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REPRODUCTIVE REGULATION IN FEMALE DAMARALAND
MOLE-RATS, *CRYPTOMYS DAMARENSIS*: PHYSIOLOGICAL AND
NEUROENDOCRINE MECHANISMS

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Reproductive regulation in female Damaraland mole-rats, *Cryptomys damarensis*: Physiological and neuroendocrine mechanisms.

by

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Reproductive regulation in female Damaraland mole-rats, *Cryptomys damarensis*: Physiological and neuroendocrine mechanisms.

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Abstract

The aim of this study was to elucidate the proximate mechanisms, both social and physiological, responsible for the anovulation observed in non-reproductive, female Damaraland mole-rats (*Cryptomys damarensis*). To identify the social cues involved in the inhibition of fertility, the histological and endocrine response of the female reproductive axis to different social environments, was investigated. The presence of corpora lutea and increased circulating progesterone concentrations indicates that non-reproductive females ovulate spontaneously when they are housed in the absence of the breeding pair. Since anovulation is due to the inhibition of fertility rather than a lack of copulatory stimulation, it has the potential to play a role in maintaining reproductive skew. The endocrine and neuroendocrine mechanisms responsible for anovulation were also investigated. In comparison to reproductive females (RF), the pituitaries of non-reproductive females (NRF) were found to be desensitised to exogenous gonadotrophin-releasing hormone (GnRH) and contained less luteinizing hormone. However, GnRH priming did not increase pituitary sensitivity. Although there was no apparent difference in the distribution and morphology of GnRH-immunoreactive structures in the brain, a significantly greater concentration of GnRH was found in the brains of NRF compared to RF. This supports the hypothesis that an inhibition of GnRH release, and consequent desensitisation of the pituitary, is responsible for infertility in NRF. Differences in pituitary sensitivity between RF and NRF were apparent following hystero-ovariectomy, in the absence of gonadal steroids. Cortisol concentrations did not differ between RF and NRF. Thus, a gonadal steroid-independent pathway, that does not involve the negative effects of stress, appears to be

responsible for the inhibition of gonadotrophin secretion. A non-steroidal mechanism does not appear to be mediated through an increased activity of endogenous opioid peptides, since the opioid antagonist, naloxone, had no effect on LH secretion. This study shows that many of the pathways leading to anovulation are common to different forms of natural infertility.

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Chapter 1

General introduction

Cooperative breeding

Sociality arises by two main routes, namely the aggregation of individuals due to the inherent advantages of group living and the delayed dispersal of offspring due to constraints on dispersal and independent breeding (Emlen 1991). In several avian and mammalian group living species, reproductive effort is not equally distributed but is restricted to one or a few individuals. Reproductive skew theory predicts that higher skew will occur as group productivity increases, as ecological constraints on dispersal and independent breeding increase, as the relatedness between a dominant and given subordinate increases and with a decrease in fighting ability of the subordinates (Keller & Reeve 1994). Non-breeding individuals may help rear non-descendent young, thereby increasing the breeding female's reproductive success (Wasser & Barash 1983). This may occur through an increase in the success of individual breeding attempts, an increase in the number of breedings per season or by enhancing breeder survivorship (Emlen 1991). In groups of closely related individuals, especially those in which some helpers never breed, this apparently altruistic behaviour may provide support for kin selection theory. Individuals that do not breed may increase their inclusive fitness by helping related individuals (Hamilton 1963). Thus, if environmental constraints on dispersal and independent reproduction are high, remaining in the colony and helping related individuals may be a better fitness option than attempting to disperse and found a new colony as a breeder. Situations in which individuals assist unrelated young are more difficult to explain in adaptive terms. In non-family groups, helping behaviour may enhance the individual's probability of survival, increase its probability of becoming a breeder in the future or increase its fecundity when it does eventually become a breeder (Emlen 1991). Alternatively, helping may merely be an unselected consequence of non-dispersal of grown young (Jamieson 1989).

If helping is important for breeding success, it is important that potential helpers do not compete by breeding independently. Models that attempt to explain reproductive skew have in common the assumption that dominant member(s) of the group control reproduction of subordinates (Vehrencamp 1983; Keller & Reeve 1994). On a proximate level, reproduction in subordinate females can be suppressed through behavioural interactions, such as by interruption of sexual behaviour by the dominant female (wolf *Canis lupus*; Packard *et al.* 1985 & marmoset monkey *Callithrix jacchus*;

Abbott 1984) or the early death of the offspring of subordinate females attributable, directly or indirectly, to behaviour of the dominant female (wild dog *Lycaon pictus*; Malcolm & Marten 1982). Social suppression can also be mediated through the physiological response to stress. This may occur through harassment induced pregnancy loss (yellow baboon *Papio cynacephalus*; Wasser & Starling 1988), harassment induced inhibition of ovulation (cynomalogous monkey *Macaca fascicularis*; Kaplan *et al.* 1986) or environmentally induced stress resulting from the exclusion of subordinates from food resources crucial for reproduction (red deer *Cervus elephas*; Clutton-Brock *et al.* 1986). The Dominant Control Model (DCM) of reproductive inhibition assumes that the dominant female gains a reproductive advantage by suppressing reproduction in non-reproductive helpers (Snowdon 1996).

In addition to behavioural interactions, the DCM assumes that pheromones can act to suppress reproduction in subordinate individuals (Snowdon 1996). However, Keller & Nonacs (1993) have questioned how queen pheromones function within social insect colonies. The authors argue that pheromonal control has never been conclusively demonstrated and that it is evolutionarily difficult to justify. If reproductive inhibition was not in the best interests of the helpers, subordinates would be able to develop mechanisms that allow them to ignore the chemical signal or the pheromonal effect on subordinates would not originally have evolved. Rather than viewing the effect of pheromones on worker behaviour as *pheromonal queen control,* whereby queens chemically manipulate workers into behaving in ways that increase the queen fitness at the workers' expense, pheromones may, in fact, be honest *pheromonal queen signals.* In this situation workers adjust their behaviour in ways that increase their own, and possibly the queen's, inclusive fitness. This is equally applicable to cooperatively breeding mammals. Furthermore, the idea of *queen signal,* as opposed to *queen control,* may provide a more appropriate framework within which to discuss reproductive inhibition in cases of socially-induced infertility in which there is little or no evidence for behavioural suppression of non-reproductive females.

Although the DCM has dominated thinking in terms of the proximate mechanisms responsible for infertility in non-reproductive individuals of cooperatively breeding species (Abbott 1988, Faulkes & Abbott 1993, Bennett 1994), the lack of behavioural

and physiological evidence supporting the DCM in marmoset monkeys led Abbott *et al.* (1997) to propose an additional physiological mechanism of rank-related reproductive inhibition that does not involve the generalised effect of stress on reproductive processes. Specialised neuroendocrine and behavioural responses to subordinate status may result in the inhibition of sexual behaviour and ovulation in non-reproductive females. Snowdon (1996) proposed the Self-Restraint Model (SRM) to explain reproductive inhibition in groups in which the dominant female does not suppress reproduction in subordinate females. Subordinate individuals may restrain themselves from breeding if conditions for successful, independent reproduction are unfavourable. According to this model, restraint by non-reproductive females functions to minimise inbreeding by restricting reproduction until an unrelated male is present, until helpers or resources are available, or until sufficient parental skills have been required. It may, therefore, pay females to forego reproduction and help rear related individuals, rather than squander energy on failed breeding attempts. In their Reproductive Suppression Model, Wasser & Barash (1983) argue that subordinate females can maximise their lifetime reproductive success by suppressing their own reproduction until a future time when conditions may be expected to be better than present ones. Thus, subordinate females may restrain themselves from breeding because it is in their best fitness interests to do so.

An analogous situation to the SRM of reproductive inhibition can be found in another form of natural infertility in which individuals restrict breeding attempts to favourable times of the year, thereby increasing the chance of a successful breeding attempt. Seasonal breeders use environmental cues (the most common being photoperiod) to suppress their own ovulation to prevent breeding during periods that are unfavourable for reproduction. In a similar way, non-reproductive females of cooperatively breeding species may respond to visual, olfactory and/or tactile cues that indicate the presence of a breeding female. These inhibitory cues are perceived by the central nervous system and translated into infertility via neuroendocrine and endocrine pathways of the Hypothalamo-Pituitary-Gonadal axis, which may function to prevent unwanted fertilisations by related males in the colony or unrelated males which may enter the group. However, the physiological pathways through which inhibitory environmental and social cues result in infertility have yet to be fully elucidated.

The Hypothalamo-Pituitary-Gonadal axis

To investigate the physiological mechanisms responsible for socially-induced infertility an understanding of the functioning of the Hypothalamo-Pituitary-Gonadal (HPG) axis is necessary (see Austin & Short 1984; Johnson & Everitt 1995). Reproduction is controlled by the central nervous system which responds to external cues and internal signals (Fig. 1.1). Gonadotrophin releasing hormone (GnRH) provides a humeral link between the neural and endocrine systems. This decapeptide is synthesised and stored in neurones in the hypothalamus. It is then released into the median eminence from where it is transported to the anterior pituitary gland via the portal system. There GnRH acts on the gonadotrophes to affect the synthesis and secretion of the glycoproteins, luteinizing hormone (LH) and follicle stimulating hormone (FSH).
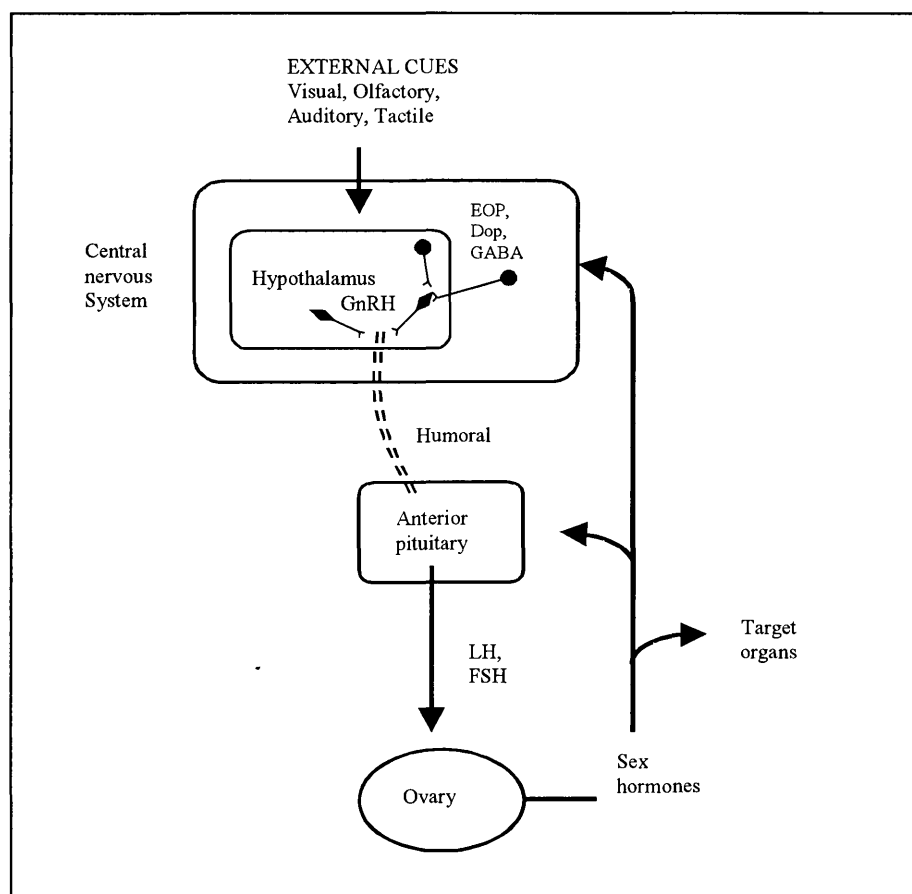
Fig. 1.1. Schematic representation of the Hypothalamo-Pituitary-Gonadal axis controlling reproduction. GnRH, gonadotrophin releasing hormone; LH, luteinizing hormone; FSH, follicle stimulating hormone; EOP, endogenous opioid peptides; Dop, dopamine; GABA γ-Aminobutyric acid (adapted from Karsch 1984).

General Introduction

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These gonadotrophins in turn, affect follicular development and ovulation in the ovary. Once follicles have developed to the antral stage, ovulation and subsequent corpus luteum formation depends on a surge of LH secreted by the anterior pituitary gland. Insufficient LH stimulation leads to atresia of the antral follicle. LH and FSH are also responsible for steroid synthesis and secretion by the ovary. These gonadal steroids play an important role in promoting sexual behaviour. In addition to affecting their target organs, progesterone and oestrogen feed back on the hypothalamus and pituitary to regulate the secretion of gonadotrophins.

Secretion of GnRH by neurones in the hypothalamus is intermittent and thus the release of gonadotrophins is pulsatile in nature. Once GnRH reaches the gonadotrophes it binds to specific receptors on the plasma membrane which triggers a cascade resulting in the gonadotrophin release (Gordon & Hodgen 1992). In addition to gonadotrophin release, pulsatile GnRH stimulation triggers additional cellular responses, including the biosynthesis of gonadotrophins (Papavasiliou *et al.* 1986; Starzec *et al.* 1986; Andrews *et al.* 1988) and regulation of GnRH receptor concentrations (DeKoning *et al.* 1978; Nett *et al.* 1981). The pulsatile release of GnRH is essential for maintaining pituitary sensitivity to GnRH.

GnRH synthesis and release from neurones in the hypothalamus is affected both positively and negatively by a number of neurotransmitters, including dopamine, serotonin, noradrenalin, adrenalin, γ-Aminobutyric acid (Kordon *et al.* 1994) and the endogenous opioid peptides (EOP; Almeida 1993). EOP have been implicated in the natural regulation of gonadotrophin secretion during normal sexual function, during the onset of puberty and in seasonal reproduction (Brooks *et al.* 1986; Almeida 1993). EOP generally have an inhibitory effect on reproductive function, an effect which can be blocked by the synthetic opioid antagonist, naloxone (Cicero *et al.* 1977; Grossman *et al.* 1981; Ferin *et al.* 1984; Leadem & Kalra 1985). Increased EOP activity could occur in response to neural signals or through mediation of the negative effects of gonadal steroids (Cicero *et al.* 1979; Wehrenberg *et al.* 1982; Currie & Rawlings 1987) and physiological stress (see Przewlocki 1993 for review).

The anovulation observed in non-reproductive females of cooperatively breeding species may result from inhibitory factors, or lack of stimulatory factors, acting at any site within the HPG axis. An understanding of the proximate mechanisms involved in reproductive inhibition is an important consideration when trying to elucidate the causes, in an ultimate sense, of socially-induced infertility in cooperatively breeding species. The Damaraland mole-rat (*Cryptomys damarensis*) provides an ideal model with which to study the proximate mechanisms involved in the suppression of the HPG axis in response to social cues.

The eusocial Damaraland mole-rat

Eusociality is commonly associated with Isopteran and Hypmenopteran insects, and refers to a social organisation in which there is cooperative care of the young, reproductive division of labour with more or less sterile individuals working on behalf of those engaged in reproduction, and an overlap of at least two generations able to contribute to colony labour (Michener 1969). However, eusociality has also been discovered in two mammalian species of the family Bathyergidae. With respect to its social system, the naked mole-rat (*Heterocephalus glaber*) was the first vertebrate to be compared to the invertebrate termites (Jarvis 1981). Since then, eusociality has also been reported in the Damaraland mole-rat (Bennett & Jarvis 1988; Jarvis & Bennett 1993).

In addition to these two eusocial species, the family Bathyergidae contains several species within the genus *Cryptomys* that exhibit varying degrees of sociality and three genera that are solitary and xenophobic, as are most other subterranean rodents (Jarvis & Bennett 1990). The range of habitats which they occupy, from mesic to arid, has provided an opportunity to examine ecological constraints driving sociality. Jarvis *et al.* (1994) proposed the Food-Aridity Hypothesis, which has subsequently been supported by a phylogenetic constraints analysis (Faulkes *et al.* 1997) to explain the evolution of sociality within the Bathyergidae. Mole-rats live in expandable, subterranean burrow systems and are herbivorous, feeding on the underground storage organs of plants (tubers, bulbs, corms) that they find while extending foraging burrows

(Brett 1991). In arid areas, much of this food source is concentrated in patches of small tubers or single large tubers, whereas in mesic areas, food is generally more evenly dispersed. Therefore, in arid areas, the probability of a single animal finding a food patch is far lower than in the mesic areas, whereas if a large number of animals are foraging, the chances of locating a food patch is greatly increased (Lovegrove & Wissel 1988; Jarvis & Bennett 1991; Lovegrove 1991).

Both the naked and Damaraland mole-rat inhabit arid areas where rainfall is sporadic and unpredictable (Jarvis & Bennett 1994). During the dry season, burrowing is severely curtailed since the soil is not easily worked, increasing the energetic cost of locating food patches. Therefore, the period following good rains may be the only opportunity for mole-rats to extend foraging burrows and locate sufficient food patches to last through the dry season. Solitary animals would find it extremely difficult to find enough food to sustain them through a drought, whereas large colonies could take advantage of favourable conditions by cooperating to find sufficient food resources (Jarvis *et al.* 1994). This is supported by the observation that larger colonies with an established workforce (n > 8) have a greater chance of surviving from one year to the next than smaller colonies (2 - 4 individuals). Jarvis & Bennett (1993) found that eight out of 16 newly founded small colonies failed in two years, whereas only one of eight established colonies failed. Thus, it may be significant that despite the high costs of digging, only small colonies extend burrows during the dry season, presumably since they can not locate sufficient food during the period when conditions were favourable for digging (Jarvis & Bennett 1993). Environmental constraints on dispersal and colony foundation mean that it may be a better fitness option for subordinates to forego independent reproduction and remain in their natal colony and help. In the case of the Damaraland mole-rat, limited food resources may be insufficient to support multiple breeders within the colony as this would result in a less effective workforce and a simultaneous increase in the number of dependent young in the colony.

Reproduction in colonies of the Damaraland mole-rat is restricted to a single breeding female (RF), which is thought to breed with only one male (Jarvis & Bennett 1981; Bennett & Jarvis 1988). In the laboratory, the RF breeds throughout the year, producing a maximum of three litters, with an average of three pups to a litter.

Subordinate animals, which are typically the offspring or close relatives of the reproductive pair (Jarvis & Bennett 1993), are reproductively inactive (Bennett *et al.* 1993). Histological and endocrine studies from both wild and captive colonies support the assertion that reproduction is limited to a single female (Bennett & Jarvis 1988; Jarvis & Bennett 1993). As would be expected of actively breeding females, urinary oestrogen and progesterone concentrations are significantly higher than those in NRF (Bennett 1994; Bennett *et al.* 1994). Follicular development was also markedly different between RF and NRF. In addition to a full range of developing follicles, the ovaries of the RF show corpora lutea and regressing corpora lutea, indicating that ovulation has occurred (Bennett *et al.* 1994). Thick, vascularised uterine walls and placental scars, characteristic of the actively breeding female are not observed in NRF. Although the ovaries of NRF contain developing and Graafian follicles, the absence of corpora lutea suggests that ovulation has not taken place. Rather, luteinized unruptured follicles (LUF), in which an intact zona pellucida clearly indicates an absence of ovulation, fill the stroma. The formation of LUF, as opposed to ovulation and formation of functional corpora lutea, may be due to insufficient LH stimulation from the pituitary gland. Both basal and GnRH stimulated plasma LH levels are significantly lower in NRF compared to RF, indicating that the pituitary is desensitised to GnRH stimulation (Bennett *et al.* 1993). Thus, reproductive function appears to be inhibited in NRF. Over 90% of the NRF live a lifetime of apparent infertility (Jarvis & Bennett 1993).

In colonies of *C. damarensis*, there appears to be a strict incest avoidance, probably based on familiarity, which prevents breeding by subordinates. Removal of the RF from the colony results in a complete cessation of reproduction until an unfamiliar, unrelated male is introduced (Bennett *et al.* 1996, Rickard & Bennett 1997). Therefore, reproductive skew is, at least in part, due to a lack of potential mates in a highly related colony. Furthermore, if the relative inactivity of the reproductive axis in NRF is socially-induced, it has may function to prevent breeding with unrelated males which enter the colony (N.C. Bennett *pers. comm.*). If this were the case, the observed reproductive skew in *C. damaresis* may result both from the absence of sexually attractive, unfamiliar males, in addition to the presence of inhibitory cues originating from the breeding female (Bennett *et al.* 1996). Both mechanisms may be explained

within the Self-Restraint-Hypothesis of reproductive inhibition, although they may occur in response to different cues and are possibly effected through different mechanisms.

Aims of the thesis

Using the Damaraland mole-rat as a model, the primary aims of this thesis are to determine the social factors affecting female reproductive function and to elucidate the endocrine and neuroendocrine mechanisms responsible for anovulation in subordinate females. **Chapter 3** focuses on the effect of the social environment on ovarian function and pituitary sensitivity in female mole-rats to determine whether infertility in subordinate females is socially-induced. Furthermore, the possible role of infertility in maintaining the reproductive skew is discussed. In **Chapter 4** the mechanisms responsible for anovulation at the pituitary level, in particular the effect of the GnRH on pituitary sensitivity, are examined. In **Chapter 5** the immunoreactive GnRH system of the Damaraland mole-rat is mapped and morphological parameters are investigated to determine whether suppression of the GnRH system is responsible for infertility in non-reproductive females. Lastly, in **Chapter 6**, possible physiological pathways involved in the inhibition of the gonadotrophin secretion at the hypothalmic or pituitary level are investigated.

Chapter 2

Materials and methods

Study Animals

Damaraland mole-rats were collected at Dordabis, Namibia (22°58'S; 17°41'E), and at Hotazel, Northern Cape Province, South Africa (27°17'; 22°58'E). To meet the needs of the study, new breeding pairs were formed in the laboratory by pairing males and females originating from different colonies. Females were classified as reproductive once they had given birth to at least one litter.

Maintenance

Colonies of *C. damarensis* were housed in plastic crates (1×0.5×0.5m) with nesting boxes. Animals were provided with wood-shavings and shredded paper towelling for nesting material, which was replaced weekly. The animals were maintained in a constant temperature room at 25°C, in continuous dark (DD) and were fed and cleaned under red light. Animals were fed daily on freshly chopped butternut, gem-squash, sweet potato, pumpkin, carrot and apple. The animals drank no free water. The general health (weight, coat condition etc.) of the animals was monitored daily at feeding time.

Experimental Procedures

Hormone administration

Gonadotrophin-Releasing Hormone (GnRH) was kindly provided by Prof. R. P. Millar (Department Chemical Pathology, UCT, RSA). The peptide was synthesised using solid phase methodology and has a purity of greater than 98% homogeneity (Millar *et al.* 1989). Naloxone hydrochloride was obtained from Sigma Chemical Company (St. Louis, USA). Peptides were dissolved in sterile physiological saline and stored at -20°C until required. GnRH and naloxone were administered in a 200µl physiological saline vehicle as a single, subcutaneous injection. The Luteinizing Hormone (LH) response to both naloxone and GnRH challenges was assessed by taking blood samples immediately before and 20min after the hormone challenge. Control animals were injected s.c. with sterile physiological saline (0.9% NaCl w/v).

Blood sampling

a) Approximately 300-400μl of whole blood was collected from veins in the feet of hand-restrained animals. Blood was collected in heparinized microhematocrit capillary tubes following venepuncture using a sterile 21gauge hypodermic needle. Prior to collecting the blood samples, individual animals were placed in buckets with wood shavings and paper towel in a temperature regulation chamber (Labex 100L) at 34°C for 45minutes. This increased blood flow, greatly facilitating blood sample collection.

b) Prior to removing the ovaries for histological examination (see below), blood samples were obtained for progesterone determination by cardiac-puncture. Approximately 2ml of whole blood was collected and kept at 4°C for less than 30min prior to centrifugation at 2500rpm, for 30min at 4°C. Plasma was stored in nunc cryo-tubes (Nalge, Denmark) at -20°C prior to radioimmunoassay.

Urine collection

To investigate progesterone profiles, urine was collected daily from three non-reproductive females. Urine was used for progesterone determination since it could be obtained via a non-invasive procedure. Animals were placed in metabolic cages until they had urinated, after which they were returned to their nesting boxes. Urine was frozen at -20°C, within one hour of being voided, until required for radioimmunoassay. Sampling was completed between 10h00 and 14h00 to minimise possible circadian fluctuations in progesterone excretion.

Hormone Assays

Luteinising hormone bioassay

Concentrations of plasma and pituitary luteinizing hormone were determined using the LH bioassay which is based on the production of testosterone by dispersed mouse Leydig cells (Van Damme *et al.* 1974). Details of the assay have been published by Harlow *et al.* (1984), Hodges *et al.* (1987) and Faulkes *et al.* (1990). This assay method has the advantage over conventional LH radioimmunoassay since it measures only biologically active LH and not structural LH, which may lead to spurious results.

Leydig cell preparation

Incubation medium, made up of 12ml Eagle's basal medium (Life Technologies Ltd., Paisley, Scotland), 2.1ml 7.5 % sodium hydrogen carbonate (Life Technologies Ltd., Paisley, Scotland), 2ml fetal calf serum (Life Technologies Ltd., Paisley, Scotland) and 100ml distilled water, was placed on ice and gassed slowly with Carbogen 5 (95% O_2:5% CO_2; BOC gases, Manchester, U.K.) for 10min. Following cervical dislocation, the testes of a male mouse (T0 strain, Bantin and Kingman, Hull, U.K.), approximately six weeks old, were removed, decapsulated and minced in 5ml incubation medium. The cells were mixed on a magnetic stirrer for 5min, filtered through nylon gauze and incubated in Carbogen 5 for one hour in a shaking water bath, preheated to 34°C. The incubated cell suspension was centrifuged at 2500rpm for 5min at 4°C. The supernatant was decanted and the pellet containing the cells was resuspended in 1.5ml incubation medium, centrifuged at 2500rpm for 5min at 4°C and decanted. The pellet was resuspended in 10ml incubation medium and the cell suspension was mixed slowly on a magnetic stirrer for 5min at 4°C. The cells were counted on a haemacytometer (WSI, depth 0.1 mm, 1/400 mm^2) using phase contrast microscopy. The cell suspension was made up with incubation medium so that the number of cells counted corresponded to the final cell suspension volume (in mls), which was stirred slowly for 5min.

Bioassay

Samples of mole-rat plasma (25µl) were prepared at a 1:20 dilution in LH buffer, containing 2.9g di-sodium hydrogen orthophosphate dodecahydrate, 0.29g sodium dihydrogen orthophosphate dihydrate, 4.38g sodium chloride (AnalaR, BDH Ltd., Poole, U.K.), made up to 1l in deionized dH_2O, with 0.1% BSA (Merck, Darmstadt, Germany). Further serial doubling dilutions (1:40, 1:80 and 1:150) were used to assay samples with higher LH concentrations. The standard curve were prepared by serially double-diluting synthetic LH (2nd International Standard 1988, Code 80/552, N.I.B.S.C., U.K.; Storring *et al*. 1993) in LH buffer over the range 200µiu.100µl^{-1} to 6.25µiu.100µl^{-1}. To the bioassay tubes was added 100µl of sample, standard, quality control (marmoset plasma at four dilutions, 1:200, 1:400, 1:800 and 1:1600) or 100µl LH buffer to obtain an estimate of total binding. Standards and total binding were assayed in triplicate whereas samples and quality controls were assayed in duplicate.

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Leydig cell incubation

Diluted cell suspension (200µl) was added to each bioassay tube and incubated in a shaking water bath at 34°C for 3hr in Carbogen 5. To terminate testosterone production, Leydig cells were killed by placing tubes in a water bath at 100°C for 15min. Tubes were placed on ice and 1.4ml phosphate buffered saline with 0.1% gelatin (AnalaR, BDH Ltd., Poole, U.K.) was added to each tube. The amount of testosterone produced during the incubation was determined by radioimmunoassay.

Testosterone radioimmunoassay

Testosterone produced by the Leydig cells in response to LH stimulation was determined by assaying 50µl, 100µl or 200µl samples from each of the bioassay tubes. In addition, estimates of total counts and non-specific binding were assayed in triplicate. Iodinated testosterone (Testosterone-3CMO(2-^{125}I)iodohistamine, Amersham, U.K.) at approximately 10000 cpm.tube^{-1} (100µl) was added to all tubes after which 100µl of antiserum (Sheep anti-testosterone no. 505, MRC, Edinburgh, U.K.) was added to all tubes except those used to estimate total counts and non-specific binding. The assay was vortexed for 1min and incubated for 2hr at room temperature. Bound and free testosterone was separated by adding 100µl of a second antibody (Anti sheep/goat IgG, Law Hospital, Lanarkshire, U.K.) to all tubes except total counts. Tubes we vortexed for 1min and incubated overnight at 4°C. To all tubes except total counts was added 1ml 0.9% saline with 0.1% triton X-100 (AnalaR, BDH Ltd., Poole, U.K.). The tubes were centrifuged at 2400rpm for 30min at 4°C. The supernatant was aspirated and the remaining pellets were counted for radioactivity in a 5005 cobra autogamma counter (Packard Instrument Company, Meriden, USA).

Validation

The bioassay has been previously validated in the Damaraland mole-rat by Bennett *et al* (1993). Serial doubling dilutions of Damaraland mole-rat plasma collected before and after exogenous GnRH stimulation were assayed and compared to the LH standard curve (Fig. 2.1a). Following Logit-Log transformation of the data (Chard 1987), parallelism of the LH standard and two serial dilutions of mole-rat plasma was tested using the *Statistica* computer package (Statsoft, Tulsa, USA). The slope of the

lines were not significantly different (ANCOVA, F_{2-8} = 2.7; P > 0.05; Fig. 2.1b). Serial doubling dilutions of pituitary LH extract from a reproductive female were made up in LH bioassay buffer and assayed. The slope of the curve was not significantly different from the standard curve of reference LH preparation (ANCOVA; F_{1-6} = 3.0; P > 0.05; Fig. 2.2). The sensitivity of the assay (determined at 90% binding) was 0.1 mi.u. per tube. The mean intra-assay coefficient of variation for estimates of repeated measures in the same assay of a unknown concentration of LH standard, determined at approximately 35% binding, was 10.2% (n = 18). Repeated measures of unknown concentrations of LH in marmoset plasma at dilutions of 1:400 and 1:800 resulted in inter-assay coefficients of variation of 8.9% (n = 17) and 10.2% (n = 17) respectively.
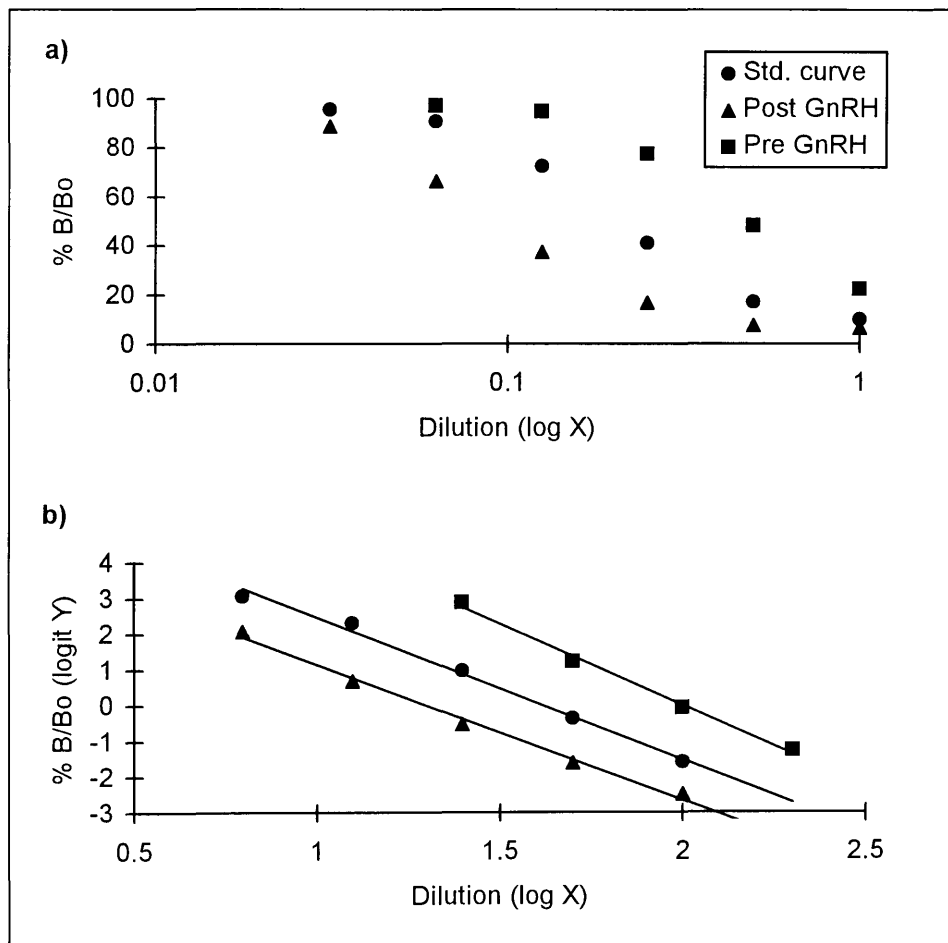
Fig. 2.1. Reference LH preparation (●) and serial doubling dilutions of Damaraland mole-rat plasma before (■) and 20min after stimulation with 2.0μg GnRH (▲) showing parallelism. **a)** Concentrations of reference LH preparation plotted on a logarithmic scale against %B (B/Bo), **b)** Concentrations of reference LH preparation plotted on a logarithmic scale against the logit of %B/Bo. Bo, total binding; B, binding.
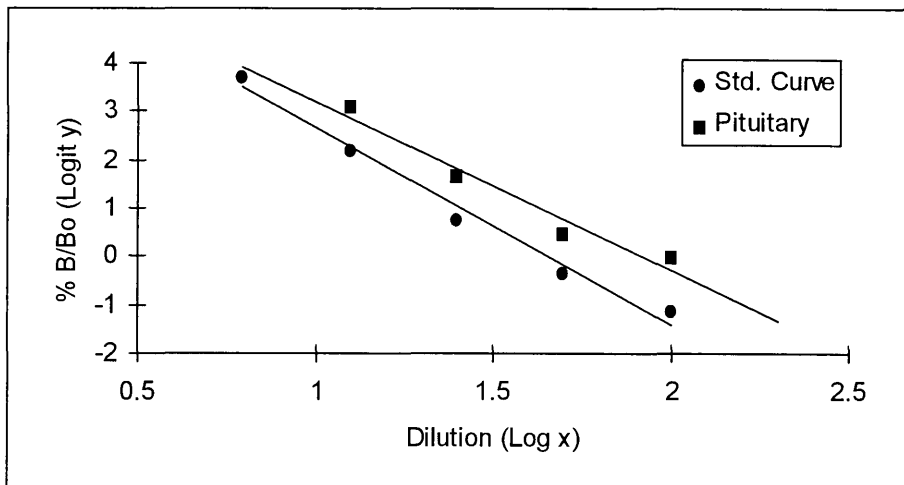
Fig. 2.2. Reference LH preparation (●) and serial doubling dilutions of Damaraland mole-rat pituitary homogenate (■) showing parallelism. Concentrations of reference LH preparation are plotted on a logarithmic scale against the logit of %B /Bo.

Progesterone radioimmunoassay

Progesterone assays were performed using the coat-a-count Progesterone Kit (Diagnostic Products Corporation, USA) as described and validated for the Damaraland mole-rat by Bennett *et al.* (1994). The antiserum is highly specific for all naturally occurring steroids with a cross-reactivity of <0.5%, with the exception of 17αDihydroprogesterone (3.4%), 11-Deoxycorticosterone (2.4%), 5β-Pregnan-3,20-dione (3.2%) and 5α-Pregnan-3,20-dione (9.0%). Duplicate 100μl urine or plasma samples and known standard concentrations, over the range 0.3 - 127.2 nmol.l^{-1}, were added to antibody coated tubes. Duplicate, uncoated tubes were used to estimate the level of non-specific binding. Estimates of total binding were obtained by adding 100μl of gel-PBS buffer (to duplicate, antibody-coated tubes. To each tube was added 1ml labelled progesterone at approximately 50000cpm, after which all tubes were vortexed for 30sec. Following a three hour incubation at room temperature, the tubes were decanted and the bound, labelled progesterone was counted in a gamma counter (Innotron). A standard curve was fitted to the data in Sigma Plot (Jandal Scientific) using the statistical model:

$$f = (a - d) / (1+(x / c)^b) + d$$

where:

a = max (y)

b = if (y [1] - y [size (y)] > 0 ,1 , -1)

c = x [int (size (y) / 2)]

d =min (y)

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Extraction

Samples were assayed without prior extraction. Three individual urine samples of 500µl were each extracted in 5ml petroleum ether. Tubes were vortexed for 5min, placed at -20°C for 60min and at -70°C for 5min. The tubes were then decanted and dried down under nitrogen gas at 34°C. Samples were reconstituted in 500µl buffer, of which 100µl was added to each assay tube. Percentage binding for the three non-extracted samples was between 50 - 60% whereas the extracted samples were all below the sensitivity of the assay. This indicates an extremely low extraction efficiency and that extraction does not increase the effectiveness of the assay.

Creatinine determination

Prior to progesterone assay, all urine samples were subject to creatinine determination as described by Bonney *et al.* (1982). Creatinine is excreted at a relatively constant rate and can therefore be used to correct for the dilution of urine. In each assay, 20µl of urine sample or creatinine standard (2.5nM) was added to 1ml dH20 and 250µl alkaline picrate. Absorbance was measured after 45min using a spectrophotometer (Novaspec II, Parmacia Biotech, Cambridge, U.K.) at a wavelength of 490nm. The inter-assay coefficient of variation was 3.06% (n = 8).

Validation

Plasma - The assay was validated for *C. damarensis* plasma by testing the slope of the curve produced using serial doubling dilutions of unextracted mole-rat plasma obtained from a pregnant female (over the range 1:1 to 1:64) against that of the standard curve. Following logit-log transformation of the data (Chard 1987), the slopes of the lines were compared using *Statistica* computer package (Statsoft, Tulsa, USA) and found not to differ significantly (ANCOVA, $F_{1-8} = 0.1$, $P = 0.75$; Fig. 2.3). In addition, mole-rat plasma spiked with cold progesterone at three concentrations yielded a recovery estimate of (109.3 ± 5.8%). The minimum detection limit of the assay was 0.36nmol.l^{-1}. Intra- and inter-assay coefficients of variation were 4.4% (n = 20).and 6.6% (n = 4) respectively.

