone-releasing hormone neurons in Syrian hamsters maintained under long or short days *Biology of Reproduction* 44: 687-692

VAN DAMME M-P, ROBERTSON DM AND DICZFALUSY E (1974) An improved *in vitro* bioassay method for measuring luteinizing hormone (LH) activity using mouse Leydig cell preparations *Acta Endocrinologica* 77: 655-671

VAN DER HORST G (1972) Seasonal effects on the anatomy and histology of the reproductive tract of the male rodent mole *Zoologica Africana* 7: 491-520

VAN DER WALT L, BENNETT NC AND SCHOEMAN AS (2001) Reproductive suppression and pituitary sensitivity to exogenous GnRH in the highveld mole-rat (*Cryptomys hottentotus pretoriae*) *Journal of Zoology, London* 254: 177-184

WALLACE E AND BENNETT NC (1998) The colony structure and social organization of the giant Zambian mole-rat, *Cryptomys mechowi* *Journal of Zoology, London* 244: 51-61

WASSER SK AND BARASH DP (1983) Reproductive suppression among female mammals: implications for biomedicine and sexual selection theory *Quarterly Review of Biology* 58: 513-538

WHITE RB, EISEN JA, KASTEN TL AND FERNALD RD (1998) Second gene for gonadotropin-releasing hormone in humans *Proceedings of the National Academy of Science, USA* 95: 305-309

WHITE RB AND FERNALD RD (1998) Genomic structure and expression sites of three gonadotropin-releasing hormone genes in one species *General Comparative Endocrinology* 112: 17-25

WHITE SA, NGUYEN T AND FERNALD RD (2002) Social regulation of gonadotropin-releasing hormone *Journal of Experimental Biology* 205: 2567-2581

WILKINSON M AND LANDYMORE KM (1989) Do brain opioid peptides regulate the onset of puberty? In: *Brain opioid systems* pp 70-91 Eds. Dyer RG and Bicknell RJ Oxford University Press, Oxford

WILSON EO (1971) The degrees of social behavior In: *Insect societies* pp 4-6 Harvard University Press, Cambridge (Massachusetts)

WILSON EO (1975) Roles and castes In: *Sociobiology: the new synthesis* pp 298-313 Harvard University Press, Cambridge (Massachusetts)

WINKLER AJ (1994) The middle/upper Miocene dispersal of major rodent groups between southern Asia and Africa In: *Rodent and Lagomorph families of Asian origins and Diversifications* pp 173-184 Eds. Tomida Y, Li C-K and Setoguchi T National Science Museum Monongraphs, Tokyo

WITKIN JW, PADEN CM AND SILVERMAN AJ (1982) The luteinizing hormone-releasing hormone (LHRH) systems in the rat brain *Neuroendocrinology* 35: 429-438

WOOD AE (1955) A revised classification of rodents *Journal of Mammalogy* 36: 165-187

YELLON SM (1989) Effect of short-day induced gonadal regression on the GnRH neuron system in the adult male Djungarian hamster *Society of Neuroscience, Abstracts* 15: 950

YELLON SM AND NEWMAN SW (1991) A developmental study of the gonadotropin-releasing hormone neuronal system during sexual maturation in the male Djungarian hamster *Biology of Reproduction* 45: 440-446

YOUNG LJ, WANG Z AND INSEL TR (1998) Neuroendocrine bases of monogamy *Trends in Neuroscience* 21: 71-75

ZUKCERKANDL E AND PAULING L (1965) Evolutionary divergence and convergence in proteins In: *Evolving genes and proteins* pp 97-165 Eds. Bryson V and Vogel HJ Academic Press, New York