HORMONAL ASPECTS OF REPRODUCTIVE SUPPRESSION IN THE NAKED MOLE-RAT, *HETEROCEPHALUS GLABER*

Erin Bianca Tuomi

Supervisor: Dr. M.J. O'Riain

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University of Cape Town
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I declare this work to be my own original work. It has not been submitted for a degree at any other university.

Erin Tuomi

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Summary

Subordinate, female naked mole-rats (*Heterocephalus glaber*) are anovulatory as a result of extremely low luteinising hormone (LH) levels. Evidence suggests that aggression from the dominant female naked mole-rat directed towards subordinates could produce these lowered levels of LH. This study examined two potential hormonal pathways of reproductive suppression in this species: endogenous opioid peptides and cortisol.

Naloxone, an opioid antagonist, was administered subcutaneously as a bolus injection to four groups of female naked mole-rats, assigned according to social status (reproductive or non-reproductive) and whether or not an animal was ovariectomized. LH levels were measured from plasma samples. Neither single nor multiple injections of naloxone had any significant effect on LH secretion in any of the groups, suggesting that EOPs are unlikely to mediate socially induced suppression in the naked mole-rat.

Plasma cortisol levels were similarly measured in four groups of female naked mole-rats, assigned according to social status (reproductive or non-reproductive) and whether or not i) the animal was ovarectomized, ii) they had been injected with naloxone or saline and, iii) they had received single or multiple injections of the latter hormones. Breeding females had significantly higher baseline cortisol levels than non-breeding subordinates suggesting that hyper-cortisolism is not a mediator of reproductive suppression in the naked mole-rat. Furthermore, dominant females and subordinates did not differ significantly in cortisol response to single or multiple saline and naloxone injections. However, multiple saline injections resulted in significantly lower cortisol levels than those exhibited after single saline injections for both intact queens and subordinates. The latter suggests that down-regulation of the

physiological stress reaction may occur under conditions of chronic stress. Thus, hypo-cortisolism may be a potential mechanism of reproductive suppression.

It is possible that exogenously applied hormones may have only a limited ability to provide further insight into the proximate mechanism(s) of reproductive suppression in naked mole-rats. This approach suffers from a number of confounding variables that can affect hormone levels (e.g. handling stress, time of day, hormonal feedback mechanisms). It is for this reason that I designed an "intelligent" burrow system which I consider to be the most effective way forward with this research endeavour. This burrow system was designed to physically separate a subordinate female from the queen, through a system of electronic sensors that regulate the opening and closing of electric doors via a preprogrammed microprocessor. The burrow system and its components were constructed and successfully tested on a colony of naked mole-rats.

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CHAPTER ONE

1.1 REPRODUCTIVE SUPPRESSION IN CO-OPERATIVELY BREEDING SOCIETIES

Co-operative breeding is the care of young by individuals other than the parent (Emlen 1982) and typically occurs when offspring remain in the natal territory and become alloparents or helpers (Brown 1987). Females that are philopatric are often reproductively suppressed by behaviourally more dominant, same-sex group members (Emlen 1991; Keller & Reeve 1994; Solomon & French 1997). The degree to which reproduction is shared among group members is termed reproductive skew (Keller & Reeve 1994), and varies along a continuum from high reproductive skew, where reproduction is monopolised by a pair of individuals (e.g. dwarf mongooses, *Helogale parvula,* Rood 1990; Keane *et al.* 1994) to low reproductive skew, where reproduction is shared more equitably among group members (e.g. banded mongooses, *Mungos mungo*, Cant 2000). Determining the mechanisms of suppression is critical to a complete understanding of why some individuals do not breed.

1.1.1 Proximate mechanisms of reproductive suppression

A number of proximate mechanisms have been proposed to explain why some subordinates do not breed. Foremost is that individuals within a family group typically lack access to opposite sex conspecifics and thus fail to reproduce because of the costs of inbreeding (Jennions & Macdonald 1994; Mumme 1997). The Self-restraint Model (SRM) proposes that reproductive suppression does not involve overt aggression between dominants and subordinates, but rather that subordinates refrain from reproducing within their natal group despite being physiologically capable of doing so (Harvey & Ralls 1986; Charlesworth & Charlesworth 1987; Boulin & Boulin

1988; Clutton-Brock 1989; Pusey & Wolf 1996; Snowdon 1996). Thus, lack of stimulation from an unrelated mate remains an important factor in inhibition of subordinate reproduction (Schoech *et al.* 1991, 1994).

More recent models, such as the Dominant-control Model (DCM) assume that control of reproduction lies with the dominant individual or individuals (Reeve & Ratnieks 1993; Snowdon 1996; Reeve *et al.* 1998; Jonhstone *et al.* 1999), where reproductive suppression is imposed by aggression directed at the subordinates by dominants. Dominant individuals do benefit from the presence of subordinates and must therefore adjust their aggression levels to ensure suppression is maintained, but subordinates are not driven out of the group (Johnstone *et al.* 1999; Cant 2000). Nevertheless, dominants seldom have complete control over subordinate reproduction (Vehrencamp 1979; Reeve & Keller 1995) and in many co-operatively breeding societies subordinates do breed although at a lower rate (Limited-control hypothesis, LCH, Clutton-Brock 1998).

Other species appear to be controlled by a combination of inbreeding avoidance and dominant suppression and thus have both DCM and SRM components. In Mongolian gerbils (*Meriones unguiculatus*), the mother is more likely to reproduce than her daughters, even if daughters have access to unrelated opposite sex conspecifics (Agren 1981; French 1994), and the ovaries of the latter typically lack corpora lutea and are atrophic (Swanson & Lockley 1978). Daughters are more likely to reproduce if their mother is removed (Payman & Swanson 1980), but ultimately require the presence of an unrelated male to initiate breeding (Clarke & Galef 2001). A similar situation occurs in pine voles (*Microtus pinetorum*). Pine voles are induced ovulators (no ovarian cyclicity) requiring a combination of physical and chemical cues from an unrelated male to induce oestrus. The lack of unrelated males is sufficient to prevent most female subordinates from reproducing within the natal group, which is typically

comprised of close relatives only. However, even if a subordinate female is presented with an unrelated male the presence of her mother can reduce litter production by up to 75% (Brant *et al.* 1998; Solomon *et al.* 2001). Thus, it is important to note that while the proximate mechanism for reproductive suppression and inbreeding avoidance differ, their ultimate effects within a group are the same and they often act in tandem.

1.1.2 Behavioural versus physiological components of suppression

Active manipulation of subordinates, by dominants, can be behavioural or physiological in nature and requires that suppression is imposed by aggression and aggression-related physiological effects. Behavioural and physiological suppression should not always be considered as discrete components as in many cases physiological suppression is directly influenced by behaviour.

Behavioural suppression may occur through the killing of subordinate offspring (infanticide), physical prevention of mating (mate guarding) and physical aggression (punishment) towards subordinates that are reproductively active. Infanticide, such as that which occurs in lions (*Panthera leo*, Packer & Pusey 1983), is an obvious way of killing a competitor's offspring, but requires discrimination between one's own offspring and other young. Hager & Johnstone (2004), using a simple game theory model, were able to show that the benefits of controlling reproduction outweigh the possibility of killing one's own young, thus making infanticide an effective option even if discrimination is imperfect. Direct interference with subordinate mating attempts (e.g. grey wolves, Seal *et al.* 1979; Packard *et al.* 1985; Creel & Creel 1991; African wild-dogs, *Lycaon pictus,* Bothma & Walker 1999; meerkats, *Suricata suricatta,* Doolen & Macdonald 1997; Clutton-Brock *et al.* 1998; and banded mongoose, *Mungos mungos,* Cant 2000) effectively prevents the onset of subordinate reproduction and thus negates the need for potentially costly downstream behaviours

such as infanticide. Physically aggressive behaviour can be highly successful in reducing successful subordinate reproduction, but requires constant vigilance and high energy output (through aggressive behaviour) from the dominant individuals if it is to maintain a high success rate. Furthermore, subordinates are often physiologically capable of reproducing if an opportunity presents itself, resulting in a number of sneak copulations (e.g. African wild-dogs, Malcolm & Marten 1982; dwarf mongooses, Keane *et al.* 1994). It is perhaps for this reason that selection has favoured a third mechanism that requires less active interference viz., the rendering of subordinates physiologically unable to reproduce. Active suppression of subordinates by dominants can take a physiological form, whereby steroid and peptide hormones in subordinates are depressed below thresholds required for reproduction. This type of suppression occurs in female common marmosets (*Callithrix jacchus,* Abbott 1984, 1987) and naked mole-rats (*Heterocephalus glaber,* Jarvis 1991), where only the dominant female shows a normal ovulatory cycle. Subordinate females' luteinising hormone levels are too low for ovulation to occur.

Alternative factors, not related to dominance, which could cause suppression of reproduction in an individual, include limited food availability and adverse climate (Abbott *et al.* 1998). Poor nutrition in particular has a strong adverse influence on the GnRH pulse generator system and GnRH release (Booth 1990; Cameron 1996; Wade *et al.* 1996). Given that dominants typically have greater control of, and access to, food resources it is not surprising that the effects of adverse nutrition on reproduction are most strongly evident in subordinates.

1.1.3 Subordinate tolerance of reproductive suppression

The question thus arises as to why subordinates tolerate suppression of reproduction instead of dispersing from the natal group. When examining the situation in terms of costs and benefits a partially satisfactory answer is revealed. Dispersal and

independent breeding has direct fitness benefits resulting from the production of offspring. Remaining in a group typically reduces direct fitness benefits, but provides much other compensation. Firstly, an individual may increase indirect fitness if related to members of the group that do breed (Stacey & Koenig 1990; Keller & Reeve 1994), or direct fitness through sneak copulations (e.g. African wild-dogs, Malcolm & Marten 1982; dwarf mongooses, Keane *et al.* 1994). Group-living is also associated with improved survival through the dilution effect i.e. the larger the group, the lower the probability of an individual being selected by a predator. Following on from this is the improved ability of groups to defend or find key food resources and the delayed benefits that an individual may receive from territory inheritance (Rood 1978; Alcock 1998). This is of great significance in saturated habitats where a dispersing individual is unlikely to obtain a territory/nest/burrow for independent breeding. Together these factors may explain why subordinates remain in the natal territory and aid reproductive individuals.

1.1.4 Dominant tolerance of subordinates

Dominant reproductives typically exercise some degree of tolerance to the presence of subordinates to obtain some genetic gain, reflected in an increase in fitness (direct or indirect). These benefits are often as a result of a low level of critical resources in the environment. In moustached tamarin monkeys (*Saguinus mystax*) the reproducing female cannot raise her twins alone, the critical resource in the environment being helpers. A dominant female can increase her offspring's chances of survival, and thus her own direct fitness by using subordinate labour. The dominant female further benefits if the helpers are reproductively suppressed, as this ensures that more of their efforts are channelled into help and less into reproduction, which may result in competition for critical resources for her offspring (Abbott 1987).

1.1.5 Outcomes of reproductive suppression

Ultimately, reproductive suppression may manifest itself in females in a variety of ways (Abbott 1987) including: a delay in puberty (e.g. house mouse, *Mus musculus,* Massey & Vandenbergh 1980; banded mongoose, Cant 2000), suppression of oestrus (e.g. house mouse, McClintock 1983), inhibition of ovulation usually related to depressed hormone levels (e.g. female common marmosets, Abbott & Hearn 1978; Abbott 1984; Abbott *et al.* 1988; Barret *et al.* 1993; Saltzman *et al.* 1997; Baker *et al.* 1999; cotton-topped tamarins, *Saguinus Oedipus,* Savage *et al.* 1988; Barrett *et al.* 1993; Damaraland mole-rats, *Cryptomys damarensis,* Bennett 1994; Bennett *et al.* 1994; and dwarf mongooses, *Helogale parvula,* Creel *et al.* 1992), block of embryo implantation and embryo re-absorption (e.g. white footed mouse, *Peromyscus leucopus,* Haigh *et al.* 1988), spontaneous abortion (e.g. house mouse, Clark *et al.* 1993; alpine marmots, *Marmota marmota,* Arnold & Dittami 1997; Hackländer & Arnold 1999) and increased subordinate infant mortality i.e. infanticide (e.g. lions, Packer & Pusey 1983; Brown 1996).

1.1.6 Physiological basis of reproductive suppression

Isolating the physiological causes of reproductive suppression becomes paramount to our understanding of reproductive suppression and its implications to co-operatively breeding societies. Early research on captive rats showed that losing a fight was stressful and resulted in an increase in glucocorticoid levels (Blanchard *et al.* 1995). Elevated glucocorticoid levels can have an adverse effect on an individual's reproductive physiology, and thus aggression may function to suppress reproduction in subordinates of co-operatively breeding mammals. However, results from captive situations can seldom be applied to wild situations. In the wild, animals are better able to avoid overt aggression and thus seldom experience levels of glucocorticoids experienced by laboratory animals. Furthermore, fights between unfamiliar individuals do not necessarily reveal hormonal patterns within a group with

a stable dominance hierarchy (Creel 2001). However, the general view is that subordination is stressful (Bronson 1973; Manogue 1975), and that this could ultimately result in reproductive failure in subordinates, as aggressive interactions can cause large and persistent increases in glucocorticoid secretion (Pottinger 1999; Sapolsky 2000).