Urine - Serial doubling dilutions of unextracted mole-rat urine (over the range 1:1 to 1:32) produced a curve, the slope of which did not differ significantly from the

standard curve (ANCOVA, F_{1-6} = 4.9, $P > 0.05$; Fig. 2.3), following logit-log transformation of the data (Chard 1987). Serial doubling dilutions of male urine (expected low concentrations of progesterone) were undetectable. The sensitivity of the assay, determined at 90% binding, was 0.4nmol.l^{-1}.
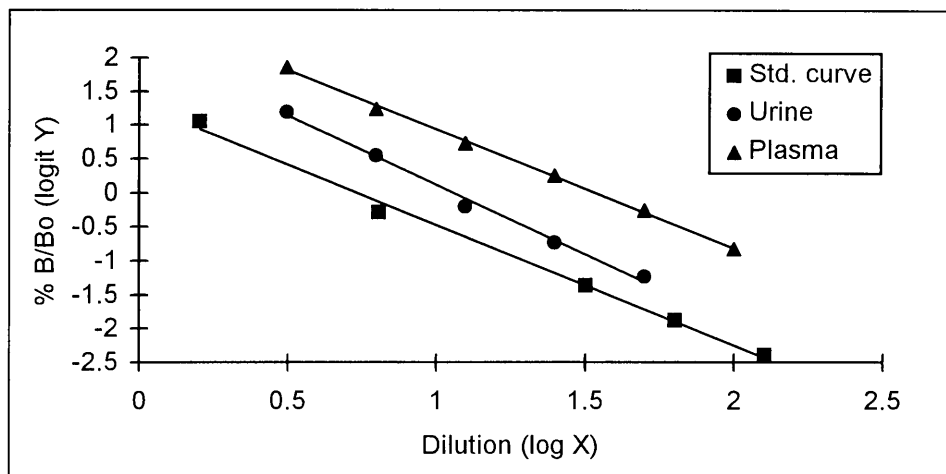
Fig. 2.3. Serial doubling dilutions of unextracted Damaraland mole-rat urine (■) and plasma (▲) and the standard curve (●), showing parallelism. Concentrations of progesterone standard are plotted on a logarithmic scale against the logit of %B/Bo.

GnRH radioimmunoassay

Tissue extraction

In order to investigate brain GnRH concentrations, brains were removed within three minutes of death, snap frozen in liquid N_2 and stored at -70°C for a period no longer than 40 days prior to assay. The brains of eight non-reproductive males were used to validate the GnRH radioimmunoassay. Ovaries and cardiac blood samples were collected from the reproductive and non-reproductive females for histological examination and progesterone determination, respectively.

GnRH extraction

Prior to RIA the brains were lyophilised and their dry weights were determined. Lyophilised brains were minced with a sterile blade and extracted individually with 5ml 2N acetic acid for 3min using a polytron homogeniser (Kinematic). All steps were performed at 4°C. The homogenates were centrifuged at 18000g for 60min and the

supernatant fluid was freeze dried overnight (Freeze mobile 6, Virtis, N.Y.). Individual brain extracts were reconstituted in 4ml TF solution (2% triethylamine, 1.05% formic acid, 96.95% dH$_2$O, pH 3.2). Extracts were sonicated (Branson sonifier 250) for 3min, and centrifuged at 10000rpm (Sigma 202MK) for 15min. The supernatants were concentrated overnight using a Savant speed-vac concentrator connected to a freeze drier (Freeze mobile 6, Virtis, N.Y.) and reconstituted in 1ml gel-PBS buffer (phosphate-buffered saline containing 0.1% gelatin) for radioimmunoassay.

Sep-pak purification

Sep-pak C-18 cartridges (Waters Associates) were activated with 2ml methanol, 5ml dH$_2$O and 4ml TF solution. Brain extract was applied to a Sep-pak column which was then washed with 3ml TF solution. Peptides adsorbed to the Sep-pak columns were eluted with 4ml acetonitrile/TF (60:40). A further two elutions were performed to determine whether the peptide was sticking to the column. In order to determine the effect of Sep-pak purification the brain extracts from five non-reproductive males were pooled and, after freeze drying, reconstituted in 10ml TF solution. This extract was divided into five, 2ml aliquots (one brain equivalent), 3 of which were purified through Sep-pak C-18 cartridges (Waters Associates). All samples were then concentrated overnight in a Savant speed-vac concentrator connected to a freeze drier (Freeze mobile 6, Virtis, N.Y.) and reconstituted in 1ml gel-PBS. To validate the GnRH radioimmunoassay for Sep-pak treated samples, a further three brain extracts were pooled and reconstituted in 6ml TF solution, of which 3ml were purified through Sep-pak C-18 cartridges. Once concentrated, samples were reconstituted in 1.5ml gel-PBS.

GnRH radioimmunoassay

Concentrations of GnRH extracted from brains of Damaraland mole-rats were determined by radioimmunoassay based on the method described by King & Millar (1986). GnRH antiserum 678 (J. A. King, Department of Chemical Pathology, UCT, RSA), which requires both the NH$_2$- and COOH-termini for binding, cross-reacts with all seven known forms of GnRH (King *et al.* 1994). Synthetic mGnRH (R. P. Millar; Department Chemical Pathology, UCT, RSA) was used as standard and ^{125}ImGnRH, iodinated using the chloramine-T method (Greenwood *et al.* 1963), as the tracer.

Iodination of mGnRH

Synthetic mGnRH (10μg) was dissolved in 80μl 0.1M NaH_2PO_4 (pH 7.6), to which was added 10μl ^{125}I (1mCi; Amersham, UK). The reduction reaction, initiated by adding 10μl chloramine T (30μg/10ml 0.1M NaH_2PO_4, Sigma Chemical Company, St. Louis, USA), was stopped after five seconds by adding 50μl $Na_2S_2O_5$ (6μg/8ml 0.1M NaH_2PO_4, Sigma Chemical Company, St. Louis, USA). The labelled peptide was purified using high performance liquid chromatography (HPLC; see below). The $^{125}ImGnRH$ was eluted over a gradient of 0 - 60% acetonitrile/0.01M ammonium acetate (v/v). The amount of free and incorporated ^{125}I collected in 30, 2ml fractions was determined by counting the radioactivity emitted by 5μl of each fraction. Following the elution of unincorporated ^{125}I in fractions 1 and 2, a peak of 3.27 × 10^6 cpm/5μl was obtained in fraction 16 (Fig. 2.4). Fraction 16 was diluted with 78ml gel-PBS and 2 × 10^{6}cpm/200μl aliquots were snap frozen at -70°C.
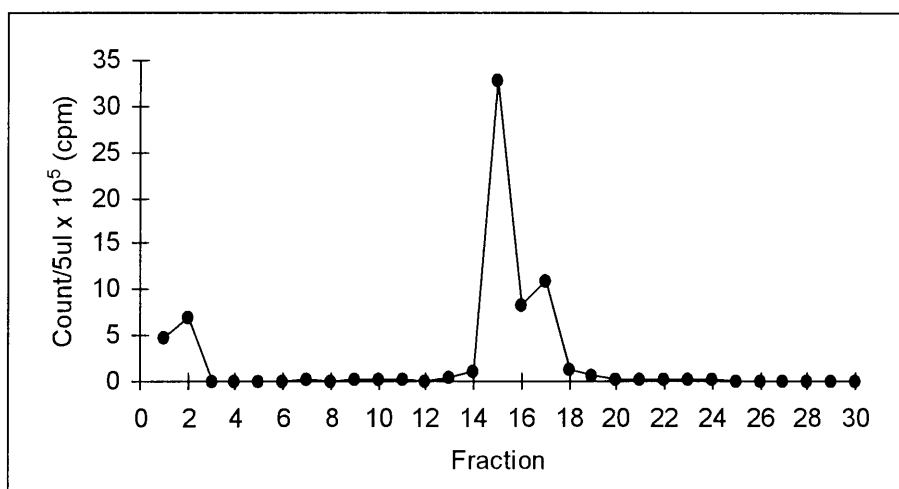
Fig. 2.4. Radioactivity (Cpm) counted in 5μl of each 2ml fraction collected during HPLC after iodination of mGnRH.

Radioimmunoassay

Serial doubling dilutions of mGnRH were prepared in gel-PBS over the range 1000 pg.ml^{-1} - 0.48 pg.ml^{-1}. The $^{125}ImGnRH$ tracer was used at approximately 15000cpm 100μl^{-1} in a total assay volume of 500μl. The mGnRH standard preparations and samples of GnRH extract, assayed at three dilutions as a routine test for parallelism,

Materials and methods 21

were assayed in duplicate. The assay was incubated at 4°C overnight. Bound and free peptide was separated using the Pharmacia Decanting Suspension (PDS) method, by adding 200µl PDS (Kabi Phamacia Diagnostics, Uppsala, Sweden) to all tubes with the exception of total counts, at 25°C. Once the tubes had been vortexed briefly and allowed to stand for 30min, 1ml dH$_2$O was added to terminate the reaction. Tubes were centrifuged at 3000rpm for 20min at 4°C and the supernatant, containing free ^{125}I, was decanted. The pellet containing the bound fraction was counted for 1min on a gamma counter (Packard Riastar 5400, Meriden, USA).

Validation

The suitability of the assay to measure GnRH from mole-rat brain extract was assessed by testing the parallelism between serial doubling dilutions of synthetic mGnRH standard and both crude extract and extract purified through Sep-pak columns (Fig. 2.5a). Following logit-log transformation of data points (Fig. 2.5b), the slopes of the tissue samples and standard displacement curve were tested for parallelism, using the *Statistica* computer package (Statsoft, Tulsa, USA), and found not to be significantly different from each other (ANCOVA, $F_{2-14} = 0.14$, $P > 0.05$).

A constant volume (100µl) of GnRH extract was spiked with known concentrations of synthetic GnRH (0.49 pg.ml^{-1} - 62.50 pg.ml^{-1}) and assayed. The GnRH extract was also assayed separately. The Spearman-Rank correlation coefficient for the relationship between expected and observed concentrations was calculated as $r_s = 1$ ($P < 0.05$, n = 8, Fig. 2.6). There was no difference in the GnRH concentrations in samples that were assayed with and without Sep-pak purification and thus crude samples were used in the assay. Peptide concentrations in the second and third elution were below the sensitivity of the assay. The intra- and inter-assay coefficient of variation was 9.6% (n = 14) and 6.6% (n = 6) respectively.
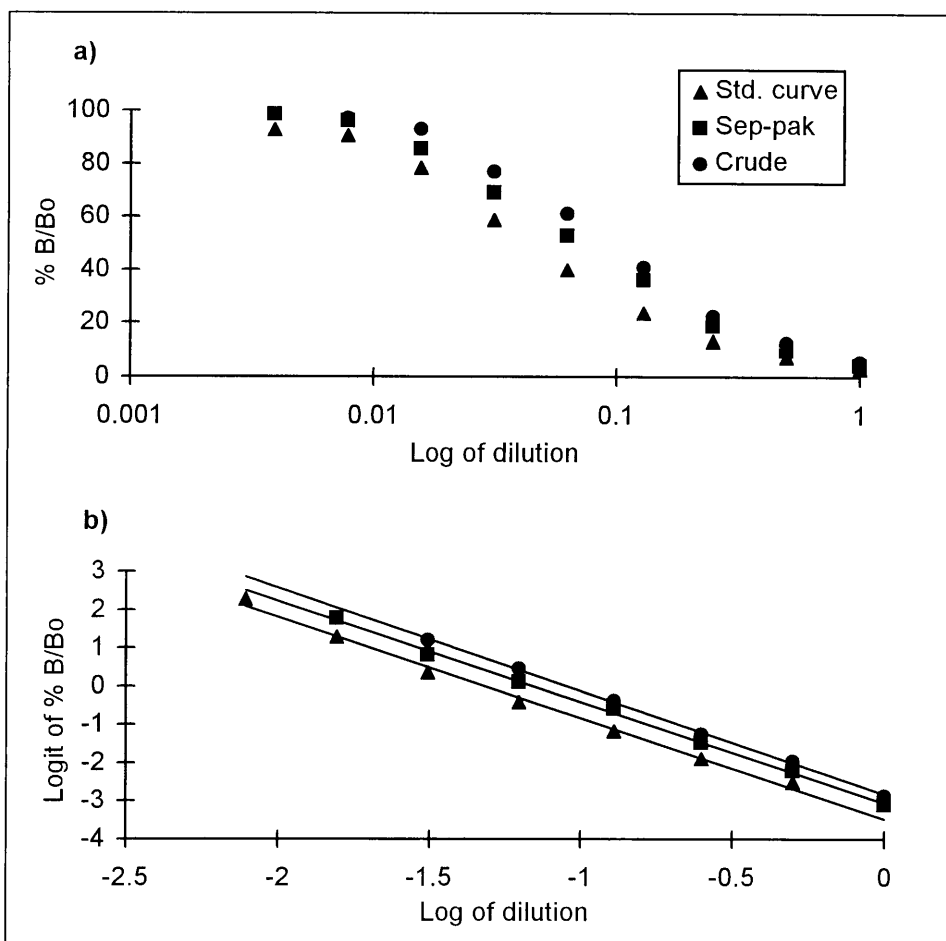
Fig. 2.5. A standard curve of synthetic mGnRH (●) compared to serial doubling dilution curves of brain extract purified through Sep-pak C18 cartridges (■) and crude brain extract (▲), showing parallelism. Concentrations of reference GnRH preparation are plotted on a logarithmic scale against the logit of %B/Bo. Bo, total binding; B: binding, NSB, nonspecific binding
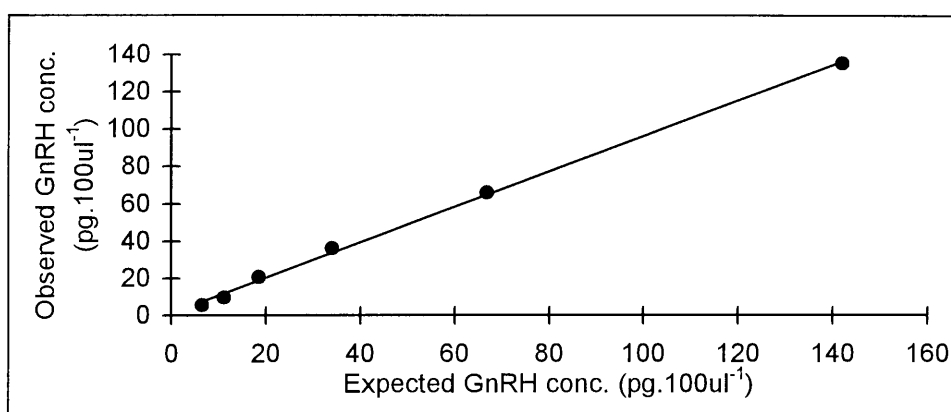
Fig. 2.6. Significant correlation between expected and observed values of known concentrations of synthetic mGnRH ($0.49pg.ml^{-1}$ - $62.50pg.ml^{-1}$) spiked with a constant volume of mole-rat brain extract (13.29 $pg.ml^{-1}$). Spearman-Rank correlation $r_s = 1$.

Materials and methods

Pituitary GnRH receptor binding assay

Tissue extraction

Pituitary glands were removed, snap frozen in liquid nitrogen and stored at -70°C until required for receptor binding assay. Pituitaries from non-reproductive males were used to determine the optimum pituitary membrane dilution and binding affinities of both mGnRH and [His^5, D-Tyr^6] GnRH for the mole-rat pituitary GnRH receptor. The methodology used to investigate binding of mGnRH or GnRH analog to pituitary receptors, has been described by Millar *et al.* (1995).

Tissue homogenisation

Individual pituitaries were homogenised in 1ml Hepes binding buffer (10^{-9} M Hepes, 10^{-4} M EDTA, 0.1% BSA, pH 7.4), using 15 strokes through a 0.8mm bore needle attached to a 10ml syringe. All steps were performed at 4°C. The homogenate was centrifuged at 15 000rpm for 30min (Sigma 202MK). The supernatant was decanted and frozen at -20°C for pituitary LH determination and the membrane pellet was reconstituted in 2.8ml Hepes binding buffer. To investigate the optimum pituitary dilution and receptor binding affinity, pituitaries from seven and four non-reproductive males respectively, were pooled prior to centrifugation.

Iodination

Due to the small size of Damaraland mole-rat pituitary glands, determination of pituitary receptor concentration was attempted using [His^5, D-Tyr^6] GnRH (Department of Chemical Pathology, UCT, RSA) as the labelled peptide in the pituitary receptor assay system, since it has a higher affinity for the GnRH receptor than conventional GnRH analogs and mammalian GnRH (Millar *et al.* 1995). The GnRH super-analog [His^5, D-Tyr^6] GnRH was labelled with ^{125}I (1mCi; Amersham, UK) using a modified chloramine-T method of Greenwood *et al.* (1963). [His^5, D-Tyr^6] GnRH (5µg), chloramine-T (30µg) and sodium metabisulphate (6µg) were made up separately in phosphate buffer (0.5M NaH$_2$PO$_4$, pH7.6) at room temperature. Chloramine-T (10µl) was added to [His^5, D-Tyr^6] GnRH (40µl) and ^{125}I (10µl) to initiate an oxidising reaction, which was subsequently terminated by adding sodium metabisulphate (50µl) after 11sec.

Purification

To separate the labelled peptide, unlabelled peptide and unbound I^{125}, three methods were attempted.

a) High Performance Liquid Chromatography

High Performance Liquid Chromatography (Spectra-Physics, Model SP 3500B) was used to separate the labelled peptide from unlabelled peptide and unincorporated ^{125}I. Peptides were eluted using a Sterisorb ODS reverse phase column (0.4×25cm; 5µm particle size; Phase Separations). The column was equilibrated with 0.01M ammonium acetate (pH 4.6) for 30min after which the iodination reaction mixture was loaded. The column was eluted on a linear gradient of 0 to 80% acetonitrile in 0.01M ammonium acetate. The gradient was repeated to determine whether any peptide had stuck to the column. Fractions (1.5ml) were collected in tubes containing 10µl 10% BSA and 5µl of each fraction was counted on a gamma counter (Packard Riastar 5400). Most of the ^{125}I eluted as a free peak in fraction three and was not incorporated into the [His^5, D-Tyr^6] GnRH peptide, as is evident from the small peak of approximately 11000 and 14000cpm/5µl in fractions 19 and 20 respectively (Fig. 2.7). Thus the iodination reaction was repeated and purification was attempted using gel filtration chromatography.
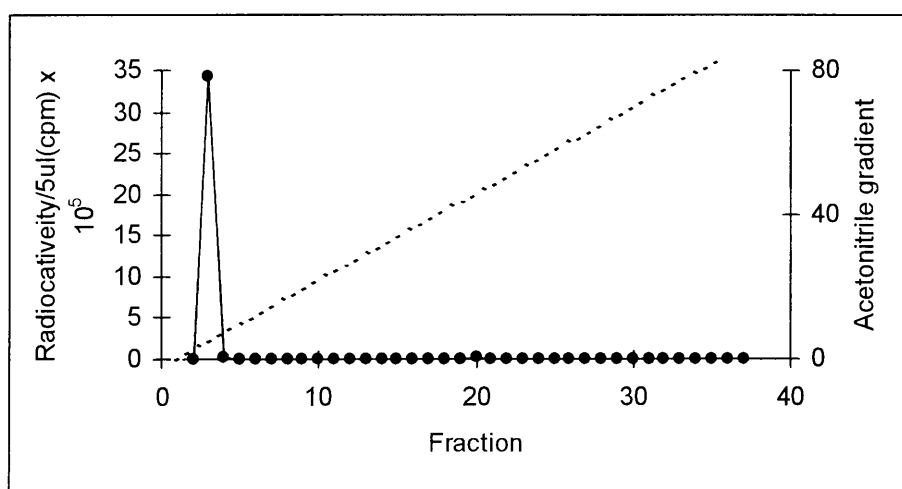
Fig. 2.7. Radioactivity (cpm) counted in 5µl of each fraction following purification using high performance liquid chromatography. - - - - represents the acetonitrile gradient of 0% - 80%.

b) QAE Sephadex

Sephadex G25 (1g) and QAE Sephadex A25 (5g; Parmacia Fine Chemicals, Uppsala, Sweden) was pre-soaked overnight at 4°C, in 100ml and 400ml dH_2O respectively. The column was constructed by pouring 10.5ml QAE Sephadex A25, followed by 2.5ml Sephadex G25, into a 10ml plastic pipette which was then equilibrated for three hours with 0.1M ammonium bicarbonate (pH 9.2). The iodination reaction mixture was placed on the column and eluted with 0.1M ammonium bicarbonate (pH 9.2). Fractions of 1ml were collected in tubes containing 10µl, 10%BSA and 400µl, 0.1M acetic acid, of which 1µl was counted (Fig. 2.8). A broad peak of radioactivity eluted in fractions 10 to 17. Label from fraction 13 (approximately 25000cpm/µl) was tested by receptor binding assay using R11 cells transfected with the human GnRH receptor. Very low binding and high filter blank values were obtained. The label was therefore discarded and the iodination was repeated using Sep-pak purification.
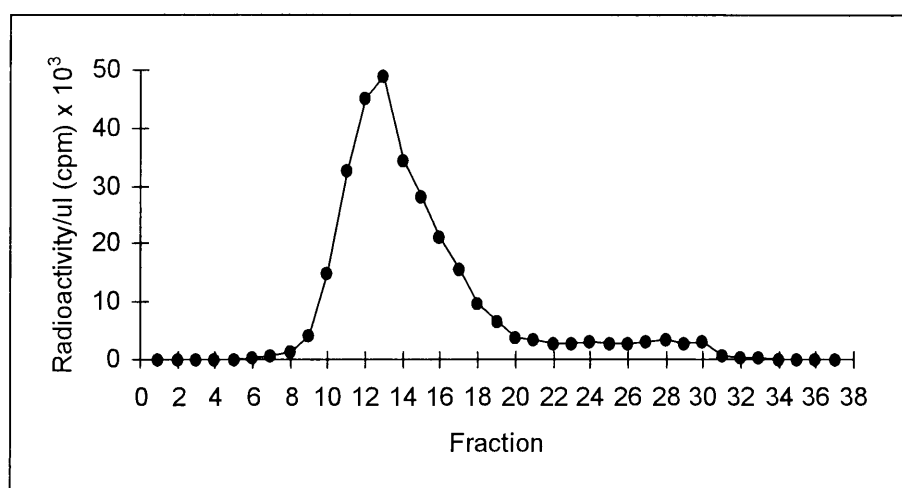
Fig. 2.8. Radioactivity (cpm) counted in 1µl of each fraction collected after Sephadex gel filtration purification of $^{125}I[His^5, D\text{-}Tyr^6]$ GnRH.

C) C-18 Sep-pak cartridge

A Sep-pak C-18 cartridge (Waters Associates) was activated with 2ml methanol followed by 5ml dH_2O and 4ml TF solution. The iodination reaction mixture was applied to the Sep-pak cartridge followed by two, 3ml TF washes. The two washes were performed to elute the free ^{125}I from the cartridge. An acetonitrile gradient

ranging from 20% to 100% acetonitrile in TF solution was applied to the cartridge. At each acetonitrile concentration, four 1ml fractions were collected. The $^{125}I[His^5$, D-Tyr^6] GnRH eluted in fractions 4 to 8, at 20% and 30% acetonitrile (Fig. 2.9). The labelled peptide from fractions 4 to 7 were tested by receptor binding assay using α-T3 cells transfected with the mouse GnRH receptor. Specific binding (total binding-non-specific binding/total counts) of 18.4% (fraction 4), 16.9% (fraction 5), 16.5% (fraction 6) and 9.8% (fraction seven) were obtained.
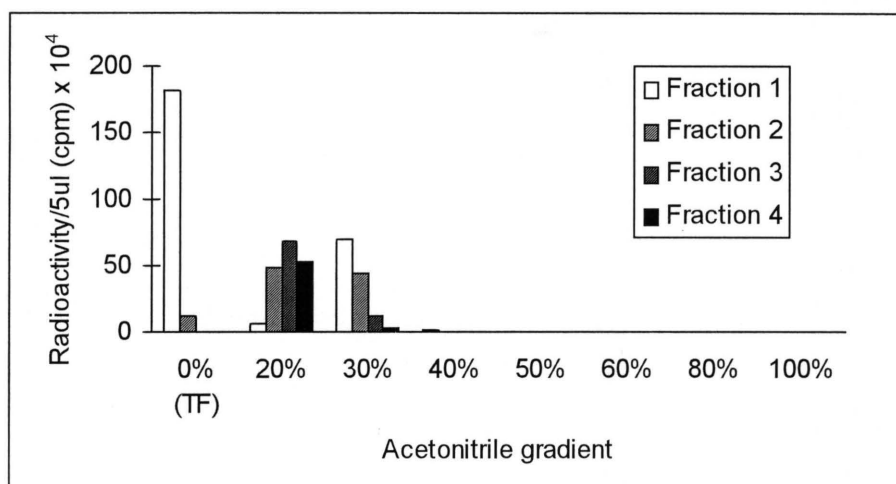
Fig. 2.9. Radioactivity (cpm) obtained by counting 5μl of each 1ml fraction, following Sep-pak separation of $^{125}I[His^5$, D-Tyr^6] GnRH and free ^{125}I over 0-100% acetonitrile/TF gradient.

Optimum pituitary concentration determination

Pituitaries from four non-reproductive males were pooled and used to determine the greatest pituitary dilution at which optimum binding (5-10%) could be obtained. Serial doubling dilutions over the range 0.5 pituitary per tube to 0.0078 pituitary per tube were assayed in triplicate. Binding of 5.1% (total binding-non-specific binding/total counts) at 1/4 pituitary per tube and 3.3% at 1/8 pituitary per tube was obtained (Fig. 2.10). Thus 1/7 pituitary per tube was used in the receptor binding assay in order to obtain estimates of both total and non-specific binding in triplicate.

SpB (%)

7
6
5
4
3
2
1
0

1/2   1/4   1/8   1/16   1/32   1/64   1/128

Pituitary dilution
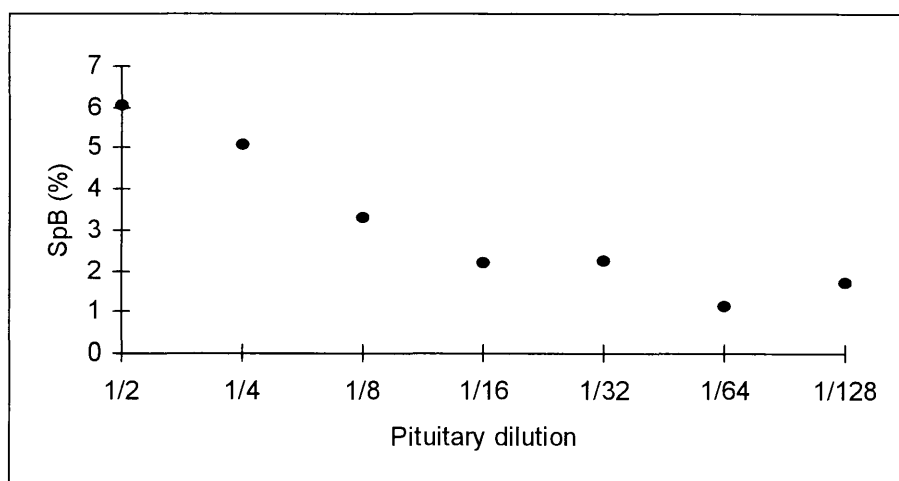
Fig. 2.10. Pituitary membrane dilution curve from 1/2 to 1/128th of a pituitary. SpB = specific binding.

Receptor binding affinity determination

The binding affinity of both mGnRH and [His^5, D-Tyr^6]GnRH for the mole-rat pituitary was investigated by incubating 50µl ^{125}I[His^5, D-Tyr^6]GnRH (100000 cpm/tube) and 400µl pooled membrane (1/6 pituitary equivalent per tube) in the presence of increasing concentrations of unlabelled peptide (50µl over the range 10^{-10} to 10^{-6}M) in 12 × 75mm glass tubes. Non-specific binding was estimated in the presence of 10^{-6}M unlabelled [D-Ala^6, NαMe-Leu^7, desGly-NH$_2$^{10}]GnRH N-ethylamide (Antagonist 26; Department Chemical Pathology, UCT, RSA) and subtracted from estimates of total binding, providing estimates of specific binding. Concentrations of unlabelled peptide were made up at 10× their final concentration. With exception of total counts, assay tubes were adjusted to a final volume of 0.5ml using Hepes binding buffer. All samples were assayed in triplicate. Incubation (4°C, overnight) was terminated by the addition of 3ml, ice-cold, 0.1% PEI buffer. Bound peptide was filtered immediately under vacuum using glass fibre filters (Whatman GF/C), pre-soaked in 0.1% PEI buffer for 5min, followed by two washes with the same buffer. Filters were folded into 5ml plastic tubes and counted for radioactivity on a gamma counter (Packard Riastar 5400).

The IC50 (the concentration of unlabelled competing ligand which inhibits 50% of the binding of the labelled ligand) which provides an indication of binding affinity, of

mGnRH and [His^5, D-Tyr^6]GnRH were 1.48 x 10^{-7}M and 1.17 x 10^{-8}M, respectively (Fig. 2.11). The binding affinities are required for receptor concentration determination in individual pituitaries.
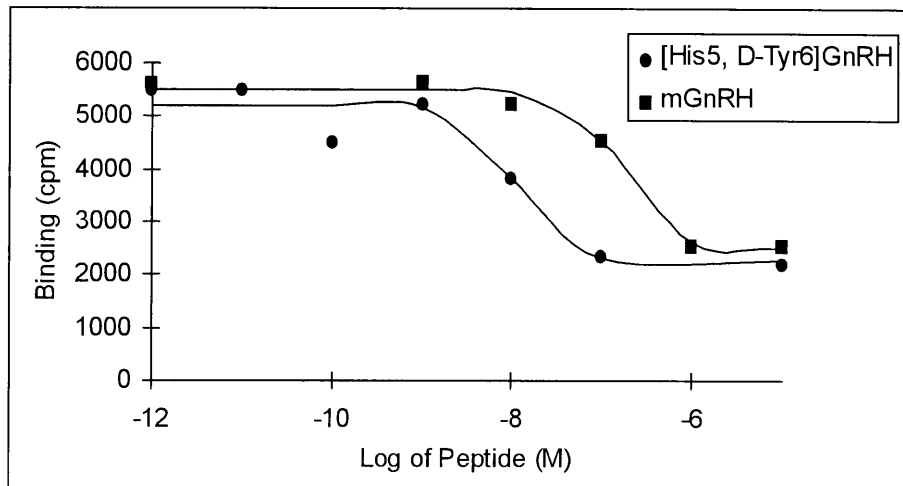
Fig. 2.11. Binding of mole-rat pituitary membrane vs. [His^5, D-Tyr^6]GnRH and mGnRH, using I^{125}[His^5, D-Tyr^6]GnRH as labelled peptide.

Receptor concentration determination

An estimate of total specific binding in individual pituitaries was obtained by incubating pituitary membrane (400µl at 1/7 pituitary per tube) with 50µl ^{125}I[His^5, D-Tyr^6]GnRH, under saturating or near saturating conditions of ligand. This was achieved by using labelled peptide at 200000 cpm/tube. For each pituitary, estimates of total binding and non-specific binding were assayed in triplicate. Estimates of non-specific binding were obtained in the presence of 10^{-6}M [D-Ala^6,NMe-Leu^7,Pro^4,NHet]GnRH (Department Chemical Pathology, UCT, RSA).

Data reduction

The method used in this study to determine receptor concentrations in individual pituitaries requires the binding affinities of the *C. damarensis* pituitary receptor for mGnRH and [His^5, D-Tyr^6]GnRH (Fig. 2.11) and estimates of total binding (Bo) and non-specific binding (NSB). Estimation of receptor concentration in individual pituitaries was attempted using the GnRH receptor binding affinity obtained from pooled non-reproductive male pituitaries and an estimate of total specific binding in

each pituitary. This method is based on the assumption that pituitary binding affinities do not differ due to sex or reproductive status. However, the results of the pituitary GnRH receptor assay (Table 2.1) can not reliably be used to determine the receptor concentrations in individual pituitaries. The estimates of non-specific binding would be expected to remain constant. It is apparent from Table 2.1 that this is not the case, and NSB values fluctuate with estimates of total binding, possibly due to the small amount of tissue used to obtain these estimates. This invalidated the use of this method to determine GnRH receptor concen-trations in individual pituitaries.

Table 2.1. Mean estimate of triplicate measures of total binding (Bo), non-specific binding (NSB) and specific binding (SB), obtained from receptor binding assay of individual pituitaries, using $I^{125}[His^5, D\text{-}Tyr^6]GnRH$ as labelled peptide and $[D\text{-}Ala^6, N \text{ } MeLeu^7, Pro^9\text{-}Net]GnRH$ as unlabelled competitor. RF, reproductive females; NRF, non-reproductive females; RM, reproductive males; NRM, non-reproductive males.

		Mean Bo	Mean NSB	Mean SB
a)	RF1	9132.6	6160.9	2971.7
	RF2	14927.2	8818.1	6109.0
	RF3	14871.2	11179.4	3691.8
	RF4	21466.8	14728.7	6738.1
	RF5	22493.9	17261.8	5232.2
	RF6	16304.9	7239.9	9065.1
	RF7	10574.0	7464.5	3109.6
	RF8	12217.8	8762.1	3455.7
	RF9	11561.8	6930.6	4631.2
b)	NRF1	5896.1	2989.9	2906.3
	NRF2	6553.0	3719.0	2834.1
	NRF3	9183.2	5515.8	3667.5
	NRF4	7744.0	4590.0	3153.9
	NRF5	8310.4	4496.2	3814.3
	NRF6	16026.6	10445.1	5581.6
	NRF7	6027.9	2653.5	3374.4
	NRF8	10619.3	8804.6	1814.7
	NRF9	10018.5	3169.8	6848.7
c)	RM1	5979.9	4867.1	1112.8
	RM2	6453.6	4737.6	1716.0
	RM3	14192.2	12672.5	1519.8
	RM4	6557.3	3702.8	2854.5
	RM5	5905.8	4616.0	1289.8
	RM6	8205.2	6492.6	1712.6
	RM7	4893.0	3152.5	1740.5
	RM8	11311.2	10722.8	588.4
	RM9	8879.1	6845.3	2033.8
d)	NRM1	4086.3	2964.0	1122.4
	NRM2	6397.0	5463.6	933.4
	NRM3	4028.4	3593.9	434.5
	NRM4	6178.8	6023.8	155.0
	NRM5	6027.8	4242.8	1785.0
	NRM6	8217.7	7625.9	591.9
	NRM7	4980.7	4745.4	235.2
	NRM8	5430.7	4820.6	610.0
	NRM9	7713.6	6509.4	1204.2

Cortisol radioimmunoassay

Glucocorticoids are secreted in a nyctohemeral (sleep-wake) cycle such that cortisol levels are typically higher in the morning than in the evening (in diurnal animals). Samples were thus obtained from mole-rats at two different times during the day. Corticosterone and cortisol have similar functions in terms of the stress response, although interspecific differences occur with respect to which is the dominant hormone. In *Spalax ehrenbergi*, a subterranean mole-rat, corticosterone is the dominant hormone produced in response to stress (Ganem & Nevo 1996). Thus, it is necessary to determine which of these glucocorticoids is the dominant stress hormone in *C. damarensis*. Surprisingly, given the general predominance of corticosterone as the stress hormone in rodents (Barlow *et al.* 1975; Bradley *et al.* 1981; Fokkema *et al.* 1988; Ganem & Nevo 1996), radioimmunoassay of *C. damarensis* plasma revealed measurable levels of cortisol but not of corticosterone (Ganem *pers. comm.*).

Plasma cortisol concentrations were determined using a commercial radioimmunoassay kit (coat-a-count, Diagnostic Products, USA). A series of known concentrations of cortisol (50µl) over the range 27.6 - 1380nmol.ml^{-1} were assayed in triplicate in addition to estimates of total and non-specific binding. Plasma samples (50µl) were added to assay tubes in duplicate. Iodinated cortisol (1ml) was added to all assay tubes, giving a total count of 50 000cpm per tube. The assay was vortexed briefly and incubated in a water bath at 37°C for 45min. Bound and free I^{125} cortisol was separated using the coated-tube methodology. Unbound tracer was decanted and tubes were counted for 1min in a gamma counter (Innotron). A curve was fitted to the range of known standards using the statistical model described for the progesterone radioimmunoassay.

Validation

Specificity of the antiserum was determined by the supplier and cross-reactivity was less than 1% with all naturally occurring steroids, with the exception of 11-Deoxycortisol (11.4%), Prednisolone (76%) and Prednisone (2.3%). Hemolysis has no significant effect on the assay. Serial doubling dilutions of mole-rat plasma in gel-PBS buffer produced a curve with a slope that was not significantly different from the standard curve, following logit-log transformation of the data (ANCOVA, $F_{1-6} = 0.08$,

$P > 0.05$; Fig. 2.12). A pool of mole-rat plasma was spiked with known amounts of cortisol at five concentrations. A comparison of observed and expected values yielded a mean recovery of 102% ($\pm$ 7.9%) indicating that the sample matrix did not interfere with cortisol determination. The sensitivity of the assay was 5.5 nmol.l^{-1}. The intra-assay coefficient of variation was 7.35% (n = 20).
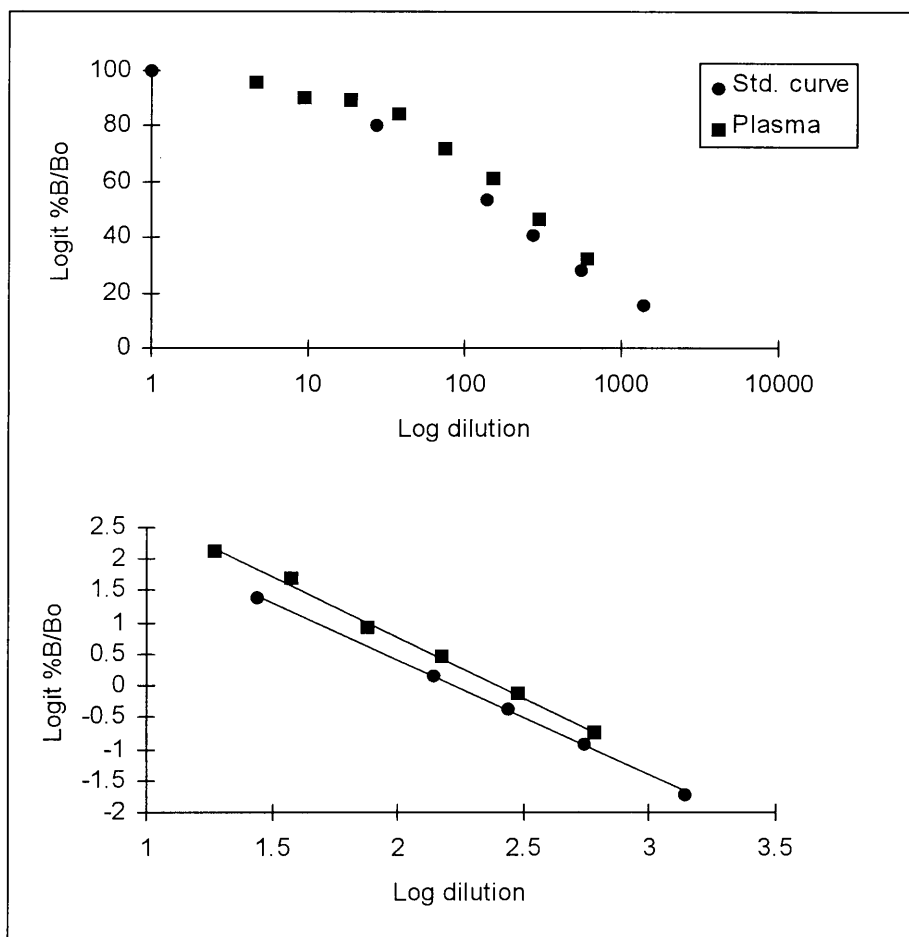
Fig. 2.12. Reference cortisol preparation (●) and serial doubling dilutions of Damaraland mole-rat plasma (■) showing parallelism. **a)** Concentrations of reference cortisol preparation plotted on a logarithmic scale against %B (B/Bo), **b)** Concentrations of reference cortisol preparation plotted on a logarithmic scale against the logit of %B/Bo. Bo, total binding; B, binding.