When closely examined, there does not appear to be any consistent relationship between social rank and stress levels among co-operatively breeding mammals (Abbott *et al.* 2003). In dwarf mongooses (Creel *et al.* 1992) and mountain gorillas (*Gorilla gorilla,* Robbins & Czekala 1992) dominants and subordinates have similar glucocorticoid levels. However, in wild dogs (Creel *et al.* 1996; Creel *et al.* 1997), grey wolves (Jameson *et al.* 1999; Sands & Creel 2004), alpine marmots (Arnold & Dittami 1997), ring-tailed lemurs (*Lemur catta,* Cavigelli 1999) dwarf mongooses and common marmosets (Creel 2001), dominant animals have higher cortisol levels than subordinates. A number of causes have been suggested to explain differences in hormonal profiles between subordinates and dominants. Firstly it has been hypothesized that higher levels of aggression among dominants, especially during the mating season, could explain their elevated glucocorticoid levels. Furthermore, differences between dominant and subordinate hormonal profiles could be a consequence of the weight and age advantage of the former or a lack of unrelated partners for subordinates (O'Riain *et al.* 2000a). For example, dominant breeding female meerkats have both higher cortisol (Carlson *et al.* 2004) and higher luteinising hormone levels than subordinates (O'Riain *et al.* 2000a). However, when controlling for weight, female age and access to unrelated partners, the rank related differences in luteinising hormone in meerkats disappear (O'Riain *et al.* 2000a). Irrespective of the cause, higher glucocorticoids in dominant reproductives is a direct contradiction of the theory that elevated glucocorticoid levels suppress reproduction. It is possible that higher cortisol levels could be an indirect consequence of being reproductively

active, with higher levels of reproductive hormones stimulating adrenal steriodogenesis (Carlson *et al.* 2004) and hence elevated glucocorticoids.

In species where subordinates do have higher glucocorticoid levels than dominants, such as female alpine marmots (Hackländer *et al.* 2003), stress-related physiological profiles are still a possible cause of reproductive suppression.

Mammals are not the only animals in which stress exerts direct and indirect effects on reproduction. Stress is also known to cause a wide number of physiological responses in fish, such as a decrease in reproductive hormone levels, reduced fecundity, smaller egg size and lower survival of eggs and larvae (Campbell *et al.* 1994). In Atlantic cod, stress reduces the number of courtships per hour and increases the number of abnormal larvae (Morgan *et al.* 1999). In carp (*Cyprinus carpio L.*), corticosteroids had a direct inhibitory affect on testicular androgen (independent of LH), but only after puberty (Consten *et al.* 2002).

Traditionally, stress is thought to have a negative impact on reproduction in reptiles (Greenburg & Wingfield 1987), but is context specific and some individuals may exhibit an inhibitory response while others don't, e.g. two lizards have two phenotypes, but only the non-territorial phenotype responds with a decrease in plasma testosterone despite both phenotypes showing an increase in cortisol (Moore & Jessop 2003). Thus, subordinates and dominants could respond differently to a similar stressor, which may explain the lack of clear correlation between social status, glucocorticoids and reproduction in social mammals. Furthermore, an individual's reaction to stress is highly variable and related to a number of factors including social affiliations, stressor frequency, displacement aggression, dominance rewards and the probability and predictability of future aggressive encounters. For example, if two rats are placed in a divided cage with a shock grid and subjected to

the same shocks, both will show a similar stress reaction. However, if one rat is given the ability to reduce the shock rate for both rats, it will show a reduced stress response while the rat without the ability to control its environment still shows a stress response similar to its original response, despite a decreased shock rate (Weiss 1970). Variability in an individual's response to a physiological stressor makes quantifying aggression-related stress effects on reproduction difficult. This does not, however, dismiss its potential role in reproductive suppression.

1.1.7 Conclusion

It is imperative for evolutionary biologists to understand why individuals within co-operatively breeding societies forgo direct fitness opportunities. Behavioural stress, and the 'stress hormones' associated therewith, appears to be a key variable in our understanding of why subordinates fail to reproduce. This thesis represents an attempt to further our understanding of the effects of stress on reproduction in the most social of mammals – the naked mole-rat. This species is characterized by extreme reproductive skew, highly aggressive breeders and marked differences in luteinising hormone levels between dominants and subordinates. Together these characteristics make the naked mole-rat an ideal study animal with which to explore the proximate mechanism(s) for reproductive suppression in a co-operatively breeding mammal.

1.2 THE STUDY ANIMAL: THE NAKED MOLE-RAT, *HETEROCEPHALUS GLABER*

1.2.1 General Biology

The naked mole-rat (Family: Bathyergidae) is a eusocial mammal that lives in colonies of between 40 and 90 individuals in the semi-arid regions of East Africa, including Somalia, Ethiopia and Kenya (Jarvis 1981; Jarvis *et al.* 1994; Brett 1986). Naked mole-rats are subterranean, excavating extensive underground burrow systems, up to four kilometres in length (Brett 1986). The burrow consists predominantly of foraging tunnels, enabling the mole-rats to search for sparsely located underground tubers and bulbs that comprise their staple diet (Jarvis & Sale 1971; Brett 1991).

The habitat in which the naked mole-rat lives is physically harsh with hard, dry soil conditions and large, randomly distributed, below ground food sources (Bennett & Faulkes 2000). Together these conditions impose high energetic costs on finding food and make dispersal and successful independent breeding a virtually impossible option (Lovegrove & Wissel 1988; Sherman *et al.* 1992; Jarvis *et al.* 1994; Brett 1991; O'Riain & Braude 2000). Together the difficulty of locating food and the high costs of dispersal have been hypothesised to be the ultimate evolutionary factors selecting for group-living in naked mole-rats (Sherman *et al.* 1992; Jarvis *et al.* 1994).

Members of a colony are highly xenophobic to conspecifics from other colonies (O'Riain & Jarvis 1997), which together with an apparent lack of incest avoidance promotes close inbreeding (Reeve *et al.* 1990; Honeycutt *et al.* 1991; Jarvis *et al.* 1994). In accordance, naked mole-rats exhibit the highest inbreeding co-efficient (F = 0.62) yet recorded among free-living mammals (Reeve *et al.* 1990). Although inbreeding is commonly thought to reduce fitness, this level of inbreeding may

ultimately be beneficial as co-adapted gene complexes and local adaptations are maintained (Shields 1993). Occasionally a dispersive morph surfaces in a colony (O'Riain *et al.* 1996). These dispersive morphs display both behavioural and morphological differences to other colony members (e.g. preference for foreign individuals and fat storage around the neck area). They disperse from their natal colony during favourable conditions and attempt to locate and reproduce with foreign conspecifics (Braude 2000; Ciszek 2000; O'Riain & Braude 2000)

Naked mole-rats exhibit perhaps the highest reproductive skew in mammals and whilst they do not have sterile castes, 95% of subordinates never breed (Faulkes *et al.* 1990a; Faulkes & Abbott 1991; Jarvis 1991). Reproduction within a colony is typically monopolised by a single dominant female (the queen) and 1 - 3 males (Jarvis 1981, 1991; Lacey & Sherman 1990). The queen is behaviourally aggressive; the only female to display sexual behaviour and can be easily identified by her prominent nipples, elongated body shape and a perforate vagina (O'Riain *et al.* 2000b). Most other females are physiologically non-reproductive, with hormone levels below the thresholds required for ovulation and do not engage in sexual behaviour (Jarvis 1991; Faulkes *et al.* 1990b; Margulis *et al.* 1995; Van der Westhuizen 1997).

Subordinate female naked mole-rats fail to ovulate (Jarvis 1991; Faulkes *et al.* 1990a) and their ovaries lack both preovulatory follicles and corpora lutea (Jarvis 1991). A similar trend exists in males, but the endocrine differences between breeders and non-breeders are far smaller and the latter are capable of viable spermatogenesis (Faulkes & Abbott 1991). Together, male and female non-breeders, attend to the construction, maintenance, food location and defence of the colony (Brett 1986; Lacey & Sherman 1990; Jarvis *et al.* 1994). Smaller colony members tend to perform 'worker' type behaviour such as tunnel digging, foraging and cleaning

while larger animals assume a defence role (Jarvis 1981; Brett 1986; Lacey & Sherman 1990; Jarvis et al. 1991).

1.2.2 Proximate mechanisms of reproductive suppression in the naked mole-rat

It is thought that the anterior pituitary in non-breeding female naked mole-rats is less sensitive to gonadatrophic releasing hormone (GnRH) than that of breeding females. This suggests that the block in ovulation may be due to a disrupted secretion of hypothalamic GnRH (Faulkes *et al.* 1990a; Faulkes *et al.* 1990b). Non-breeders, of both sexes, can become sexually functional through removal from the colony and/or removal/death of the breeding female (Jarvis 1991; Faulkes *et al.* 1991; Margulis *et al.* 1995; Clarke & Faulkes 1998). Removal of a non-breeding female can initiate ovarian cyclicity in as little as seven days and pregnancy can occur within the first oestrous cycle (Faulkes *et al.* 1990a; Faulkes *et al.* 1991). If a breeding female is removed from a stable colony most of the larger, older females within the colony will compete for breeding status with a single female attaining dominance through behavioural aggression (Jarvis 1991; Margulis *et al.* 1995). There is no consistent pattern dictating which female will become the next queen (Margulis *et al.* 1995). The alacrity and ease with which a breeding female is replaced is important so breeding can continue producing sufficient colony members to search for food and maintain tunnels in a food scarce environment.

Non-breeding males also show clear endocrine differences from breeders. When removed from a colony, males show a marked increase in testosterone. However, when paired with a female tonic testosterone levels drop and only peak during the female's late luteal and early follicular stage of ovulation. It is unknown whether this affects spermatogenesis (Faulkes & Abbott 1991). Thus, in the absence of incest avoidance mechanisms, all colony members are potential mates, raising the question, as to how the queen maintains her monopoly on reproduction.

It was originally suggested that primer pheromones in the breeding female's urine could have a suppressive effect (Jarvis 1981), as is the case in many rodent species e.g. pine voles (*Microtus pinetorum,* Lepri & Vanderbergh 1986) and wild mice (*Mus musculus*, Massey & Vanderbergh 1980). Naked mole-rats show extensive grooming in the toilet area, an ideal location for odour communication. Furthermore, animals regularly sniff head and anogenital regions of other colony members (Lacey *et al.* 1991) lending support to the idea that pheromones could play a role in suppression. Faulkes and Abbott (1993) subsequently refuted this hypothesis by showing that singly-housed females commenced ovarian cyclicity even when exposed to urine (including the queen's) from the colony toilet chamber. It could, however, be argued that levels of queen pheromones in the soiled bedding were diluted by pheromones from other colony members and therefore were not strong enough to physiologically suppress a subordinate female. In meadow voles (*Microtus pennsylvanicus)*, chemical signals from an unrelated male can result in implantation failure. This failure is more likely if physical contact with the male is allowed (Storey 1986), suggesting that chemical cues presented in conjunction with additional tactile cues may provide a stronger suppressive effect. Smith *et al.* (1997) demonstrated that removal from the physical presence of the queen, but maintaining contact with non-breeding members and colony odour does not maintain reproductive suppression.

Results from other studies (Lacey & Sherman 1990; Faulkes & Abbott 1991; Margulis *et al* 1995, Clarke & Faulkes 1997) have suggested that aggression directed at subordinates by the queen and other older members of the colony suppresses reproduction in the majority of smaller, younger subordinates. Naked mole-rat queens are behaviourally dominant to male and female subordinates, shoving, biting and even occasionally killing a close competitor. The queen performs the majority of the shoving (up to 96%) within a colony (Margulis *et al.* 1995) and typically targets a subset of the larger, older females (Jarvis 1991).

Studies at the University of Cape Town have explored the relationship between the levels of luteinising hormone (LH) in subordinates and the amount and type of aggression they received from the queen (Van der Westhuizen 1997, 2002). Van der Westhuizen (1997) hypothesised that females with higher LH levels (necessary for ovulation) would be aggressively targeted by the queen in an attempt to suppress their LH levels. The results obtained from this study were ambiguous and failed to separate cause and effect. Thus, if a subordinates exhibited low LH levels and a high shove rate, then it could be interpreted as either (i) having a low LH level due to a high shove rate (physiological suppression because of high aggression), or (ii) that the shove rate by the queen is not influenced by LH levels in subordinates, because individuals with low LH levels have a high shove rate.