Histology

Ovarian histology

Two groups of females, namely reproductive females and non-reproductive females were killed by decapitation, since the brains and pituitaries were required for GnRH radioimmunoassay and GnRH receptor binding assay. A further three groups, namely of females housed in the absence of the breeding pair, females housed in the presence of (but physically separated from) unfamiliar males and females paired with unfamiliar males, were sacrificed by intra-muscular injection of an excess dose (50µg) ketamine hydrochloride (Ketalar, Warner-Lambert Research Laboratories, South Africa). Ovaries were immediately removed and placed in Bouins fixative for 14 hours, washed twice in water, once in 70% ethanol and stored in 70% ethanol until sectioning. Prior to sectioning, the ovaries were processed in a Jung Histokinette 2000 processor using standard histological techniques. Serial sections of 5µm were cut through the equatorial region of each ovary. Every seventh section was mounted on a glass slide and counter-stained with haematoxylin and eosin. Ovarian size and the degree of follicular development were examined using a Reichert-Jung Microstar 110 stereo microscope (American Optical, USA). The maximum ovarian length and breadth was recorded using an ocular graticule at 10x magnification or digital callipers. Ovarian volume was calculated using the formula for the volume of an ellipsoid; $V = 4/3\pi ab^2$, where a = ½ maximum length and b = ½ maximum breadth (Woodall & Skinner 1989). The degree of vascularisation of the uterine horns was also noted. The presence of the following ovarian structures, as defined by Wheater *et al.* (1994) and Bennett *et al.* (1994), were noted:

1) *Primordial follicle* - a primary oocyte surrounded by a single layer of follicular cells, containing a large nucleus and little cytoplasm.

2) *Primary follicle* - An enlarged follicle with an enlarged oocyte surrounded by one or more layers of cuboidal follicular cells. In more advanced primary follicles the zona pellucida is present between the oocyte and follicular cells, which have proliferated to form the zona granulosa. The external layer of cells can be differentiated into the theca interna and theca externa.

3) *Secondary follicle* - The zona granulosa proliferates and the follicular antrum,

Materials and methods 33

which is filled with follicular fluid, starts to appear. The theca interna and externa are well developed.

4) *Tertiary/Graafian follicle* - The oocyte stops growing and the follicular antrum is visible as a large, continuous space, around which the zona granulosa forms an even layer. The oocyte is situated within the follicular antrum and is surrounded by a layer of cells, the corona radiata, which is attached to the zona pellucida via a thin bridge of cells.

5) *Corpus luteum* - Following ovulation, the zona pellucida is lost with the ovum and the large, remaining structure forms the corpus luteum, the granulosa cells of which become steroid secreting.

6) *Luteinized unruptured follicles (LUF)* - In contrast to the corpus luteum, LUF form in the absence of ovulation, which can be deduced by the presence of an intact zona pellucida. LUF stain positively for 3β-hydroxysteroid dehydrogenase, the enzyme responsible for the conversion of pregnenolone to progesterone, indicating that these structures contribute to the measurable levels of circulating progesterone observed in non-reproductive females.

GnRH Immunocytochemistry

Perfusion and tissue processing for ICC

Following anaesthesia using halothane (Fluothane, Zeneca, Woodmead, RSA) followed by intra-muscular injection of 0.2ml ketamine (Anaket-V, Premier Pharmaceutical Labs, Bryanston, RSA) and 0.1ml rompun (Bayer, Leyverkusen, Germany), individual mole-rats were perfused through the aorta with approximately 150ml 0.1M phosphate buffered saline (PBS), followed by approximately 300ml 4% paraformaldehyde (Associated Chemicals Enterprises, Glenvista, RSA) in 0.1M PBS. Solutions were filtered immediately prior to use. Heads were removed, immersion fixed in 4% paraformaldehyde for approximately 10-12 hours and stored in 0.1M phosphate buffered saline. Brains were removed from the cranium after one week and placed in 15% and 30% sucrose (BDH Chemical Company, Poole, England). Brains were frozen on dry ice and stored at −70° C until sectioning.

The brains were sectioned on a freezing microtome (Bright Instrument Company, Huntingdon, England) at 30μm on a coronal plane for comparative analysis between the three groups of females and one brain was sectioned on the sagittal plane. Every fifth section from the septal region (at the juncture of the two hemispheres) to the posterior hypothalamus was processed for ICC. In addition one male brain was sectioned at 15μm and all adjacent sections containing the subfornical organ were processed.

Immunocytochemistry

After washing in 1M PBS, free-floating sections were treated with 0.5% triton X-100 (BDH Chemical Company, Poole, England), to increase antibody penetration, and 0.02% H_2O_2 (Sigma Chemical Company, St. Louis, USA), to block endogenous peroxidase activity, for 30min. Sections were placed in 2% normal donkey serum (Sigma Chemical Company, St. Louis, USA) for 30min to minimise non-specific binding of the antibodies. Sections were incubated first in mouse anti-LHRH antibody (1:1000; kindly supplied by Dr. David Silverside) overnight and, after washing in 1M PBS, in biotinylated goat anti-mouse antibody (Vektor Laboratories, Peterborough, England), diluted 1:1000 in standard antibody diluent, for 2hr at room temperature. Standard antibody diluent contained 0.1g $MgCl_2.6H_2O$, 1.02g $NaH_2PO_4.2H_2O$, 6.1g Na_2HPO_4, 2.0g NaCl, 0.5g Sodium azide (BDH Chemical Company, Poole, England) and 5.0ml normal donkey serum (Sigma Chemical Company, St. Louis, USA) in 400ml DH_2O. Following treatment with Avidin Biotin Complex (Vektor Laboratories, Peterborough, England) for 90min, the sections were washed in 0.1M PBS and 0.05M Tris buffer (Trizma 7.6, Sigma Chemical Company, St. Louis, USA) to prevent nickel precipitates. A blue-black reaction product was formed by a 6min incubation with NH_4NiSO_4, activated by H_2O_2. Sections were rinsed in 0.1M PBS and mounted on non-coated slides using elvanol (Sigma Chemical Company, St. Louis, USA), cover-slipped using DPX mountant (BDH Chemical Company, Poole, England) and viewed under a light microscope (Zeiss) at 400 – 1000X magnification.

Analysis and data reduction

Distribution, morphology, size and number (total, preoptic area and mediobasal hypothalamus) of immunoreactive GnRH perikarya were determined and compared between reproductive, non-reproductive and removed non-reproductive females. The number of processes were counted on all cells in the preoptic area and compared between the three groups. An estimate of perikarya size of 20 randomly selected cells in the preoptic area of each individual using image analysis software (NIH Image Analysis). Once an image had been digitised at 400x magnification using a Panasonic video camera (WV-E550) connected to a light microscope (Zeiss, Germany), the program's outline tool was used to determine the cell soma area. For cell somas where it was not readily apparent where the cell body ended and the process began, the boundary was drawn from the point where an imaginary oval would curve round to meet the opposite side of the cell (Robinson *et al.* 1997). Soma area was compared within and between groups of females. The number of neurones in the whole brain were counted, in addition to those in the preoptic area and medial basal hypothalamus.

In situ hybridisation

Brains were sectioned at 30μm on a freezing microtome. All steps were performed under RNAse free conditions. Sections were immediately thaw mounted on poly-L-lysine (Sigma Chemical Company, St. Louis, USA) coated slides and allowed to air-dry for 30min. Slides were washed in 0.1M phosphate buffered saline (PBS) and dehydrated for 10min in 0.025M triethanolamine/0.1M acetic anhydride in 0.9%NaCl (Sigma Chemical Company, St. Louis, USA) followed by 5min in 70%, 95% and 100% EtOH. Chloroform (BDH Chemical Company, Poole, England) was used for delipidation. Following a final dehydration step in 100% EtOH, sections were air-dried for 30min and stored at −70°C until hybridisation. Immediately prior to hybridisation, sections were dehydrated in sequential steps of 70%, 95%, 100% ethanol. Slides were air-dried for 30min. The riboprobe (synthesised by Dr Imre Kallo) was defrosted at 90°C for 5min, quenched on ice and added (30μl) to each section. Slides were cover-slipped using parafilm and incubated at 50°C overnight in a humid chamber, constructed by placing filter paper, moistened with 2X SSC (Sigma Chemical

Company, St. Louis, USA) in a sealed container. Coverslips were removed in 2xSSC with 10mM β-mercaptoethanol (Sigma Chemical Company, St. Louis, USA). During the post hybridisation wash, sections were placed in 4x SSC with 10mM β-mercaptoethanol for 30min, treated with RNase A for 30min at 37°C, washed in 2x SSC for 30min, 1x SSC for 15min, 0.5x SSC for 15min, and 0.1x SSC at 60°C for 30min, followed by two rinses in 2x SSC. Sections were dehydrated in 50%, 70% and 90% ethanol with 0.3M ammonium acetate for 5min and air dried.

Slides were dipped in emulsion (ILFORD K5), allowed to gel on a chilled plate for approximately 10min and dry overnight. Following a six week exposure in a light sealed box at 4°C, sections were developed for 10min using Kodak D19 developer (80g/l; Sigma Chemical Company, St. Louis, USA), washed for 5min in dH_2O and placed in fixative (250g/l $Na_2S_2O_3.5H_2O$; BDH Chemical Company, Poole, England) for 10min. Sections were stained using 0.25% toluedine blue (BDH Chemical Company, Poole, England) in 0.2M PBS and dehydrated in increasing concentrations of ethanol from 50 - 100%. Once the ethanol had been removed by two washes in xylene, sections were mounted using DPX (BDH Chemical Company, Poole, England).

Although a preliminary *in situ* hybridisation study aimed at investigating GnRH mRNA, using perfused *C. damarensis* brains, was unsuccessful, control sections from unperfused rat tissue showed a strong hybridisation signal (Plate 2.1). It is possible that perfusion of the tissue affected the mRNA or that the mRNA degraded during storage. Ronnekleiv *et al.* (1989) found that mRNA hybridisation signal started to decrease in tissue stored for longer than 3-4 weeks.

Following page:
Plate 2.1. *In situ* hybridisation signal in control rat sections. **a)** 25x magnification, scale bar = 500μm and **b)** at 400x magnification, scale bar = 50μm. AC, anterior commisure; OC, optic chiasm. Plate **(b)** corresponds to the area highlighted by the rectangle in Plate **(a)**.
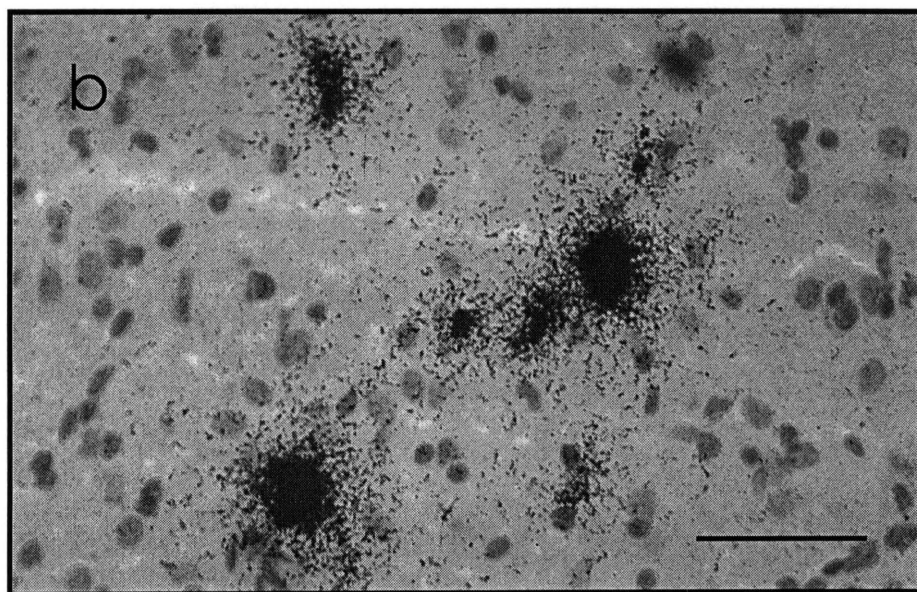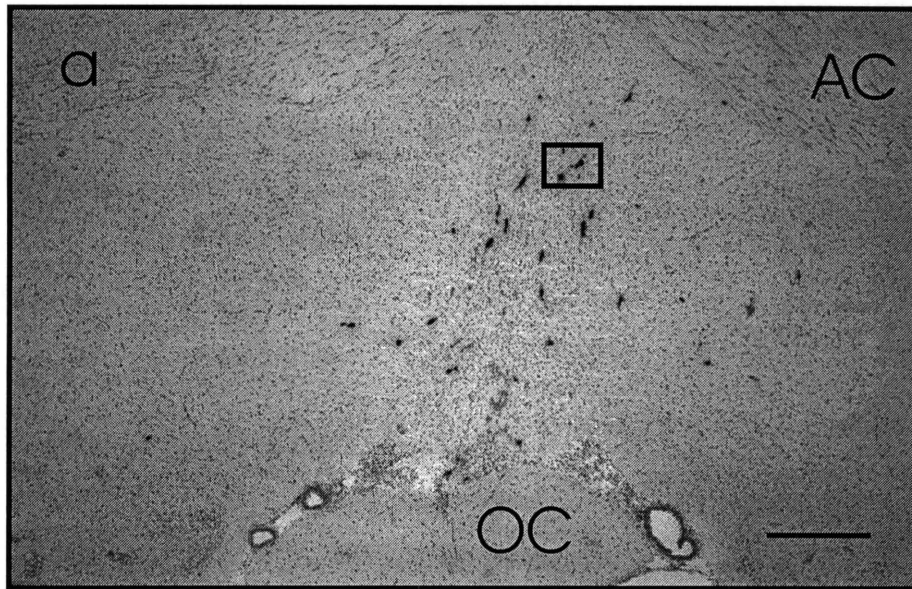
Plate 2.1.

Chapter 3

The effect of the social environment on female reproductive function

Abstract

The proximate, social cues responsible for anovulation in subordinate females were investigated by examining the effect of the social environment on endocrine and histological parameters in female Damaraland mole-rats. Luteinizing hormone secretion in response to an exogenous gonadotrophin-releasing hormone challenge (0.5µg, 1.0µg and 2.0µg GnRH) in non-reproductive females housed in the absence of the breeding pair (rNRF) was significantly higher than in non-reproductive females in the colony (NRF), but was significantly lower than in reproductive females (RF). Corpora lutea were present in the ovaries of rNRF, but not in NRF. Circulating levels of progesterone were significantly higher in rNRF compared to NRF. This indicates that ovulation occurs spontaneously in females housed in the absence of the breeding pair as a result of increased pituitary sensitivity. However, urinary progesterone profiles obtained from rNRF failed to provide evidence for ovarian cyclicity. Breeding females exhibited higher plasma progesterone concentrations and greater ovarian volume compared to rNRF. This may either simply be a physiological consequence of pregnancy or possibly indicates that the corpora lutea in rNRF are non-functional. It can be concluded that female Damaraland mole-rats are spontaneous ovulators and that anovulation is due to inhibitory social cues within the colony environment, not to a lack of copulatory stimulation. Therefore, subordinate females are infertile and inhibition of the hypothalamo-pituitary-gonadal axis could potentially play a causal role in maintaining reproductive skew in colonies of *C. damarensis*.

Introduction

Colonies of the Damaraland mole-rat (*Cryptomys damarensis*) exhibit an extreme reproductive skew characterised by a single breeding female in colonies typically containing between 2 and 14 individuals, although as many as 41 have been recorded (Jarvis & Bennett 1993). The contribution of a physiological mechanism to the maintenance of the reproductive skew is the subject of debate and has yet to be fully resolved (Bennett 1994; Bennett *et al.* 1994; Burda 1995; Bennett *et al.* 1996). In many cooperatively breeding species, inhibitory social cues cause subordinate females to remain in an anovulatory state. Subordinate females in the group exhibit a socially-induced infertility since removal of the dominant, breeding female results in the

initiation of ovarian cyclicity and ovulation e.g. in the Callitrichid monkeys (Epple & Katz 1984; Abbott 1988; Abbott *et al.* 1988; Savage *et al.* 1988) and naked mole-rat (*Heterocephalus glaber*, Faulkes *et al.* 1990). In *C. damarensis*, the finding that the reproductive systems of non-reproductive females (NRF) and reproductive females (RF) exhibit marked physiological and endocrine differences has been interpreted as evidence that NRF are socially suppressed by the RF (Bennett 1994; Bennett *et al.* 1994) and that anovulation indicates a state of socially-induced infertility. However, the authors failed to show NRF in the colony are in fact infertile and did not demonstrate the existence of inhibitory, social cue/s that could affect the reproductive system of subordinate females.

Burda (1995) challenged this interpretation and suggested that NRF are not infertile and anovulation is not responsible for maintaining reproductive skew. Working on the less social common mole-rat from Zambia, *C. hottentotus*, Burda (1995) proposed that subordinate females of the genus *Cryptomys* are in fact not socially suppressed, but that their pseudopregnant-like state can be interrupted by frequent, multiple matings with an unrelated male, leading to ovulation. He proposed that the reproductive skew is maintained solely through a behavioural incest avoidance. However, Burda (1995) paired the females, which subsequently conceived, with an unfamiliar male outside the confines of the colony. This may have resulted in a relaxation of a physiological inhibition operating within the social environment of the colony. Furthermore, species differences exist with respect to reproductive physiology within the genus *Cryptomys*. For example, in contrast to *C. damarensis*, non-breeding Mashona mole-rats (*C. darlingi*) are not anovulatory (Bennett *et al.* 1997), making generalisations within the genus inappropriate.

Consequently, Bennett *et al.* (1996) investigated the effect of the removal of the RF on the reproductive endocrinology of NRF and found that luteinizing hormone (LH) secretion in response to a gonadotrophin-releasing hormone (GnRH) challenge increased, whereas progesterone values decreased, significantly in NRF in the absence of the RF. Although this suggests that the social environment affects the reproductive axis of NRF, the study did not determine whether ovulation had in fact occurred in females housed in the absence of the RF. LH levels remained significantly lower than

those of the RF, suggesting that the presence of an unrelated male may be necessary. It is possible that elements of both hypotheses are required to explain the reproductive state observed in NRF. Thus one could envisage a system wherein a dual mechanism comprising inhibition (due to the presence of breeding individuals) and a lack of stimulation (due to the absence of unrelated individuals) could result in the observed state of reproductive quiescence in NRF.

The aim of this chapter was, therefore, to investigate the effect of the social environment on the reproductive axis of *C. damarensis* females to determine whether the endocrine and histological differences between RF and NRF could be explained by reproductive inhibition due to the presence of the breeding pair, as opposed to lack of stimulation by unfamiliar conspecifics.

Materials and methods

Study animals

Single GnRH challenges

Five groups of mole-rats, in which individuals were grouped according to sex, reproductive status and social environment, were used to investigate plasma LH concentrations measured prior to and 20min after a single challenge of 2.0μg, 1.0μg and 0.5μg of exogenous GnRH. RF (n = 12) were obtained from seven wild caught colonies and from five colonies generated in the laboratory by pairing females with unrelated males. NRF (n = 29) from seven colonies were maintained in the presence of an actively breeding female. A third group of non-reproductive females were housed in the absence of the breeding pair (rNRF; n = 13). They were obtained from three colonies, in one of which the breeding pair had been removed for at least three years. The two remaining colonies were captured without the RF and were maintained in the laboratory for at least six and 12 months respectively. Since the reproductive male (RM) is typically the heaviest individual in the colony (Jacobs *et al.* 1991) large males were removed, whereas smaller male siblings were left in the colony. These colonies were reproductively quiescent for the duration of the study. RM (n = 10) were obtained from eight colonies formed in the lab and from two wild caught colonies. In the two wild caught colonies, there was a clear distinction between a single large male

(> 170g) and a few smaller male offspring. Non-breeding males (NRM; n = 10) were obtained from four wild caught colonies.

Ovarian histology

Ovaries from five groups of females housed in different social environments were examined. RF (n = 8) were obtained either by pairing females with unfamiliar males in the lab (n = 6), in which case they were used only once they had given birth to at least one litter, or were identified as RF in wild caught colonies (n = 2). RF were killed irrespective of the stage of the oestrus cycle or pregnancy. NRF (n = 10) were obtained from three colonies of wild caught individuals (n = 9) and were killed approximately one week after capture. One female originated from a colony that had been generated in the laboratory by pairing unrelated individuals. rNRF (n = 6) were obtained from two colonies, both of which were captured without the RF and maintained in the laboratory for at least 10 and 18 months respectively. Both colonies contained male siblings. In addition, two NRF were removed from their colony and housed alone for approximately six months. Of the final two groups, the first comprised non-breeding females that were housed singly in the presence of, but physically separated by wire mesh, from an unfamiliar male (p/UM; n = 6). The second group was comprised of non-breeding females paired singly with an unfamiliar reproductive male (pUM; n = 6). The females in both groups were sacrificed after 30 days. In addition to removing the ovaries, blood samples were obtained by cardiac puncture from females in all five groups for plasma progesterone determination.

Urinary progesterone profiles

Urine samples were collected daily for progesterone determination from four non-breeding females. Urine samples were obtained from two females for nine days while they were housed in their colony and for 44 days subsequent to removal from the colony. On removal from the colony one female was housed alone and the other was housed in the presence of, but physically separated by wire mesh, from an unrelated male. In addition, urine samples were collected over 46 and 57 days, respectively, from two rNRF which had been housed in the absence of the breeding female for more than three years.

Results

Plasma LH concentrations

Mean plasma LH concentrations in the three groups of females (RF, NRF and rNRF) and two groups of males (RM and NRM) increased following the administration of 0.5, 1.0 and 2.0μg exogenous GnRH (Fig. 3.1). The difference between basal (pre-GnRH) and GnRH-stimulated LH levels was significant at all GnRH doses in all five groups (Table 3.1). There was no significant difference between plasma LH concentrations before and after a control injection of physiological saline in any of the five groups (Table 3.2).

The relationship between mean GnRH-stimulated LH concentrations in the three groups of females was similar at each concentration of GnRH (0.5, 1.0 and 2.0μg GnRH; Fig. 3.1). Statistical values are provided in Tables 3.3a-c. Plasma LH concentrations following a GnRH challenge were significantly higher in RF compared to NRF, at all three doses of GnRH (Table 3.4). Similarly, mean plasma LH concentrations in rNRF were significantly higher than those in NRF, but significantly lower than those in RF. Although mean plasma LH concentrations following a GnRH challenge were consistently higher in NRM compared to RM, the differences were not significant (Table 3.5).

Within each group, the magnitude of the LH response to GnRH administration was not significantly different between the three doses of GnRH, with one exception. In NRM, the LH response to a 0.5μg GnRH challenge was significantly higher than the response to a 1.0μg challenge (Table 3.6).
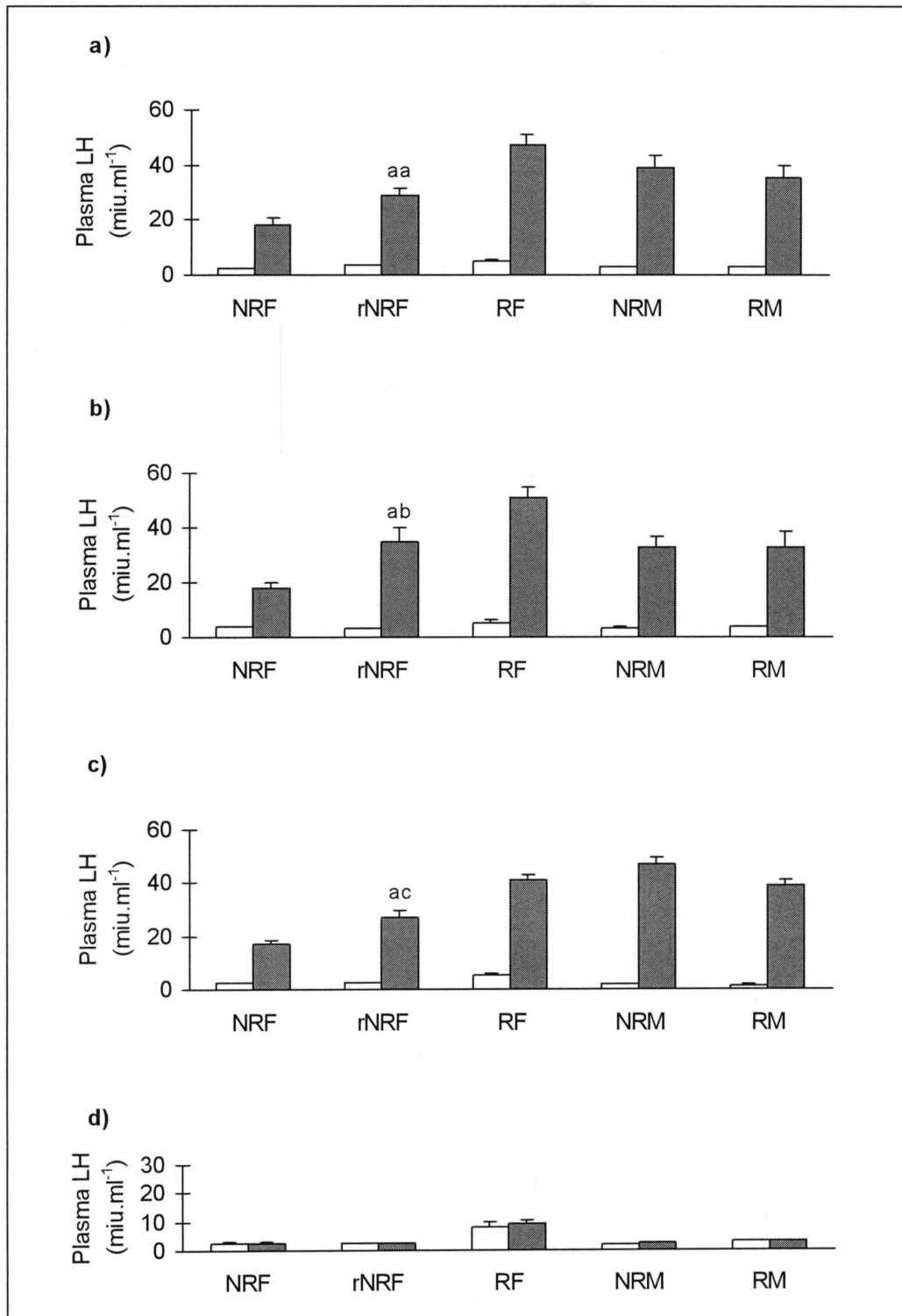
Fig. 3.1. Bioactive plasma LH concentrations (mean ± sem) immediately before (clear bars) and 20min after (shaded bars) a single challenge (s.c.) of **a)** 0.5μg, **b)** 1.0μg and **c)** 2.0μg GnRH and **d)** a saline control. NRF, non-reproductive females; rNRF, non-reproductive females housed in the absence of the breeding pair; RF, reproductive females; NRM, non-reproductive males; RM reproductive males. $P < 0.001$ for all **(a-c)** post-GnRH values *vs.* pre-GnRH values. $P > 0.05$ for all post-saline **(d)** values *vs.* pre-saline values. aa: $P < 0.01$ *vs.* non-breeding females; $P < 0.001$ *vs.* breeding females; 2.0μg GnRH. ab: $P < 0.01$ *vs.* non-breeding females; $P < 0.05$ *vs.* breeding females; 1.0μg GnRH. ac: $P < 0.01$ *vs.* non-breeding females; $P < 0.001$ *vs.* breeding females; 0.5μg GnRH (Mann-Whitney U test).

Ovarian structure

Significant differences in ovarian size were observed between the five groups of females (Kruskal Wallis; H_4 = 16.9, P < 0.05). The ovaries of RF could be clearly distinguished from those of the NRF housed in the colony, both macro- and microscopically (Plate 3.1a & 3.2b). The ovaries of RF had a significantly greater mean volume than those of the NRF (Mann-Whitney U test; U = 14, P < 0.05, N_1 = 8, N_2 = 10; Fig. 3.2). Large corpora lutea (Plate 3.2), which filled the entire stroma in seven out of the eight RF (a corpus albicans was observed in the eighth RF) resulted in the macroscopic granular appearance of the ovary. Few luteinized unruptured follicles (LUF) were observed. The mean ovarian volumes of the three pregnant females were among the four largest of the eight RF.
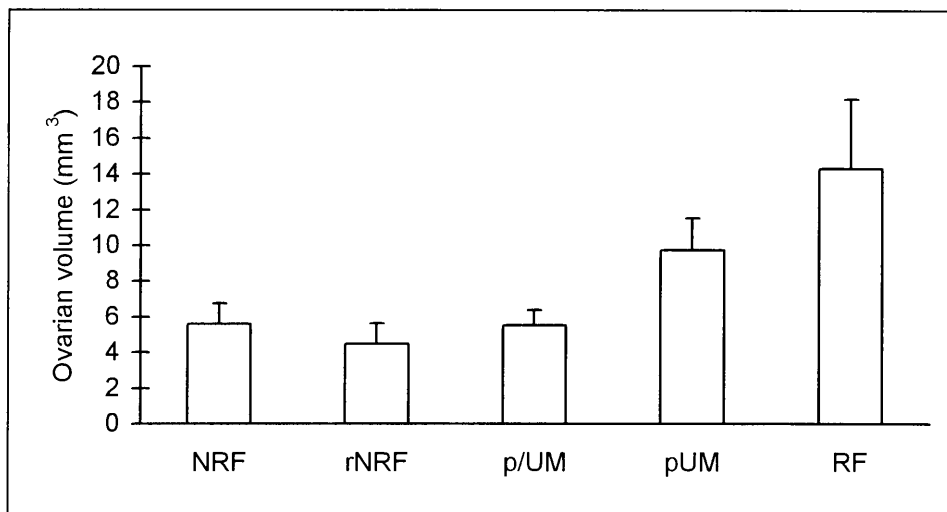
Fig. 3.2. Ovarian volume (mean ± sem) in five groups of females. RF, reproductive females; NRF, non-reproductive females; rNRF, non-reproductive females housed in the absence of the breeding animals; p/UM, non-reproductive females housed singly in the presence of, but physically separated from an unrelated male, for 30 days; pUM, non-reproductive females housed with an unrelated male for 30 days. See text for statistical comparisons.

Primordial, primary, secondary and Graafian follicles were observed in the ovaries of all NRF (Plate 3.1a). The stroma was packed with LUF resulting in a "paved" appearance. The ovaries of two of the 10 NRF, which originated from the same colony, each contained one corpus luteum (Plate 3.1b). The two females originated from the same colony. Despite the fact that corpora lutea were found in seven of the

eight rNRF (Plate 3.2b), mean ovarian volume was not significantly greater than that of NRF (Mann-Whitney U test; $U = 32$, $P > 0.05$, $N_I = 10$, $N_2 = 8$; Fig. 3.2). LUF were observed in the stroma not occupied by CL. The corpora lutea appeared to be smaller than those of RF (compare Plate 3.2a & b), possibly accounting for the difference in ovarian size between the two goups ($U = 6$, $P < 0.01$, $N_I = 8$, $N_2 = 8$). None of the females were pregnant and no placental scars were observed in the uterus.

The ovaries of both p/UM (four of the six females) and pUM (four of the six females) contained CL, in addition to a full range of developing follicles. Mean ovarian volume of p/UM was not significantly different from either NRF (Mann-Whitney U test; $U = 30$, $P > 0.05$, $N_I = 6$, $N_2 = 10$) or rNRF ($U = 15$, $P > 0.05$, $N_I = 6$, $N_2 = 8$), but did differ significantly from both groups in which females were paired with males, pUM ($U = 4$, $P < 0.05$, $N_I = 6$, $N_2 = 6$) and RF ($U = 6$, $P < 0.05$, $N_I = 6$, $N_2 = 8$). As expected, ovarian volume of pUM did not differ significantly from that of RF ($U = 17$, $P > 0.05$, $N_I = 6$, $N_2 = 8$), but was significantly greater that rNRF ($U = 6$, $P < 0.05$, $N_I = 6$, $N_2 = 8$). As in RF, larger ovaries were associated with pregnancy.

Following pages:
Plate 3.1. Ovarian structure of **a)** a typical non-reproductive female and **b)** a non-reproductive female with a single corpus luteum (CL). Magnification = 40x.

Plate 3.2. Ovarian structure of a typical **a)** reproductive female and **b)** non-reproductive female housed in the absence of the breeding pair. CL, Corpus luteum. Magnification = 40x.
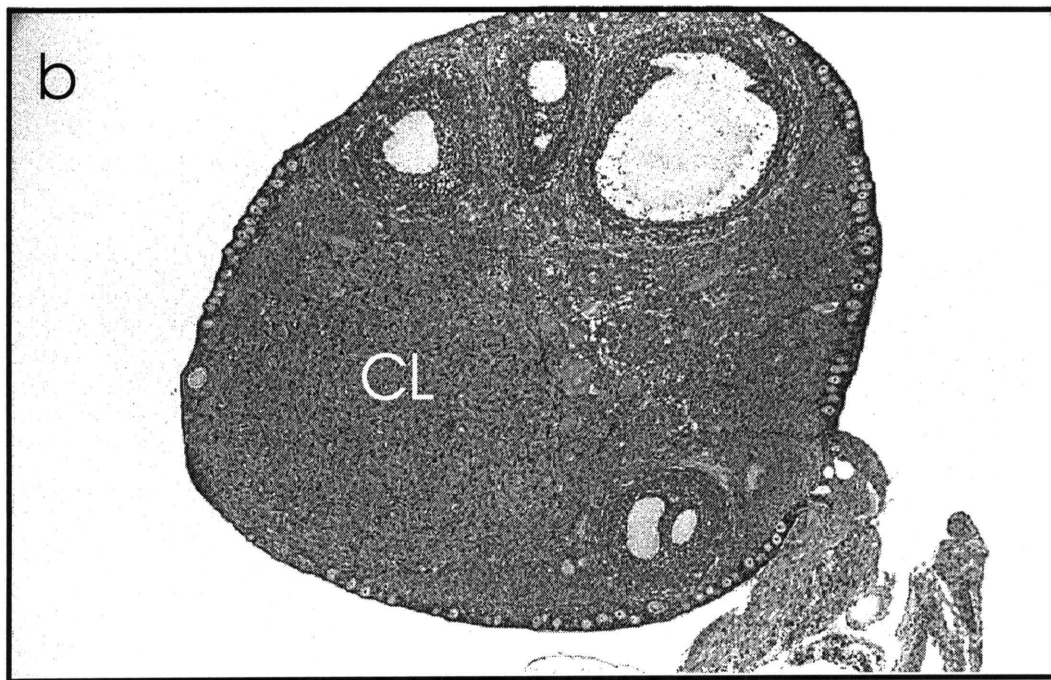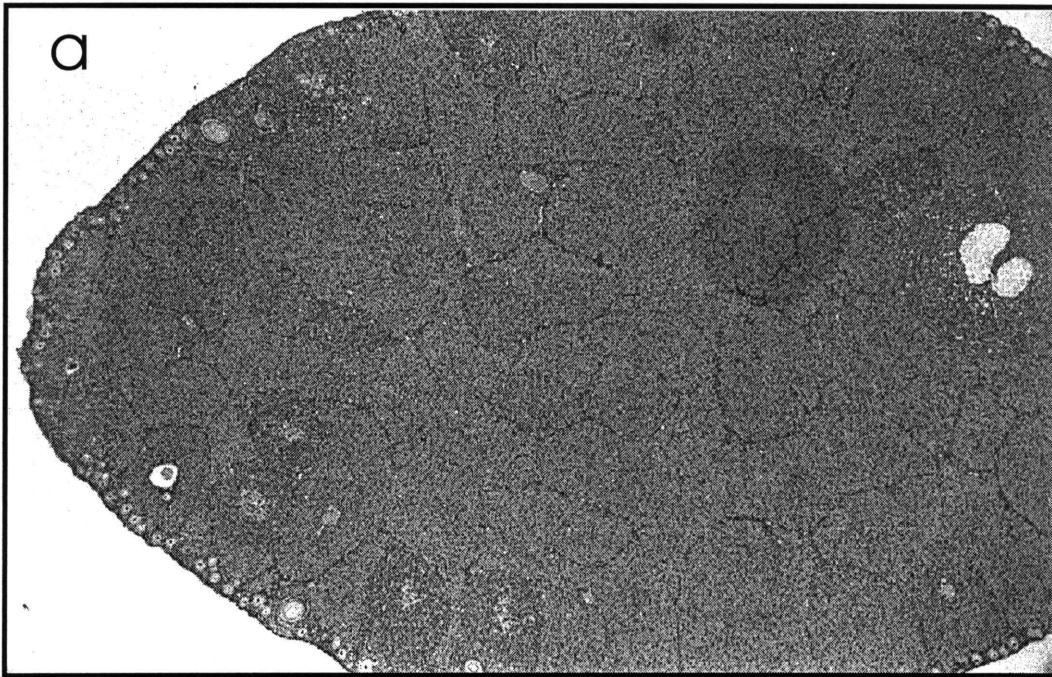
Plate 3.1
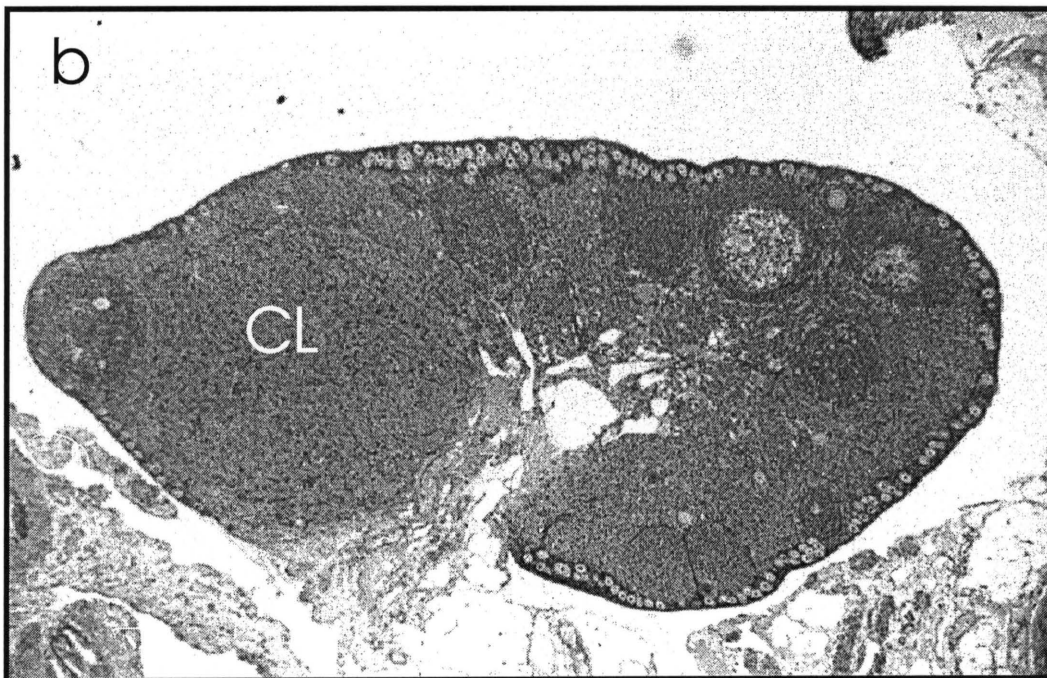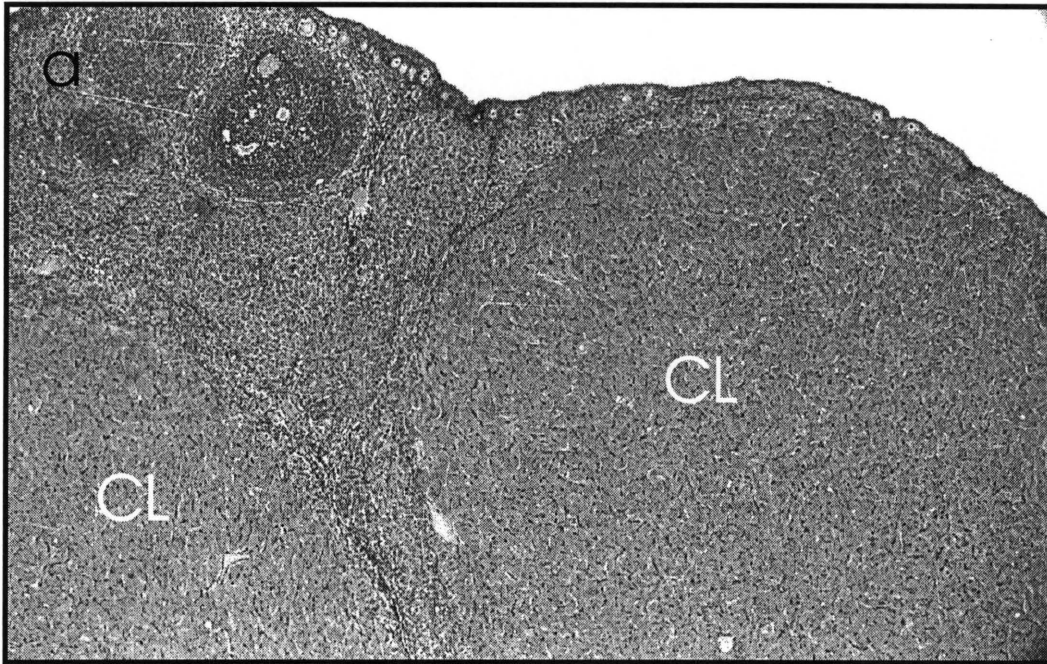
Plate 3.2
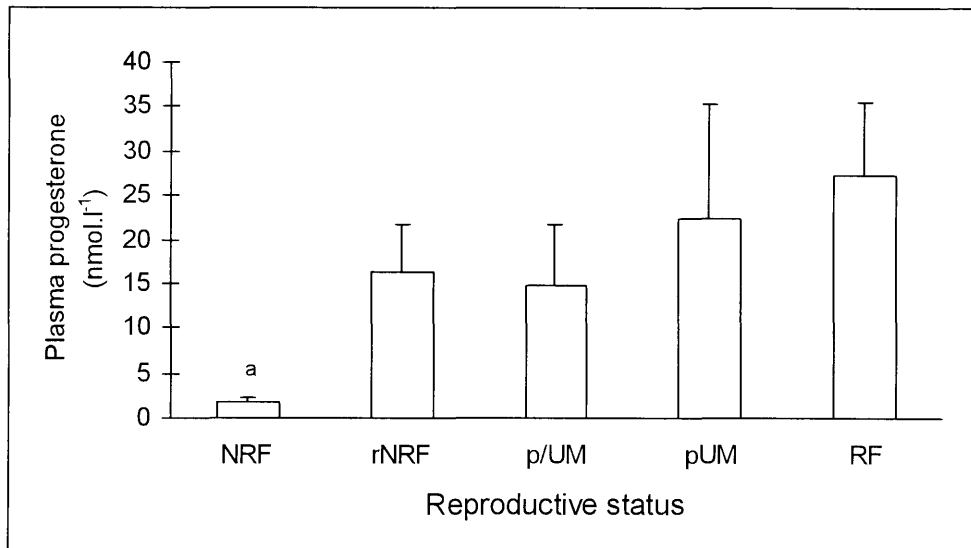
Fig. 3.3. Mean (± sem) plasma progesterone concentrations for single samples obtained from five groups of females. RF, reproductive females; NRF, non-reproductive females; rNRF, non-reproductive females housed in the absence of the breeding animals; p/UM, non-reproductive females housed singly in the presence of, but physically separated from an unrelated male, for 30 days; pUM, non-reproductive females housed with an unfamiliar male for 30 days. a: $P < 0.05$ vs. rNRF; $P < 0.05$ vs. p/UM; $P < 0.05$ vs. RF (Mann-Whitney U test following Kruskal Wallis analysis).

Plasma progesterone concentrations

Mean plasma progesterone concentrations for the five groups of females are presented in Fig. 3.3. Kruskal Wallis analysis ($H_2 = 16.4$, $P < 0.05$) revealed significant differences in circulating progesterone concentrations between the five groups of females. Circulating progesterone concentrations in NRF were significantly lower than in rNRF, p/UM and RF, but not in pUM (Table 3.8). Significant differences were not observed between NRF and pUM due to high variation in latter values. Progesterone concentrations from the three pregnant RF and two pregnant pUM were above the upper detection limit of the assay and were assigned values of 63 nmol.l^{-1}, corresponding to approximately 15% binding on the assay standard curve. Insufficient volumes prohibited the re-assay of these samples at an appropriate dilution. In contrast to NRF in which progesterone concentrations did not exceed 5.0nmol.l^{-1}, only three out of 10 RF exhibited progesterone concentrations below 10.0nmol.l^{-1}.

Urinary progesterone profiles

Urinary progesterone profiles were obtained from two NRF that were removed from the colony after 10 days of sampling. One female was subsequently housed alone and

the second in the presence of an unrelated male. Sampling continued for approximately 44 days (Fig. 3.4a & b). In neither female was there any evidence of cyclicity in progesterone concentrations. Progesterone concentrations ranged from below the sensitivity of the assay (1.2nmol.mmol^{-1} Cr) to an upper limit of 9.1nmol.mmol^{-1} Cr in the female housed alone (Fig. 3.4a) and from 2.4 to 10.5nmol.mmol^{-1} Cr in the female housed in the presence on the unrelated male (Fig. 3.4b). In two rNRF that had been housed alone for more than three years, progesterone concentrations fluctuated from 4.1nmol.l^{-1} - 36.5 nmol.mmol^{-1} Cr and 2.7 - 26.7nmol.mmol^{-1} Cr, respectively (Fig. 3.5a & b).
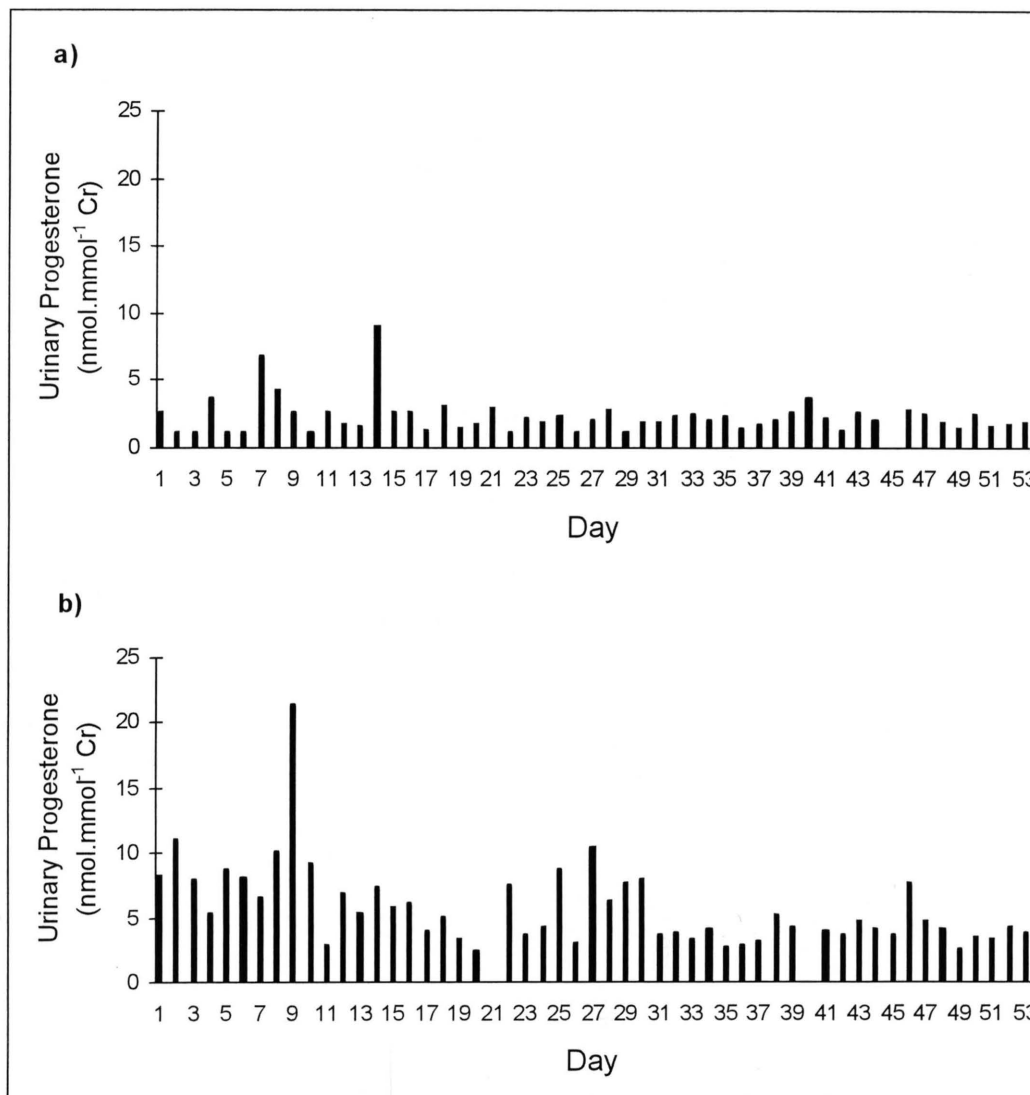
Fig. 3.4. Urinary progesterone concentrations collected daily from **a)** a non-reproductive female removed from the colony and housed alone 10 days after sampling commenced and **b)** a non-reproductive female removed from the colony and housed in the presence of, but physically separated (by wire mesh) from an unfamiliar male 10 days after sampling commenced.
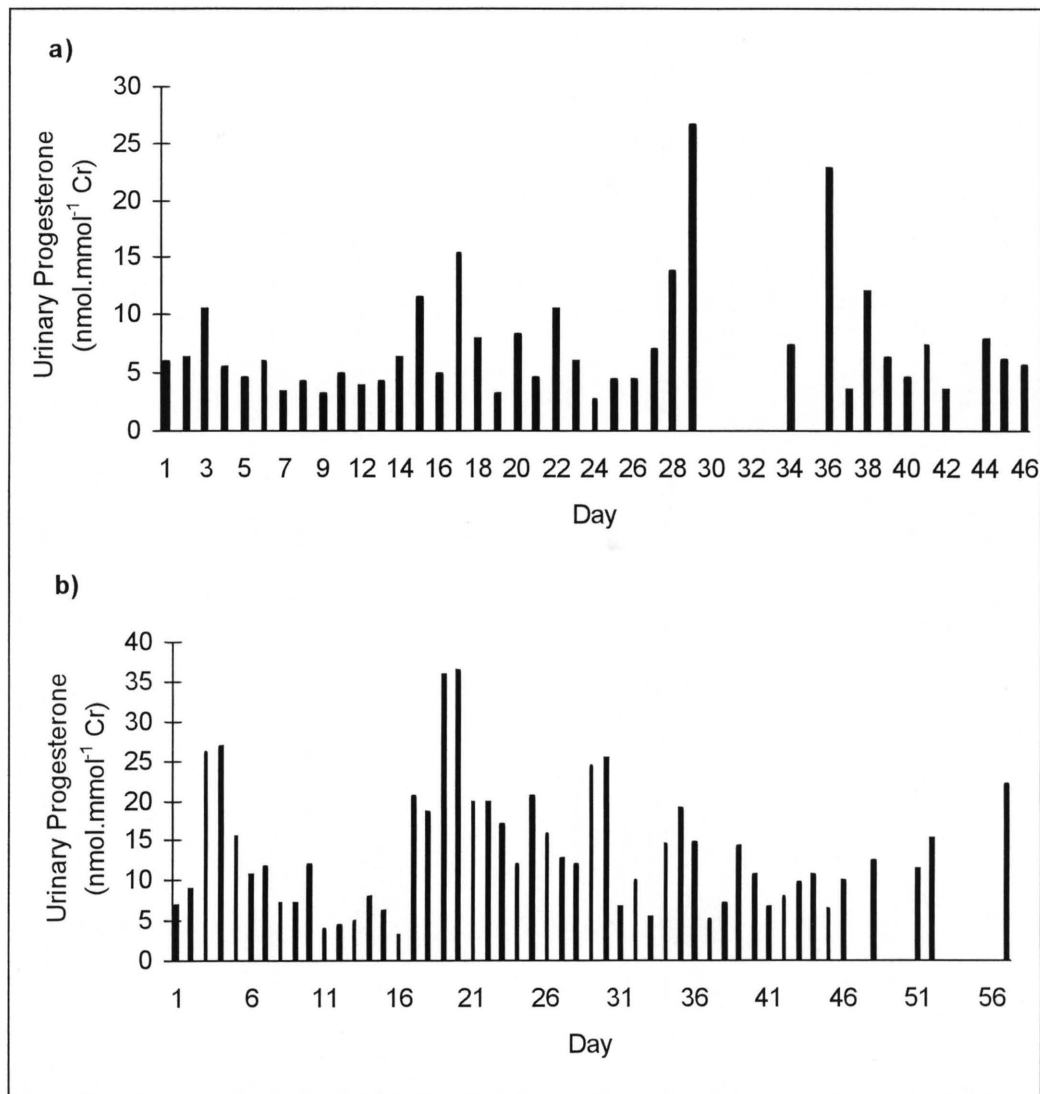
Fig. 3.5. Urinary progesterone concentrations collected daily from two non-reproductive females that had been housed in the absence of the breeding female for more than three years.

Discussion

Anovulation: Infertility or lack of copulatory stimulation?

Anovulation in non-breeding female Damaraland mole-rats is associated with decreased pituitary sensitivity (Bennett *et al.* 1993; Bennett *et al.* 1994). Pituitary sensitivity in NRF is affected by the social environment since GnRH-stimulated LH secretion was found to be significantly greater in rNRF than NRF (Fig. 3.1). In contrast to the dose-dependent effect of GnRH on LH secretion in RF and NRF naked mole-rats (Faulkes *et al.* 1990), this effect was observed irrespective of the dose of GnRH administered. Thus, pituitary sensitivity is affected by inhibitory social cues

resulting from the presence of one or both of the breeders. However, this does not, in itself, indicate that ovulation occurs in rNRF. Pituitary sensitivity in rNRF was not equivalent to that of RF (Fig. 3.1) and it is possible that a higher degree of pituitary sensitivity (such as that observed in RF) is required to produce sufficient LH to initiate ovulation. Despite the increase in pituitary sensitivity in rNRF compared to NRF, ovulation may nevertheless need to be induced, possibly through copulation with an unrelated male. Circulating progesterone concentrations in these females suggest, however, that this is not the case. In contrast to a previous study in which progesterone concentrations decreased significantly in NRF when the breeding female was removed from the colony (Bennett *et al.* 1996), progesterone concentrations were found to be significantly lower in NRF than in rNRF (Fig. 3.3). The primary difference between the two studies was that plasma progesterone was measured in the present study whereas urinary progesterone was measured in the previous study. The difference may, therefore, be due to the physiology of progesterone excretion in *C. damarensis*, but this requires further investigation. The most likely origin of the elevated progesterone concentrations observed in this study is the formation of corpora lutea, suggesting that ovulation occurs in rNRF. In cooperatively breeding naked mole-rats, strong evidence that ovulation is initiated in NRF on removal of the RF was obtained through cyclical progesterone secretion, which remarkably, may occur in as little as 7 days (Faulkes *et al.* 1990). The Bathyergidae have strong hystricomorph affinities (Jarvis & Bennett 1991) and in a review of their reproductive characteristics, Weir (1974) found most species to be spontaneous ovulators.

Ovarian cyclicity could not be confirmed through progesterone profiles in the Damaraland mole-rat (Fig. 3.4 & 3.5), suggesting that urinary progesterone is not a suitable metabolite to use in this species. However, histological examination of ovarian structure confirms that non-breeding females ovulate spontaneously in the absence of the breeding individuals. As found in a previous study (Bennett *et al.* 1994), although follicular development occurs, the absence of corpora lutea in eight of the ten females examined indicates that ovulation does not typically occur in NRF. Since LH is required for both follicular development and ovulation (Johnson & Everitt 1995), the anterior pituitary of NRF appears to be capable of releasing sufficient LH necessary for

follicular development but not for the LH surge that is required for ovulation. This is supported by the presence of high densities of LUF in the ovaries of *C. damarensis* females, since deficient LH secretion is thought to be a primary factor leading to the formation of LUF in women (Hamilton *et al.* 1985; Schenken *et al.* 1986) rats (Mattheij & Swarts 1995) and rabbits (Bomsel-Helmreich *et al.* 1989). In contrast to NRF, corpora lutea were observed in the ovaries of seven of the rNRF examined. Therefore, despite the apparently lower sensitivity of the pituitary of rNRF when compared to RF (Fig. 3.1), the pituitary is nevertheless capable of producing sufficient LH to stimulate ovulation. The fact that rNRF were not paired with unfamiliar males suggests that anovulation is not merely due to a lack of copulatory stimulation but, rather, results from inhibitory social cues and, therefore, reflects a socially-induced infertility. This refutes Burda's (1995) hypothesis that an absence of copulation can explain the anovulation observed in NRF. Since subordinate females are infertile while they remain in the colony, anovulation has the potential to play a causal role in maintaining reproductive skew.

The single CL was found in two NRF and was unexpected. However, ovulation in non-breeding females of other cooperatively breeding species that exhibit an impairment of fertility, is not unknown. Abbott (1984) observed that one daughter ovulated in at least 50% of common marmoset family groups. Urinary oestrogen and plasma progesterone peaks have also been found in other NRF Callitrichids (Tardif 1984; French & Stribley 1987). In the present study, comparison of the GnRH-stimulated LH levels in the two NRF with other NRF, revealed that they were among the highest in the group and resembled LH levels found in rNRF. If one includes the results obtained by Bennett *et al.* (1994), in which 12 NRF from two colonies were examined, only two out of 22 NRF Damaraland mole-rats exhibited evidence of ovulation. This suggests that although the CL observed in two NRF, ovulation does not seem to be a typical occurrence in NRF but may rather be due to specific factors within that colony. In this regard, it may be significant that the colony, from which both females originated, was wild-caught and its social composition unknown. In contrast to the follicular development observed in the ovaries of NRF Damaraland mole-rats, the ovaries of NRF naked mole-rats are akin to prepubescent rodent ovaries

with very little follicular development occurs in the ovaries of NRF (Kayanja & Jarvis 1971). Since naked mole-rats typically inbreed, a more stringent inhibition of ovarian function may be required to maintain reproductive skew in the presence of a large number of potential mates within the colony. In contrast, Damaraland mole-rats exhibit a strong inhibition to incest, which prevents breeding between familiar colony members (Bennett *et al.* 1996; this study).

Since ovulation occurs spontaneously in rNRF, what is responsible for the difference in pituitary sensitivity between rNRF and RF (Fig. 3.1)? The lack of clear evidence of ovarian cyclicity in rNRF (Fig. 3.4 & 3.5) may be due to the formation, following ovulation, of corpora lutea that are not fully functional. The laboratory rat, mouse and hamster require the act of coitus to produce a fully functional corpus luteum (see Short 1984). The smaller size of the ovaries and corpora lutea in rNRF (compare Plate 3.2a & b) when compared to RF may be significant in this regard. Alternatively, the greater pituitary sensitivity in RF compared to rNRF may merely be a consequence of actively breeding. An increase in corpus luteum size and steroid output is associated with pregnancy in the rat (Heap & Flint 1984). In the group of RF, three of the four largest ovaries originated from the three pregnant females. The greater LH response to a GnRH challenge, larger size of the ovaries and greater circulating progesterone concentrations in breeding *C. damarensis* females may merely be an effect of pregnancy, rather that a prerequisite for breeding.

Infertility and the maintenance of reproductive skew

If the inhibition of ovarian function and ovulation plays a role in maintaining reproductive skew, through what mechanism could this be effected? It is well known that ovarian hormones play a critical role in the expression of reproductive behaviour in many species (see Johnson & Everitt 1995). Since female Damaraland mole-rats typically solicit copulations from males during the mating sequence (Bennett 1990) one possibility is that inadequate gonadal steroid levels result in the absence of proceptive sexual behaviour and, thus, fertilisation in subordinate females. In the dwarf mongoose (*Helogale parvula*), low oestrogen levels lead to low mating rates that cannot be explained by direct aggression by dominant females (Creel *et al.* 1992). However,

pairing of NRF Damaraland mole-rats with unrelated males immediately following removal from the colony typically results in rapid proceptive and receptive behaviour on the part of the female. Presumably, this occurs too rapidly for an increase in ovarian hormone levels to occur in response to the unrelated male or removal from the RF. Furthermore, pairing of hystero-ovariectomised females, in which oestrogen and progesterone levels would be expected to be extremely low, with unrelated males results in sexual behaviour and copulation (*pers. obs.*). This suggests either that, unlike most rodents, progesterone and oestrogen are not essential for reproductive behaviour in *C. damarensis*, or more probably, that the levels of ovarian hormone in NRF are sufficient to promote sexual behaviour. This does not mean that an absence of sexual behaviour is not responsible for preventing pregnancy in NRF, merely that an absence of sexual behaviour would not be due to their physiological state. An alternative mechanism through which infertility could prevent pregnancy, is a direct result of the lack of ovulation following mating. Even if unrelated males mate with NRF, the absence of ova would prevent fertilisation. Whether NRF actually mate with unrelated males in the colony requires investigation in order to determine how inhibition of the reproductive axis functions in maintaining reproductive skew.

As concluded above, infertility in NRF has the potential to play a role in maintaining reproductive skew. Whether it does, in fact, function in this regard has yet to be determined. Recrudescence of the reproductive axis on removal of the RF from the colony does not preclude the same effect from occurring in response to an unrelated male entering the colony. Colony members are typically highly related (Jarvis & Bennett 1993) and despite the recrudescence of ovarian activity in rNRF, a strict incest avoidance prevents them from breeding with colony members (this study; Bennett *et al.* 1996; Rickard & Bennett 1997, Jarvis *et al.* 1994). A similar incest taboo, in which offspring show no sexual interest in each other, has been observed in colonies of cooperatively breeding marmoset monkeys (Abbott 1984). Thus, the reproductive axis of subordinate females may be inactive due to a lack of mating opportunities within the colony, rather than social inhibition in the presence of the dominant female. The recrudescence of sexual activity on removal of the breeding pair may merely occur in anticipation of dispersal and independent breeding. In the absence of sexually

attractive, unrelated males, subordinate females may benefit by not ovulating due to energetic constraints. It is, therefore, possible that the suppressed reproductive axis plays no role in the maintaining reproductive skew, which may occur entirely through incest avoidance. However, the fact that multiple RF have never been found in a wild caught colony suggests that if NRF are stimulated by unrelated males entering the colony, NRF disperse with the new male and attempt to found a new colony. Indeed, this may be an important mechanism whereby mate location, dispersal and colony foundation is achieved. Thus, although the infertility observed in NRF is socially-induced and has the potential to play a role in maintaining reproductive skew, further investigation into both the behavioural and physiological response of NRF to an unrelated male entering the colony is required to test this hypothesis.

Appendix

Table 3.1. Mean (± sem) plasma LH concentrations immediately prior to (0min) and 20min after a single injection of 2.0, 1.0 and 0.5μg GnRH. The U, Z and probability values following the Mann-Whitney U test performed between the two groups (pre and post GnRH) are presented. Statistical significance ($\alpha = 0.05$) is denoted by *.

Dose	Reproductive status	Plasma LH (mIU.ml^{-1}) 0min	N	Plasma LH (mIU.ml^{-1}) 20min	N	U	Z	P
2.0μg	Reproductive female	5.2 ± 0.6	10	47.6 ± 3.3	10	0	-3.8	1.6×10^{-4} *
	Non-reproductive female	2.7 ± 0.1	28	18.5 ± 2.2	26	0	-6.3	3.0×10^{-10} *
	Removed non-reproductive female	3.8 ± 0.1	10	29.1 ± 2.8	10	0	-3.8	1.6×10^{-4} *
	Reproductive male	3.2 ± 0.2	10	35.6 ± 4.3	10	0	-3.8	1.6×10^{-4} *
	Non-reproductive male	2.9 ± 0.1	10	39.4 ± 4.2	10	0	-3.8	1.6×10^{-4} *
1.0μg	Reproductive female	5.4 ± 1.2	10	50.8 ± 4.3	10	0	-3.8	1.6×10^{-4} *
	Non-reproductive female	3.8 ± 0.1	10	17.9 ± 2.2	10	0	-3.8	1.6×10^{-4} *
	Removed non-reproductive female	3.2 ± 0.1	10	34.9 ± 5.2	9	0	-3.7	2.4×10^{-4} *
	Reproductive male	3.7 ± 0.05	10	32.6 ± 5.9	9	0	-3.6	3.5×10^{-4} *
	Non-reproductive male	3.5 ± 0.1	10	33.2 ± 3.5	10	0	-3.8	1.6×10^{-4} *
0.5μg	Reproductive female	5.0 ± 1.0	10	41.1 ± 2.0	10	0	-3.7	2.4×10^{-4} *
	Non-reproductive female	2.7 ± 0.1	10	17.1 ± 1.4	10	0	-3.8	1.6×10^{-4} *
	Removed non-reproductive female	2.4 ± 0.1	10	27.0 ± 2.9	10	0	-4.0	7.1×10^{-5} *
	Reproductive male	1.61 ± 0.1	10	38.6 ± 2.4	10	0	-3.8	1.6×10^{-4} *
	Non-reproductive male	2.2 ± 0.04	10	46.6 ± 2.8	10	0	-3.8	1.6×10^{-4} *

Table 3.2. Mean (± sem) plasma LH concentrations immediately prior to (0min) and 20min after a single, physiological saline injection. The U, Z and probability values following the Mann-Whitney U test performed between the two groups (pre and post saline) are presented.

Reproductive state	Plasma LH (mIU.ml⁻¹) 0min	N	Plasma LH (mIU.ml⁻¹) 20min	N	U	Z	P
Reproductive female	8.0 ± 1.5	6	9.0 ± 1.8	6	15	-0.5	0.6
Non-reproductive female	2.7 ± 0.2	6	2.7 ± 0.1	6	16	-0.3	0.7
Removed non-reproductive female	2.2 ± 0.2	6	2.2 ± 0.1	6	15.5	-0.4	0.7
Reproductive male	3.2 ± 0.1	6	3.1 ± 0.1	6	15	-0.4	0.6
Non-reproductive male	2.2 ± 0.1	6	2.5 ± 0.1	6	9	-1.44	0.1

Table 3.3. Mean (± sem) plasma LH concentrations **a)** immediately before and **b)** 20min after a 2.0µg GnRH s.c. injection. $N1$ and $N2$ refer to the sample sizes of the first and second group respectively, as defined under reproductive state. The U, Z and probability values following the Mann-Whitney U test are presented. Statistical significance ($\alpha = 0.05$) is denoted by *.

	Reproductive state	$N1$	$N2$	U	Z	P
a	Reproductive female (1) vs. Non-reproductive female (2)	10	28	30.5	3.6	0.13
	Reproductive female (1) vs. Removed non-reproductive female (2)	10	10	30	1.5	0.1
	Non-reproductive female (1) vs. Removed non-reproductive female (2)	28	10	4	4.5	6.6×10^{-6} *
b	Reproductive female (1) vs. Non-reproductive female (2)	10	26	9	-4.3	1.9×10^{-5} *
	Reproductive female (1) vs. Removed non-reproductive female (2)	10	10	9	-3.1	0.002 *
	Non-reproductive female (1) vs. Removed non-reproductive female (2)	26	10	51	-2.8	0.005 *

Table 3.4. Mean (± sem) plasma LH concentrations **a)** immediately before and **b)** 20min after a 1.0 μg GnRH s.c. injection. *N1* and *N2* refer to the sample sizes of the first and second group respectively, as defined under reproductive state. The *U, Z* and probability values obtained following the Mann-Whitney *U* test are presented. Statistical significance (α = 0.05) is denoted by *.

	Reproductive state	$N1$	$N2$	U	Z	P
a	Reproductive female (1) *vs.* Non-reproductive female (2)	10	10	50	0	1.0
	Reproductive female (1) *vs.* Removed non-reproductive female (2)	10	10	50	0	1.0
	Non-reproductive female (1) *vs.* Removed non-reproductive female (2)	10	10	10	3.0	0.002 *
b	Reproductive female (1) *vs.* Non-reproductive female (2)	10	10	1	-3.7	0.0002 *
	Reproductive female (1) *vs.* Removed non-reproductive female (2)	10	9	21	-2.0	0.05 *
	Non-reproductive female (1) *vs.* Removed non-reproductive female (2)	10	9	17	-2.3	0.02 *

Table 3.5. Mean (± sem) plasma LH concentrations obtained **a)** immediately before and **b)** 20min after a 0.5 μg GnRH s.c. injection. *N1* and *N2* refer to the sample sizes of the first and second group respectively, as defined under reproductive state. The *U, Z* and probability values following the Mann-Whitney *U* test are presented. Statistical significance (α = 0.05) is denoted by *.

	Reproductive state	$N1$	$N2$	U	Z	P
a	Reproductive female (1) *vs.* Non-reproductive female (2)	10	10	33	-1.0	0.3
	Reproductive female (1) *vs.* Removed non-reproductive female (2)	10	10	16	2.4	0.02 *
	Non-reproductive female (1) *vs.* Removed non-reproductive female (2)	10	10	19.5	2.3	0.02 *
b	Reproductive female (1) *vs.* Non-reproductive female (2)	10	10	0	-3.8	0.0002 *
	Reproductive female (1) *vs.* Removed non-reproductive female (2)	10	10	10	-3.2	0.002 *
	Non-reproductive female (1) *vs.* Removed non-reproductive female (2)	10	10	17.5	-2.6	0.008 *

Table 3.6. Mean (± sem) plasma LH concentrations **a)** immediately before and **b)** 20min after a 2.0. 1.0 and 0.5 µg GnRH s.c. injection. *N1* and *N2* refer to the sample sizes of the first and second group respectively, as defined under reproductive state. The *U, Z* and probability values following the Mann-Whitney *U* test are presented. Statistical significance ($\alpha = 0.05$) is denoted by *.

Dose		Reproductive state	*N1*	*N2*	*U*	*Z*	*P*
2.0µg	a	Reproductive male (1) *vs.* Non-reproductive male (2)	10	10	29.5	-1.5	0.1
	b	Reproductive male (1) *vs.* Non-reproductive male (2)	10	10	41	-0.7	0.5
1.0µg	a	Reproductive male (1) *vs.* Non-reproductive male (2)	10	10	22.5	-1.8	0.1
	b	Reproductive male (1) *vs.* Non-reproductive male (2)	9	10	41	-0.3	0.7
0.5µg	a	Reproductive male (1) *vs.* Non-reproductive male (2)	10	10	0	-3.8	0.0002 *
	b	Reproductive male (1) *vs.* Non-reproductive male (2)	10	10	22	-2.1	0.03 *

Table 3.7. Mean (± sem) plasma LH concentrations obtained following the administration of three doses of GnRH (2.0μg, 1.0μg and 0.5μg) within each group **a)** 2.0μg vs. 1.0μg GnRH **b)** 2.0μg vs. 0.5μg and GnRH **c)** 1.0μg vs. 0.5μg GnRH. The U, Z and probability values following the Mann-Whitney U test are presented. Statistical significance (α = 0.05) is denoted by *.

a)	Reproductive status	N1 (2.0μg)	N2 (1.0μg)	U	Z	P
	Reproductive females	10	10	40.5	-0.7	0.5
	Removed non-reproductive females	10	9	36.0	-0.7	0.5
	Non-reproductive females	26	10	120.0	-0.4	0.7
	Reproductive males	10	9	36.0	-0.7	0.5
	Non-Reproductive males	10	10	35.0	-0.8	0.4

b)	Reproductive Status	N1 (2.0μg)	N2 (0.5μg)	U	Z	P
	Reproductive females	10	10	26.0	-1.8	0.07
	Removed non-reproductive females	10	10	50.0	-0.4	0.7
	Non-reproductive females	26	10	117.5	-0.4	0.7
	Reproductive males	10	10	39.0	-0.8	0.4
	Non-Reproductive males	10	10	32.0	-1.4	0.2

c)	Reproductive Status	N1 (1.0μg)	N2 (0.5μg)	U	Z	P
	Reproductive females	10	10	31.0	-1.4	0.2
	Removed non-reproductive females	9	10	35.0	-1.1	0.3
	Non-reproductive females	10	10	48.0	-0.2	0.9
	Reproductive males	9	10	28.0	-1.4	0.2
	Non-Reproductive males	10	10	19.0	-2.1	0.03 *

Table 3.8. Mean (± sem) plasma progesterone concentrations between five groups of females. RF reproductive females, NRF non-reproductive female, rNRF non-reproductive females removed from the breeding pair, p/UM females housed in the presence of an unrelated male for 30 days, pUM females housed with an unrelated male for 30 days. *N1* and *N2* refer to the sample sizes of the first and second group respectively, as defined under reproductive state. The *U, Z* and probability values using the Mann-Whitney *U* test (following Kruskal Wallis analysis) are presented. Statistical significance ($\alpha = 0.05$) is denoted by *.

Reproductive state	Progesterone n.mol^{-1}	*N1*	Progesterone n.mol^{-1}	*N2*	*U*	*Z*	*P*
RF (1) *vs.* NRF (2)	27.4 ± 8.1	10	1.8 ± 0.4	9	0	-3.7	0.0002*
RF (1) *vs.* rNRF (2)	27.4 ± 8.1	10	16.4 ± 5.2	6	24	-0.7	0.5
NRF (1) *vs.* rNRF (2)	1.8 ± 0.4	9	16.4 ± 5.2	6	1	-3.1	0.002*
RF (1) *vs.* p/UM (2)	27.4 ± 8.1	10	14.8 ± 6.7	6	17	-1.4	0.2
NRF (1) *vs.* p/UM (2)	1.8 ± 0.4	9	14.8 ± 6.7	6	11	-1.9	0.1
rNRF (1) *vs.* p/UM (2)	16.4 ± 5.2	6	14.8 ± 6.7	6	15	-0.5	0.6
RF (1) *vs.* pUM (2)	27.4 ± 8.1	10	22.4 ± 12.8	6	14	-1.7	0.1
NRF (1) *vs.* pUM (2)	1.8 ± 0.4	9	22.4 ± 12.8	6	14	-1.5	0.1
rNRF (1) *vs.* pUM (2)	16.4 ± 5.2	6	22.4 ± 12.8	6	12	-1.0	0.3
p/UM (1) *vs.* pUM (2)	14.8 ± 6.7	6	22.4 ± 12.8	6	17	-0.2	0.9

Chapter 4

Inhibitory pathways at the anterior pituitary level

Abstract

To investigate pituitary sensitivity in female Damaraland mole-rats, pituitary luteinizing hormone (LH) was measured in non-reproductive females (NRF) and reproductive females (RF) and the LH response to multiple gonadotrophin-releasing hormone (GnRH) challenges was determined in NRF and non-reproductive females housed in the absence of the breeding pair (rNRF). Pituitary LH concentrations were significantly lower in NRF than RF. Attempts to measure GnRH receptor concentrations were unsuccessful. Two different GnRH priming regimes were used in an attempt to sensitise the pituitary to exogenous GnRH. Hourly priming for 10 hours with 0.5μg GnRH resulted in an increase in the basal plasma LH levels in both NRF and rNRF. A similar increase in the basal plasma LH levels in NRF occurred after priming with 2.0μg GnRH every eight hours, for 120 hours. This supports the hypothesis that reduced GnRH stimulation of the pituitary, as opposed to the action of an inhibitory factor, is responsible for reproductive inhibition in NRF. However, both priming regimes resulted in a significant decrease in GnRH-stimulated LH secretion in NRF and rNRF. Removal of the negative feedback effects of gonadal steroids did not alter the depressive effects of GnRH priming on pituitary sensitivity to exogenous GnRH in hystero-ovariectomised NRF. Priming may cause a desensitisation of the pituitary due to the negative effects of high concentrations of GnRH on pituitary LH and GnRH receptor concentrations. Further priming studies using lower doses of GnRH are needed to investigate the potential role of reduced GnRH stimulation of the anterior pituitary gland in the reproductive inhibition of NRF.