One solution to this problem would be to experimentally manipulate the hormone levels of subordinates and to observe whether the queen changes her aggression levels in accordance. The first effort in this regard (Hamblin & O'Riain 2000) attempted to sexually activate subordinate females that were rarely shoved by the queen, by injecting them with 17β oestradiol. The hypothesis was that if sexually activated, these females would become the focus of queen aggression. However, the injections failed to activate the subordinate females highlighting the difficulties associated with exogenous hormone treatments. It is further possible that these results were confounded by the negative feedback mechanism associated with the hypothalamus-pituitary axis and oestrogen.

Together the literature discussed in this review points to a lack of clarity in our understanding of the role of cortisol in co-operatively breeding mammals. At present most studies that have examined the relationship between hormone levels and the degree of suppression in subordinates have been correlational. The problem with this approach is that it fails to distinguish between cause and effect (*sensu* Van der

Westhuizen 1997, 2002). The objective of this thesis is to firstly establish baseline levels of the key stress (cortisol) and reproductive (LH) hormone levels in intact colonies of naked mole-rats and then to manipulate both the animal and the animal's immediate environment to provide insight into the role of stress on suppression.

1.2.3 Outline of the present study

In this study I investigated two possible hormonal pathways of reproductive suppression in the naked mole-rat (*Heterocephalus glaber*): endogenous opioid peptides (EOPs) and cortisol. Both hormones play an integral role in stress physiology. Endogenous opioid peptides are released under stress conditions and act to reduce pain, while cortisol released from the adrenal cortex acts to restore homeostasis in the body (Reeder & Kramer 2005). However, despite these positive functional responses both hormones exert an inhibitory effect on reproductive function through down-regulation of GnRH release (opioids, Almeida 1993 & cortisol, Chrousos & Gold 1992). Thus, in a species such as the naked mole-rat, characterized by high reproductive skew and high levels of aggression between breeders and non-breeders, it is likely that reproductive suppression may potentially be mediated by stress hormones.

In chapter two, I investigate the role of endogenous opioid peptides in reproductive suppression in the naked mole-rat; particularly its potentially suppressive effects on luteinising hormone. The protocol was based on Molteno & Bennett (2002), who examined the role of EOPs in the Damaraland mole-rat (*Cryptomys damarensis*). Their study concluded that EOPs do not play a role in reproductive suppression in this species. Damaraland mole-rats exhibit high reproductive skew, but in contrast to naked mole-rats, display low levels of aggression between breeders and non-breeders. Thus, due to high levels of aggression between dominants and

subordinates the naked mole-rat was considered a better species in which to examine the role of EOPs in reproductive suppression.

In chapter three, I investigate the role of cortisol in reproductive suppression in the naked mole-rat. Plasma cortisol levels were measured in four groups of female naked mole-rats, assigned according to social status (reproductive or non-reproductive) and whether or not i) the animal was ovarectomized, ii) they had been injected with naloxone or cortisol and, iii) they had received single or multiple injections of the latter hormones.

Based on the results obtained in chapters two and three, in collaboration with two electrical engineers I designed an "intelligent" burrow system which I propose is the way forward in our attempts to understanding the proximate mechanisms of reproductive suppression in naked mole-rats (chapter four).

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CHAPTER TWO

THE POTENTIAL ROLE OF ENDOGENOUS OPIOID PEPTIDES IN REPRODUCTIVE SUPPRESSION OF THE FEMALE NAKED MOLE-RAT (*HETEROCEPHALUS GLABER*)

2.1 Abstract

Subordinate female naked mole-rats (*Heterocephalus glaber)* are anovulatory due to markedly low plasma luteinising hormone (LH) levels. It is generally accepted that reproductive suppression in subordinate naked mole-rats is mediated through aggressive behaviour by the dominant female. Endogenous opioid peptides (EOP's) have been implicated in the suppression of LH secretion and naturally induced infertility in a variety of mammals. The influence of EOPs on plasma LH levels, in female naked mole-rats was examined to investigate a potential hormonal mechanism of suppression. Naloxone, an opioid antagonist, was administered to four groups of females, assigned according to social status (reproductive or non-reproductive) and whether or not the animal was ovariectomized. All groups exhibited a significant increase in LH following a single GnRH injection. However, there was no difference between queen and subordinate post-GnRH, LH levels. Neither single nor multiple injections of naloxone had any significant effect on LH secretion in any of the groups. These results imply that EOPs are unlikely to mediate socially induced suppression in the naked mole-rat.

Key words: reproductive suppression, endogenous opioid peptides

2.2 Introduction

Social mammals vary greatly in the degree to which reproduction is shared among group members. While reproduction in social mammals is positively correlated with social status, not all adult members compete for reproduction within the group. Many examples of high reproductive skew can be attributed to inbreeding avoidance, with individuals refraining from reproducing within their natal group despite being behaviourally and physiologically capable of reproduction (Harvey & Ralls 1986; Charlesworth & Charlesworth 1987; Boulin & Boulin 1988; Clutton-Brock 1989; Snowdon 1996; Pusey & Wolf 1996; Bennett *et al.* 1996; Clarke *et al.* 2001). Conversely, in other species, subordinate reproduction appears to be directly controlled by the dominant individuals (marmosets, *Callithrix jacchus,* Abbott 1984, 1987).

Active, behavioural suppression of subordinate reproduction is typically under the control of the dominant individuals. Behavioural suppression may occur through the killing of subordinate offspring (infanticide), physical prevention of mating (mate guarding) and physical punishment of subordinates (wolves, *Canus lupis*, Seal *et al.* 1979; Packard *et al.* 1985; Creel & Creel 1991; meerkats, *Suricata suricatta,* Doolen & Macdonald 1997). Active suppression of subordinates by dominants can also take a physiological form, whereby peptide hormones in subordinates are depressed below thresholds necessary for reproduction (marmosets, *Callithrix jacchus,* Abbott 1984, 1987; naked mole-rats, *Heterocephalus glaber,* Faulkes *et al.* 1990; Margulis *et al.* 1995; Van der Westhuizen 1997).

Endogenous opioid peptides (EOPs) have been proposed as a potential pathway through which infertility in subordinate mammals can be induced by dominants (Molteno & Bennett 2002). Under stress conditions, the adrenal glands produce EOP's, with analgesic properties, to ameliorate pain. However, despite the obvious

benefits of opioids it has long been known that they have an inhibitory effect on reproductive function, down-regulating the release of various pituitary hormones (Aurich *et al.* 1996), such as GnRH from the GnRH neurons (Almeida 1993). EOPs have been implicated during many natural periods of infertility in the mammalian reproductive cycle (associated with lowered luteinising hormone levels), such as seasonal anestrus (Aurich *et al.* 1994) and lactation (Mattioli *et al.* 1986).

By using naloxone, an opioid antagonist, luteinising hormone concentrations can be restored to functional levels, as reported in a variety of mammal species e.g., male rats (Bruni *et al.* 1977); female rats (Petraglia *et al.* 1986); sheep (Currie and Rawlings 1987); pigs (Barb *et al.* 1986); cattle (Short *et al.* 1987); male humans (Agmo & Paredes 1988; Veldhuis *et al.* 1984); rams (Lincoln *et al.* 1987) and hamsters (Chen *et al.* 1984). Thus, in co-operatively breeding mammals characterized by overt aggression towards subordinates by dominants and high reproductive skew, it is possible that an opioid antagonist such as naloxone may be used to release subordinates from suppression.

Molteno & Bennett (2002) investigated the role of endogenous opioid peptides on LH secretion in non-reproductive female Damaraland mole-rats, *Cryptomys damarensis*. The Damaraland mole-rat is a co-operatively breeding subterranean rodent that lives in colonies of up to 41 individuals (Jarvis & Bennett 1993) with reproduction restricted to a pair of individuals. In the event of the death of a breeder reproduction ceases within the colony until the arrival of foreign conspecifics through immigration. Within the Damaraland mole-rat, there appear to be two components to socially induced infertility. Incest avoidance explains most of the colony variance in reproduction, but the presence of the breeding female does result in significantly lower baseline LH levels in subordinate females (Bennett *et al.* 1996). However, subordinate females

did not show a significant increase in LH after single and multiple naloxone injections. This suggests that EOPs do not play a role in social induced infertility in this species. However, aggression is not particularly marked in this species and subordinate reproduction is largely restricted by inbreeding avoidance rather than social suppression by the dominant female. Thus, it is arguable that this species is not the ideal study animal with which to assess the role of EOPs in socially induced reproductive suppression. By contrast, a member of the same family (Bathyergidae), the naked mole-rat, which is characterized by routine inbreeding and high levels of aggression between breeders and non-breeders (Jarvis 1981, 1991; Faulkes *et al.* 1990; Lacey & Sherman 1991), is well suited to such a study. Evidence suggests that in this species the queen controls subordinate reproduction through overt aggression (Faulkes 1990, Lacey & Sherman 1991; Margulis *et al* 1995; Clarke & Faulkes 1997), with most subordinate females having peptide hormone levels below the threshold required for ovulation (Faulkes *et al.* 1990; Margulis *et al.* 1995; Van der Westhuizen 1997). Hence, a socially-induced suppression of LH through the action of EOPs would be more likely in the naked mole-rat.

The aim of this paper was to examine, through the use of the opioid antagonist naloxone, whether endogenous opioids play a critical role in the suppression of reproduction in non-breeding female naked mole-rats. We predicted that if EOPs are implicated in the physiological suppression of subordinate female naked mole-rats, LH should increase significantly after a single or multiple injections of naloxone. Furthermore, we predicted that subordinates would show a greater LH increase following a naloxone injection than queens, as the former are physiologically suppressed. We further tested the effects of naloxone on EOPs by removing the ovaries of queens and subordinates to remove the negative feedback effects of progesterone and oestrogen, with respect to LH, on the hypothalamic pituitary axis (Molteno & Bennett 2002). We predicted that ovarectomized females would show a

greater increase after single or multiple naloxone injections when compared to intact females.

2.3 Materials and Methods

2.3.1 General

The experiments were performed on captive colonies of naked mole-rats maintained in the Department of Zoology at the University of Cape Town. The animals were housed in artificial Perspex® and glass burrow systems (durable, transparent, easily cleaned) linked with a nest, food and toilet chamber as described by Jarvis (1991). Toilet and food chambers were cleaned daily. Animals were provided with a variety of freshly chopped vegetables and fruit each day. The animals were maintained in constant temperature rooms at 28°C.

2.3.2 Study animals and surgical procedure

In this paper a reproductive female (queen) was defined as a female that had produced at least five litters at the onset of the experiment. A non-reproductive female (subordinate) was an adult female that had not bred (O'Riain *et al.* 2000). Six non-reproductive and six reproductive female naked mole-rats were ovarectomized by a registered veterinarian. Animals were returned to their original colonies within two hours of their operation and a minimum of six weeks were allowed for recovery before further experimentation (Molteno & Bennett 2002).

Animals were divided into one of four groups based on their social status (queen, subordinate) and presence or absence of ovaries (ovarectomized, intact). Group 1 animals consisted of six intact queen naked mole-rats (IQ). Group 2 animals consisted of six intact subordinate naked mole-rats (IS). Group 1 and 2 originated from six colonies, an IS and IQ from each (Colony A, 1200, 7000, 7700, 7100, 2000).

The IS and IQ, from each colony, were matched as closely as possible for body mass (Mass: IQ mean 58.21 ± 36.2; IS mean 42.63 ± 19.6). Group 3 animals consisted of six hystero-ovarectomized queen naked mole-rats (OvQ). Group 4 animals consisted of six hystero-ovarectomized subordinate naked mole-rats (OvS). Group 3 and 4 originated from a further six colonies, an OvS and OvQ from each (Colony B, C, D, S, 408, 100). The OvQ and OvS, from each colony, were matched for body mass (Mass: OvQ mean 57.26 ± 18.2; OvS mean 47.09 ± 24.4).

2.3.3 Weighing of animals

Experimental animals were weighed weekly for the duration of the study. Two randomly selected animals from each colony served as controls and were weighed at the same time as experimental animals (Table 1 & 2).

2.3.4 Experimental design

Three different experiments were conducted on each of the four groups.

Experiment 1: To illustrate differential pituitary secretion of LH in breeding and non-breeding female naked mole-rats, the LH response to a single injection of GnRH was measured. All animals in groups 1-4 were administered a single, subcutaneous dose of 0.5μg of GnRH in 200μl physiological saline solution. Blood was taken immediately before and 20 minutes after the GnRH injection

Experiment 2: The effect of a single dose of naloxone followed by a GnRH injection as above was investigated in groups 1-4. Animals received a single injection of 250μg naloxone in 250μl physiological saline. The dosage of 250μg in 250μl was based on the amount administered for a 40g animal (similar dosage to that used in rats by Johnson & Crowley (1984) and Leposavic *et al.* (1991)). A GnRH challenge (0.5μg in 200μl physiological saline) was performed 20 minutes after the naloxone

injection. Blood was taken immediately before the naloxone injection and 20 minutes after the GnRH challenge. Control animals (n=8) received equal volumes of physiological saline.

Experiment 3: The effect of frequent low doses of naloxone was investigated in groups 1 through 4. Animals received hourly injections of 125μg of naloxone in 125μl of physiological saline for 10 hours. A GnRH (0.5μg in 200μl physiological saline) challenge was performed before the start of the naloxone injections and 20 minutes after naloxone priming. Blood was taken 20 minutes after the first GnRH challenge and 20 minutes after the second GnRH challenge. Control animals (n= 8) received equal volumes of physiological saline. The dosage of 125μg in 125μl was based on the amount administered for a 40g animal (similar dosage to that used in rats by Johnson & Crowley (1984) and Leposavic *et al.* (1991)).

Ampoules of 0.5μg of GnRH in 200μl of sterile physiological saline or sterile 200μl physiological saline only (control) were stored at –20°C until required. The GnRH was synthesized in the laboratory of R.P. Millar using solid phase methodology (the purity of GnRH was greater than 98% homogeneity) (Millar *et al.* 1989). Powdered naloxone was stored at -4°C and mixed with saline before experiments. All injections were administered subcutaneously as a bolus.

Blood was obtained by piercing a superficial blood vessel in the hind-foot of the animal using a sterilised needle. The blood was then collected using heparinized microhaemacrit tubes. Animals were returned to their colonies after bleeding. The blood samples were then transferred to Nunc^{R} tubes and centrifuged (500***g*** for 6 minutes). After centrifuging the plasma was then be pippetted off into new Nunc^{R} tubes and stored at –20°C before the radioimmunoassay.

2.3.5 Luteinising Hormone Assay

LH concentrations were measured using an *in vitro* bioassay based on the production of testosterone by mouse Leydig cells (Van Damme *et al.* 1974). Plasma samples were assayed at a dilution of 1:20 LH pituitary preparation (2nd International Standard 1988, code no. 80/552, NIBSC, UK) was used over the range 200-1.4 miu.ml. The testosterone was measured by radioimmunoassay as described by Bennett (1994). Checks for parallelism to the standard curve were carried out to validate the LH assay after GnRH administration. Following logit-log transformation of the data (Chard 1987) parallelism of the LH standard and the serial dilution of squirrel plasma was tested using the Statistica computer package (Statsoft, Tulsa, OK). The curve was parallel and not significantly different from the reference preparation. Sensitivity of the assay (determined at 90% binding) was 12.7miu/tube or 2.5 miu/ml. Standard curves were modelled using the curve fit option of Sigma plot. Intra and inter-assay coefficient of variation for repeated measurement of a quality control were 8 (n = 4) and 11 % (n = 8) respectively. The assay has been validated for use in the naked mole-rat by van der Westhuizen *et al.* (2002).

2.3.6 Statistics

All means are reported with standard deviation (SD). Statistical analyses were preformed using *Statistica 7.0.* Data was checked for normality (Shapiro-Wilk W Test) and equal variance (Levene's Test). Data that were normally distributed were compared using paired t-tests. When data were not normally distributed Wilcoxon-matched pair's tests were used. Elevation rates were calculated using correlations and R^2 values were then compared between groups. The significance level was taken at $p<0.05$ (Zar 1999).

2.4 Results

Experiment 1: Single GnRH injection

Ovarectomized queens (OvQ), ovarectomized subordinates (OvS), intact queens (IQ) and intact subordinates (IS) showed a significant increase in LH ($p=0.027$, $n= 6$, $z=2.201$; $p=0.028$, $n=6$, $z= 2.201$; $p=0.005$, $n= 5$, $t= -7.58$; and $p<0.027$, $n= 6$, $z=2.201$ respectively), after administration of a single GnRH injection (Figure 1).

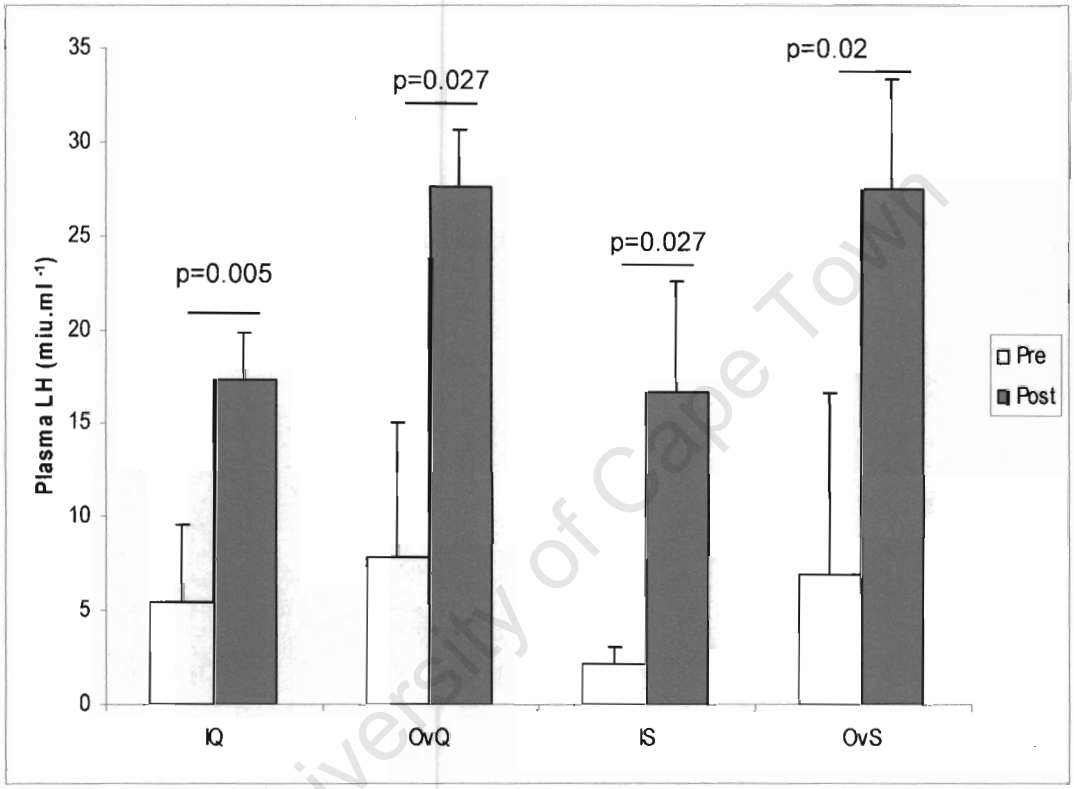

Figure 1: Mean (±SD) basal plasma LH (pre) and the plasma LH response (post) to a single GnRH injection (0.5μg in 200μl saline) for intact queens (IQ), ovarectomized queens (OvQ), intact subordinates (IS) and ovarectomized subordinates (OvS), (n=6 for all groups). The horizontal line denotes significant differences between the columns below them.

There were no significant between-group differences for either baseline or post-GnRH challenge LH levels (Figure 1).

Experiment 2: Single naloxone injection

OvQ, OvS, IQ and IS showed a significant increase in LH (p=0.001, n= 6, t= -6.40; p=0.027, n=6, z=2.201; p=0.001, n=5, t=-9.661; p=0.043, n=6, z=2.022, respectively), after administration of a single naloxone injection (Figure 2).

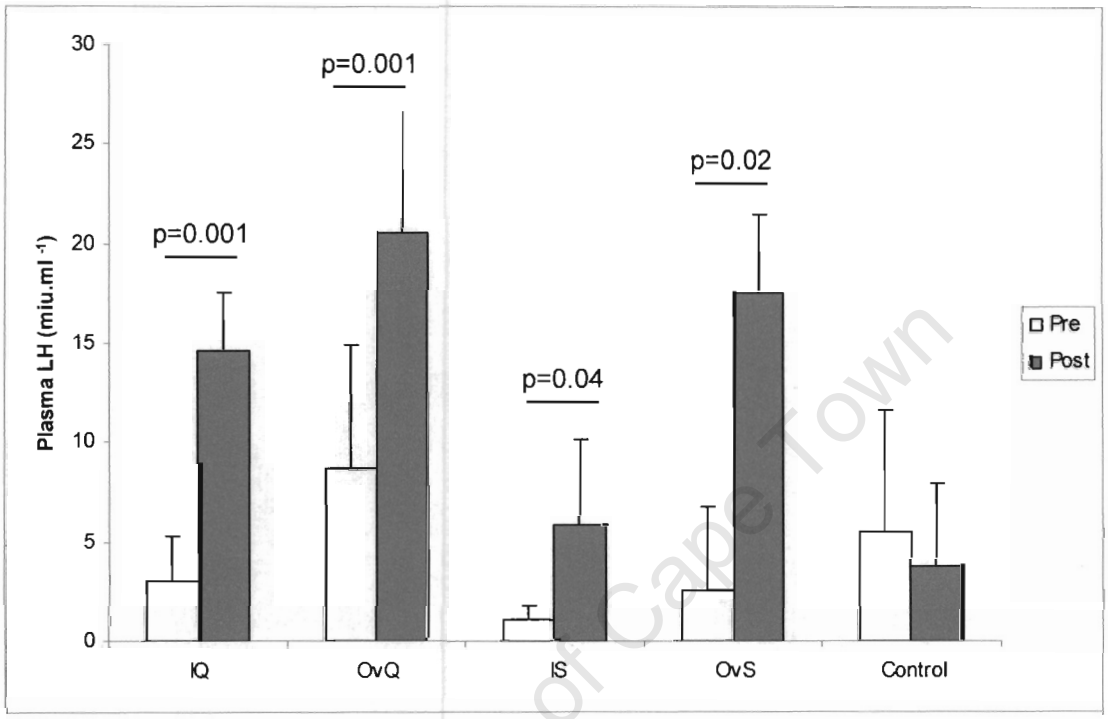

Figure 2: Mean (±SD) basal plasma LH (pre) and the plasma LH response (post) to a single naloxone injection (250μ*g* in 250μ*l* saline) followed by a single GnRH injection (0.5μ*g* in 200μ*l* saline) for intact queens (IQ), ovarectomized queens (OvQ), intact subordinates (IS) and ovarectomized subordinates (OvS). Controls received equal volumes of saline (n=6 for all groups except IQ n =5, controls n=8). The horizontal line denotes significant differences between the columns below them.

However, as the experimental procedure included a GnRH priming injection its effects cannot be excluded. Thus, the mean plasma LH levels after the naloxone and GnRH injection (Experiment 1) was compared with mean plasma LH after a single GnRH injection (Experiment 2). When thus compared; OvQ, OvS, IQ and IS

did not show a significant increase in LH (Figure 3). There was no significant difference in pre and post LH levels in control animals (p= 0.07, n=7, z = 1.825).

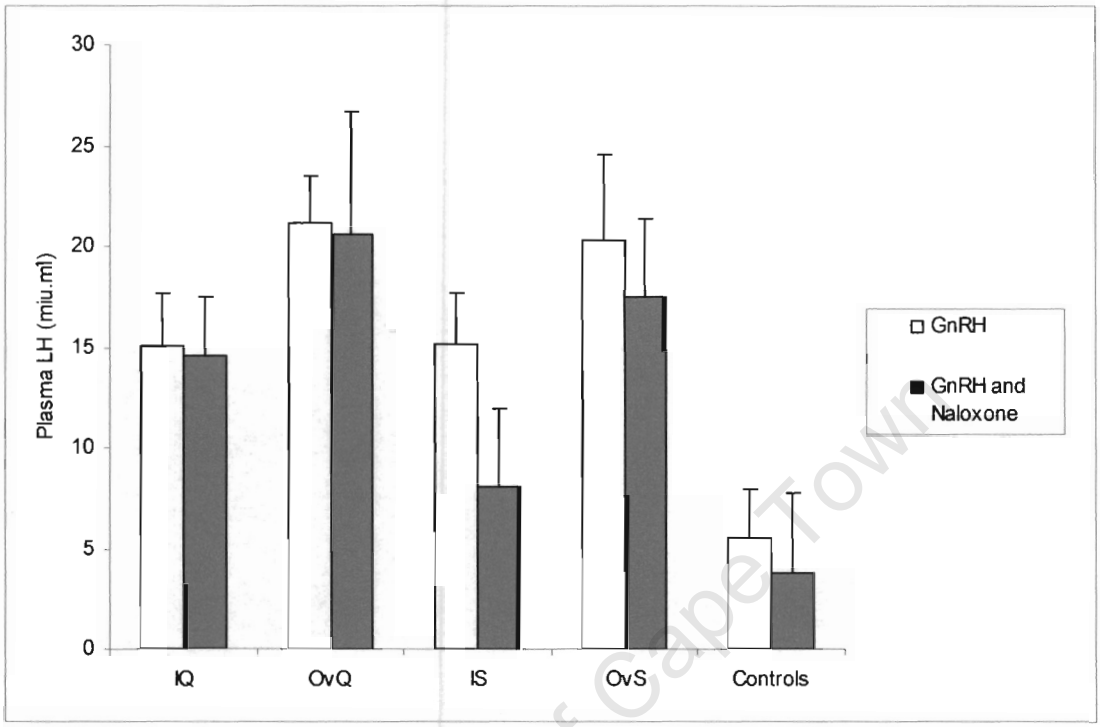

Figure 3: Mean (±SD) GnRH (0.5μg in 200μl saline) stimulated plasma LH (GnRH) and the GnRH (0.5μg in 200μl saline) stimulated plasma LH response to a single naloxone (250μg in 250μl saline) injection (GnRH and naloxone) for intact queens (IQ), ovarectomized queens (OvQ), intact subordinates (IS) and ovarectomized subordinates (OvS). Controls received equal volumes of saline. (n=6 for all groups except IQ n =5, controls n=8).