Introduction

Infertility in subordinate females of cooperatively breeding species appears to be due to inadequate stimulation of the ovary by luteinizing hormone (LH), suggesting that suppression occurs at a supra-gonadal level (Faulkes *et al.* 1990; Bennett *et al.* 1993; Abbott 1997; Chapter 3, Fig. 3.1). Neuroendocrine control of reproductive function originates in the hypothalamus (Johnson & Everitt 1995). Gonadotrophin releasing hormone (GnRH) provides a humoral link between the neural and endocrine systems. This regulatory neuropeptide is synthesised and stored in neurones in the medial basal

hypothalamus and is released in a pulsatile manner into the median eminence. It is transported via a portal blood system to the anterior pituitary gland, where it acts on the gonadotrophes to affect the secretion of the glycoproteins, LH and follicle stimulating hormone. In addition to gonadotrophin release (Gordon & Hodgen 1992), GnRH triggers additional cellular responses, including the biosynthesis of gonadotrophins (Papavasiliou *et al.* 1986; Starzec *et al.* 1986; Andrews *et al.* 1988; Conn 1994) and upregulation of GnRH receptors via increased receptor synthesis (Nett *et al.* 1981; Laws *et al.* 1990; Braden & Conn 1992). Consistent with this hypothesis is the observation that high GnRH receptor concentrations are associated with periods of high GnRH secretion (Marian *et al.* 1981; Khalid *et al.* 1991; Braden & Conn 1991; Conn 1994). Thus, although low basal plasma LH concentrations and pituitary desensitisation in non-reproductive female (NRF) mole-rats could be the result of the direct action of inhibitory factors on the anterior pituitary, blocking the signal transduction system at a post-receptor site, insufficient stimulation of the gonadotrophes by GnRH may also lead to reduced biosynthesis of LH and down-regulation of pituitary GnRH receptors.

The aim of this chapter was to determine the cause of pituitary sensitivity in NRF Damaraland mole-rats by investigating pituitary GnRH receptor concentrations, pituitary LH concentrations and the pituitary response to multiple administration of low doses of exogenous GnRH in intact and ovariectomised females. However, the lack of a response to GnRH priming does not necessarily mean that insufficient GnRH stimulation in not responsible for a reduction in pituitary sensitivity and may merely reflect an inadequate experimental protocol.

Materials and methods

Study animals

Pituitary LH bioassay, GnRH radioimmunoassay and GnRH receptor binding assay

Reproductive females (RF; n = 10) were obtained either by pairing females with unfamiliar males in the lab (n = 8), in which case they were used only once they had given birth to at least one litter, or were identified as RF in colonies captured in the

wild (n = 2). RF were killed irrespective of the stage of the oestrous cycle. NRF (n = 10) were obtained from two colonies of wild caught individuals which were sacrificed approximately one week after capture and from one colony that had been generated in the laboratory. RF and NRF were the same animals as those used for histological examination (see Chapter 3). Reproductive males (RM; n = 10) were obtained from the same colonies as the reproductive females and non-reproductive males (NRM; n = 18) were taken from three wild-caught colonies. Large males were not used to minimise the chance of selecting reproductive males.

GnRH priming

NRF (n = 21) from eight colonies, six of which were wild-caught and two generated in the laboratory, were maintained in the presence of an actively breeding female. RF (n = 10) resulted from pairings with unrelated males in the laboratory. A third group of females, comprised of non-reproductive females housed in the absence of the breeding individuals (rNRF; n = 13), originated from three colonies without reproductive individuals. In one colony the breeding pair had been removed for at least three years. The two remaining colonies were captured without the reproductive female and were maintained in the laboratory for at least six months and one year, respectively. Hystero-ovariectomised non-reproductive females (ovxNRF; n = 10) were obtained from four wild caught colonies and two colonies created in the laboratory. After hystero-ovariectomy, animals were allowed to recover for two months prior to experimentation.

Experimental design

Two experimental designs were used in an attempt to sensitise pituitaries to exogenous GnRH challenges in both intact and hystero-ovariectomised NRF. OvxNRF were used to determine whether sensitisation of the pituitary could be achieved by priming with exogenous GnRH in the absence of the negative feedback effects of gonadal steroids.

Experiment 1

LH secretion in response to a 2.0μg GnRH challenge was measured prior to and after priming with eight challenges of 0.5μg GnRH, administered s.c. every hour, in NRF (n

= 10) and rNRF (n = 10). Control animals (n = 6 in each group) received physiological saline instead of 0.5μg GnRH.

Experiment 2

NRF (n = 10) and rNRF (n = 10) were injected with 2.0μg GnRH, every eight hours for 120 hours (16 challenges). Blood samples were collected immediately before and 20min after the first and 20min after last GnRH challenge. This was repeated in ovxNRF (n = 10). Control animals (n = 6 in each group) received physiological saline instead of GnRH after the first and before the last GnRH challenge.

Results

Pituitary LH concentrations

Mean pituitary LH concentrations from NRF, RF, NRM and RM are presented in Fig. 4.1. There was significantly more LH in the pituitaries of RF than in NRF (Mann-Whitney U test; $U = 27$, $P < 0.001$, $N = 10$). In contrast, pituitary LH concentrations were significantly higher in NRM when compared to RM ($U = 27$, $P < 0.001$, $N = 10$).
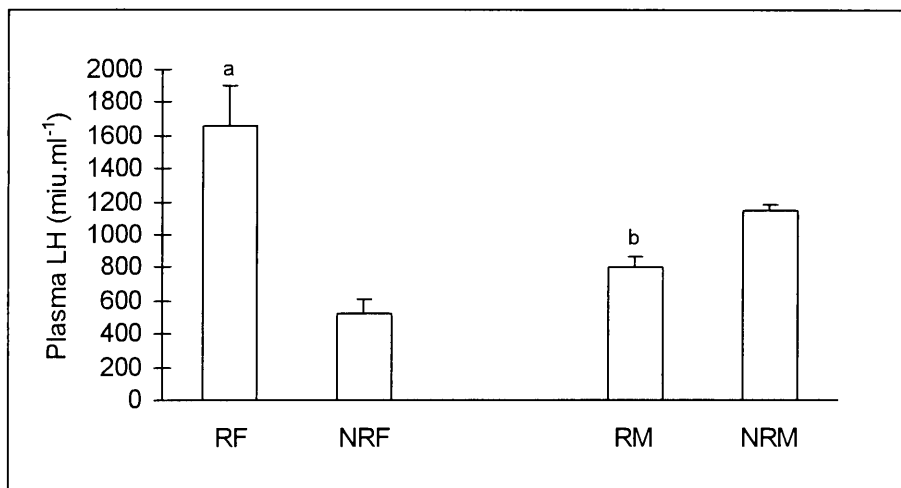
Fig. 4.1. Concentrations (mean ± sem) of pituitary LH. RF, reproductive females; NRF, non-reproductive females; RM, reproductive males; NRM, non-reproductive males. a: $P < 0.0002$ *vs.* non-reproductive females. b: $P < 0.001$ *vs.* non-reproductive males (Mann-Whitney U test).

GnRH priming

Experiment 1 - As shown in Fig. 4.2, basal levels of LH increased significantly after short term priming (hourly for eight hours) with a low dose of GnRH (0.5μg) in both intact NRF and rNRF. Statistical values are presented in Table 4.1. However, the plasma LH response to a 2.0μg GnRH decreased significantly after priming in both NRF and rNRF. There was no difference in either basal or GnRH-stimulated plasma LH levels before and after saline administration in control animals.
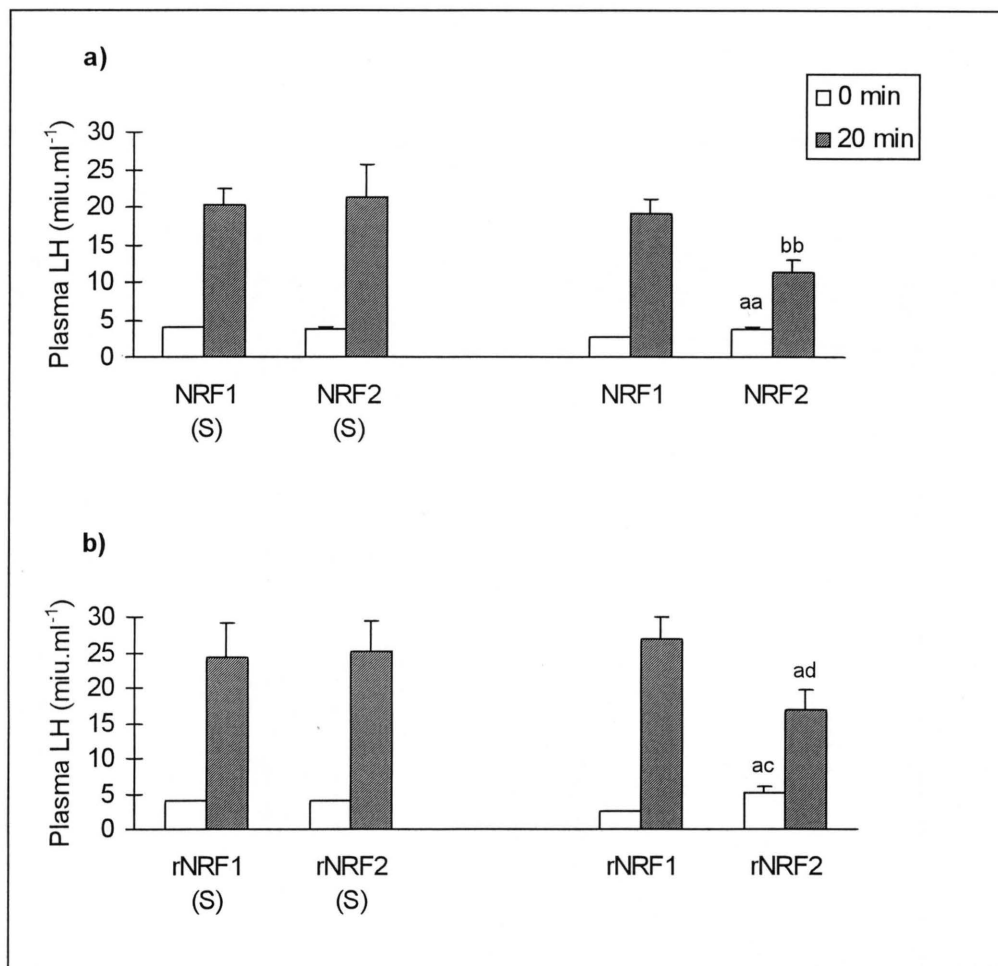
Fig. 4.2. Basal (0min) and GnRH-stimulated (20min) plasma LH levels (mean ± sem) before (1) and after (2) eight, hourly, 0.5μg GnRH challenges or saline injections (S). **a)** NRF, non-reproductive females; **b)** rNRF, non-reproductive females housed in the absence of the breeding pair. aa: $P < 0.0002$ *vs.* basal plasma LH before priming in non-reproductive females. ab: $P < 0.005$ *vs.* GnRH-stimulated plasma LH before priming in non-reproductive females. ac: $P < 0.004$ *vs.* basal plasma LH before priming in removed non-reproductive females. ad: $P < 0.02$ *vs.* GnRH-stimulated plasma LH before priming in removed non-reproductive females (Mann-Whitney U test).

Experiment 2 - Basal plasma LH secretion increased significantly in NRF following priming with 2.0μg GnRH, every eight hours, for 120 hours (Fig. 4.3). Statistical values are presented in Table 4.2. In contrast, rNRF showed no significant difference in basal LH levels before and after priming. Both NRF and rNRF showed a significant decrease in GnRH-stimulated LH secretion after priming. Control animals showed no difference in basal or GnRH-stimulated LH levels following saline administration. The experiment was replicated on ovxNRF, with the difference that basal values of LH were not measured following the priming, and yielded similar results (Fig. 4.4). GnRH-stimulated plasma LH levels decreased significantly after priming with GnRH but showed no significant difference in control animals (Table 4.3).
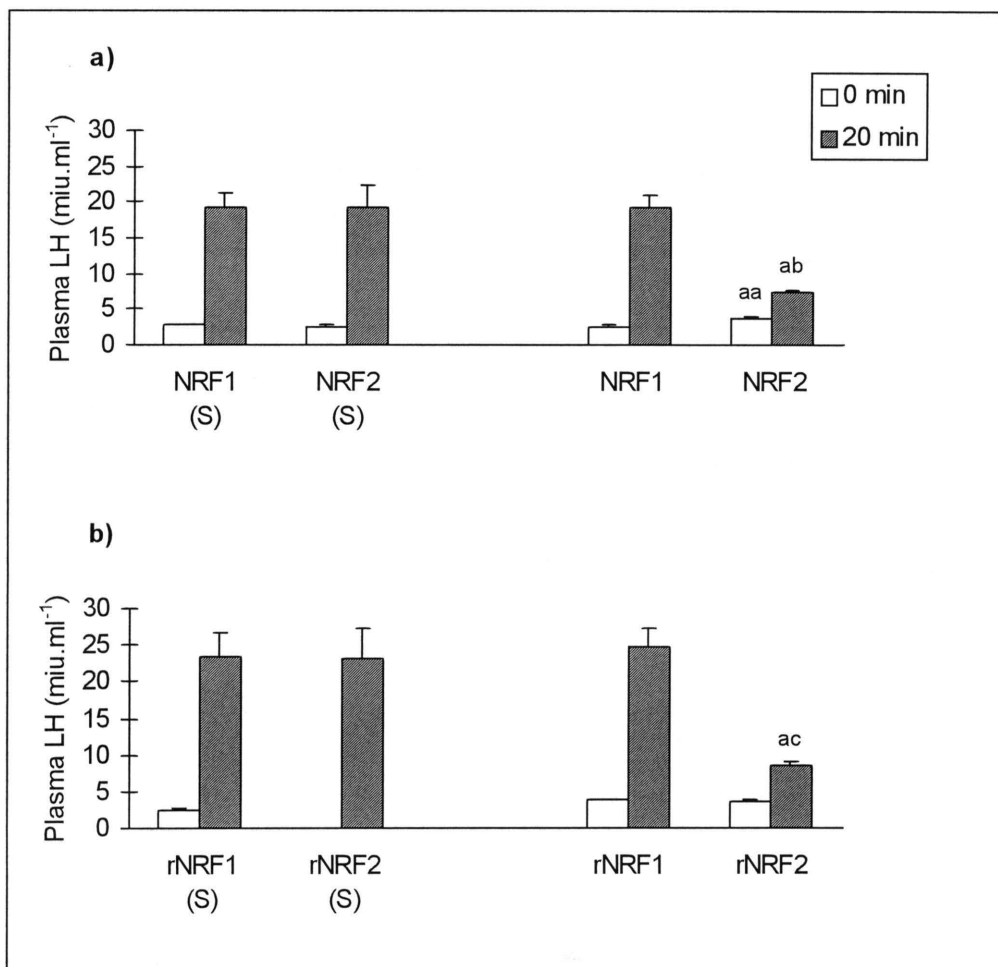
Fig. 4.3. Mean (± sem) basal LH concentrations (0min) and mean LH response to a 2.0μg GnRH challenge (20min) prior to (1) and 120 hours after (2) priming with 2.0μg GnRH or saline (S) every eight hours. **a)** NRF, non-reproductive females; **b)** rNRF, non-reproductive females housed in the absence of the breeding pair. aa: *P* < 0.005 *vs.* basal plasma LH before priming in non-reproductive females. ab: *P* < 0.0002 *vs.* GnRH-stimulated plasma LH before priming in non-reproductive females. ac: *P* < 0.0002 *vs.* GnRH-stimulated plasma LH before priming in removed non-reproductive females (Mann-Whitney U test).
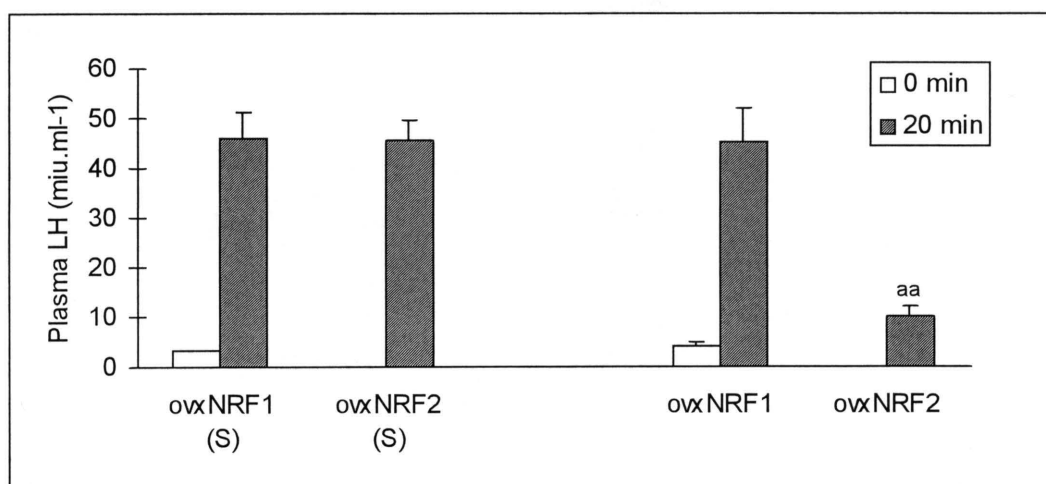
Fig. 4.4. Mean (± sem) basal LH concentrations (0min) and mean LH response to a 2.0μg GnRH challenge (20min) prior to (1) and 120 hours after (2) priming with 2.0μg GnRH or saline (S) every eight hours. OvxNRF, hystero-ovariectomised non-reproductive females. aa: $P < 0.001$ vs. GnRH-stimulated plasma LH before priming (Mann-Whitney U test).

Discussion

The pituitary desensitivity observed in NRF (Chapter 3, Fig. 3.1) may be due to reduced levels of releasable LH in the pituitary gonadotrophes and lower concentrations of pituitary GnRH receptors. Pituitary LH concentrations in non-reproductive *C. damarensis* females were found to be significantly lower than those in RF (Fig. 4.1). This finding is consistent with the hypothesis that GnRH secretion and subsequent stimulation of the anterior pituitary gland is not sufficient for ovulation in NRF, since it affects LH biosynthesis (Papavasiliou *et al.* 1986; Starzec *et al.* 1986; Andrews *et al.* 1988; Conn 1994). Deficient or absent hypothalamic GnRH in hypogonadal mice (Young *et al.* 1983) is associated with a reduced pituitary LH content (Charlton *et al.* 1983). Although attempts to determine GnRH receptor concentrations in pituitaries of *C. damarensis* were unsuccessful (Chapter 2, Table 2.1), low GnRH receptor concentrations are associated with naturally induced infertility in seasonally anoestrous ewes (Khalid *et al.* 1987) and lactating rats (Lee *et al.* 1989).

Although low pituitary LH in NRF may result from inadequate GnRH binding, the possibility that an inhibitory factor, acting independently of GnRH directly at the

pituitary level, needs to be addressed. If exogenous GnRH administration results in sensitisation of the pituitary and increased gonadotrophin secretion, the action of an inhibitory factor is unlikely. Basal levels of plasma LH increased significantly after priming with 0.5µg GnRH for eight hours in both NRF and rNRF (Fig. 4.2). A similar increase in basal LH levels occurred following priming with 2.0µg GnRH for 120 hours in NRF (Fig. 4.3). However, this was not observed in rNRF (Fig. 4.3), possibly due to the small sample size of females (n = 4) from which these samples were obtained. Studies on hypogonadal mice (hypothalamic GnRH is deficient or absent; Young *et al.* 1983) show that low basal levels of LH are increased by exogenous GnRH administration (Iddon *et al.* 1980). This evidence appears to support the contention that a lack of endogenous GnRH stimulation may be responsible for pituitary desensitivity in physiologically suppressed NRF Damaraland mole-rats. In contrast, GnRH-stimulated LH secretion decreased following both of the priming regimes in intact NRF and rNRF (Fig. 4.2 & 4.3). A similar effect was observed in ovxNRF following priming with 2.0µg GnRH every eight hours (Fig. 4.4). Pituitary desensitisation does not appear to be due to the experimental procedure since control animals in all experiments showed no difference in GnRH stimulated LH secretion in response to saline administration. Furthermore, the fact that this effect was observed using different priming regimes, in ovxNRF and in intact females of different reproductive states, indicates that the GnRH priming decreases pituitary sensitivity. This observation does not, however, disprove the hypothesis that inadequate GnRH secretion is responsible for the difference in pituitary sensitivity between NRF and RF. The decrease in pituitary sensitivity may be due to the amount of GnRH used during the priming. Constant exposure to GnRH or its agonists results in the pituitary gland becoming refractory to further stimulation by GnRH with respect to LH release (DeKoning *et al.* 1978). Desensitisation may occur partly through down-regulation of receptors (Nett *et al.* 1981) as a result of physical internalisation of the agonist-receptor complex (Braden *et al.* 1989) and a reduction in receptor synthesis (Wu *et al.* 1994). However, other mechanisms of desensitisation, for example uncoupling of second messenger systems, are also likely to occur (Goropse & Conn 1987). In non-reproductive *C. damarensis* females, exhaustion of the available stores of releasable LH in the pituitary may also play a role.

In non-breeding female naked mole-rats (*Heterocephalus glaber*), priming with a low dose of GnRH results in a significant increase in the LH response to a GnRH challenge, which was comparable to that observed in reproductive females (Faulkes *et al.* 1990). In an elegant study, Ruiz *et al.* (1986) used long-term, pulsatile infusions of GnRH to initiate LH release and ovarian cyclicity in non-breeding female marmoset monkeys (*Callithrix jacchus*). Termination of GnRH infusion results in a return to the hypogonadotrophic state. Pituitary sensitisation and/or ovulation has also been achieved using pulsatile GnRH administration during seasonal infertility in sheep (Legan & Karsch 1979) and lactational infertility in women (Glasier *et al.* 1986) and rats (Lee *et al.* 1989). Thus, there is strong evidence to suggest inactivity of the reproductive axis of females exhibiting various forms of natural infertility appears to result from a lack of a stimulatory factor (GnRH), rather than the action of an inhibitory factor acting independently of GnRH at the level of the pituitary.

Although probable, an increase in pituitary sensitivity and/or resulting ovulation through exogenous GnRH administration does not necessarily indicate a suppression of GnRH secretion. Decreased stimulation by GnRH could conceivably result from a natural antagonist that binds competitively to the GnRH receptors or to GnRH itself, thereby preventing GnRH from binding to its receptor. If this were the case, multiple injections of exogenous GnRH may result in upregulation simply by increasing the concentration of GnRH so that it "out-competes" the competitive binder. Thus, it is still not certain that reduced pituitary desensitivity in NRF is due to an suppression of GnRH secretion from the hypothalamus. To confirm the implied disruption or inhibition of hypothalamic GnRH release a more direct investigation into the nature of the differences in the GnRH system and its potential role in meditating the effects of the social environment on fertility in subordinate *C. damarensis* females is required. This is dealt with in Chapter 5.

Appendix

Table 4.1. Plasma LH concentrations (mean ± sem) immediately before (0min) and 20 minutes after (20min) the first and last 2.0μg GnRH challenge of **a)** hourly priming for 10 hours with 0.5μg GnRH and **b)** control saline injections over the same period. NRF, intact non-reproductive females; rNRF, non-reproductive females housed in the absence of the breeding pair. The results of the Mann-Whitney U test are presented. Statistical significance (α = 0.05) is denoted by *.

		Reprod. status	Plasma LH $(mIU.ml^{-1})$ pre GnRH	N	Plasma LH $(mIU.ml^{-1})$ post GnRH	N	U	Z	P
a)	0min	NRF	2.7 ± 0.02	10	3.8 ± 0.2	10	0	-3.8	0.00021*
	0min	rNRF	2.6 ± 0.04	10	5.1 ± 0.9	10	12	-2.9	0.004*
	20min	NRF	19.2 ± 1.8	10	11.4 ± 1.6	10	13	-2.8	0.005*
	20min	rNRF	26.8 ± 3.1	10	16.7 ± 3.1	10	19	-2.3	0.02*
b)	0min	NRF	3.9 ± 0.1	6	3.8 ± 0.1	6	13.5	-0.7	0.5
	0min	rNRF	3.9 ± 0.1	6	4.0 ± 0.1	6	14.5	-0.6	0.6
	20min	NRF	20.4 ± 2.0	6	21.2 ± 4.5	6	18	0	1
	20min	rNRF	25.7 ± 4.0	6	25.2 ± 4.2	6	16.5	-0.2	0.81

Table 4.2. Plasma LH concentrations (mean ± sem) immediately before (0min) and 20 minutes after (20min) a single 2.0μg GnRH challenge, prior to (pre priming) and two hours after (post priming) **a)** eight hourly priming for 120 hours with 2.0μg GnRH and **b)** control saline injections over the same period. NRF, intact non-reproductive females; rNRF, non-reproductive females housed in the absence of the breeding pair. The results of the Mann-Whitney U test are presented. Statistical significance (α = 0.05) is denoted by *.

		Reprod. status	Plasma LH $(mIU.ml^{-1})$ pre GnRH	N	Plasma LH $(mIU.ml^{-1})$ post GnRH	N	U	Z	P
a)	0min	NRF	2.7 ± 0.02	10	3.7 ± 0.1	10	0	-3.8	0.0002*
	0min	rNRF	3.8 ± 0.1	10	3.6 ± 0.1	10	14.5	-0.8	0.4
	20min	NRF	19.2 ± 1.8	10	7.4 ± 0.3	10	0	-3.8	0.0002*
	20min	rNRF	24.9 ± 2.3	4	8.5 ± 0.7	4	0	-2.8	0.005*
b)	0min	NRF	2.7 ± 0.04	6	2.6 ± 0.1	6	13	-0.8	0.4
	0min	rNRF	2.6 ± 0.06	6	2.6 ± 0.07	6	13	-0.8	0.4
	20min	NRF	19.2 ± 1.9	6	19.3 ± 3.2	6	17	-0.2	0.9
	20min	rNRF	23.5 ± 3.2	6	23.2 ± 4.2	6	16	-0.3	0.7

Table 4.3. Plasma LH concentrations (mean ± sem) 20 minutes after a single 2.0μg GnRH challenge, prior to (pre priming) and two hours after (post priming) **a)** eight hourly priming for 120 hours with 2.0μg GnRH and **b)** control saline injections over the same period. OvxNRF, hystero-ovariectomised non-reproductive female. The results of the Mann-Whitney U test are presented. Statistical significance ($\alpha = 0.05$) is denoted by *.

	Reproductive status	Plasma LH $(mIU.ml^{-1})$ pre GnRH	N	Plasma LH $(mIU.ml^{-1})$ post GnRH	N	U	Z	P
a)	OvxNRF	45.2 ± 6.8	10	10.1 ± 1.7	10	7	-3.3	0.001*
b)	OvxNRF	45.9 ± 5.4	6	45.5 ± 4.3	6	16	-0.3	0.7

Chapter 5

Inhibition of the gonadotrophin-releasing hormone (GnRH) system

Abstract

The gonadotrophin-releasing hormone (GnRH) system in the brains of reproductive (RF), non-reproductive (NRF) and removed non-reproductive (rNRF) females was investigated to determine whether it plays a role in the social inhibition of fertility. GnRH-ir structures formed a loose continuum from the septal areas and diagonal band of Broca (DBB), rostrally, through the medial preoptic area (MPOA) to the posterior hypothalamus, caudally. GnRH-ir cells and fibres were also found in the subfornical organ. Two areas of dense GnRH-ir fibre staining were observed, at the level of the organum vasculosum of the lamina terminalis (OVLT) and in the medial basal hypothalamus (MBH). Relatively few of the total number of cells were found in the MBH (14.8% in RF, 13.2% in NRF, 15.6% in rNRF). In contrast, dense clumping of cells was found in the in the DBB and MPOA at the level of the OVLT (38.6% in RF, 40.9% in NRF, 42.1% in rNRF). Cells were typically unipolar or bipolar, the numbers of which did not differ between groups. No differences were found in either the number of cells (in the POA, MBH or in the total number) or the soma area between the three groups of females. However, GnRH concentrations, measured in whole brains by radioimmunoassay, were significantly higher in NRF than RF, whereas those in reproductive and non-reproductive males did not differ. This suggests that although no differences were found in GnRH-ir morphology between the three groups of females, GnRH release is inhibited in NRF, resulting in the accumulation of GnRH, possibly in the nerve terminals in the median eminence.

Introduction

Investigations into the neuroendocrine mechanisms responsible for various forms of natural infertility have lead to the hypothesis that an alteration in the functioning of the gonadotrophin-releasing hormone (GnRH) system, and subsequent lack of pituitary stimulation, is responsible for the observed anovulation or gonadal regression. This hypothesis has received support from priming studies in which the hypothalamic "site of suppression" is bypassed by the administration of exogenous GnRH, leading to an increase in the sensitivity of the pituitary gland and even ovulation (McLeod *et al.* 1982; Ruiz *et al.* 1986). However, it is possible that inhibitory factors (e.g. gonadal steroids) may act directly at the level of the pituitary (Johnson & Everitt 1995), the

"GnRH pulse generator" being uncompromised in infertile individuals. Although attempts to understand the neuroendocrine pathways responsible for anovulation in socially suppressed individuals of cooperatively breeding species using indirect methods have implicated suppression of GnRH release (see Chapter 4), this hypothesis has not been supported by direct investigation of the GnRH system (Saltzman *et al.* 1995).

The possible disruption of GnRH secretion could be investigated in various ways. One approach involves the direct measurement of GnRH release. However, since low concentrations prohibit the measurement of GnRH in the peripheral circulation (Clarke & Cummins 1982; Ferin, Van Vugt & Wardlaw 1984) it is necessary to determine the release of pulsatile GnRH in the hypophysial portal blood, restricting the use of this technique. Saltzman *et al.* (1995) measured the release of GnRH from the hypothalamus into the portal blood system in reproductive and subordinate female marmosets (*Callithrix jacchus*). Contrary to the expected results, the GnRH concentrations and dynamic patterns of release were very similar between the two groups of females, suggesting that GnRH secretion is not compromised in subordinate females. A second approach involves the use of immunocytochemical techniques to visualise the GnRH neuronal system. Morphological parameters of GnRH-immunoreactive (GnRH-ir) neurones (e.g. size and number) can vary with sex (e.g. springbok *Antidorcas marsupialis*, Robinson *et al.* 1997) and endocrine state e.g. following gonadectomy in rats (King *et al.* 1987) and administration of melatonin (mink *Mustela vison*, Ntoumi *et al.* 1994), following puberty (Djungarian hamster *Phodopus sungorus*, Yellon & Newman 1991; ferret, Tang & Sisk 1982), mating (musk shrew *Suncus murinus*, Dellovade *et al.* 1995) and ovulation (little brown bat *Myotis lucifugus*, Anthony *et al.* 1989). Furthermore, alterations in morphological characteristics of GnRH-ir neurones in cases of naturally induced infertility (white footed mouse *Peromyscus leucopus*, Glass 1986, Syrian hamster *Mesocricetus auratus*, Urbanski *et al.* 1991, African teleost fish *Haplochromis burtoni*, Francis *et al.* 1993; musk shrew *Suncus murinus*, Dellovade *et al.* 1995) illustrate the plasticity of the GnRH system. The variation is probably not due to differences in actual number of neurones, but in the quantity of immunoreactive material within the neurones, thereby resulting in differential visualisation. These differences in morphology or distribution of

GnRH neurones imply an alteration in the functioning (synthesis and/or release) of the GnRH system.

The primary aim of this study was, therefore, to investigate whether socially-induced infertility in the eusocial Damaraland mole-rat could be attributed to differences in the GnRH system by comparing the distribution, morphology, number and size of immunoreactive neurones between reproductive (RF), non-reproductive (NRF) and non-reproductive females housed in the absence of the breeding pair (rNRF). Furthermore, the concentrations of GnRH in whole brains of RF and NRF, as well as reproductive (RM) and non-reproductive males (NRM), were compared. Since the distribution and morphology of GnRH neurones and their projections exhibit interspecific variation, a further aim of this chapter was to characterise the morphology and distribution of the GnRH-ir system in the brain of this species.

Materials and Methods

Study animals

Immunocytochemistry

Following capture, female mole-rats were maintained in the laboratory for up to 12 months and were assigned to one of three groups (n = 7 for each group), according to their reproductive status and the social environment in which they were maintained. All RF originated from colonies maintained in the laboratory and had given birth to at least one litter prior to being killed. All of the rNRF originated from five wild caught colonies that were captured without the reproductive female. Large males were removed, as the breeding male is typically one of the largest males in the colony (Jacobs *et al.* 1991). These colonies were maintained in the laboratory for 11 weeks prior to being killed. NRF were obtained from one wild caught colony (n = 4) and from three colonies in which the females were born in captivity (n = 3). Two males were also sacrificed for the study.

GnRH radioimmunoassay

RF (n = 10) were obtained either by pairing females with unfamiliar males in the lab (n = 8), in which case they were used only once they had given birth to at least one litter, or were identified as reproductive females when wild caught (n = 2). RF were killed irrespective of the stage of the oestrous cycle. Three females were pregnant. NRF (n = 10) were obtained primarily from two colonies of wild caught individuals which were sacrificed approximately one week after capture. One female was bred in the laboratory. RF and NRF were the same as those used for histological examination (Chapter 3). RM (n = 10) were obtained from the same colonies as the reproductive females and NRM (n = 18) were taken from three wild caught colonies. Smaller males were not used in this group to minimise the chance of spuriously selecting reproductive males.

Results

Morphology and distribution of GnRH-ir neurones

GnRH neurones in the brain of *C. damarensis* are typically unipolar or bipolar cells (Plate 5.1a & b) and measure approximately 7μm in diameter. A small proportion of cells were apparently non-polar (Fig.5.1). Kruskal Wallis analysis revealed that the number of unipolar ($H = 0.2$; $P > 0.05$; $N = 21$) and bipolar cells ($H = 1.0$; $P > 0.05$; $N = 21$) were not significantly different between the three groups. Only two cells with a more complex morphology were observed in this study. Although the entire soma of most cells was stained black, some cells contained a dark circular object within a more lightly stained soma. Cells possessed a smooth contour and appeared to occur singly without forming associations with neighbouring cells.

Following page:
Plate 5.1. Examples of a typical **a)** unipolar neurone and **b)** a bipolar neurone. Magnification = 400x. Scale bar = 25μm.
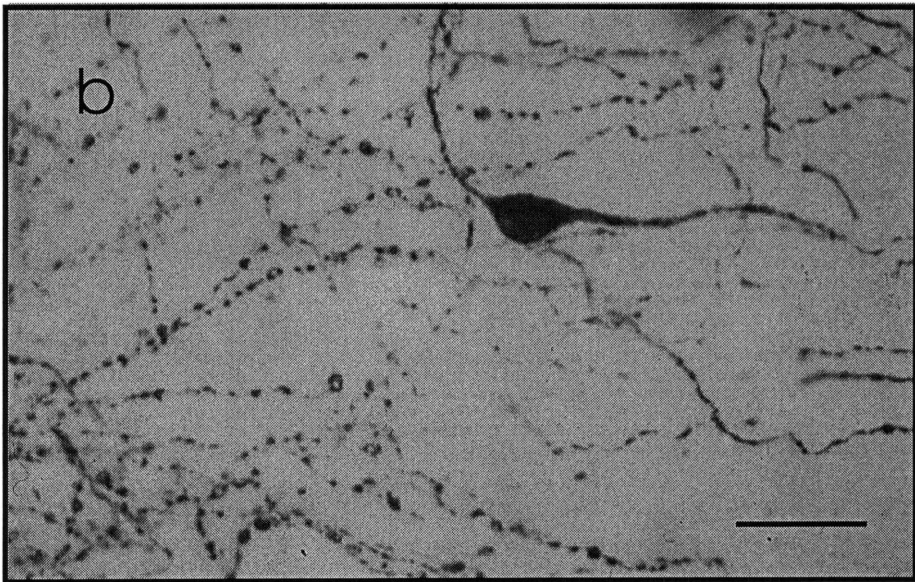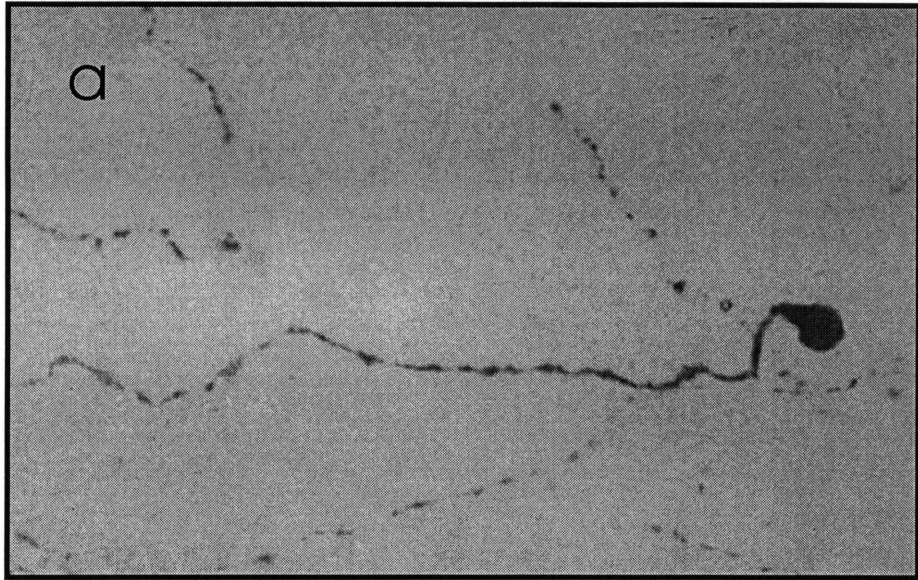
Plate 5.1
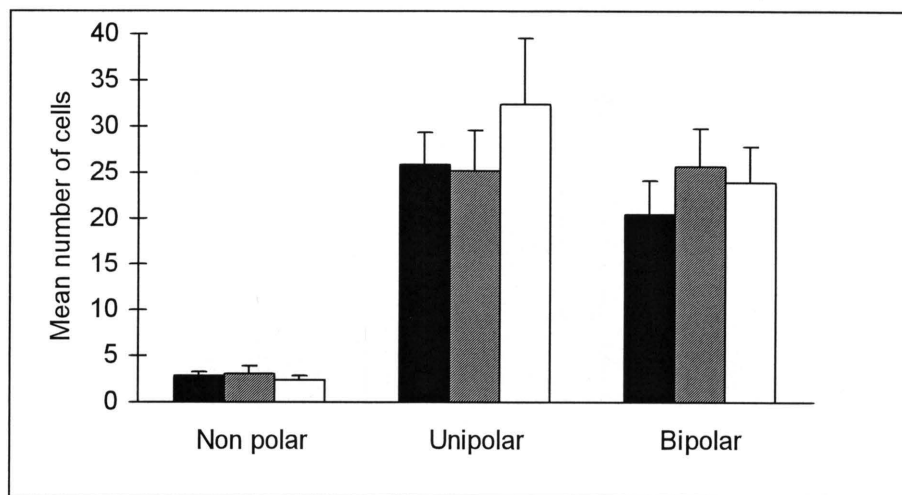
Fig. 5.1. Comparison (mean ± sem) between the number of non-polar, unipolar and bipolar cells from the preoptic region of reproductive (solid bars), non-reproductive (shaded bars) and non-reproductive females housed in the absence of the breeding pair (clear bars).