Experiment 3: Multiple naloxone injection

There were no significant differences for OvQ, OvS, IQ and IS in baseline LH and post-injection regime LH concentrations (p=0.202, n=6, t=-0.509; p<0.46, n= 6, t=-0.807; p=0.317, n=6, t=-1.110; p=0.57, n=6, t=0.597 respectively), after administration of multiple naloxone injections. There were no significant between-

group differences. There was no significant difference in pre and post LH levels in control animals ($p<0.878$, $n= 7$, $z= 0.157$) (Figure 4).

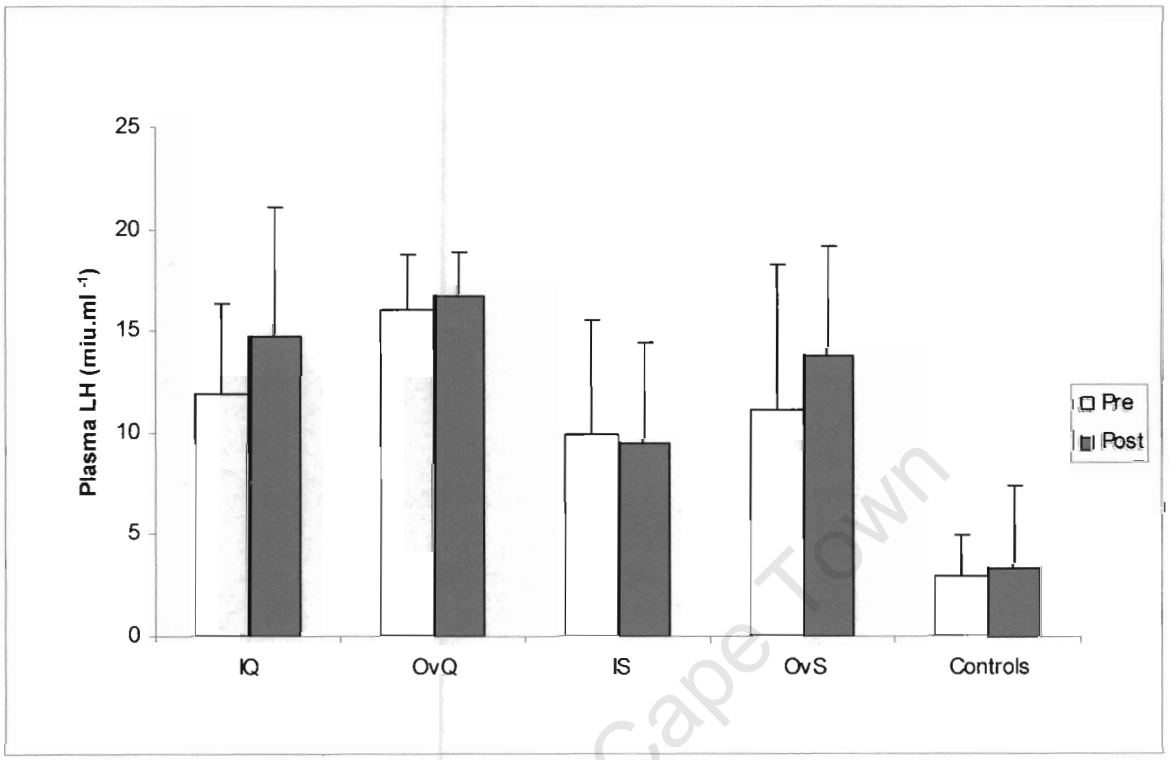

Figure 4: Mean (±SD) GnRH (0.5μg in 200μl saline) stimulated plasma LH (pre) and the GnRH (0.5μg in 200μl saline) stimulated plasma LH response (post) after 8, 1 hourly naloxone injections (125μg in 125μl saline) for intact queens (IQ), ovarectomized queens (OvQ), intact subordinates (IS) and ovarectomized subordinates (OvS). Controls received equal volumes of saline (n=6 for all groups except OvS n =5, controls n=7).

Mass data

Changes in mass of experimental animals during the course of the experiment, of IQ, IS, OvQ and OvS ($p=0.0214$, $r^2= 0.338$; $p=0.2267$, $r^2= 0.5652$; $p=0.132$, $r^2= 0.510$; and $p=0.793$, $r^2= 0.771$ respectively) did not differ significantly from the control animals ($r^2= 0.742$).

Loss of animals

Four intact females (three subordinates, one queen) and three ovarectomized females (one subordinate, two queens) died (e.g. illness and fighting) during the experimental period. These animals were replaced with individuals from the same colony and were as closely matched for mass to the original animal.

2.5 Discussion

All experimental groups (1-4) showed a significant increase in plasma LH after a single injection of GnRH. Interestingly, there was no significant difference between basal LH levels and post-GnRH challenge LH levels in IQ and IS ($p=0.97$). This is contrary to Faulkes *et al.* (1991) who showed that IQ's had significantly higher baseline and post-GnRH challenge levels. Surprisingly, our level of administrated GnRH was higher (0.5μg in 200μl of saline versus 0.1μg in 200μl saline) and one would expect a similar, if not exaggerated, difference in LH levels for IQ and IS. Furthermore, Faulkes *et al.* (1991) used a comparable number of individuals (IQ n=6; IS n=5) to this experiment, so it is unlikely that sample size biased the results. It is unclear if Faulkes *et al.* (1991) matched IQ and IS for mass and age. This could prove an important factor in LH levels. Our animals were matched as closely for mass and age as possible to exclude the influence of mass on hormonal profiles (as queens tend to be older and heavier). Consequently, our subordinates were most probably older and heavier than those used in Faulkes *et al.* (1991) which could explain the relatively high LH levels, resulting in a lack of significant difference between IQ and IS.

There was no significant increase in LH levels (over that produced by GnRH alone) in any of the groups (1-4), following single or multiple injections of naloxone. This is contrary to our prediction that naloxone would increase LH levels, and to a number of studies that indicate that opioids play a role in the suppression of LH secretion (male

rats, Bruni *et al.* 1977; female rats, Petraglia *et al.* 1986; ewes, Currie and Rawlings 1987; rams, Lincoln *et al.* 1987; and hamsters, Chen *et al.* 1984). The lack of any significant increase in LH levels after single or multiple naloxone injections in this study suggests that EOPs do not play a significant role in socially-induced reproductive suppression in female naked mole-rats. Furthermore, reproductive females consistently showed a greater LH response to injections of naloxone than non-breeders which is converse to what would be expected if EOPs were important in maintaining reproductive suppression in subordinates. Abbott (1988) suggested that a short-term blockade (e.g. a single injection of naloxone) of opioid receptors is insufficient to stimulate an increase in plasma LH. However, neither single nor multiple naloxone injections (over an eight hourly period) resulted in a significant LH increase in any of the groups, further supporting our contention that EOPs are unlikely to be pivotal to suppression in subordinate naked mole-rats. These results are similar to those obtained by Molteno & Bennett (2002) for Damaraland mole-rats.

OvQ and OvS had consistently higher (not significant) basal and response plasma LH levels than IQ and IS in all of the experiments (Figure 1- 4). This finding was predicted as ovariectomy relieves the hypothalamic-pituitary axis from the negative feedback effects of oestrogen and progesterone on LH secretion (Molteno & Bennett 2002), thus resulting in higher LH levels. In contradiction, Aurich *et al.* (1996) and Lincoln *et al.* (1987) have shown that naloxone had no effect on LH secretion in ovarectomized mares and castrated rams, unless testosterone and oestrogen titers were artificially restored to normal baseline levels. These findings suggest that in some species opioid inhibition of LH release is influenced positively by gonadal steroids in both males and females. Our results for females do not support this pattern, as ovarectomized animals exhibited increased LH levels following naloxone injections.

Increased cortisol is another possible endocrine pathway through which reproductive suppression could occur in the naked mole-rat. Cortisol, produced in the adrenal cortex is a principle component of the physiological response to stress (Dunn & Berridge 1990; Chrousos & Gold 1992). The reproductive system is directly linked to the stress system and is profoundly affected by changes to it. Naloxone blocks the opioid inhibitory action on CRF neurons, thus inducing a rise in ACTH and cortisol (Tsagarakis *et al.* 1990, Jackson *et al.* 1995, Martin del Campo *et al.* 1994). The physiological response to naloxone has been compared to that of a normal stress response (Oswald *et al.* 2004). In the case of this experiment, the effects of naloxone may have been counteracted if cortisol and opioids acted concurrently, resulting in reduced LH levels. Opioids are known to modulate ACTH and cortisol both in the stressed and unstressed state (Yamamoto *et al.* 2003; Williams *et al.* 2005). This is supported by the increase of LH, although not significant, in groups 1-4 after administration of multiple naloxone injections. Perhaps then, opioids cannot be so readily dismissed as having no role in socially induced reproductive suppression in either the naked or the Damaraland mole-rat. Furthermore, cortisol responses to a stress event need to be examined so we can assess its relationship with LH in subordinate naked mole-rat females.

2.6 Tables

Table 1: Mean mass, range and mass at the onset and termination of the experiment, for intact queens and subordinates. C^1 and C^2 are the randomly selected control animals for each colony. Control weights are in italics.

Colony	Animal	Mean (g)	Range (g)	Initial Mass (g)	End Mass (g)
A	QA	58	20.5	70.7	62.3
	SA	40.2	7.2	40.1	43.5
	C^1	*43.2*	*11.1*	*40.1*	*49.7*
	C^2	*37.7*	*7.4*	*34.2*	*40.2*
1200	Q1200	48.2	10.8	54.8	48.6
	S1200	56.3	9.1	55.4	58.3
	RQ	60.2	6.1	58.9	61
	RS	41.7	2.1	40.6	42.7
	C^1	*44.2*	*7.2*	*42*	*48.7*
	C^2	*44.2*	*10.9*	*42.3*	*30.6*
7000	Q7000	69.6	25.3	76.2	81.4
	S7000	43.2	4.5	43.8	46.1
	RS	38.5	0.8	40	38.9
	C^1	*50.9*	*13.5*	*41*	*54.2*
	C^2	*23.9*	*0.8*	*23.2*	*23.9*
7700	Q7700	47.6	18.8	48.7	42.6
	S7700	44.9	3.1	42.8	43.6
	C^1	*44.3*	*3*	*44.2*	*44.7*
	C^2	*38.4*	*6.1*	*39.7*	*38.8*
7100	Q7100	69.9	19.7	56.9	71.5
	S7100	44.7	6.3	46.7	45.8
	C^1	*39.8*	*7.8*	*39.7*	*39.8*
	C^2	*28.8*	*5.1*	*27*	*30.8*
2000	Q2000	41.6	4.8	44.6	36.8
	S2000	46.9	14.3	44.2	45.2
	RQ	56.6	3.5	54.9	58.4
	RS	40	41	37.2	37.1
	C^1	*47.2*	*6.5*	*45*	*50.8*
	C^2	*39.7*	*6.3*	*36.5*	*45.2*

Table 2: Mean mass, range and mass at the onset and termination of the experiment, for ovarectomized queens and subordinates. C^1 and C^2 are the randomly selected control animals for each colony. Control weights are in italics.

Colony	Animal	Mean (g)	Range (g)	Intial Mass (g)	End Mass (g)
B	QB	55.2	14.6	62.8	59.2
	SB	52.9	5.3	53.3	49.8
	C^1	*29.2*	*4.4*	*28.5*	*27.9*
	C^2	*37.7*	*8.6*	*36.9*	*42.9*
C	QC	53.5	25.4	66	57.5
	SC	47.9	8	51.2	50.6
	C^1	*37.2*	*8.9*	*56.5*	*36.5*
	C^2	*40*	*6.8*	*37*	*42.6*
D	QD	45.6	6.1	47.8	46.1
	SD	54.7	19.6	49.8	61.6
	RQ	55.7	13	59.5	57.8
	C^1	*75.65*	*12.1*	*70.1*	*79.6*
	C^2	*29.1*	*14.1*	*27.5*	*34.1*
S	QS	50	5	39.9	41.3
	SS	35.5	7.3	51.9	38.4
	C^1	*39.9*	*5.1*	*39.8*	*42.1*
	C^2	*55.7*	*6.3*	*55.6*	*58.2*
408	Q408	50.7	8.3	56.4	52.3
	S408	33.3	3.5	35.1	34
	C^1	*51.29*	*3.4*	*50.6*	*51.9*
	C^2	*46.3*	*2.9*	*46*	*46.3*
100	Q100	58.7	7.6	64.6	58.4
	S100	54.4	4.5	51.3	46.4
	SR	39.2	8.1	40.8	37.8
	C^1	*50.1*	*11.4*	*52.6*	*59.5*
	C^2	*29.47*	*5.6*	*31.5*	*29.3*

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CHAPTER THREE

THE EFFECTS OF STATUS AND STRESS REGIMES ON CORTISOL LEVELS IN FEMALE NAKED MOLE-RATS, *HETEROCEPHALUS GLABER*

3.1 Abstract

Subordinate female naked mole-rats have strikingly lower LH levels than dominant females. It is generally accepted that dominant females suppress subordinate female reproduction through aggressive behaviour, particularly shoving. Cortisol, the primary glucocorticoid in mammals, is released under stressful situations and has been implicated in the suppression of LH and FSH secretion. In this study, the role of plasma cortisol on LH secretion in female naked mole-rats was examined to investigate a potential hormonal mechanism of suppression. Naloxone, an opioid antagonist, and saline were administered to four groups of females, assigned according to social status (reproductive or non-reproductive) and whether or not the animals were ovariectomized. Both intact and ovarectomized queens had significantly higher baseline cortisol levels than their subordinate counterparts. Both intact queens and subordinates exhibited a significant increase in cortisol following a single saline injection. However, there was no significant change in cortisol for intact queens and subordinates following multiple saline injections. Only ovarectomized queens and subordinates exhibited a significant increase in cortisol following a single naloxone injection, whilst none of the groups exhibited any change following multiple naloxone injections. These results reveal that queens have higher baseline cortisol, suggesting that elevated cortisol levels are unlikely to play a role in reproductive suppression in subordinates. However, multiple injections, indicative of a chronic stress regime, resulted in down-regulation of cortisol levels in both queens and subordinates. This suggests that hypo-cortolism may play a role in reproductive suppression of subordinates.

Keywords: reproductive suppression, cortisol, stress, hypo-cortolism

3.2 Introduction

When an animal experiences a stressful situation (e.g. an aggressive attack), immediate physiological changes occur within the body to redirect energy (nutrients and oxygen) away from long-term body functions such as growth, reproduction and digestion to the central nervous system and muscles. This provides an animal with immediate energy to cope with a stressful situation and ultimately restore homeostasis to the body (Sapolsky 2000). Within a matter of seconds, corticotrophin releasing hormone (CRH), a principle component of the hypothalamic-adrenal axis response system, is released and sets in motion a series of physiological responses to the stressor (Dunn & Berridge 1990; Chrousos & Gold 1992). CRH stimulates pituitary ACTH (adrenocorticotropic hormone), which in turn stimulates release of glucocorticoids (GC's) from the adrenal cortex. Cortisol is the primary glucocorticoid found in most mammals (Reeder & Kramer 2005). Much of the cortisol produced during a stress reaction is bound in the blood to a binding globulin; the remaining unbound cortisol is biologically active instigating the numerous changes that occur in the body (Lynn *et al.* 2003). An acute stress reaction is highly beneficial in restoring homeostasis of the body and cortisol itself feeds back negatively at the level of the pituitary, hypothalamus and hippocampus, reducing CRH secretion and consequently cortisol secretion (Reeder & Kramer 2005). However, if cortisol levels remain chronically elevated a broad range of negative consequences become evident such as reduced immune function, reproductive suppression and loss of muscle mass (Chrousous & Gold 1992, Creel 2001).

Chronic elevation of cortisol levels can decrease sex steroid secretion and it has been hypothesized that if subordinates experience a greater stress load it might explain reproductive failure (Wingfield *et al.* 1991). The reproductive system is profoundly affected by the stress system. CRH, despite being implicated in a number of reproductive functions including ovulation, luteolysis, implantation and parturition,

actually inhibits the LH releasing hormone neurons of the hypothalamus and GnRH secretion, while glucocorticoids (cortisol) inhibit pituitary LH release and render target tissues of the sex hormones resistant to oestradiol (Chrousos & Gold 1992; Chrousos *et al.* 1998; Dobson & Smith 2000; Wingfield & Sapolsky 2003; Kalantaridou *et al.* 2004). Glucocorticoid administration significantly reduces the LH peak response to GnRH in humans (Sakakura *et al.* 1975), and inhibits oestradiol-stimulated uterine growth in rabbits (Rabin *et al.* 1990). The stress response is highly adaptive when stress is acute. Chronic stress, on the other hand, may have numerous adverse affects and has been attributed to complete inhibition of human female reproduction (Bromberger *et al.* 1997). Prolonged exposure to GC's has been demonstrated to reduce the GnRH content of the brain (Consten *et al.* 2001), decrease pituitary responsiveness to GnRH (Rivier & Rivest 1991) and decrease gonadal responsiveness to LH despite circulating levels (Charpenet *et al.* 1981). In the male rat GC's suppress the post castration rise in circulating LH by reducing pituitary sensitivity to GnRH (Suter *et al.* 1988), while in gonadectomized sheep an *in vitro* infusion of cortisol suppresses LH secretion (Porter *et al.* 1990; Daley *et al.* 1999). In primates, exogenous cortisol inhibits LH and FSH secretion in ovarectomized rhesus monkeys, but inhibition of the sex hormones only becomes evident after three to four weeks (Dubey & Plant 1985).

In many social species that show high reproductive skew there are rank related differences in basal plasma cortisol levels. The general view is that subordination is stressful, as in the olive baboon (*Papio anubis*) society (Sapolsky 1990) and in spotted hyena (*Crocuta crocuta*) clans (Goymann *et al.* 2001) where subordinates show higher cortisol levels than dominants. However, subordinates are not always characterized by higher cortisol levels than dominants. In wild dogs (*Lycaon pictus*), alpine marmots (*Marmota marmota*), dwarf mongooses (*Helogale parvula*) (Creel 2001), wolves (*Canis lupis,* Sands & Creel 2004), female common marmosets

(*Callithrix jacchus,* Abbott *et al.* 1997; Saltzman *et al.* 1994, 1996, 1998) and cotton top tamarins (*Saguinus Oedipus,* Ginther *et al.* 2001), dominants consistently show greater cortisol levels than subordinates (Creel 2001). In naked mole-rats, the mammal with arguably the highest reproductive skew, results are contradictory. Faulkes & Abbott (1997) indicated subordinates had higher cortisol levels than dominants, while Clarke & Faulkes (1997) indicated that cortisol levels in dominants (both males and females) were greater than subordinates. Furthermore, Clarke & Faulkes (1998) found no relationship between dominance and cortisol titres in males. In addition, Clarke & Faulkes (2001) found no correlation between cortisol titres and the rate an individual was shoved, suggesting social stress does not suppress reproductive function while, Faulkes (1990) found non-breeding subordinates ovulated when removed from their colonies despite cortisol remaining high. Contradictory results may be related to a number of external factors such time of day, sexual status, age, experience and social stability within the colony. Rank related cortisol differences in naked mole-rats are thus still ambiguous.

In the naked mole-rat circumstantial evidence suggests that the queen controls subordinate reproduction through overt aggression (Lacey & Sherman 1991; Faulkes & Abbott 1991; Margulis *et al* 1995; Clarke & Faulkes 1997), with most females having luteinising hormone levels below the threshold required for ovulation (Faulkes *et al.* 1990; Margulis *et al.* 1995; Van der Westhuizen 1997). Under these circumstances the endocrine pathway of socially-induced infertility is likely to involve the stress system with the hypothalamic-pituitary-adrenal axis (HPA axis) presumed to play a central role in the mediation of stress-induced gonadal inhibition (Rivier & Rivest 1991).

In this study, we examined rank-related difference in basal plasma cortisol levels, hypothesizing that subordinates, the primary recipients of queen aggression should

experience a higher stress load and hence have higher basal levels of cortisol. Ultimately these chronic levels of stress could result in lowered LH levels and reproductive suppression in subordinate females. Furthermore we predicted that subordinates and queens should show a differing stress response to a novel stressor (saline/naloxone injection regime), both acute (single injection) and chronic (multiple injections), with subordinates exhibiting a more acute stress response when compared to dominants. We predicted that a multiple injection regime would cause a greater increase in cortisol levels in both queens and subordinates when compared to a single injection regime. We further predicted that subordinates would experience a significantly greater rise in cortisol than queens to both injection regimes. The physiological response to naloxone has been likened to a normal stress response, so plasma obtained from naloxone experiments was included in the results. Again we predicted that subordinates would show a greater cortisol response than queens to naloxone injections.

3.3 Methods

3.3.1 General

The experiments were performed on captive colonies of naked mole-rats, maintained in the Department of Zoology at the University of Cape Town. The animals were housed in artificial Perspex® and glass burrow systems (durable, transparent, easily cleaned) linked with a nest, food and toilet chamber as described by Jarvis (1991). Toilet and food chambers were cleaned daily and animals were provided *adlibitum* with variety of freshly chopped vegetables and fruit each day. The animals were maintained in constant temperature rooms at 28°C.

3.3.2 Study animals and surgical procedure

In this paper a reproductive female (queen) is defined as a female that had produced at least five litters at the onset of the experiment. A non-reproductive female (subordinate) is an adult female that had not bred (O'Riain *et al.* 2000). Six non-reproductive and six reproductive female naked mole-rats were ovarectomized by a registered veterinarian. Animals were then returned to their original colonies within two hours of their operation and a minimum of six weeks were allowed for recovery before the onset of experimentation. Removal of the ovaries served to remove the negative feedback effects of progesterone and oestrogen on the hypothalamic pituitary axis (Molteno & Bennett 2002).

Animals were divided into one of four groups based on a) their social status (queen, subordinate) and b) the presence or absence of functional ovaries (i.e. hystero-ovarectomized or intact). Group 1 animals consisted of six intact queen naked mole-rats (IQ). Group 2 animals consisted of six intact subordinate naked mole-rats (IS). Group 1 and 2 originated from six colonies, an IS and IQ from each (Colony A, 1200, 7000, 7700, 7100, 2000). The IS and IQ, from each colony, were matched for body mass and age (Mass: IQ mean 58.21 ± 36.2; IS mean 42.63 ± 19.6). Group 3 animals consisted of six hystero-ovarectomized queen naked mole-rats (OvQ). Group 4 animals consisted of six hystero-ovarectomized subordinate naked mole-rats (OvS). Group 3 and 4 originated from a further six colonies, an OvS and OvQ from each (Colony B, C, D, S, 408, 100). The OvQ and OvS, from each colony, were matched for body mass and age (Mass: OvQ mean 57.26 ± 18.2; OvS mean 47.09 ± 24.4).

3.3.3 Experimental design

Additional blood obtained in chapter two was used to explore the effects of multiple and single injections of naloxone and saline on cortisol levels in female naked mole-

rats. It provided us with an opportunity to examine rank-related baseline cortisol differences between subordinates and queens, and their response to a variety of injection regimes. Primarily our aim was to examine responses to saline injections, a standardised stressor with no exogenous hormonal interference, but due to available plasma we were able to include cortisol levels after single and multiple naloxone injections. Thus, we have used a similar protocol to the previous chapter.

Four different experiments were conducted on each of the groups. All injections were administered subcutaneously as a bolus. Ovarectomized animals were excluded from experiment 1 and 2 due to an insufficient sample size.

Experiment 1: To illustrate possible differential cortisol secretion in breeding and non-breeding female naked mole-rats, the cortisol response to two injections of physiological saline solution was measured. All animals in group 1 and 2 were administered two subcutaneous doses of 200μl physiological saline solution. Blood was taken immediately before and 20 minutes after the saline injection. For comparison with the single naloxone injections, experiment one was referred to as single saline injections.

Experiment 2: The effect of frequent doses of physiological saline on cortisol secretion was investigated in groups 1 and 2. Animals received hourly injections of 200*u*l physiological saline over 10 hours. Blood was taken immediately before and 20 minutes after the last saline injection.

Experiment 3: The effect of a single physiological stressor (naloxone) on cortisol secretion was investigated in groups 1, 2, 3 and 4. All animals in group 1-4 were administered a single, subcutaneous dose of 250μg naloxone in 250μl physiological

saline. A GnRH (0.5μg in 200μl physiological saline) challenge was preformed before the naloxone injection and 20 minutes after the naloxone injection. Blood was taken 20 minutes after the first GnRH challenge and 20 minutes after the second GnRH challenge.

Experiment 4: The effect of frequent low doses of naloxone was investigated in groups 1 through 4. Animals received hourly injections of 125μg of naloxone in 125μl in physiological saline over 8 hours. A GnRH (0.5μg in 200μl physiological saline) challenge was preformed before the start of the naloxone injections and 20 minutes after naloxone priming. Blood was taken 20 minutes after the first GnRH challenge and 20 minutes after the second GnRH challenge. The dosage of 125μg in 125μl was calculated on the amount administered for a 40g animal (similar dosage to that used in rats by Johnson & Crowley (1984) and Leposavic *et al.* (1991)).