GnRH-ir neurones were distributed in a loose continuum along the Septo-Preoptico-Infundibular pathway (Plate 5.2). Rostrally, cells containing GnRH-ir material were located in the septal region, orientated primarily with their long axes in the dorsoventral plane. Isolated cells were also observed more laterally in the peripheral cortex ventral to the anterior commissure. Relatively high concentrations of immunoreactive cell bodies (38.6% of the total number in RF, 40.9% in NRF and 42.1% in rNRF) were found in the horizontal and vertical diagonal band of Broca (DBB), in the region of the organum vasculosum of the lamina terminalis (OVLT; Plate 5.2b) extending caudally into the medial preoptic area (MPOA; Plate 5.2c). Dorsally these cells were located close to the midline whereas in the ventral area, cells were distributed in a more lateral position (Plate 5.2 b & c). GnRH cells were found in the MPOA and in the lateral and anterior hypothalamus. GnRH-ir cells were also observed in the subfornical organ. Consecutive coronal sections (15μm) from a male brain revealed 14 GnRH perikarya within the 15 sections (spanning approximately 225μm) that contained GnRH-ir material in this organ (Plate 5.3). Relatively few of the total number of cells (14.7% of the total number in RF, 13.2% in NRF and 15.6% in rNRF)were located in the medial-basal hypothalamus (MBH). These cells occurred in the retrochiasmatic area, arcuate nucleus and median eminence (ME) and as far caudally as the mammillary recess of the third ventricle. At the level of the ME, the cell bodies did not generally lie within the dense fibre network (see Plate 5.2f) but displayed a more lateral distribution.

Morphology and distribution of GnRH-ir fibres

Many GnRH immunoreactive fibres had a characteristically beaded appearance (beads up to 3.7μm in diameter) in both the MPOA and MBH (Plate 5.1b). In many bipolar cells one fibre was thicker than the other allowing the axon and dendrite to be differentiated (Plate 5.1b). GnRH-ir fibres were far more widespread throughout the brain than the cell bodies. Although fibres were apparent in the olfactory lobe, the major tract of fibres followed the septo-preoptico-infundibular pathway, similar to that of the cell bodies. Fibres were seen close to the ventral midline, rostral to and within the vertical and horizontal limb of the DBB. Fibres in the horizontal limb of the DBB ran primarily in a rostrocaudal plane. Approaching the region of the OVLT, fibres congregated in the first of two densely stained regions. Fibres ran in both a dorsoventral plane from the medial septum and in the rostrocaudal plane along the ventral midline within the horizontal and vertical DBB (Plate 5.2a). The dense network of fibres in the region of the OVLT (Plate 5.2b) continued into the MPOA, primarily along the midline in a dense aggregation beneath the third ventricle (Plate 5.2c). Note the absence of an optic chiasm at the base of the brain. Further caudally, far fewer fibres were apparent but could be seen in a thin band of cells beneath the third ventricle (the band of cells is broken in Plate 5.2d). A greater density of fibres was present in a retrochiasmatic area running in the rostrocaudal plane toward the second region of high GnRH-ir fibre density, namely the ME in the MBH. Fibres could also be found running close to the ventral surface in the tuber cinereum. In the MBH the GnRH fibres formed a dense peripheral zone (Plate 5.2e) but became more diffuse in the lateral (Plate 5.2f) and central areas of the median eminence and extended into the infundibular stem itself (Plate 5.2g). Caudal to the infundibular stem the fibres again formed a dense peripheral zone (Plate 5.3h). A network of GnRH-ir fibres was present within the subfornical organ (Plate 5.3).

Following pages:
Plate 5.2. Coronal sections (**a** - **g**), rostral to caudal, through a NRF brain and **h**) sagittal section through the midline of a rNRF brain, showing areas of GnRH immunoreactive cells and fibres. **a**) diagonal band of Broca (DBB) **b**) region of the organum vasculosum of the lamina terminalis (OVLT) **c**) MPOA with high density of staining ventral to the third ventricle (IIIv) **d**) thin band of cells below the third ventricle, note the absence of the optic chiasm **e**) retrochiasmatic area (RCh) approaching the median eminence (ME), note the peripheral dense band of fibres **f**) median eminence and **g**) infundibular stem (IS). Magnification = 63x. Scale bar = 100μm.

Plate 5.3. GnRH-ir network of neurones and fibres in the subfornical organ below the fornix at **a**) 25x magnification, scale bar = 400μm and **b**) 100x magnification scale bar = 100μm. Plate (**b**) corresponds to the area highlighted in the rectangle in Plate (**a**).
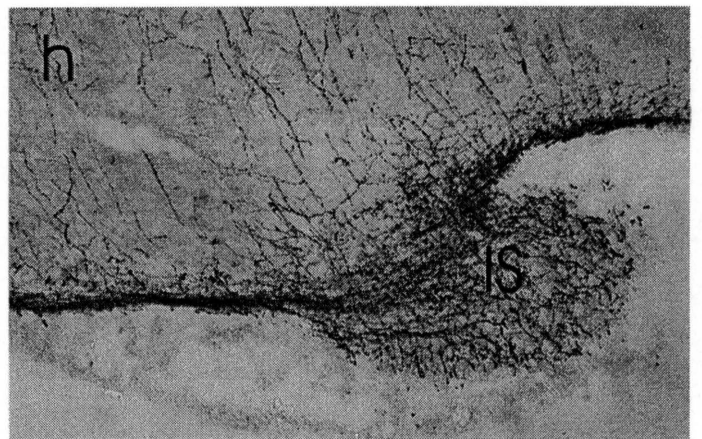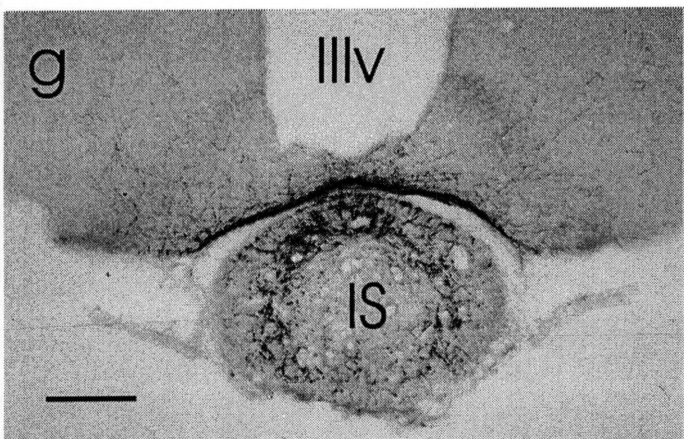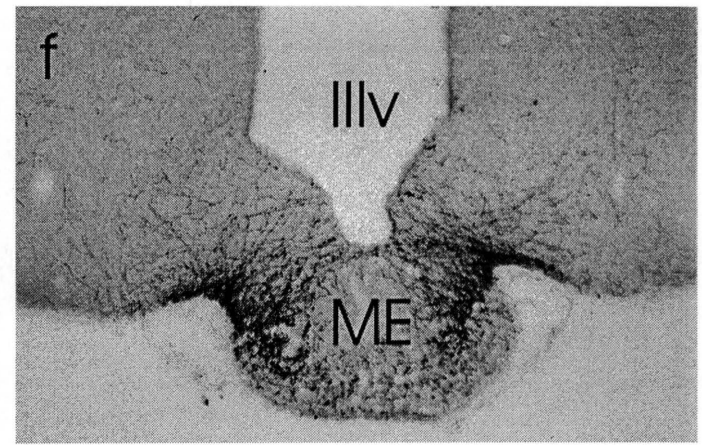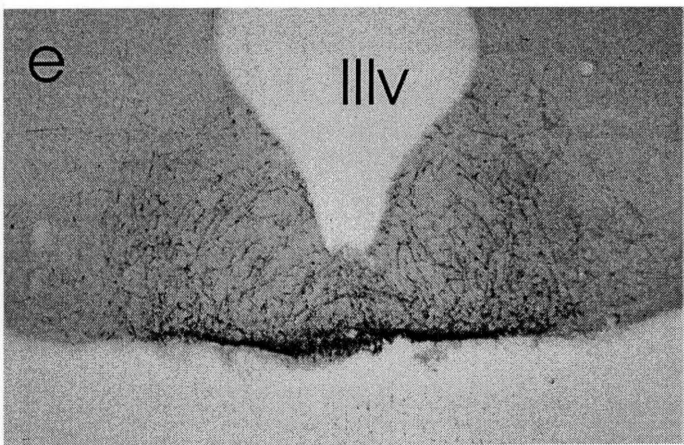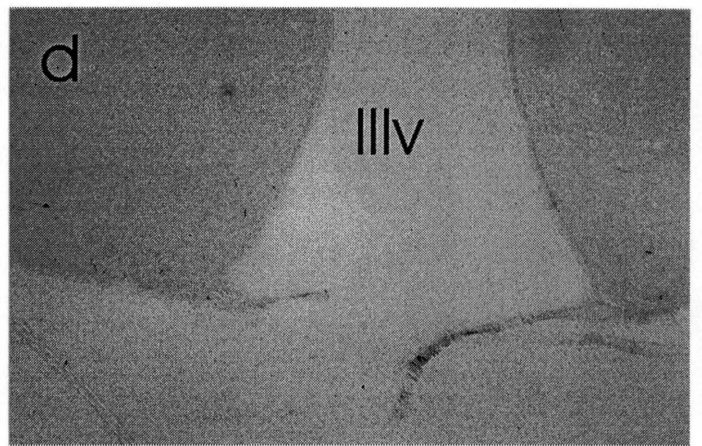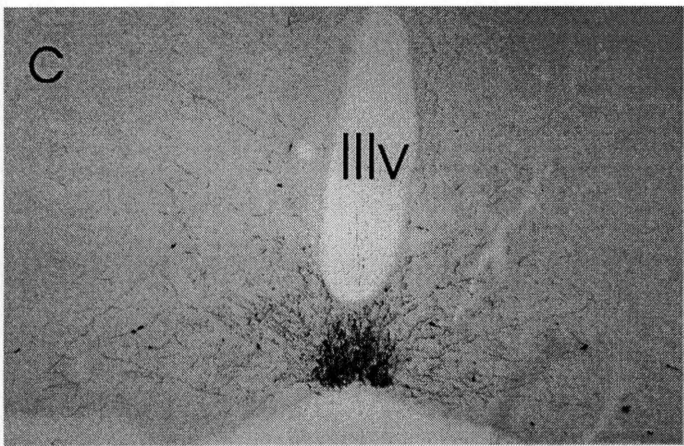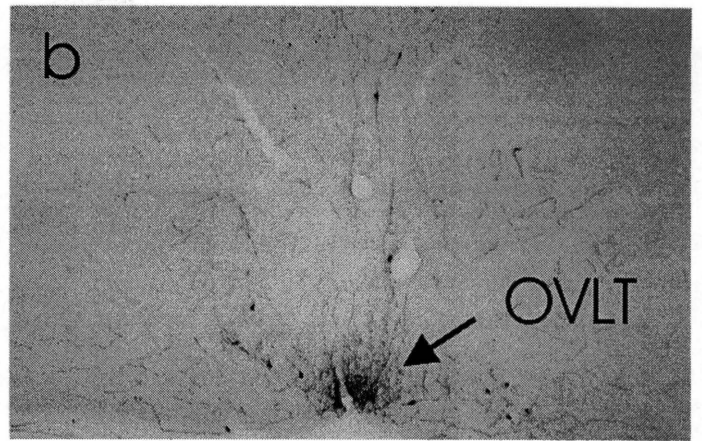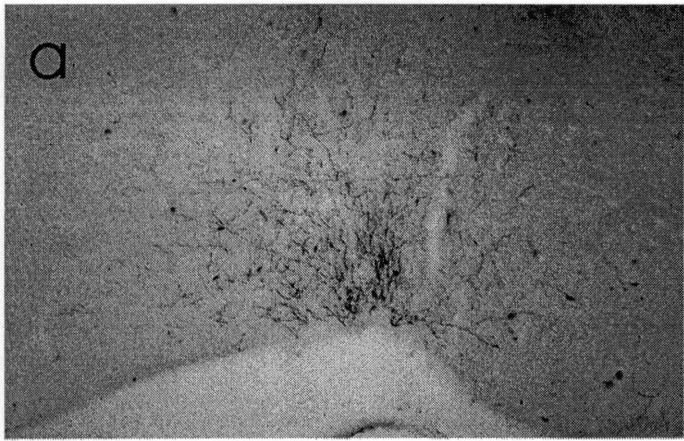
Plate 5.2.

Number and size of cell bodies

A large individual variation in the number of GnRH cells counted in the entire brain was observed, ranging from 74-172 in NRF, 76-173 in RF and 79-234 in rNRF. Since every fifth section was processed, an individual mean of 130 neurones (counted across all three groups), translated into approximately 650 cells for the entire brain (from the confluence of the two hemispheres rostrally to the posterior hypothalamus caudally). The mean number of GnRH-ir cell bodies in the whole brain (Kruskal Wallis; $H_2 = 0.12$; $P = > 0.05$; $N = 21$), the preoptic area ($H_2 = 0.27$; $P > 0.05$; $N = 21$) and in the medial basal hypothalamus ($H_2 = 0.56$; $P > 0.05$; $N = 21$; Fig. 5.2). did not differ between RF, NRF and rNRF. Similarly, there was a large variation in GnRH-ir soma size, ranging from 40.6-154.2μm^2 (mean = 89.7 ± 2.0μm^2) in RF, 47.9-140.4μm^2 (mean 86.0 ± 1.7μm^2) in NRF and 36.4-168.2μm^2 in rNRF (mean 88.3 ± 1.9μm^2). ANOVA revealed that soma size differed significantly between females within the groups of NRF ($F_{133} = 3.9$ $P < 0.05$; $N = 140$) and rNRF ($F_{133} = 3.4$ $P < 0.05$; $N = 140$) but not within the group of RF ($F_{133} = 1.4$ $P > 0.05$; $N = 140$). The mean soma areas were not significantly different between the three groups of females (ANOVA; $F = 0.97$ $P > 0.05$; $N = 420$).
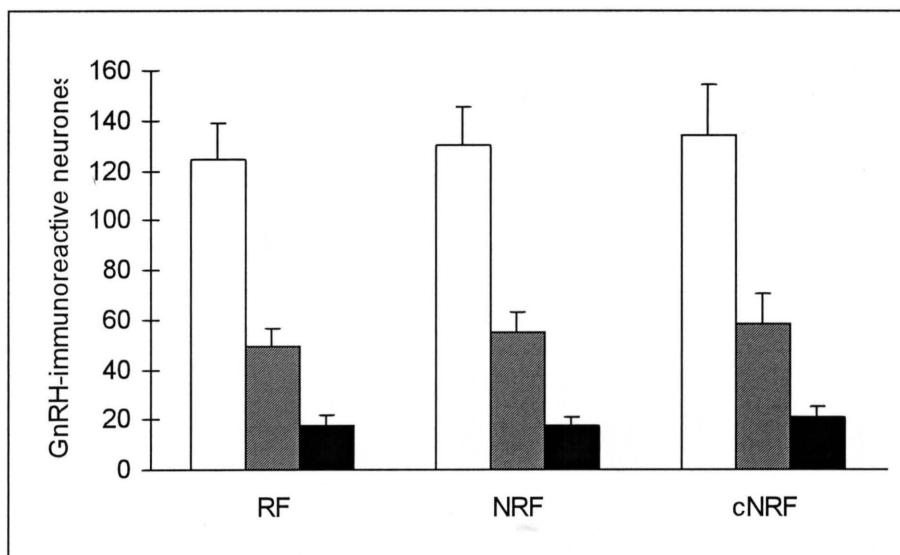
Fig. 5.2. Mean (± sem) number of GnRH immunoreactive neurones in the total brain from the confluence of the two hemispheres rostrally, to the posterior hypothalamus (clear bars), preoptic area (shaded bars) and medial basal hypothalamus (solid bars). RF, reproductive females; NRF, non-reproductive females; RM, reproductive males; NRM, non-reproductive males.
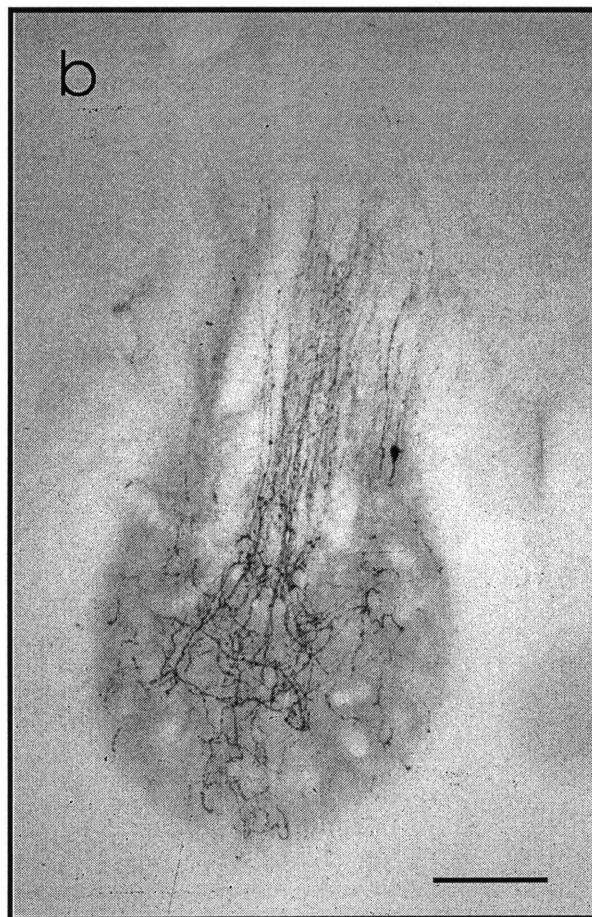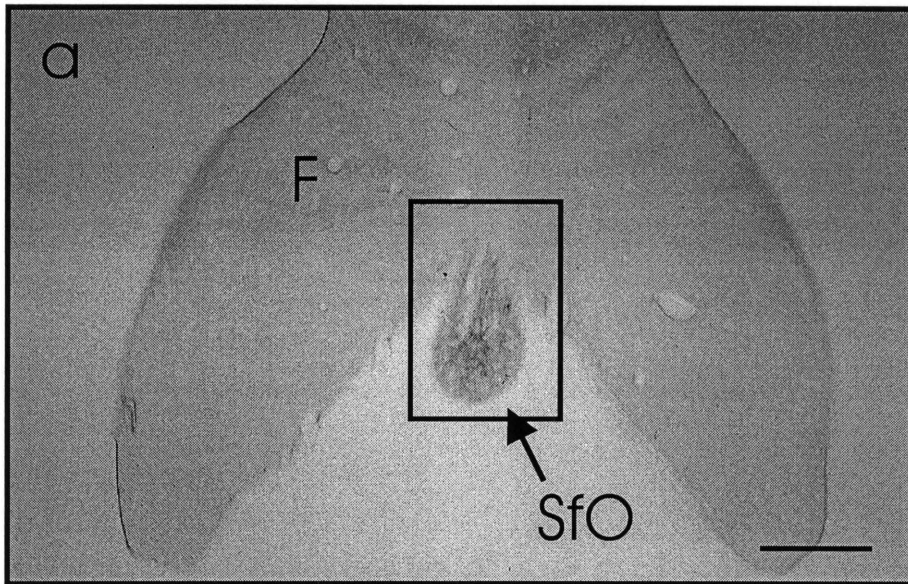
Plate 5.3.

Brain GnRH concentrations

Mean concentrations of GnRH in the brains of RF, NRF, RM and NRM are presented in Fig. 5.3. The GnRH concentrations were significantly higher in NRF than in RF (Mann-Whitney U test; $U = 4$, $P < 0.005$, $N = 9$). In contrast, although the concentrations of GnRH in the brains of reproductive males were higher than those in non-breeding males, the difference was not significant (Mann-Whitney U test; $U = 27$, $P > 0.05$, $N = 9$).
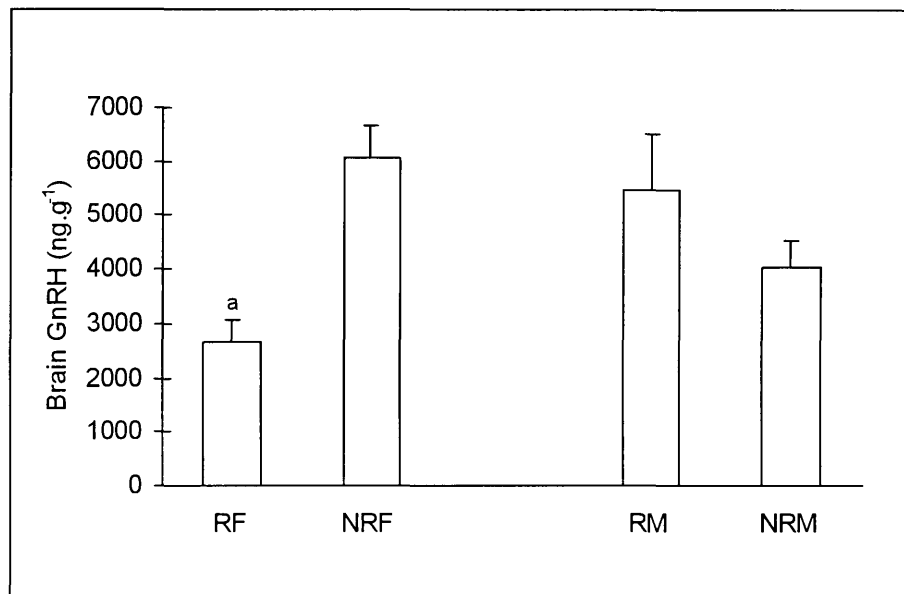
Fig. 5.3. Concentrations (mean ± sem) of GnRH in whole brains. RF, reproductive females; NRF, non-reproductive females; RM, reproductive males; NRM, non-reproductive males. a: $P < 0.005$ *vs.* NRF (Mann-Whitney U test).

Discussion

The distribution of GnRH-ir neurones and their projections in the brain of *C. damarensis* forms a loose continuum along the septo-preoptico-infundibular pathway. Although this is generally typical of most mammals, interspecific differences in distribution of GnRH neurones do occur, especially with respect to the degree to which they migrate caudally (Silverman *et al.* 1994). This may be partly due to their unusual origin. GnRH neurones originate in the olfactory placode and migrate to their final location during neonatal development (Schwanzel-Fukuda & Pfaff 1989). In the opposum (*Monodelphis domestica*) cell bodies do not migrate into the preoptic area

(Schwanzel-Fukuda *et al.* 1988) whereas in the rat, GnRH perikarya do not extend into the MBH (Merchenthaler *et al.* 1984; Witkin *et al.* 1982). In contrast, in the mink approximately 7% of the cells are located in the POA and 80% in the MBH (Ntoumi *et al.* 1982). Nevertheless, in most mammals in which cell bodies migrate into the MBH these cell usually constitute a relatively small percentage of the total cells (Silverman *et al.* 1994). This is true of the Damaraland mole-rat, in which only approximately 14% of the total number of neurones were found in the MBH. Intraspecific comparison of the distribution of GnRH immunoreactive cells and fibres did not reveal any noticeable variation and the number of neurones in the POA and MBH did not differ between RF, NRF and rNRF.

As found in all mammalian species studied to date, two main areas of dense GnRH-ir staining, the OVLT and the ME, were observed in the mole-rat (Plate 5.2b & f). Both fibres and cell bodies were also found in the subfornical organ (Plate 5.3). These three structures are known as circumventricular organs, described by Silverman *et al.* (1994) as, "sites within the CNS in which ependymal lining of the ventricular space is highly specialised and where the capillaries forming the blood supply are fenestrated, thereby permitting access of blood-borne substances into the nervous tissue". The fact that all three circumventricular organs contain GnRH-ir structures may provide insight into the mechanism whereby neurones, migrating during neonatal development, locate the ME, the site at which transfer of GnRH from the neural system to the circulatory system occurs. During migration, GnRH neurones may "accumulate" in areas that have a weak blood brain barrier, including the OVLT and subfornical organ, even though they do not appear to be important sites in terms of GnRH release. These neurones may, nevertheless, contribute to GnRH released into the ME. In the rat, surgical isolation of the MBH results in the elimination of gonadotrophin secretion, suggesting a rostral origin for the GnRH released into the median eminence (Brownstein *et al.* 1976). In the guinea pig, although only cells in the arcuate region are necessary to maintain LH pulsatility, neurones in the preoptic area contribute to the GnRH input into the median eminence (Krey & Silverman 1978; Silverman & Krey 1978).

GnRH soma diameter, approximately 7μm in *C. damarensis*, appears to be smaller

than that found in most other mammals, including the white-footed mouse (8μm; Glass 1986), rat (10μm; Witkin *et al.* 1982) sheep (15 - 20μm; Lehman *et al.* 1986) and springbok (10-20μm; Robinson *et al.* 1997). The greater diameter of neurones in larger species is mirrored by the general correlation between absolute GnRH cell number and brain size in different species (Yellon & Newman 1991). A mean neurone number of 300-400 is calculated for the Djungarian hamster (Yellon & Newman 1991) and 650-750 for the Syrian hamster (Jennes & Stumpf 1980). In larger species including ungulates (Lehman *et al.* 1986; Robinson *et al.* 1997) and primates (Marshall & Goldsmith 1980; Silverman *et al.* 1982) neurones number in the thousands. The mean number of 650 neurones calculated for the Damaraland mole-rat is consistent with this observation. At an intraspecific level, a significant correlation was found between mean soma size and body length in an African teleost fish *Haplochromis burtoni* (White & Fernald 1993). This illustrates the importance of considering the potential role of body size related effects when comparing parameters that are used as indicators of GnRH system activity.

The neurones in the Damaraland mole-rat are predominantly unipolar and bipolar, as found in many mammals including the rat (Witkin *et al.* 1982, Merchenthaler *et al.* 1984), white-footed mouse (Glass 1986), Syrian hamster (Jennes & Stumpf 1980) and mink (Ntoumi *et al.* 1992). In contrast, those found in the sheep (Lehman *et al.* 1986), springbok (Robinson *et al.* 1997) and rhesus monkey (Silverman *et al.* 1982) have a far more complex morphology. The number of bipolar neurones is certainly an underestimate due to the orientation of the bipolar cells perpendicular to the plane of the section. Furthermore, many of the apparently unipolar cells in the rat brain have in fact shown to be bipolar using silver-gold intensification of the immunocytochemical staining (Merchenthaler *et al.* 1984). In the male Djungarian hamster, the ratio of these subtypes changes during sexual maturation. The number of unipolar, but not bipolar cells, in the DBB doubles during puberty and the number of bipolar cells increases significantly following puberty in the DBB as well as the lateral hypothalamus (Yellon & Newman 1991). The authors relate these changes in cell subtype number to differential GnRH storage and release during development. In this study, the number of unipolar and bipolar cells did not vary with female breeding status.

No significant differences in the number of immunoreactive cell bodies or in mean soma area were found between the groups of RF, NRF and rNRF mole-rats (no correlation was found between mean soma area and body size within the groups of females in this study). This is contrary to the expected results since previous immunocytochemical studies suggest that morphological differences in GnRH-ir structures within the GnRH system exist in individuals of species that are naturally infertile. Thus, in the seasonally breeding white footed mouse, the number of cell bodies and the optical density for staining of immunoreactive cells in specific brain regions increased in mice exposed to a short photoperiod. In contrast, in the seasonally breeding Syrian hamster, Urbanski *et al.* (1991) did not find any differences in the number of immunopositive GnRH neurones in individuals maintained under long and short daylength regimes, but did find that the area of the soma was larger in short day maintained animals. As was found in the seasonally anoestrous sheep (Lehman *et al.* 1986) and between reproductive and bachelor male springbok (Robinson *et al.* 1997), no difference was observed either in cell number or in mean soma area between reproductively active and inactive Damaraland mole-rats. This suggests that an alteration in the functioning of the GnRH system may occur in some species in response to inhibitory environmental cues, but there appears to be interspecific variation in the effect of these cues on GnRH cell morphology and number. Nevertheless, a general increase in the levels of GnRH in the brain of non-breeding individuals supports the hypothesis that there is a reduction in the rate of GnRH release.

It is clear from the amount of interspecific variation in the effect of environmental and social cues on the morphology of the GnRH-ir system that the lack of differences in neurone size or number observed in this study does not necessarily indicate that the effect of inhibitory social cues on fertility is not mediated through the GnRH system. Significant differences in cell soma size were found between individuals within the groups of NRF and rNRF. It is possible that this variation, some of which may be due to procedural effects, may mask real differences between the groups. Alternatively, the lack of differences in neurone size and number may be real and differences may occur elsewhere in the GnRH system. Although no differences were observed in GnRH-ir morphology, radioimmunoassay of brain GnRH revealed significant differences between NRF and RF (Fig. 5.3). Higher concentrations of GnRH in NRF support the

hypothesis that GnRH release is inhibited in these females. In contrast to the Damaraland mole-rat, the seasonally breeding Syrian hamster shows no difference in hypothalamic concentrations of GnRH in animals transferred to an inhibitory short daylength, despite the fact that differences were found in an immunocytochemical study (Urbanski *et al.* 1991). In *C. damarensis*, it is possible that GnRH is transported to the nerve terminals in the ME and an inhibition of release results in an accumulation of peptide in the fibres rather than the cell soma, accounting for the greater GnRH concentrations in NRF. A higher fibre density in the ME has been observed in female musk shrews following copulation (Tai *et al.* 1997) and in the seasonally breeding white footed mouse maintained under an inhibitory, short photoperiod (Glass 1986). Although the above evidence does not provide definitive answers regarding the neuroendocrine mechanisms responsible for anovulation in NRF Damaraland mole-rats, it does strongly suggest that there is an alteration in the functioning of the GnRH system, possibly due to an inhibition of GnRH release.

The majority of studies (including the present study) support the hypothesis that natural infertility is mediated through an inhibition of GnRH release, resulting in an accumulation of peptide in GnRH neurones and, consequently, a suppression of the reproductive axis. However, an inhibition of GnRH release is not the only mechanism through which GnRH secretion could be affected. In the African teleost fish *H. burtoni*, an increase in GnRH-ir soma size occurs when males become territorial and a decrease occurs if males loose their territorial status (Francis *et al.* 1993). It is difficult to explain these differences in terms of a reduction in GnRH release, since it is the reproductively active fish which have larger neurones. Rather, non-territorial males may exhibit a decreased level of synthesis and release of GnRH. Sites at which possible regulation of GnRH system include gene transcription, mRNA translation, post-translational processing and neuronal transport of the vesicles from the cell soma to the nerve terminals in addition to the aforementioned block to GnRH release. Further *in situ* hybridisation studies using frozen tissue, to investigate GnRH mRNA levels, and immunocytochemical studies using antibodies directed against pro-GnRH and GnRH in colchicine treated animals (neuronal transport of GnRH is blocked), could provide further insight into the role of differential GnRH synthesis and processing in the mediation of the social environment on fertility.

Chapter 6

Endocrine and neuroendocrine factors affecting gonadotrophin secretion

Abstract

To investigate the mechanisms responsible for the inhibition of GnRH/LH, the role of endogenous opioid peptides (EOP), gonadal steroids and cortisol was examined. The β-endorphin antagonist, naloxone, was administered to intact reproductive (RF), non-reproductive (NRF) and removed non-reproductive females (rNRF) as well as hystero-ovariectomised reproductive (ovxRF) and non-reproductive females (ovxNRF), to determine its effect on plasma LH levels. A single injection of naloxone had no significant effect on LH secretion in either intact or hystero-ovariectomised females. Priming with naloxone failed to affect either basal or GnRH stimulated LH secretion in any group of females. A significant response to a single naloxone injection was obtained in NRF following GnRH priming. However, a similar result was obtained in rNRF, suggesting that socially-induced infertility is unlikely to be mediated through the EOP system. Plasma progesterone concentrations were significantly lower in NRF than in rNRF. This suggests that greater negative feedback effects due to increased progesterone secretion are not responsible for infertility in NRF. GnRH stimulated LH secretion was greater in hystero-ovariectomised compared to intact females, but there was a significant difference in GnRH stimulated LH secretion between ovxRF and ovxNRF. This indicates that suppression of gonadotrophin secretion occurs independently of gonadal steroids. No differences were observed in plasma cortisol levels between RF and NRF. Thus, socially-induced stress does not appear to be responsible for inhibition of the reproductive axis. Although this does not rule out reproductive inhibition through dominant control by the RF, it is possible that NRF restrain themselves from breeding because it is in their best interests to do so.

Introduction

Previous chapters have provided evidence that socially-induced infertility in female Damaraland mole-rats is due to inadequate gonadotrophin secretion, which appears to be attributable to an inhibition of GnRH secretion from the hypothalamus. Studies involving socially and environmentally induced infertility suggest that both a steroid-dependent and independent mechanism operate to suppress gonadotrophin secretion (Karsch *et al.* 1984; Abbott 1988). Bennett *et al.* (1996) proposed that the endocrine milieu in non-reproductive female (NRF) Damaraland mole-rats is comparable to that

of pseudopregnancy and, thus, the negative feedback effects of increased progesterone play a role in suppressing gonadotrophin secretion in NRF. Progesterone is thought to control the oestrous cycle and ovulation primarily through its negative effect on the frequency of the GnRH pulse generator (Karsch *et al.* 1984). Alternatively, activation of the hypothalamo-pituitary-adrenal axis in response to stress may result in the suppression of gonadotrophin secretion (Rivier & Vale 1984; Dunbar 1985; Nikolarakis *et al.* 1986; Rivier *et al.* 1986; Olster & Ferin 1987). Release of adrenocorticotrophin from the pituitary, which acts on the adrenal cortex to release the glucocorticoids, cortisol and corticosterone, may be activated directly by stressors or may be regulated by corticotrophin-releasing hormone (CRH; Przewlocki 1993). Possible pathways through which the effects of stress may affect the HPG axis include the inhibition of GnRH secretion by CRH and glucocorticoids, a glucocorticoid mediated decrease in the responsiveness of the pituitary to GnRH and a direct action of glucocorticoids on the gonads (Johnson *et al.* 1992). Thus, rank-related social infertility may result from the physiological effects of stress imposed by a dominant, reproductive female (Macann 1995 in Abbott *et al.* 1997; Wasser & Starling 1988).

The negative effects of both gonadal steroids and adrenal corticosteroids on gonadotrophin secretion may be mediated through endogenous opioid peptides (EOP). EOP are neurosecretory peptides that negatively affect gonadotrophin secretion (Almeida 1993). EOP play a role in the regulation of gonadotrophin secretion during normal sexual function in mammals. The first concrete evidence for the opioidergic control of reproduction was the demonstration that administration of morphine, an opioid agonist, inhibited ovulation in the rat when administered on the afternoon of pro-oestrus (Barraclough & Sawyer 1955). Observations made during different phases of the oestrous cycle provide evidence for opioidergic mediation of the negative feedback effects of gonadal steroids on gonadotrophin secretion. In general, the highest concentration of the opioid β-endorphin (Wehrenberg *et al.* 1982; Ferin *et al.* 1984) and maximal LH responses to the opioid antagonist naloxone (Quigley & Yen 1980; Barb *et al.* 1986; Currie & Rawlings 1987; Behrens *et al.* 1993) occur during the luteal phase in a variety of species. Furthermore, concentrations of β-endorphin in the hypophyseal portal blood, which are undetectable following ovariectomy, can be

restored by progesterone replacement (Wardlaw *et al.* 1982; Wehrenberg *et al.* 1982; Schoupe *et al.* 1985; Brooks *et al.* 1986). This indicates a high level of opioid binding and subsequent inhibition of LH secretion during periods corresponding to high levels of progesterone secretion, suggesting that EOP mediate the negative effects of progesterone on gonadotrophin secretion. EOP may also mediate the negative effects of stress on the reproductive axis (see review by Prezewlocki 1993). CRH stimulates the release β-endorphin from anterior pituitary corticotrophs (Johnson *et al.* 1992), whereas blockade of CRH receptors with α-helical CRF_{9-41} results in a significant decrease in opioid levels and a concomitant increase in GnRH secretion. Withdrawal of the CRH antagonist causes an increase in opioid and decrease in GnRH levels (Almeida *et al.* 1987). Furthermore, CRH induced gonadotrophin suppression can be reduced or reversed by naloxone treatment in rats, monkeys and humans (Almeida & Herz 1986; Gindoff & Ferin 1987; Barbarino *et al.* 1989), and treatment with antibodies that recognize endogenous opioids, stops the decrease in serum LH levels following CRH administration (Petraglia *et al.* 1986; Almeida *et al.* 1987).

EOP have been implicated in the suppression of LH secretion in many forms of naturally induced infertility, including seasonal anoestrus (Roberts *et al.* 1985; Brooks *et al.* 1986; Roberts *et al.* 1987; Aurich *et al.* 1994), stress induced infertility (Johnson *et al.* 1992) and lactation (Sirinathsinghji & Martini 1984; Mattioli *et al.* 1986). Furthermore, differences in the levels of β-endorphin in the cerebro-spinal fluid of male talapoin monkeys (*Miopithecus talapoin*) were found to be related to dominance status (Martensz *et al.* 1986). EOP are, therefore, prime candidates in the suppression of gonadotrophin secretion and fertility in NRF Damaraland mole-rats. The opioid antagonist naloxone is a valuable tool which can be used to study the role of the opioid, β-endorphin, in the suppression of the reproductive axis. However, since the potential of the anterior pituitary to secrete LH is reduced in NRF Damaraland mole-rats, it may be necessary to sensitise the pituitary using exogenous GnRH or naloxone or increased endogenous GnRH secretion that occurs following hystero-ovariectomy.

The aim of this chapter was to investigate the mechanisms through which GnRH and/or LH secretion is suppressed. More specifically, the following three hypotheses

were tested: Firstly, an increase in the activity of EOP is responsible for mediating the negative effects of the social environment on the reproductive axis. Secondly, social suppression of fertility is mediated solely through the negative feedback effects of gonadal steroids. Thirdly, socially imposed stress and a consequent increase in cortisol levels contributes to the suppression of the reproductive axis in non-reproductive females. The three hypotheses are not mutually exclusive.

Materials and methods

Study animals

For GnRH and naloxone challenges, 10 NRF originating from three wild caught colonies and 11 non-reproductive females housed in the absence of the breeding pair (rNRF), which originated from two colonies that had been caught without the breeding female, were used. The largest males were removed from the colonies, which were then maintained in the laboratory for six months and one year, respectively, before experimentation. Reproductive females (RF; n = 10) were obtained from five wild caught colonies and from five colonies that were formed in the laboratory by pairing unrelated, opposite sexed individuals. Hystero-ovariectomised non-reproductive females (ovxNRF; n = 10) originated from four wild caught colonies and two colonies created in the laboratory. Hystero-ovariectomised reproductive females (ovxRF; n = 10) originated from one wild caught colony and from 9 colonies created in the laboratory. After hystero-ovariectomy, animals were allowed to recover for two months prior to experimentation. For cortisol determination, blood samples were collected from 16 NRF from 7 wild caught colonies and 12 RF, 10 of which were wild caught and two laboratory generated.