Blood was obtained by piercing a superficial blood vessel in the hind-foot of the animal using a sterilised needle. The blood was then collected using heparinized microhaemacrit tubes. Animals were returned immediately to their colonies after bleeding. The blood samples were then transferred to nunc tubes and centrifuged (500***g*** for 6 minutes). After centrifuging the plasma was then be pippetted off into new nunc tubes and stored at –20°C before the radioimmunoassay.

3.3.4 Cortisol Assay

Plasma cortisol concentrations were determined using a commercially available kit Coat-a-Count Cortisol (TRKCO2 Diagnostic Products Corporation, Los Angeles CA) as described in Molteno (1999). The assay was able to determine cortisol concentrations of between 1.26 nmol/L and 1380 nmol/L. The cross-reactivity of the antiserum was less than 1% with all naturally occurring steroids, with the exception of

11-deoxycortisol (11.4%), prednisolone (76%) and prednisone (2.3%). The assay was validated for plasma by testing for parallelism using serial doubling dilutions of mole-rat plasma obtained from an individual with high cortisol concentrations (range 1:1 to 1:32). The slope of the lines did not differ significantly (ANCOVA $F_{1\text{-}6} = 4.7$ and $p<0.05$). The sensitivity of the assay (90% binding) was 6.1ng ml^{-1}. Intra and inter assay coefficient of variation was 2.3 % and 4.1% respectively.

3.3.5 Statistics

All means are reported with standard deviation (SD). Statistical analyses were done using *Stastica 7.0*. Data were checked for normality (Shapiro-Wilk W Test) and equal variance (Levene's Test). The significance level was taken at $p<0.05$ (Zar 1999). Data that were normally distributed were compared using paired and independent t-tests. When data were not normally distributed Wilcoxon-matched pair's and Mann-Whitney U tests were used. Differences between experiment regimes were also investigated.

3.4 Results

Baseline cortisol

Queens, both intact and ovarectomized, had significantly higher baseline cortisol levels than their subordinate counterparts ($p=0.027$, $Z=2.2156$, $n=12$; $p=0.0496$, $Z=1.962991$, $n=12$). There were no significant differences between ovarectomized and intact queens or subordinates (Figure 1).

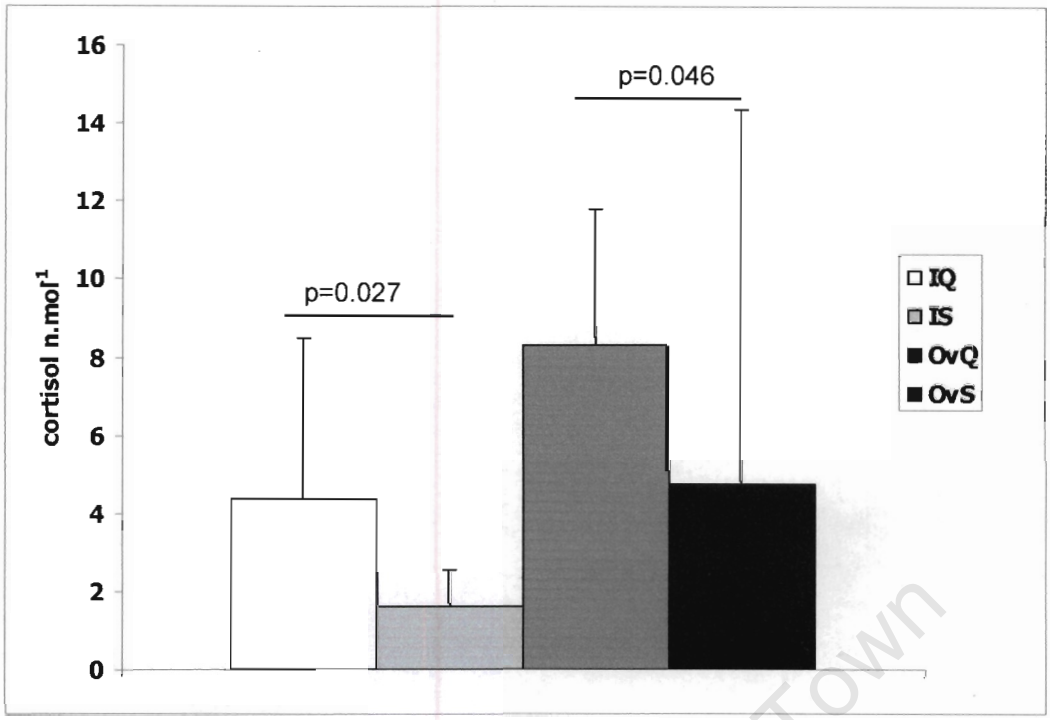

Figure 1: Mean (±SD) basal plasma cortisol from intact queen (IQ), intact subordinate (IS), ovarectomized queen (OvQ) and ovarectomized subordinate (OvS) female naked mole-rats (n=12 for all groups). The horizontal line denotes significance differences between the columns below them.

Experiment 1

Both intact queens and intact subordinates exhibited a significant increase in cortisol following a single saline injection (p=0.03, n=6, z=2.202 and p=0.02, n=6, z=2.01) respectively). There was, however, no significant difference between the post-cortisol levels of queens and subordinates (p= 0.582, n=6, t=-0.568) (Figure 2).

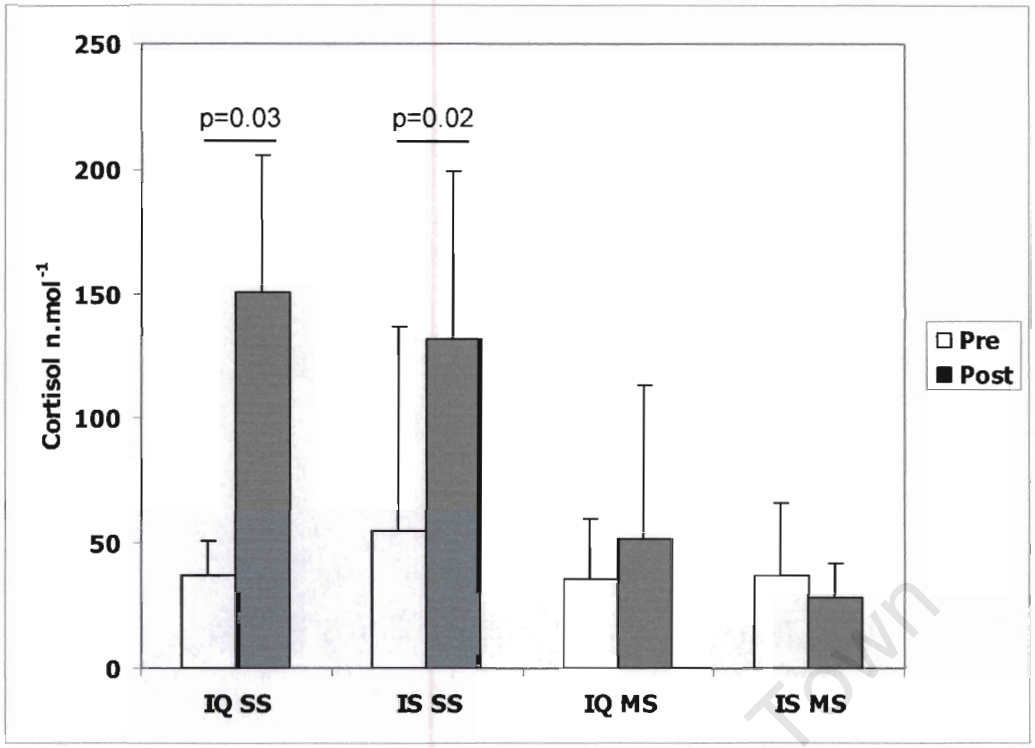

Figure 2: Mean (±SD) basal plasma cortisol (pre) and cortisol response to single (SS - experiment 1) and multiple saline (MS - experiment 2) injections for intact queens (IQ) and intact subordinates (IS) (n=6 for all groups). The horizontal line denotes significance differences between the columns below them. SS post levels are significantly greater than MS post level for queens and subordinates (p=0.028 and p=0.024 respectively)

Experiment 2

Neither queens nor subordinates exhibited a significant change in cortisol levels after multiple saline injections (p=0.584, n=6, t=-0.584 and p=0.500, n=7, t=0.717 respectively). There was no significant difference between post-injection cortisol levels of queens and subordinates (p=0.481, n=6, t=-0.911). Post-injection cortisol levels were significantly greater in experiment 1 than experiment 2 for both queens and subordinates (p=0.028, n=6, Z=2.201 and p=0.024, n=6, z=-2.241) (Figure 2).

Experiment 3

Only ovarectomized queens and subordinates showed a significant increase in cortisol after administration of a single injection of naloxone (p= 0.019, n=5, t= -3.785 and p= 0.001, n= 5, t=-5.591 respectively). Intact queens and subordinates showed no significant difference (p=0.08, Z=1.79252, n=6). Ovarectomized queens exhibited a significantly greater post-injection cortisol level when compared to intact queens (p=0.016, n=5, z=-2.402) (Figure 3).

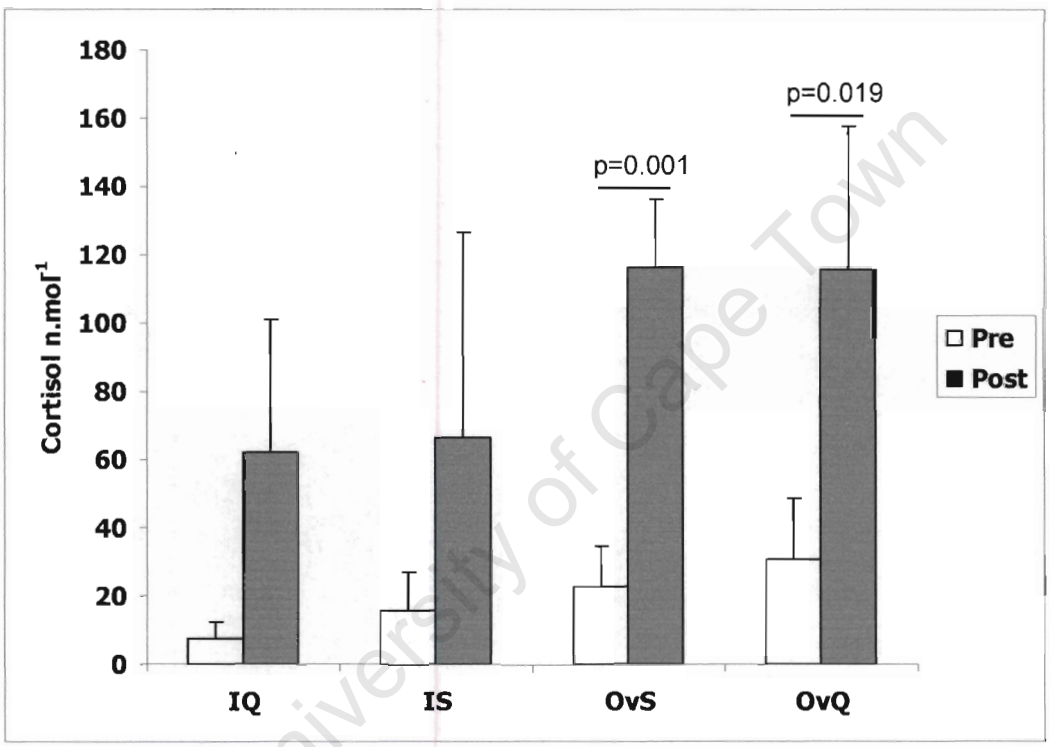

Figure 3: Mean (±SD) basal plasma cortisol (pre) and the plasma cortisol response (post) to a single naloxone injection (250μg in 250μl saline) followed by a single GnRH injection (0.5μg in 200μl saline) for intact queens (IQ), intact subordinates (IS), ovarectomized subordinates (OvS) and ovarectomized queens (OvQ) (n=6 for all groups).

Experiment 4

There was no significant difference in cortisol levels for intact queens, intact subordinates, ovarectomized queens or subordinates (p=0.345, n=6, Z=0.943;

p=0.196, n=6, t=1.552; p=0.861, n=6, t=-0.183 and p=0.660, n=6, t=0.467 respectively), following multiple naloxone injections (Figure 4).