Experimental design

Experiment 1

a) Single naloxone challenge

To investigate the possible opioidergic suppression of GnRH/LH secretion, the LH response to a single, 500µg naloxone challenge was measured in RF, NRF, rNRF,

ovxRF and ovxNRF (n = 10 in each group). Control animals (n = 6 in each group) received physiological saline.

b) Naloxone challenge after priming with GnRH

Since pituitary desensitivity may play a role in masking the effects of naloxone on GnRH secretion, NRF (n = 10) and rNRF (n = 10) were primed with 2.0µg GnRH every eight hours for 96 hours in an attempt to sensitise the pituitary to naloxone. Basal plasma LH and the response to a 500µg naloxone challenge were determined immediately before and two hours after the final GnRH challenge. The experiment was repeated in ovxNRF. Control animals (n = 6 in each group) received physiological saline instead of GnRH.

c) GnRH challenge after priming with naloxone

In a related experiment, two experimental designs were used in an attempt to sensitise the pituitary to an exogenous 2.0µg GnRH challenge through naloxone priming. NRF (n = 10) and rNRF (n = 10) were primed with 500µg naloxone, every eight hours for 96 hours. Basal levels of LH and the plasma LH responses to a single, 2.0µg GnRH challenge were determined immediately before the first and two hours after the last naloxone injection. The experimental protocol was repeated using ovxRF (n = 10) and ovxNRF (n = 10). In the second experiment, ovxRF (n = 10) and ovxNRF (n = 10) received hourly priming with 250µg naloxone for ten hours. The plasma LH response to a single, 2.0µg GnRH challenge immediately before the first and one hour after the last naloxone injection was used to determine the effect of naloxone priming on pituitary sensitivity. Control animals (n = 6 in each group) in both experiments received physiological saline instead of naloxone.

Experiment 2

To investigate the role of ovarian steroids in the suppression of LH secretion in NRF, basal and 2.0µg GnRH stimulated LH secretion in NRF (n = 27) and RF (n = 10) presented in Chapter 3 (Fig. 3.1a) were compared to ovxNRF (n = 10) and ovxRF (n = 10), respectively. In addition, plasma progesterone concentrations in NRF (n = 9), rNRF (n = 6) and RF (n = 10), measured in Chapter 3 (Fig. 3.3), are presented.

Experiment 3

Cortisol determination

Blood samples were collected from RF (n = 12) and NRF (n = 16) at two different times of the day (at least five days apart), from 08:00 to 09:30 and from 16:00 to 17:30, to minimise variation resulting from the nyctohemeral cycle of cortisol secretion.

Results

Experiment 1

Single naloxone challenge

Plasma LH secretion in response to a single naloxone challenge did not differ from basal LH levels in NRF, rNRF or RF (Fig. 6.1). Statistical values are provided in Table 6.1. Similarly, no significant differences were found between basal LH levels and the LH response to a single control saline injection.
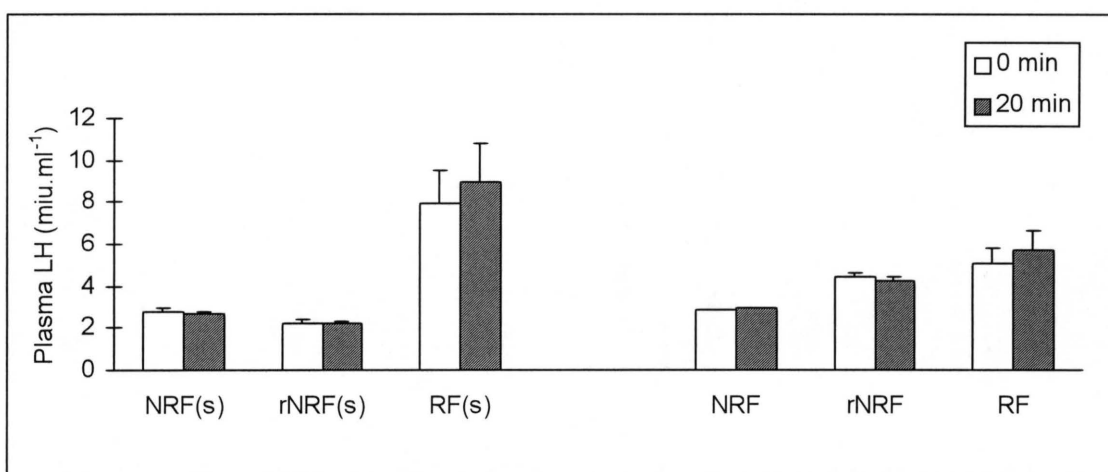
Fig. 6.1. Mean (± sem) basal plasma LH (0min) and the plasma LH response (20min) to a single, 500µg naloxone injection or single injection of a placebo saline control (s). NRF, non-reproductive females; rNRF, non-reproductive females housed in the absence of the breeding pair; RF, reproductive females.

Single naloxone challenge following GnRH priming

The LH response to a naloxone challenge was significantly higher than basal LH levels after GnRH priming in both NRF and rNRF (Fig. 6.2). LH concentrations and

statistical values are presented in Table 6.2. Control animals showed no LH response to a naloxone challenge.
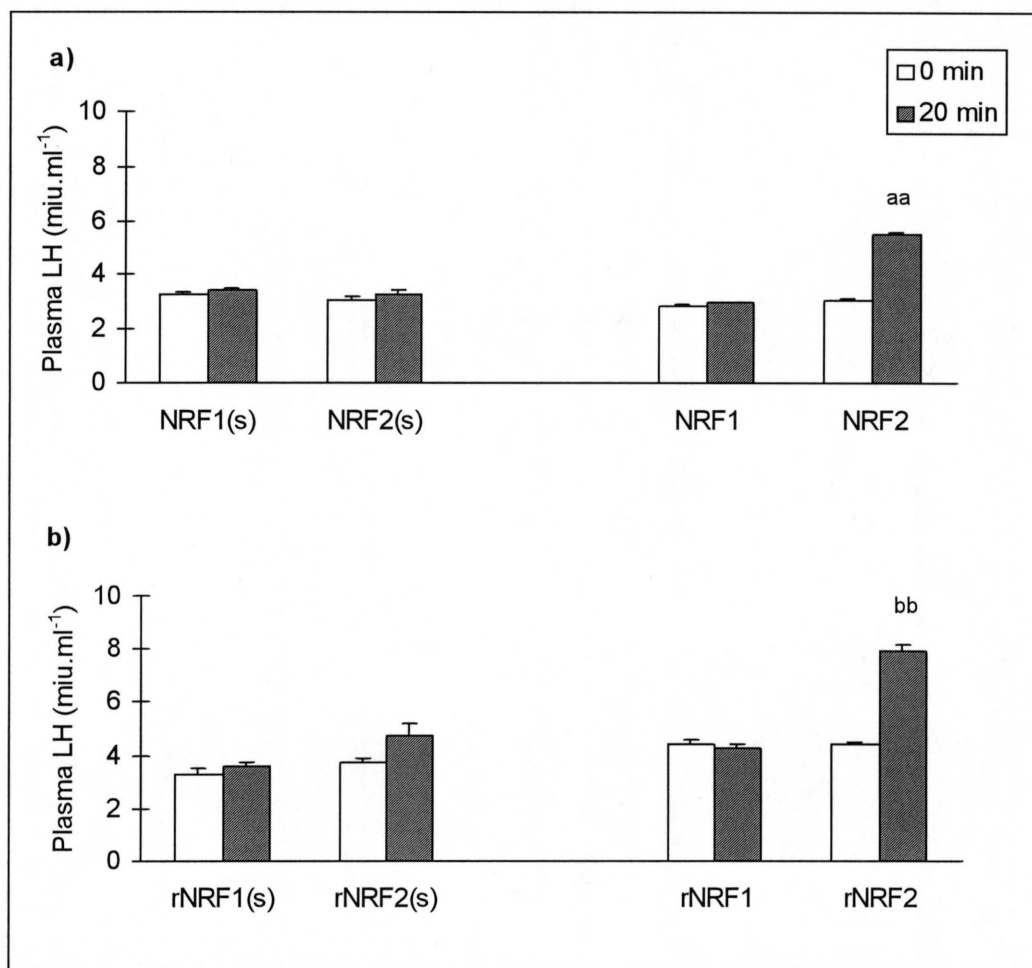
Fig. 6.2. Mean (± sem) basal plasma LH (0min) and the plasma LH response (20min) to a single, 500μg naloxone challenge immediately before (1) and two hours after 2.0μg GnRH priming (2), every eight hours for 96 hours, or after multiple injections of a placebo saline control (s). **a)** NRF, non-reproductive females; **b)** rNRF, non-reproductive females housed in the absence of the breeding pair. aa: *P* < 0.005 *vs* 0min, NRF2, 2.0μg dose; bb: *P* < 0.005 *vs* 0min, rNRF2, 2.0μg dose (Mann-Whitney U test).

Single GnRH challenge following naloxone priming

No difference was found between basal plasma LH concentrations before and one hour after naloxone priming every eight hours for 96 hours, in either NRF or rNRF (Fig. 6.3). LH secretions and statistical comparisons are presented in Table 6.3. LH concentrations in response to a 2.0μg GnRH challenge did not differ significantly

before or after naloxone priming in intact NRF or rNRF. Similarly, treatment with placebo saline resulted in no difference between basal or GnRH stimulated LH values in NRF and rNRF.
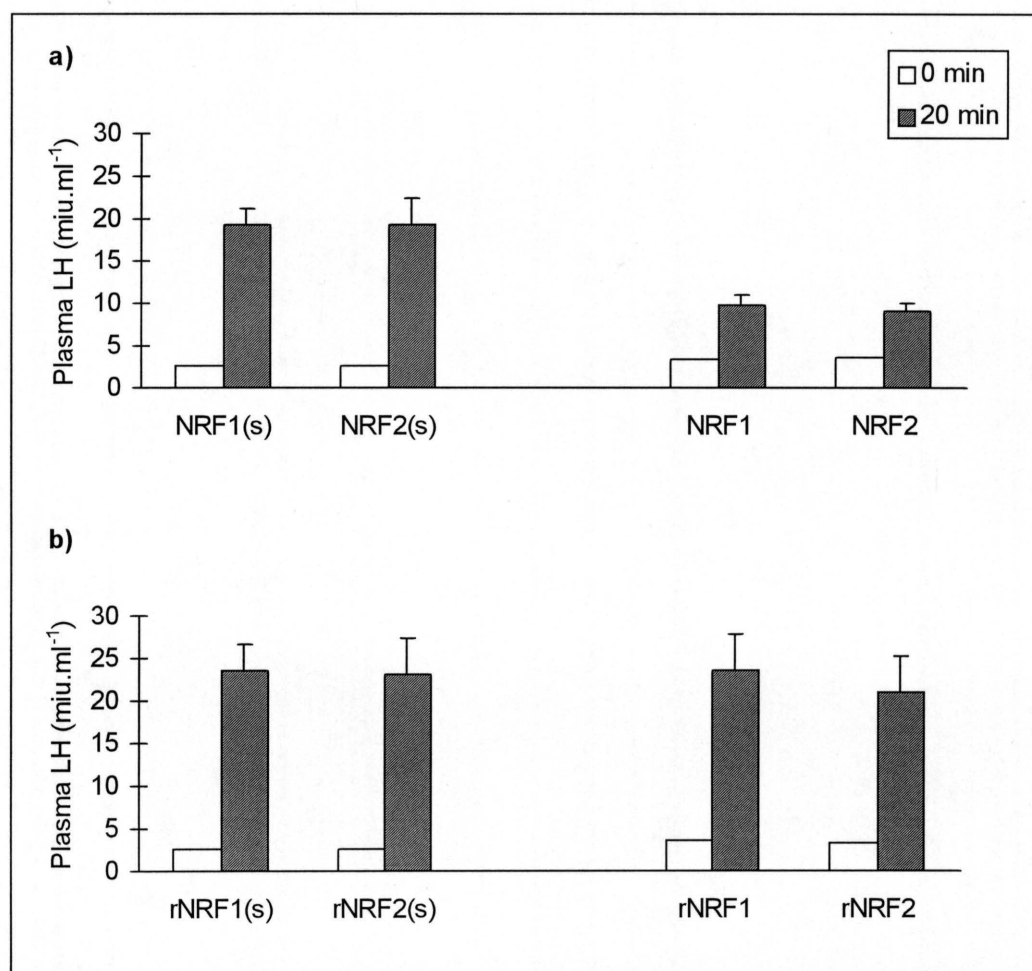
Fig. 6.3. Mean (± sem) basal (0min) and 2.0μg GnRH stimulated (20min) levels of plasma LH before (1) and one hour after (2) either naloxone priming, 500μg, eight hourly for 96 hours or injections of a saline control (s). **a)** NRF, non-reproductive females **b)** rNRF, non-reproductive females removed from the presence of the breeding pair.

Naloxone challenge following GnRH priming in hystero-ovariectomised females

Neither ovxRF nor ovxNRF showed a significant difference between plasma LH levels before and after a 500μg naloxone challenge (Fig. 6.4). Similarly, control animals showed no response to a saline injection. LH concentrations and statistical values are presented in Table 6.4. Priming with exogenous 2.0μg GnRH every eight hours for 96

hours in ovxNRF did not result in a significant difference between LH concentrations before and after a 500µg naloxone challenge (Fig. 6.5). There was no response to a naloxone challenge in control animals (Table 6.5).
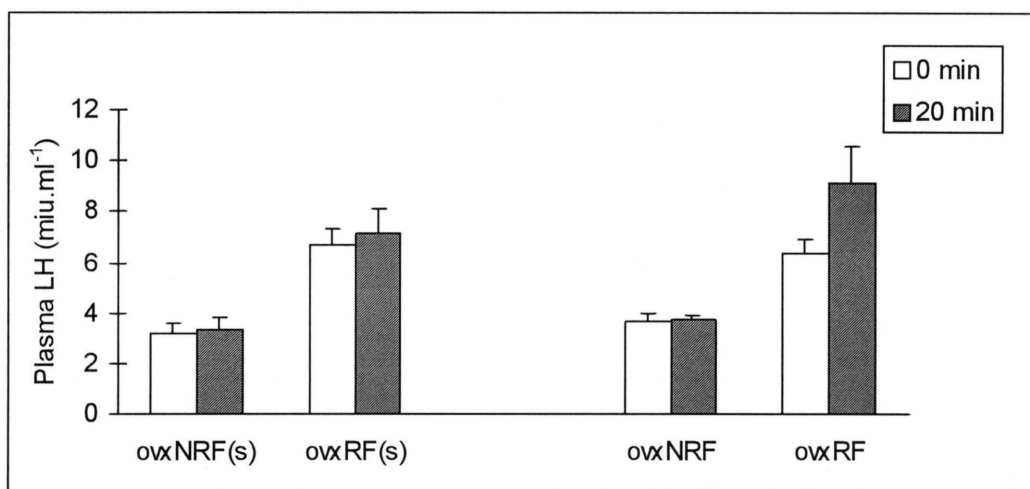
Fig. 6.4. Mean (± sem) basal plasma LH (0min) and the plasma LH response (20min) to a single, 500µg naloxone injection or single injection of a placebo saline control (s). ovxNRF, hystero-ovariectomised non-reproductive females; ovxRF, reproductive females.
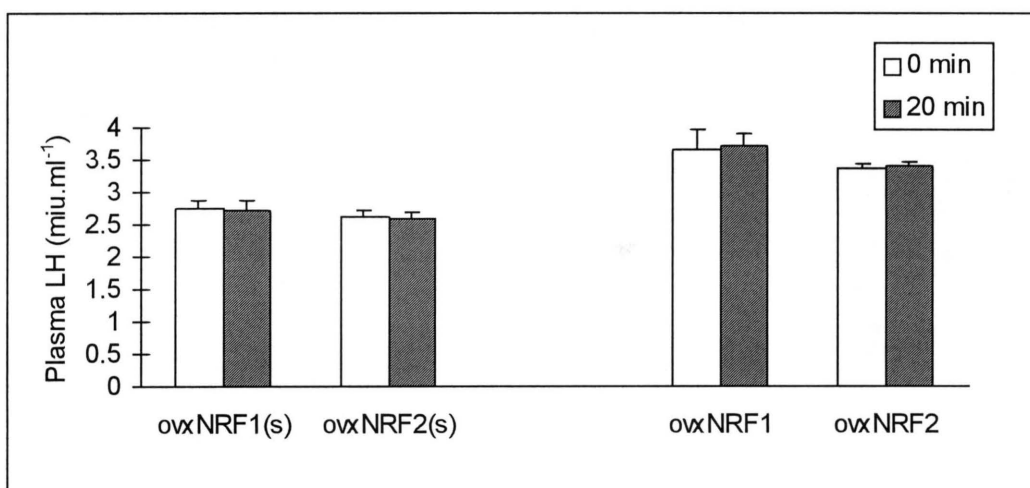
Fig. 6.5. Mean (± sem) basal plasma LH (0min) and the plasma LH response (20min) to a single, 500µg naloxone challenge immediately before (1) and two hours after 2.0µg GnRH priming (2), every eight hours for 96 hours, or after multiple injections of a placebo saline control (s). ovxNRF, hystero-ovariectomised non-reproductive females.

GnRH challenge following naloxone priming in hystero-ovariectomised females

Neither ovxNRF nor ovxRF showed a significant change in GnRH stimulated LH secretion following hourly naloxone priming (250µg) for ten hours (Fig. 6.6). Similar results were obtained using placebo saline in control animals. LH concentrations and statistical values are provided in Table 6.6. In ovxNRF primed every eight hours for 96 hours with 500µg naloxone, GnRH stimulated LH concentrations were significantly lower than prior to naloxone priming (Fig. 6.7). Although GnRH stimulation naloxone LH secretion following priming was also lower in ovxRF, the difference was not significant. Control animals showed no difference between GnRH stimulated LH secretion before and after saline treatment (Table 6.6).
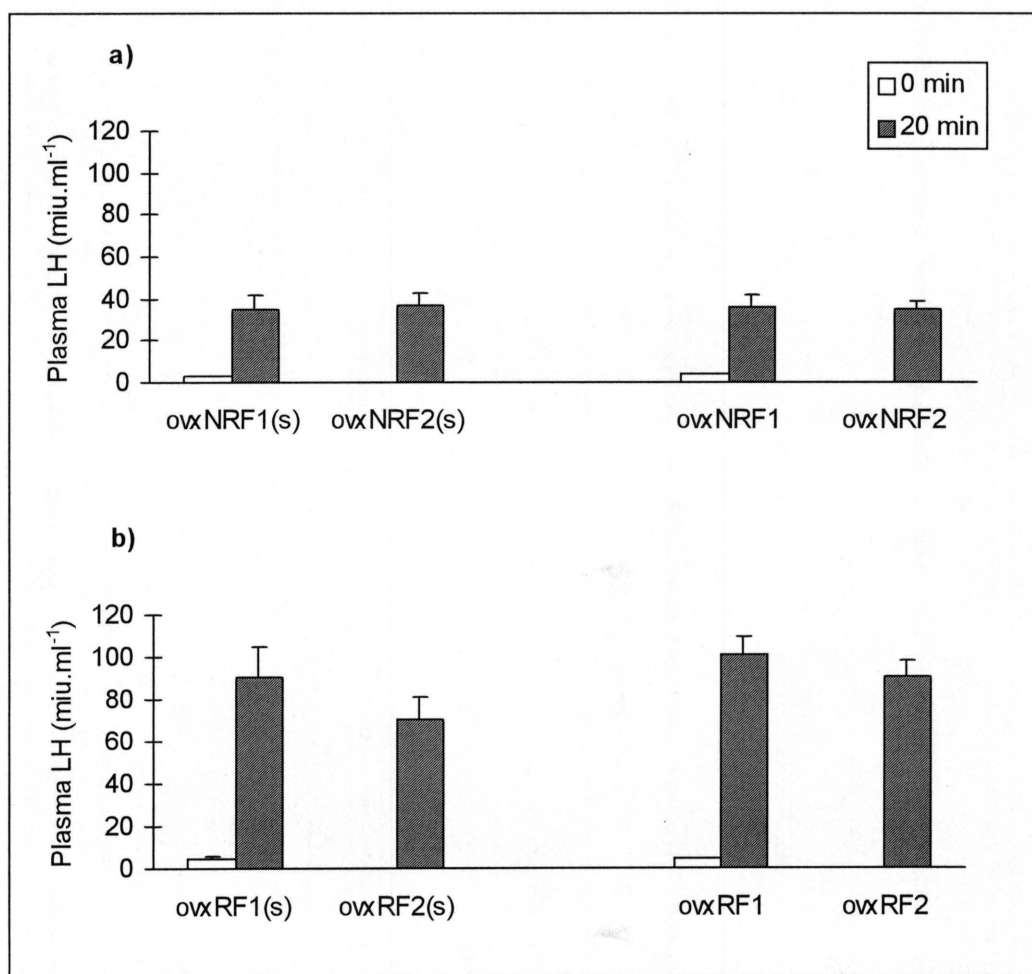
Fig. 6.6. Mean (± sem) basal (0min) and 2.0µg GnRH stimulated (20min) levels of plasma LH before (1) and one hour after (2) either naloxone priming, 250µg, hourly for 10 hours, or injections of a saline control (s). **a)** ovxNRF, hystero-ovariectomised non-reproductive females; **b)** ovxRF, hystero-ovariectomised reproductive females.
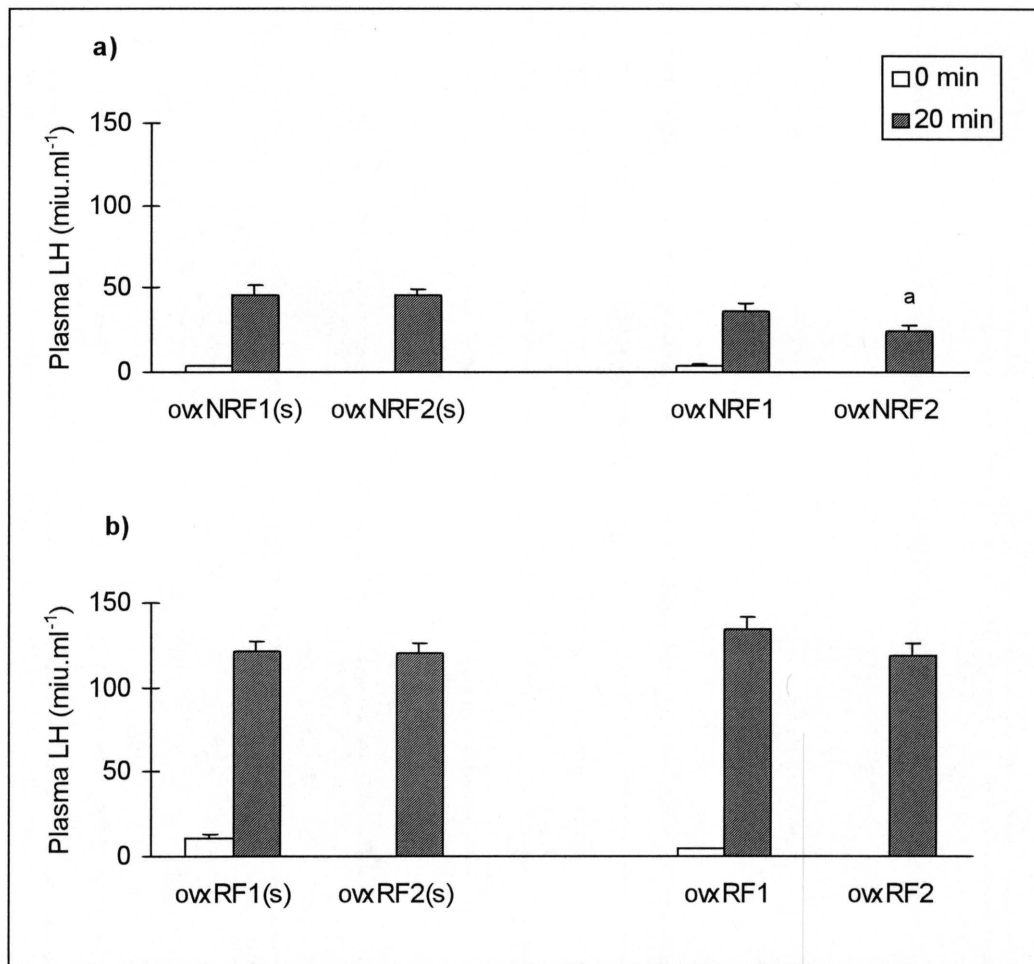
Fig. 6.7. Mean (± sem) basal (0min) and 2.0μg GnRH stimulated (20min) levels of plasma LH before (1) and one hour after (2) either naloxone priming, 500μg, eight hourly for 96 hours, or injections of a saline control (s). **a)** ovxNRF, hystero-ovariectomised non-reproductive females; **b)** ovxRF, hystero-ovariectomised reproductive females. a: $P < 0.05$ *vs* 20min, NRF1, 2.0μg dose (Mann-Whitney U test).

Experiment 2

GnRH challenges

Basal and GnRH stimulated LH secretion before and after hystero-ovariectomy in reproductive and non-reproductive females are presented in Fig. 6.8. The response to an exogenous, 2.0μg GnRH challenge was significantly higher than basal plasma LH levels in both ovxRF and ovxNRF (Table 6.7). There was no response to a saline injection in control ovxRF and ovxNRF. Intact RF and NRF showed significantly lower GnRH (2.0μg) stimulated LH secretion than ovxRF and ovxNRF, respectively (Table 6.8). Although the basal levels of plasma LH were significantly different between ovxNRF and NRF, those of reproductive females were not. As observed in

intact females, both basal and GnRH stimulated concentrations in ovxNRF were significantly lower than the respective concentrations in ovxRF (Table 6.9).
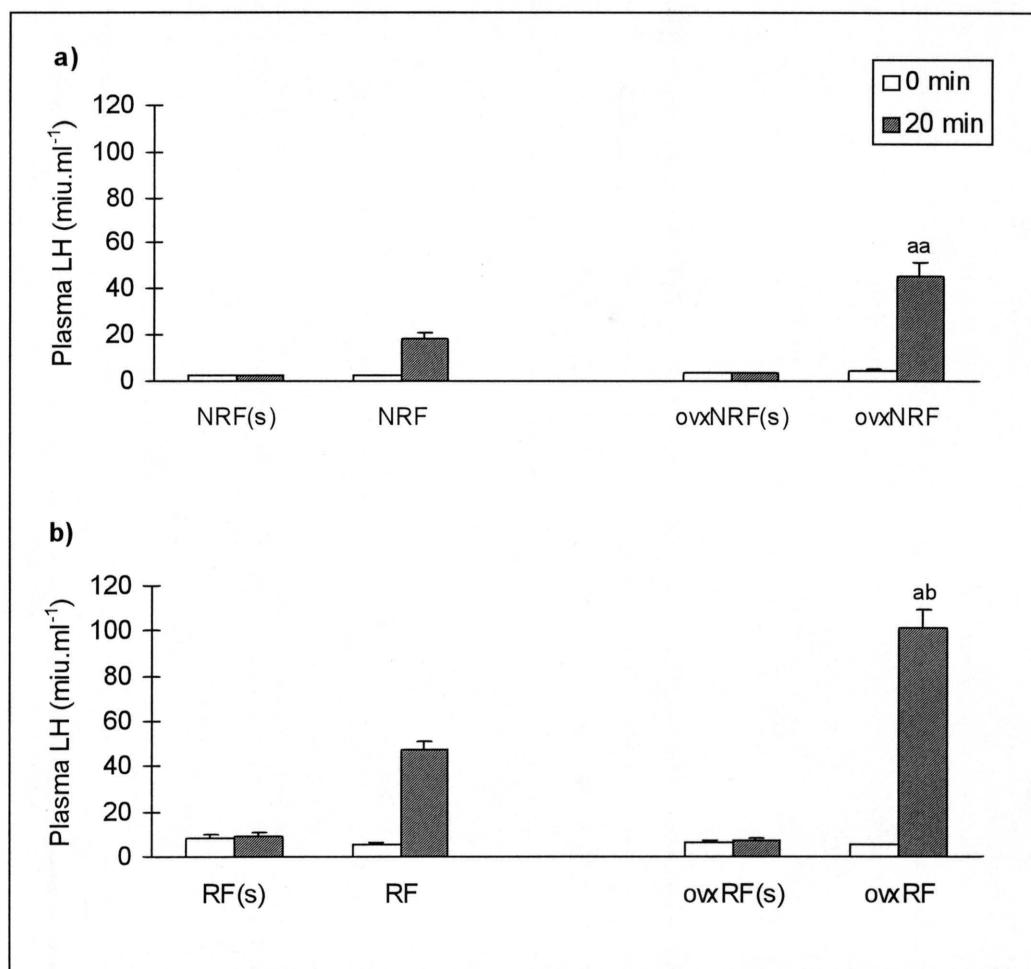
Fig. 6.8. Plasma LH concentrations (mean ± sem) before (0min) and after (20min) a single, 2.0µg GnRH challenge or a single saline injection (s). **a)** NRF, intact non-reproductive female; ovxNRF, hystero-ovariectomised non-reproductive female; **b)** RF, reproductive female; ovxRF, hystero-ovariectomised reproductive female. aa: $P < 0.0002$ *vs.* 0min, ovxNRF; $P < 0.01$ *vs.* 20min, NRF. ab: $P < 0.0002$ *vs.* 0min, ovxRF; $P < 0.001$ *vs.* 20min, RF; $P < 0.0005$ *vs.* 20min, ovxNRF (Mann-Whitney U test).

Progesterone concentrations

Circulating progesterone concentrations ranged from 0.9 - 4.2nmol.l⁻¹ in NRF, 3.8 - 33.9nmol.l⁻¹ in rNRF and from 4.7nmol.l⁻¹ to more than 60nmol.l⁻¹ in RF. Progesterone concentrations in the three pregnant RF exceeded the upper limit of the assay and were assigned concentrations of 60nmol.l⁻¹, which corresponds to values obtained at approximately 15% binding on the assay standard curve. The mean progesterone

concentration in rNRF was significantly higher than in NRF (Mann-Whitney U test, U = 1, $P < 0.005$, $N_1 = 6$, $N_2 = 9$; Fig. 6.9). In contrast to NRF in which progesterone concentrations did not exceed 5.0nmol.l^{-1}, only three out of 10 RF had progesterone concentrations below 10.0nmol.l^{-1}. The mean progesterone concentration in RF was significantly higher than NRF (Mann-Whitney U test, $U = 10$, $P < 0.0005$, $N_1 = 9$, $N_2 = 10$).
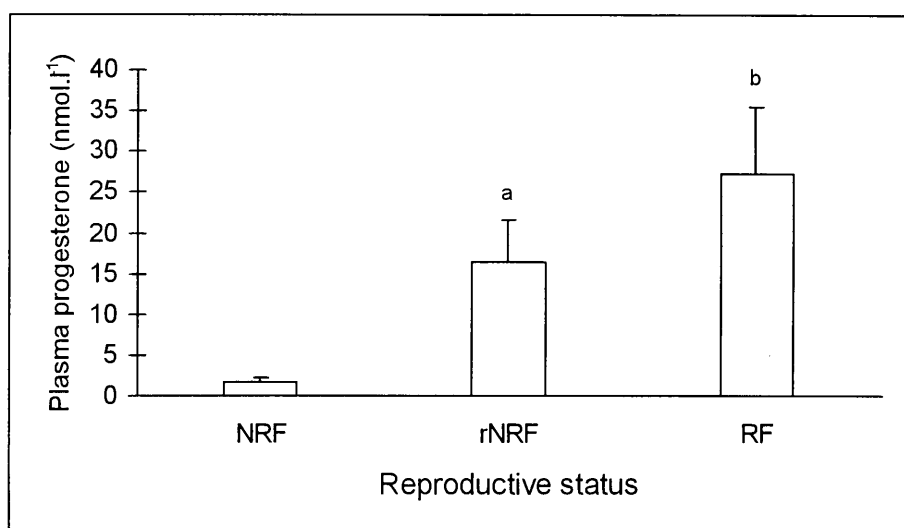
Fig. 6.9. Mean ($\pm$ sem) circulating progesterone concentrations. NRF, non-reproductive females; rNRF, non-reproductive females removed from the breeding pair; RF, reproductive females that have produced young. a: $P < 0.005$ vs. non-breeding females. b: $P < 0.0005$ vs. non-breeding females (Mann-Whitney U test following Kruskal-Wallis analysis).

Experiment 3

Plasma cortisol concentrations

Plasma cortisol concentrations, measured in NRF and RF between 08:30 and 09:00 hours and 16:00 - 16:30 hours, are presented in Fig. 6.10. There were no significant differences in the mean cortisol concentrations between the two groups of females at either time period (ANOVA, $F_{1-3} = 1.49$, $P > 0.05$, $N = 56$). The mean cortisol concentrations are characterised by a high level of variation within each group.
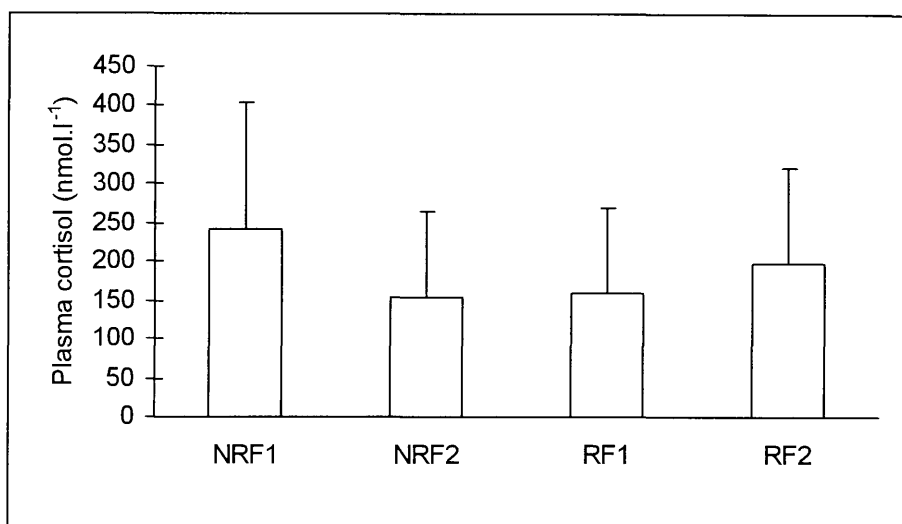
Fig. 6.10. Circulating concentrations of cortisol (mean ± sem) sampled between 08:30 and 09:00 hours (1) and 16:00 and 16:30 hours (2). NRF, non-reproductive females; RF, reproductive females.

Discussion

Endogenous opioid peptides

The results obtained in this study suggest that EOP do not play a role in socially-induced suppression of gonadotrophin secretion in NRF Damaraland mole-rats. No change in plasma gonadotrophin levels was found in response to a single injection of naloxone in intact NRF, rNRF or RF (Fig. 6.1). Comparable results were obtained in intact, subordinate marmoset monkeys (Abbott 1988) and naked mole-rats (Faulkes 1990). Abbott (1988) suggested that short term blockade of opiate receptors may be insufficient to stimulate an increase in plasma LH levels. In seasonal breeders a constant infusion of naloxone for three hours resulted in an increase in LH secretion (Schillo *et al.* 1984). The reduced potential of the pituitary to secrete LH in NRF Damaraland mole-rats (Chapter 3 & 4) may contribute to the absence of a LH response to naloxone. However, attempts to sensitise the pituitary to GnRH using naloxone priming were unsuccessful (Fig. 6.3). The reason for the low GnRH stimulated LH values in NRF compared to those obtained in the saline control is unknown, but may possibly be due to degradation of the exogenous GnRH prior to the challenges. Although naloxone administration had a stimulatory effect on LH secretion in NRF after priming with GnRH, it is likely that this reflects general EOP activity during normal sexual function, rather than a role in social suppression of fertility, since naloxone had a similar stimulatory effect on plasma LH secretion in rNRF (Fig. 6.2).

The increase in endogenous GnRH secretion that is a consequence of ovariectomy results in sensitisation of the pituitary and may provide a more appropriate model with which to study the effects of naloxone on gonadotrophin secretion. In contrast to intact female marmosets, a similar, significant increase in LH secretion occurs in response to the administration of a single injection of GnRH and naloxone in ovariectomised, subordinate females (Abbott *et al.* 1987). This suggests that EOP play a role in the social suppression of fertility in marmosets and, furthermore, that they act independently of ovarian steroid negative feedback. Naloxone administration did not, however, result in an increase in GnRH stimulated plasma LH levels in ovxNRF Damaraland mole-rats (Fig. 6.4), even after GnRH priming (Fig. 6.5). Furthermore, priming with naloxone using two different priming regimes did not increase basal or GnRH stimulated LH levels (Fig. 6.6 & 6.7). Thus, there is no evidence to suggest that EOP play a role in the suppression of gonadotrophin secretion in NRF Damaraland mole-rats, via either a gonadal dependent or independent mechanism. However, an inadequate experimental procedure, e.g. an incorrect dose and/or priming regime may be responsible for the lack of an LH response to naloxone administration. Alternatively, a different class of opioids e.g. dynorphins or enkephalins, rather than the endorphins, may be involved in mediating the effects of inhibitory social cues on gonadotrophin secretion .

Negative feedback effects of gonadal steroids

Circulating progesterone levels were found to be significantly higher in rNRF than in NRF and were comparable to those of RF (Fig. 6.9). This contradicts an earlier study by Bennett *et al.* (1996) which shows that urinary progesterone concentrations in subordinate females decrease significantly when the breeding female is removed from the colony. The authors postulate that progesterone produced by LUF contributes to suppression of gonadotrophin secretion in NRF via increased negative feedback effects due to the higher levels of progesterone. However, the present study suggests that this hypothesis is incorrect. Although LUF produced measurable quantities of progesterone in NRF, progesterone produced by the corpora lutea in rNRF exceeded these levels (Fig. 6.9). Thus, if progesterone does play a role in the suppression of gonadotrophin secretion, increased negative feedback effects of progesterone in NRF may be due to

an increased sensitivity of the pituitary and/or hypothalamus to these effects, rather than an increased concentrations of progesterone. Thus, suppression of gonadotrophin secretion may result from a modification of the sensitivity of the hypothalmo-pituitary axis to gonadal steroid negative feedback. The development of an increased sensitivity to the negative feedback effects of oestrogen, via its negative effect on LH pulse amplitude, has been demonstrated in socially suppressed marmoset monkeys (Abbott 1988). In ovariectomised subordinate females, a low dose oestradiol implant results in a large decrease in plasma LH levels whereas there is no decrease in plasma LH levels of dominant female (Abbott *et al.* 1988). A number of studies have demonstrated a similar increase in sensitivity to the negative feedback effects of gonadal steroids in seasonally breeding, ovariectomised ewes (Legan *et al.* 1977; Martin *et al.* 1983; Karsch *et al.* 1984). Marked seasonal differences in the effect of oestrogen on the GnRH pulse generator occurs in ovariectomised ewes treated with a constant release Silastic implant of oestradiol. Thus, it is possible that both progesterone and oestrogen play a role in suppression of the reproductive axis, progesterone through its effect on the frequency of the GnRH pulse generator and oestrogen through its negative effect on the LH pulse amplitude.

In this study, hystero-ovariectomy and consequent absence of gonadal steroid negative feedback resulted in a significant increase in the sensitivity of the pituitary to GnRH in both ovxNRF and ovxRF. However, as found in intact females (Chapter 3, Fig. 3.1), GnRH stimulated LH secretion was still significantly lower than in reproductive females (Fig. 6.8). Thus, although gonadal steroids clearly play an important role in determining the plasma LH levels in intact females via their negative feedback effects, hystero-ovariectomy does not abolish the suppression of gonadotrophin secretion in NRF. This strongly suggests that a gonadal steroid independent mechanism is involved in the suppression of gonadotrophin secretion, although it is possible that steroids produced by the adrenal gland may effect of LH secretion. Moreover, this does not preclude the presence of a gonadal dependent mechanism. Interestingly, in addition to an increased sensitivity to negative feedback of oestrogen, both socially suppressed marmoset monkeys (Abbott 1988) and seasonally anoestrous ewes exhibit a decrease in the frequency of LH pulses during the anoestrous season in ovariectomised females,

suggesting a gonadal independent mechanism may also be involved in the seasonal control of LH release (Legan *et al.* 1977; Goodman *et al.* 1982).

Stress induced infertility

The well known negative effect of stress on fertility (Dunbar 1985, Johnson *et al.* 1992) makes stress imposed by the RF a prime candidate in the suppression of fertility in NRF. In subordinate female gelada baboons (*Theropithecus gelada*), overt harassment by the dominant females is associated with elevated circulating and urinary cortisol levels and a decreased frequency of ovulation (McCann 1995 in Abbott *et al.* 1997). Similarly, increased aggression toward subordinate male talapoin monkeys results in increased cortisol and prolactin in subordinates and an inhibition of sexual behaviour (Keverne *et al.* 1984). However, in this study, circulating levels of cortisol in NRF were not significantly different from those in RF (Fig. 6.10). High individual variation in cortisol concentrations was observed in each group. This may be a result of the nyctohemeral cycle of cortisol secretion, despite sampling at two different times during the day, or may be due to differential stress responses to the sampling procedure. Cortisol concentrations obtained from individuals in two freshly trapped colonies revealed similar results to those obtained in this study (N.C. Bennett & C. Faulkes, *unpubl. data*). In common marmosets and cotton-top tamarins, urinary cortisol levels were in fact significantly higher in dominant compared to subordinate females (Abbott *et al.* 1981; Ziegler *et al.* 1995). This contrasts with predictions based on the Dominant Control Model of reproductive suppression (Snowdon 1996), which has commonly been invoked to explain socially-induced infertility in cooperatively breeding mammals (Abbott 1987; Faulkes & Abbott 1993; Bennett 1994). The lack of overt harassment or elevated cortisol or prolactin concentrations in subordinates marmoset monkeys and naked mole-rats, led Abbott *et al.* (1997) to hypothesise that a specialised neuroendocrine mechanism, independent of generalised stress response, may be responsible for the inhibition of sexual behaviour and ovulation. This may well also be the case in *C. damarensis*, since, although a dominance hierarchy exists, overt acts of aggression by the dominant female towards subordinates seldomly occur (Bennett *et al.* 1994), which is consistent with the Self-Restraint Model of reproductive inhibition (Snowdon 1996).

Although reproductive inhibition through dominant control by the RF is not ruled out, subordinate females of some cooperatively breeding species may use the presence of the RF as a cue (be it via visual, tactile or olfactory cues) not to breed, in a similar manner to the use of inhibitory photoperiodic cues by seasonal breeders that abstain from reproducing during unfavourable times of the year. The presence of the RF in colonies of *C. damarensis* may represent a suboptimal breeding environment to NRF if one considers that multiple breeding females may increase demand for food (at a colony level) at a rate greater that that which it could be found. This would occur as a result of a decrease in the number of 'workers' and a simultaneous increase in number of young. One could thus envisage that the reproductive axis of both seasonally and socially infertile females may respond to different but equivalent extrinsic cues (presence of the breeding female and photoperiod respectively) in a similar manner. In this regard it is interesting to keep in mind the similarities between the physiological and neuroendocrine mechanisms that result in seasonally and socially-induced infertility. As elegantly expressed by Abbott *et al.* (1997), "It is intriguing to speculate that in species that encounter environmentally determined reproductive constraints, there has been convergent evolution of adaptive neuroendocrine responses."

Appendix

Table 6.1. Plasma LH concentrations (mean ± sem) before (0min) and 20 minutes after (20min) **a)** a single 500μg naloxone challenge and **b)** a control saline injection. The U, Z and probability values obtained following the Mann-Whitney U test are presented. NRF, intact non-reproductive females; rNRF, non-reproductive females housed in the absence of the breeding pair; RF, reproductive females.

	Reproductive status	Plasma LH (mIU.ml^{-1}) 0min	N	Plasma LH (mIU.ml^{-1}) 20min	N	U	Z	P
a)	NRF	2.8 ± 0.06	10	3.0 ± 0.4	10	9.5	-1.4	0.2
	rNRF	4.5 ± 0.1	10	4.3 ± 0.1	10	10	-1.3	0.2
	RF	5.1 ± 0.7	10	5.7 ± 0.9	10	41.5	-0.6	0.5
b)	NRF	8.0 ± 1.5	6	9.0 ± 1.8	6	15	-0.5	0.6
	rNRF	2.7 ± 0.2	6	2.7 ± 0.1	6	16	-0.3	0.7
	RF	2.2 ± 0.2	6	2.2 ± 0.1	6	15.5	-0.4	0.7

Table 6.2. Plasma LH concentrations (mean ± sem) before (0min) and 20 minutes after (20min) a single 500μg naloxone challenge **a)** two hours after eight hourly priming for 96 hours with GnRH (2.0μg) and **b)** two hours after control saline eight injection over the same period. Results of the Mann-Whitney U test are presented. NRF, intact non-reproductive females; rNRF, non-reproductive females housed in the absence of the breeding pair. Statistical significance (α = 0.05) is indicated by *.

	Reproductive status	Plasma LH (mIU.ml^{-1}) 0min	N	Plasma LH (mIU.ml^{-1}) 20min	N	U	Z	P
a)	NRF	3.1 ± 0.07	10	5.5 ± 0.1	10	0	-3.8	0.0002*
	rNRF	4.4 ± 0.1	10	8.0 ± 0.2	10	0	-3.8	0.0002*
b)	NRF	3.1 ± 0.1	6	3.3 ± 0.2	6	12	-1	0.3
	rNRF	3.8 ± 0.2	6	4.8 ± 0.5	6	6.5	-1.9	0.1

Table 6.3. Plasma LH concentrations (mean ± sem) immediately before (0min) and 20 minutes after (20min) a single 2.0μg GnRH challenge, prior to (pre naloxone) and two hours after (post naloxone) a) eight hourly priming for 96 hours with 500μg naloxone and b) control saline eight injections over the same period. Results of the Mann-Whitney U test are presented. NRF, intact non-reproductive females; rNRF, non-reproductive females housed in the absence of the breeding pair.

		Reproductive status	Plasma LH $(mIU.ml^{-1})$ pre naloxone	N	Plasma LH $(mIU.ml^{-1})$ post naloxone	N	U	Z	P
a)	0min	NRF	3.3 ± 0.04	10	3.5 ± 0.1	10	37	-1	0.3
	0min	rNRF	3.5 ± 0.1	10	3.4 ± 0.1	10	33.5	-1.2	0.2
	20min	NRF	9.8 ± 1.1	10	9.0 ± 0.9	10	46.5	-0.3	0.8
	20min	rNRF	23.6 ± 4.3	10	20.9 ± 4.2	10	42	-0.6	0.5
b)	0min	NRF	2.7 ± 0.04	6	2.6 ± 0.1	6	13	-0.8	0.4
	0min	rNRF	2.6 ± 0.06	6	2.7 ± 0.06	6	12	-1	0.3
	20min	NRF	19.2 ± 1.9	6	19.3 ± 3.2	6	17	-0.2	0.9
	20min	rNRF	23.5 ± 3.2	6	23.2 ± 4.2	6	16	-0.3	0.7

Table 6.4. Plasma LH concentrations (mean ± sem) before (0min) and 20 minutes after (20min) a) a single 500μg naloxone challenge and b) a single control saline injection. Results of the Mann-Whitney U test are presented. OvxNRF, hystero-ovariectomised non-reproductive; OvxRF, reproductive females.

	Reproductive status	Plasma LH $(mIU.ml^{-1})$ 0min	N	Plasma LH $(mIU.ml^{-1})$ 20min	N	U	Z	P
a)	OvxNRF	3.7 ± 0.3	10	3.7 ± 0.2	10	31	-0.8	0.4
	OvxRF	6.3 ± 0.6	10	9.1 ± 1.4	10	29	-1.6	0.1
b)	OvxNRF	3.2 ± 0.4	6	3.3 ± 0.5	6	14	-0.6	0.5
	OvxRF	6.7 ± 0.6	6	7.2 ± 0.9	6	14	-0.6	0.5

Table 6.5. Plasma LH concentrations (mean ± sem) before (0min) and 20 minutes after (20min) a single 500μg naloxone challenge **a)** two hours after eight hourly priming for 96 hours with GnRH (2.0μg) and **b)** two hours after control saline injections over the same period. Results of the Mann-Whitney U test are presented. OvxNRF, hystero-ovariectomised non-reproductive; OvxRF, reproductive females.

	Reproductive status	Plasma LH (mIU.ml^{-1}) 0min	N	Plasma LH (mIU.ml^{-1}) 20min	N	U	Z	P
a)	OvxNRF	3.4 ± 0.7	10	3.4 ± 0.7	10	44.5	-0.4	0.7
b)	OvxNRF	2.6 ± 0.1	6	2.6 ± 0.1	6	11	-0.3	0.8

Table 6.6. Plasma LH concentrations (mean ± sem) 20 minutes after (20min) a single GnRH (2.0μg) challenge **a)** one hour after hourly priming for 10 hours with 250μg naloxone or **b)** physiological saline **c)** two hours after eight hourly priming for 96 hours with naloxone (500μg) or **d)** physiological saline. Results of the Mann-Whitney U test are presented. OvxNRF, hystero-ovariectomised non-reproductive; OvxRF, reproductive females. Statistical significance (α = 0.05) is indicated by *.

	Reproductive status	Plasma LH (mIU.ml^{-1}) pre naloxone	N	Plasma LH (mIU.ml^{-1}) post naloxone	N	U	Z	P
a)	OvxNRF	35.5 ± 5.7	10	35.2 ± 3.8	10	39.5	-0.4	0.7
	OvxRF	101.0 ± 8.7	10	90.2 ± 7.7	10	37.5	-0.9	0.3
b)	OvxNRF	35.2 ± 5.9	6	36.3 ± 6.3	6	16.5	-0.2	0.8
	OvxRF	90.6 ± 14	6	71.0 ± 11.0	6	12	-0.9	0.3
c)	OvxNRF	35.7 ± 4.6	10	23.6 ± 4.0	10	21	-2.2	0.02*
	OvxRF	134.3 ± 7.8	10	118.9 ± 7.5	10	29	-1.6	0.1
d)	OvxNRF	45.9 ± 5.4		45.5 ± 4.3	6	16	-0.3	0.7
	OvxRF	120.6 ± 6.0		120.2 ± 5.3		17.5	-0.1	0.9

Table 6.7. Plasma LH concentrations (mean ± sem) before (0min) and 20 minutes after (20min) **a)** a single 2.0µg GnRH challenge and **b)** physiological saline. Results of the Mann-Whitney U test are presented. OvxNRF, hystero-ovariectomised non-reproductive; OvxRF, reproductive females. Statistical significance ($\alpha = 0.05$) is denoted by *.

	Reproductive status	Plasma LH (mIU.ml⁻¹) 0min	N	Plasma LH (mIU.ml⁻¹) 20min	N	U	Z	P
a)	OvxRF	4.9 ± 0.3	10	134.3 ± 7.8	10	0	-3.8	0.0002*
	OvxNRF	4.0 ± 0.5	10	35.7 ± 4.6	10	1.0	-3.7	0.0002*
b)	OvxRF	6.7 ± 0.6	6	7.2 ± 0.9	6	16.0	-0.32	0.75
	OvxNRF	3.2 ± 0.4	6	3.3 ± 0.5	6	14.0	-0.64	0.52

Table 6.8. A comparison of plasma LH concentrations (mean ± sem) between intact and hystero-ovariectomised (Ovx) reproductive and non-reproductive females prior to (0min) and 20 minutes after (20min) a single 2.0µg GnRH challenge. Results of the Mann-Whitney U test are presented. Statistical significance ($\alpha = 0.05$) is indicated by *.

		Intact females Plasma LH (mIU.ml⁻¹)	N	Ovx females Plasma LH (mIU.ml⁻¹)	N	U	Z	P
0min	Reproductive Female	5.2 ± 0.6	10	4.9 ± 0.3	10	46	-0.3	0.8
0min	Non-reproductive female	2.7 ± 0.02	27	4.0 ± 0.5	10	29.5	-3.7	0.0003*
20min	Reproductive Female	47.5 ± 3.3	10	134.3 ± 7.8	10	2	-3.6	0.0003*
20min	Non-reproductive female	19.2 ± 1.8	26	35.7 ± 4.6	10	40	-3.2	0.0001*

Table 6.9. Comparison of basal (0min) and 2.0µg GnRH stimulated (20min) LH concentrations (mean ± sem) between hystero-ovariectomised reproductive and non-reproductive females. Results of the Mann-Whitney U test are presented. Statistical significance ($\alpha = 0.05$) is indicated by *.

	Reproductive females Plasma LH (mIU.ml⁻¹)	N	Non-reproductive females Plasma LH mIU.ml⁻¹	N	U	Z	P
0min	4.9 ± 0.3	10	4.0 ± 0.5	10	22	-2.1	0.03
20min	134.3 ± 7.8	10	35.7 ± 4.6	10	4	-3.5	0.0005*

Chapter 7

Synthesis

The proximate mechanisms responsible for the anovulatory state of non-reproductive female (NRF) Damaraland mole-rats were investigated on both the social and physiological level. The effect of the social environment on the reproductive axis was examined to determine whether anovulation does, in fact, reflect infertility in subordinate females (Chapter 3). Histological and endocrine evidence suggests that NRF ovulate spontaneously when housed in the absence of the breeders. Thus, anovulation in NRF appears to result from inhibitory social cues and cannot simply be explained by a lack of copulatory stimulation. Since NRF are not induced ovulators but are infertile while they remain in the colony, anovulation has the potential to play a role in maintaining reproductive skew. However, the fact that removal of inhibitory social cues results in an increase in the activity of the hypothalamo-pituitary-gonadal axis in NRF does not preclude the same effect from occurring in response to an unfamiliar male entering the colony. Therefore, the effect of an unfamiliar male on NRF in the colony, both physiologically and behaviourally, requires further investigation to determine whether anovulation does, in fact, contribute to reproductive skew.

The endocrine and neuroendocrine mechanisms responsible for the infertility in NRF Damaraland mole-rats formed the primary focus of this thesis (Chapter 4, 5, & 6). The observation that follicular development, but not ovulation, occurs in the ovaries of NRF suggests that the pituitary does not secrete sufficient luteinizing hormone (LH) to produce an LH surge. The study shows that anovulation results from a desensitised anterior pituitary gland, which, in turn, implicates inadequate gonadotrophin-releasing hormone (GnRH) secretion in the inhibition of fertility, since GnRH stimulates LH biosynthesis and GnRH receptor upregulation. Although the distribution and morphology of GnRH-immunoreactive neurones and fibres does not appear to differ between RF and NRF, an accumulation of GnRH in the hypothalamus of NRF supports the hypothesis that GnRH release into the median eminence is inhibited. The potential inhibition of synthesis and processing of GnRH remains to be investigated. There is no evidence to suggest that endogenous opioid peptides are involved in the inhibition of GnRH release, but further studies, using alternative experimental protocols, are necessary.

There is growing evidence to support the theory that the inhibition of gonadotrophin

secretion in different forms of natural infertility is mediated through mechanisms that operate both dependently and independently of the negative feedback effects of gonadal steroids (Karsch *et al.* 1984; Abbott 1988). The assertion that increased concentrations of progesterone produced by LUF result in a pseudopregnant-like state and, consequently, infertility (Bennett *et al.* 1994), is not supported by this study. This does not, however, discount a gonadal steroid mediated mechanism from operating via an increased sensitivity to negative feedback. Strong evidence from hystero-ovariectomised females does, however, show that a gonadal steroid-independent mechanism is involved in the suppression of gonadotrophin secretion.

Infertility in NRF occurs in the absence of elevated levels of the adrenal steroid, cortisol, the hormone commonly associated with stress. If one considers that very little overt aggression is directed towards NRF by the RF, it seems improbable that socially-induced infertility in female Damaraland mole-rats is mediated through the physiological response to stress and, thus, that NRF are suppressed by the dominant reproductive female. Rather, it is emerging from this and other studies that there are common pathways leading to anovulation in various forms of natural infertility. These studies are providing insight into the ultimate causes of infertility in socially-suppressed species. NRF may be responding to social cues, in a similar way to which seasonal breeders respond to inhibitory photoperiods, via a specialised neuroendocrine response that is independent of the generalised response to stress. Thus, NRF may restrain themselves from breeding in sub-optimal conditions and may delay reproduction until a more favourable time. Alternatively, they may never breed, gaining fitness benefits entirely through kin selection. Although dominant control by the RF may operate independently of the generalised stress response, the Self-Restraint Model is an alternative hypothesis to explain reproductive inhibition in cooperative breeding mammals in which subordinate females are infertile (Snowdon 1996; Abbott *et al.* 1997). Rather than being suppressed by the dominant female against their best fitness interests, subordinate females may be responding to sub-optimal breeding conditions because it is in their best interests to do so. Whichever the case, the social species within the family Bathyergidae continue to provide an excellent model with which to study current hypotheses regarding the proximate and ultimate factors involved in maintaining reproductive skew in closely related animal societies.

Appendix 1

The effect of relaxed dispersal constraints on reproductive inhibition in non-reproductive females

Introduction

Damaraland mole-rats are obligate outbreeders and exhibit a strict incest avoidance within the colony. In the absence of a reproductive individual, siblings do not breed and the colony becomes reproductively quiescent until an unrelated individual is introduced (Jarvis & Bennett 1993; Bennett *et al.* 1996; Rickard & Bennett 1997; Chapter 3). Preliminary evidence from a field study conducted by Jarvis & Bennett (1993) suggests that colony fragmentation follows the death of a breeder, but this dispersal event is delayed until the next good rains. Although most individuals remain in their natal colony, there is heightened dispersal during the sporadic and unpredictable rainfall periods since burrowing is greatly facilitated. Little is known about how mole-rats disperse. Field evidence suggests that dispersal may occur above ground (Bennett, Jarvis & Hazell *unpubl. data*). In addition to forming new colonies, unrelated males may enter new colonies and either take over the colony as the breeder or find a mate and subsequently leave to found a new colony. Roaming males have been observed in the field (N. C. Bennett *pers. comm.*). Alternatively, dispersal may occur in small groups (Jarvis & Bennett 1993), which may facilitate colony foundation due to constraints on digging and locating sufficient food patches (Lovegrove & Wissel 1988). During rainy periods in which constraints on dispersal are reduced, reproductive inhibition in non-reproductive females may be relaxed due to the greater probability of successful independent reproduction. To investigate this hypothesis, the activity of the reproductive axis in non-reproductive females was investigated during the dry and rainy season.

Materials and methods

Data on body mass and gonadotrophin-releasing hormone (GnRH) stimulated plasma luteinizing hormone (LH) concentrations were obtained for 10 reproductive males (RM), 10 non-reprodutive males (NRM) and 10 non-reproductive females (NRF) that had been maintained in the laboratory for at least one month. The mean plasma LH response 0.5, 1.0 and 2.0μg GnRH challenge in each of these groups are presented in Chapter 3 (Fig. 3.1). The relationship between GnRH stimulated LH concentrations and body weight was investigated in both RM and NRM at all three doses of GnRH to

determine whether a body mass related effect on pituitary sensitivity exists, in the absence of possible confounding factors that may be present in females e.g. reproductive inhibition in NRF or pregnancy in RF. The same relationship was tested in NRF to determine whether there are size related differences in the degree of reproductive inhibition.

GnRH stimulated (2.0μg) LH concentrations and body mass were determined in two groups of NRF approximately one week after capture. The first group of females (n = 18), from six colonies, was captured during September 1996 at the end of the dry period and the second group (n = 8), from two colonies, was captured during the rainy period in February 1997.

Results

No significant correlations were found between body weight and GnRH-stimulated LH concentrations at 0.5μg, 1.0μg and 2.0μg GnRH in RM and NRF (Fig. 8.1) nor in NRF that had been maintained in the lab for at least one month (Fig. 8.2). Statistical values are presented in Table 8.1. Similarly, in the two groups of freshly captured non-reproductive females caught in September 1996 (Fig. 8.3a) and February 1997 (Fig. 8.3b), no correlation was found between body weight and LH concentrations measured after a 2.0μg GnRH challenge. However, when the data from the two groups were pooled, the correlation between body weight and LH concentration was significant (Fig. 8.3c). Indeed, Mann-Whitney U tests revealed significant differences in both mean body weight ($U = 11$, $P = 0.0007$, $N_1 = 18$, $N_2 = 8$) and mean GnRH-stimulated LH concentrations ($U = 15.5$, $P = 0.002$, $N_1 = 18$, $N_2 = 8$) between the two groups of females. The LH response to a 2.0μg GnRH between NRF caught in February 1997 and non-reproductive females housed in the absence of the breeding pair (rNRF) was not significantly different ($U = 38$, $P = 0.85$, $N_1 = 10$, $N_2 = 8$).
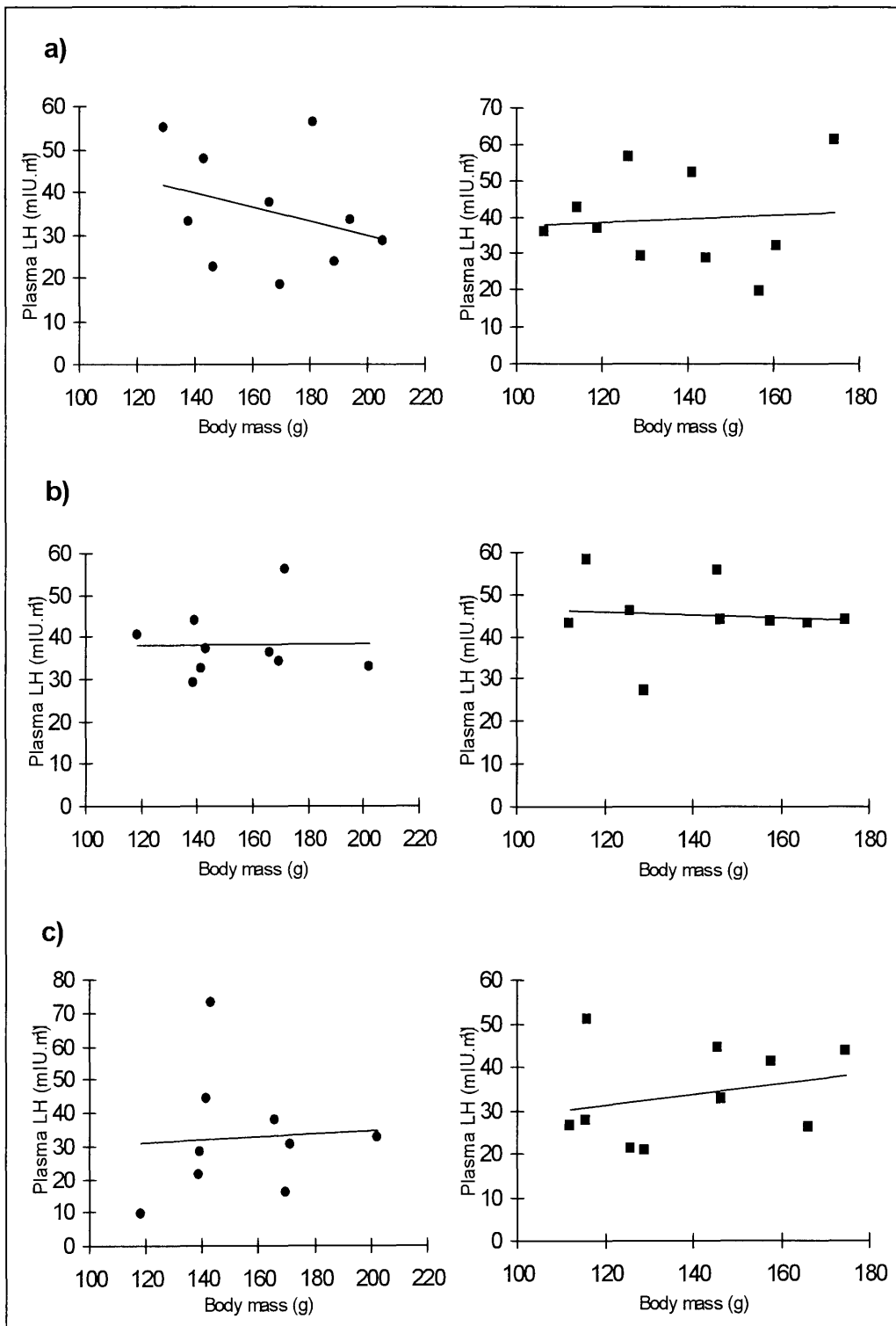
Fig. 8.1. Relationship between body weight and the plasma LH response to **a)** 2.0μg, **b)** 1.0μg and **c)** 0.5μg GnRH challenge in reproductive (●) and non-reproductive (■) males.
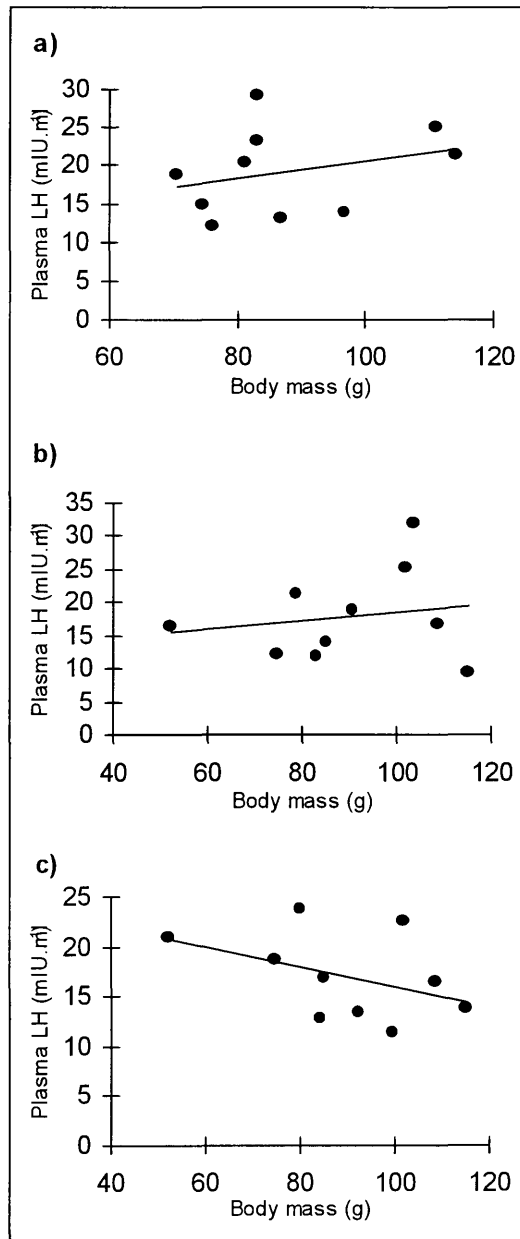
Fig. 8.2. Relationship between body weight and the plasma LH response to **a)** 2.0µg, **b)** 1.0µg and **c)** 0.5µg GnRH challenge in non-reproductive females.
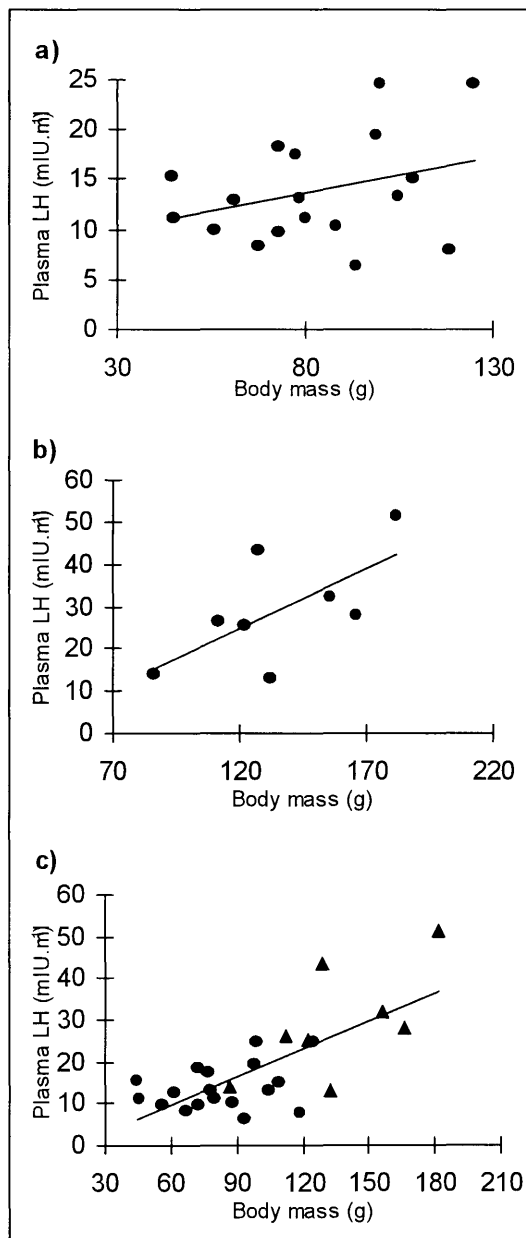
Fig. 8.3. Relationship between body weight and the plasma LH response to 2.0μg GnRH challenge in freshly captured non-reproductive females. Females were captured in **a)** September 1996, **b)** February 1997 and **c)** combined September 1996 (●) and February 1997 (σ) data. The correlation between body weight and the plasma LH response for the combined data **c)** was significant (Spearman Rank Correlation; r_s = 0.60; P < 0.05; N = 26).

Discussion

There was no relationship between body mass and the degree of pituitary sensitivity in either of two groups of newly caught NRF, trapped in September 1996 and February 1977. However, when the data from the two groups were pooled, a significant correlation was found (Fig. 8.3c), indicating that females caught in February (1997) were heavier and exhibited a greater pituitary sensitivity to exogenous GnRH than females caught in September (1996). Indeed, a significant difference was found in both mean body mass and the plasma LH response to a GnRH challenge between the two groups of females. Since there were no significant correlations in either RM or NRM at any of the three doses GnRH (Fig. 8.1), the degree of pituitary sensitivity does not appear to be a direct physiological consequence of body size. Furthermore, the degree of reproductive inhibition in NRF is not directly related to body size since there were no positive correlations between body size and pituitary sensitivity in NRF maintained in the laboratory (Fig. 8.2). Thus, there appears to be a temporal component responsible for the alteration in the activity of the reproductive axis (as indicated by a more sensitive pituitary) which does not appear to be dependent on, but is correlated to, body size.

It is possible that there is a relaxation of reproductive inhibition in the females caught in February (1997) due to a temporal factor relating to the change in ecological constraints upon dispersal. Consider that the first group of females was caught in September 1996 and the second group in February of the following year. During the intervening four months (October 1996 - January 1997), 90 % (282.6mm) of the annual rainfall fell, measured over the three years from January 1995 to December 1997 (Fig. 8.4). This is significant, since only 363.6mm of rain fell over the 20 months preceding this period (January 1995 - September 1996). During periods following good rainfall, at least 25mm of rain in a short period is required, burrowing through the sandy Kalahari arenosols is facilitated and field data suggests that dispersal is restricted to these periods (Jarvis & Bennett 1993). This may, therefore, have been the first opportunity for dispersal in more than 20 months, resulting in rapid weight gain and an enhanced activity of the reproductive axis. These NRF exhibit a pituitary sensitivity comparable to that of NRF housed in the absence of the breeding pair, indicating that it

may be possible for NRF females to "ignore" the inhibitory social cues. A larger body size may increase the probability of successful dispersal. Field evidence suggests that dispersers are generally larger than the population mean (Bennett, Jarvis & Hazell *unpubl. data*). Thus, body size and pituitary sensitivity increase independently in reponse to the same environmental cues and for the same purpose, namely dispersal. O'Riain *et al.* (1997) found that dispersing morphs exist in naked mole-rat (*Heterocephalus glaber*) colonies, that are both larger and have a greater pituitary sensitivity to GnRH than other non-reproductives. Although it is not known how dispersal and mate location is achieved, the above evidence supports the contention that dispersal occurs following sufficient rain and that reproductive inhibition in NRF is relaxed during these periods in anticipation of possible independent reproduction, in this obligate outbreeder.
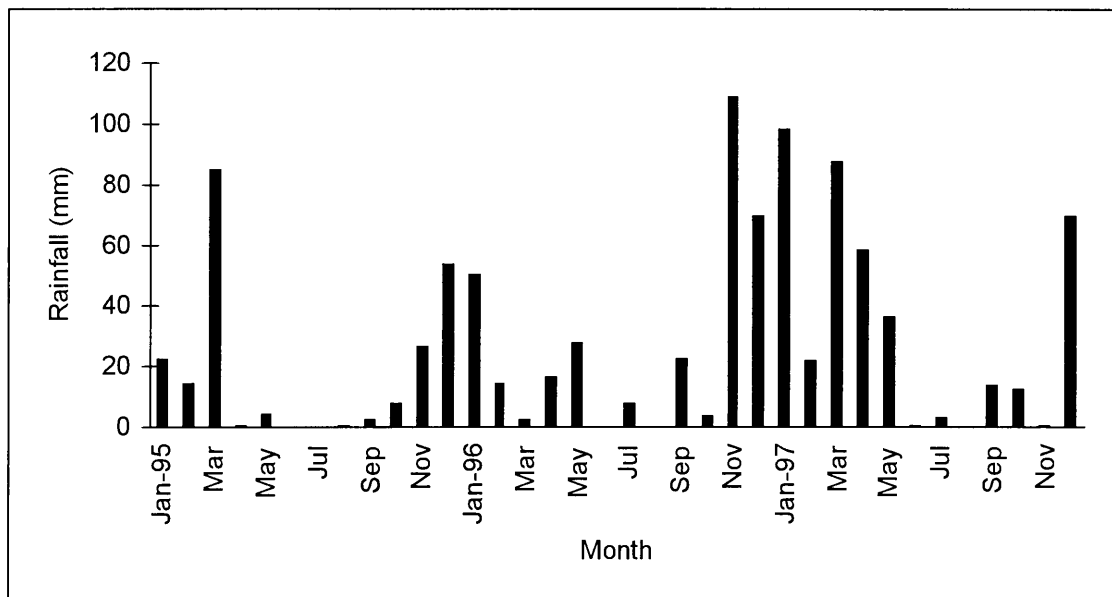
Fig. 8.4. Mean monthly rainfall for the area in which *C. damarensis* were trapped from January 1996 to December 1997 (South African Weather Bureau).

Appendix

Table 8.1. Relationship between body mass and the plasma LH response to a 2.0, 1.0 and 0.5µg GnRH challenge. **a)** Individuals housed maintained in the laboratory. **b)** Freshly captured individuals. δ - Non-reproductive females caught in September 1996; φ - non-reproductive females caught in February 1997; ψ - combined group of δ and φ non-reproductive females. The results of a Spearman-Rank correlation are presented. Statistical significance ($\alpha = 0.05$) is denoted by *.

	Reproductive state	GnRH dose	N	r_s	P
a)	Reproductive male	2.0µg	10	-0.3	0.45
	Non-reproductive male	2.0µg	10	-0.1	0.78
	Non-reproductive female	2.0µg	10	0.3	0.4
	Reproductive male	1.0µg	9	0.05	0.90
	Non-reproductive male	1.0µg	9	-0.1	0.77
	Non-reproductive female	1.0µg	10	0.1	0.8
	Reproductive male	0.5µg	9	0.4	0.36
	Non-reproductive male	0.5µg	10	0.2	0.65
	Non-reproductive female	0.5µg	10	-0.4	0.3
b)	Non-reproductive female[δ]	2.0µg	18	0.2	0.4
	Non-reproductive female[φ]	2.0µg	8	0.6	0.1
	Non-reproductive female[ψ]	2.0µg	26	0.6	0.001*

Summary

The proximate mechanisms, both social and physiological, responsible for the anovulation observed in non-reproductive, female Damaraland mole-rats (*Cryptomys damarensis*) were investigated. Non-reproductive females that were housed in the absence of the breeding pair (rNRF) showed greater pituitary sensitivity and higher levels of circulating progesterone concentrations than non-reproductive females housed in the colony (NRF), indicating an increase in the activity of the reproductive axis. Indeed, the presence of corpora lutea in the ovaries of rNRF indicate that non-breeding females start ovulating spontaneously when housed in the absence of the breeding pair. Since anovulation is due to the inhibition of fertility rather than a lack of copulatory stimulation, it has the potential to play a role in maintaining reproductive skew. Physiologically, anovulation in NRF appears to be due to a desensitised pituitary. The significantly lower luteinizing hormone (LH) response to exogenous gonadotrophin-releasing hormone (GnRH) in NRF, compared to reproductive females (RF), may partly be due to inadequate stores of releasable LH, as the pituitaries of NRF contained less luteinizing hormone than RF. This suggests that the GnRH system may be suppressed in NRF, since GnRH stimulates gonadotrophin biosynthesis as well as LH release. Although there was no apparent difference in the distribution and morphology of GnRH-immunoreactive structures in the brain, a significantly greater concentration of GnRH was found in the brains of NRF compared to RF. This supports the hypothesis that an inhibition of GnRH release, and consequent desensitisation of the pituitary, is responsible for infertility in NRF. Strong evidence that a gonadal steroid-independent mechanisms plays a role in suppressing gonadotrophin secretion in NRF was obtained in hystero-ovariectomised females. Despite the general increase in pituitary sensitivity in both RF and NRF in the absence of gonadal steroids, RF showed a significantly greater LH response to a GnRH challenge. A gonadal steroid-independent mechanism does not, however, involve the adrenal steroids, since cortisol concentrations did not differ between RF and NRF. A neural mechanism does not appear to be mediated through an increased activity of endogenous opioid peptides, since administration of the opioid antagonist, naloxone, had no effect on LH secretion. Although differences exist, this study shows that similar physiological pathways lead to anovulation in different forms of natural infertility.

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