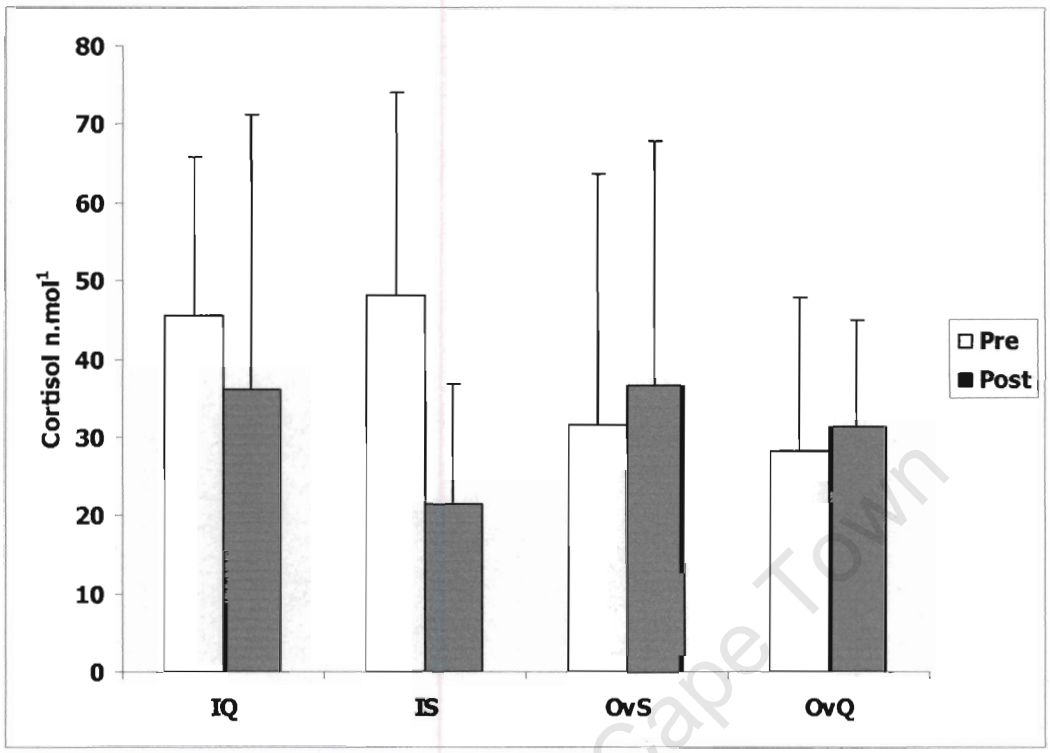

Figure 4: Mean (±SD) GnRH (0.5μg in 200μl saline) stimulated plasma cortisol (pre) and the GnRH (0.5μg in 200μl saline) stimulated plasma cortisol response (post) after 8, 1 hourly naloxone injections (125μg in 125μl saline) for intact queens (IQ), intact subordinates (IS), ovarectomized subordinates (OvS) and ovarectomized queens (OvQ) (n=6 for all groups).

Naloxone versus saline injections

Intact subordinates showed no significant difference between saline and naloxone injections in both the multiple and single injection regimes (p=0.475, n=7, t=-0.7408; p=0.092, n=6, t=-1.8573 respectively).

Intact queens showed no significant difference between multiple injections of saline and naloxone (p=0.872, n=6, t=-0.1601). However, cortisol levels after multiple saline injections were significantly greater than cortisol levels following multiple naloxone injections (p=0.044, n=5, z= -2.0083).

3.5 Discussion

Baseline

Faulkes & Abbott (1997) showed that subordinates had significantly greater baseline cortisol levels than dominants, while Clarke & Faulkes (1997) indicated a significantly negative correlation between dominance and cortisol titers (thus, queens had higher cortisol levels than subordinates). In this study both ovarectomized and intact queens had significantly higher baseline levels than their subordinate counterparts (see figure 1). Our results are contradictory to Faulkes & Abbott (1997) and hence to our prediction that baseline cortisol would be greater in subordinates. Both ovarectomized and intact queens had significantly higher baseline levels of cortisol than their subordinate counterparts (see figure 1). Our results concur with Clarke & Faulkes (1997).

These results are similar to those found in the common marmoset (*Callithrix jacchus*), where subordinate females are reproductively suppressed and show greatly reduced cortisol levels when compared with breeding females (Saltzman *et al.* 1998). Oestradiol has been shown to elevate circulating cortisol levels in several primate species (Coe *et al.* 1986), thus the fact that queens have higher oestrogen levels could explain their higher cortisol levels. However, a reduction of oestrogen through ovariectomy causes a reduction in circulating cortisol levels in several species (e.g. rhesus macaques, Smith & Norman 1987), thus one would expect that if oestrogen mediates cortisol, ovariectomy would lead to a reduction in cortisol levels. In this study, baseline cortisol levels from ovarectomized queens and subordinates were consistently higher than those of intact queens and subordinates. Again this is similar to common marmosets; although unlike our study, breeding female marmosets have higher cortisol levels than ovarectomized females (Abbott *et al.* 1997, 1998; Saltzman *et al.* 1998). Thus, in both naked mole-rats and marmosets, lowered cortisol levels cannot be solely attributed to lower oestrogen levels.

Saltzman *et al.* (1998, 2000) and Abbott *et al.* (1998) suggest that reduced cortisol levels are due to a reduced responsiveness to ACTH and reduced levels of ACTH, possibly mediated by LH or progesterone as both these hormones can influence HPA activity. LH can stimulate GC release from both rat and opossum adrenal tissue *in vitro* (Vinson and Renfree 1975; Vinson *et al.* 1976). Abbott et al. (1998) suggests that under these circumstances hypocortolism is an adaptation to a hypo-oestrogenic adult state and could possibly protect the body from the negative effects of low oestrogen levels such as bone loss. Support for this hypothesis is provided by the apparent absence of low-oestrogen bone loss in hypo-corticol marmosets (Colman *et al.* 1997). It is possible that a similar system occurs in the naked mole-rat. Further investigation is required, focusing in particular on changes of cortisol throughout the reproductive cycle, as baseline levels of cortisol are shown to increase during pregnancy in some species (e.g. humans, Lightman *et al.* 2001). If oestrogen does have some effect on cortisol we would expect more variation in cortisol levels among intact queens when compared with ovarectomized queens, subordinates and intact subordinates.

Experiment 1, 2, 3 and 4

Surprisingly, only OvQ and OvS exhibited a significant increase in cortisol following a single naloxone injection. Furthermore, OvQ post-injection cortisol levels were significantly greater than IQ post injection cortisol levels ($p=0.01$). Naloxone is known to block the opioid inhibitory action on CRF neurons, thus inducing a rise in ACTH and cortisol (Tsagarakis *et al.* 1990; Jackson *et al.* 1995; Martin del Campo *et al.* 1994). The physiological response to naloxone has been compared to that of a normal stress response (Oswald *et al.* 2004). It is possible that hypo-estrogenic, ovarectomized females reacted differently to naloxone, inducing a greater rise in cortisol levels when compared to their intact counterparts. Unfortunately, ovarectomized animals were not used for the single saline injections, so we were

unable to examine differing responses to a physical stressor, thus possibly isolating naloxone as contributing variable.

As predicted, both IQ and IS showed a significant increase in cortisol following a single saline injection. However, when cortisol levels, after a single naloxone injection, are compared to cortisol levels after a single saline injection, no significant differences were evident for IS ($p=0.092$). This suggests that the animals were reacting to a physical stressor, the injection, rather than either saline or naloxone. Unexpectedly, cortisol levels after a single saline injection were significantly greater than after a single naloxone injection for IQ ($p=0.044$). There was no significant difference between queens and subordinates for either single naloxone or saline injections.

Following multiple saline injections, IS cortisol levels were depressed below baseline, while IQ cortisol levels showed no change. Thus, our prediction that multiple saline injections would cause a greater increase in cortisol than single injections was not supported. Furthermore, there was no significant difference between cortisol levels after multiple saline injections for both queens and subordinates. Combined with the lack of significant differences between queens and subordinates for single injections, one can assume that there is no difference in the stress response between subordinates and queens.

What is apparent when examining multiple versus single injection regimes is that down-regulation of cortisol does appear to occur in the naked mole-rat. As an animal learns that a stressor is not a threat, the novel quality of a stressor is lost. A reduction in the stress reaction follows, thus limiting the deleterious consequences of chronic levels of cortisol (Sapolsky 2002). This is known to occur in rhesus macaques (*Macaca mulatta)* after three to four days of regular chair restraint. The macaques not

only decreased behavioural indicators of stress but showed depressed HPA activity. If chair restraint is repeated after a break of up to six months, depressed cortisol levels continue (Ruys *et al.* 2004). In the naked mole-rat cortisol depression occurs more rapidly, in our experiment it was evident 10 hours after the onset of multiple injections. This suggests that naked mole-rats show a predisposition towards down-regulation of cortisol during a chronic stress condition. This is not unexpected, for subordinates in particular, which experience routinely high levels of aggression from the queen. Thus, hypo-cortisolism rather than hyper-cortisolism may play a role in reproductive suppression of subordinate naked mole-rats. Queens within a stable colony do not experience high levels of aggression so may remain unaffected by aggression related stress. Furthermore, it has been suggested that if the hypothalamic-gonadal (HPG) axis is already activated, then the hypothalamic-pituitary axis cannot inhibit an already stimulated system. This has been shown to occur in rats in which stress procedures stimulating the HPA axis did not inhibit the HPG axis (Lemaire *et al.* 1997) when they were already sexually activated. Therefore, even under a stress condition the already reproductively activated queen is unlikely to lose her reproductive monopoly in the colony.

There is a large degree of ambiguity around hormone studies due to the large number of variables that can affect hormonal systems. Cortisol, in particular, can be affected by a number of factors such as immune function (Hermus & Sweep 1990), food intake (Ausman *et al.* 1989) and reproductive function (Saltzman *et al.* 1998), so it remains difficult to obtain unbiased basal levels of cortisol. Clarke & Faulkes (1997, 1998) and Faulkes & Abbott (1997), give no indication when during the day urine samples were taken. As many mammals (e.g. humans, Kolevska *et al.* 2003; red-backed voles, *Clethrionomys grapperi*, Kramer & Sothern 2001) show a daily cortisol circadian rhythm, where levels tend to decrease after 1 pm, the time of day cannot be excluded from variables affecting baseline cortisol. All animals in this study were

bled before 11 am to avoid this confounding variable. Variation in the time of day when samples were taken cannot be excluded as an explanation for the differing results between the studies. Clearly, cortisol levels in the naked mole-rat, and its interactions with the reproductive axis needs to be examined further, with special regard to changes in cortisol through the reproductive cycle and the potential role of hypo-cortisolism to suppression of reproduction in subordinate females.

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CHAPTER FOUR

THE WAY FORWARD

4.1 Synthesis

The results from chapters one and two in this study suggest that endogenous opioid peptides do not play a role in reproductive suppression in subordinate naked mole-rats. Furthermore, queens exhibited significantly higher cortisol levels than subordinates, suggesting that hyper-cortisolism is not the hormonal pathway leading to low LH levels in subordinate females. It is possible that hypo-cortisolism may serve an important role in reproductive suppression in this species, as down-regulation of cortisol occurred in intact queens and subordinates subjected to multiple saline injections. It has been suggested that if the hypothalamic-gonadal (HPG) axis is already activated, then the hypothalamic-pituitary axis cannot inhibit an already stimulated system. Therefore, even under a stress condition the already reproductively activated queen is unlikely to lose her reproductive monopoly in the colony, while the subordinates may become reproductively suppressed.

Both of the experiments in this study involved the handling of animals in addition to subjecting them to exogenous hormone regimes. While this approach was deemed preferable to purely correlative data (*sensu* Van der Westhuizen 1997; Van der Westhuizen *et al.* 2002) it is arguably a relatively crude approach when considering the complexity of the mammalian reproductive axis and its interface with stress.

While we have not succeeded in unequivocally elucidating the proximate mechanism of reproductive suppression in naked mole-rats, we have a better understanding of the role of both EOPs and cortisol. It is obvious from previous removal experiments (e.g. Faulkes *et al.* 1990) that the physical presence of the queen is central to our understanding of reproductive suppression in subordinates. It is thus my suggestion that we should attempt to design an experiment in which one controls for all variables

except direct physical contact with the queen. This approach would control for all potentially suppressive odour signals from other colony members and enable the experimenter to test the effects of direct contact with the queen alone. This could be achieved by designing an 'intelligent burrow system' that is able to separate a queen and subordinate without either of their removal from the colony.

The above experimental design may also achieve what previous researchers have attempted unsuccessfully (Hamblin and O'Riain 2000, Chapter 2 this study), *viz.* sexually activating a subordinate through removal from direct contact with the queen. Faulkes *et al.* (1990) have shown that temporary removal of a subordinate female from the colony results in her becoming sexually active within 7-10 days. Unfortunately, re-introduction of an animal into its original colony following a period of absence will result in aggression, from all colony members, not exclusively associated with her new sexual status. Each colony has a unique odour and any animal with a foreign smell is readily detected and then attacked, often fatally. O'Riain & Jarvis (1997) demonstrated that loss of the colony odour through physical separation occurred within twelve hours of removal. These individuals were attacked despite no changes in their sexual status (summarised in figure 1). Clearly it is only through the design of an "intelligent" burrow system that one will be able to achieve this objective.

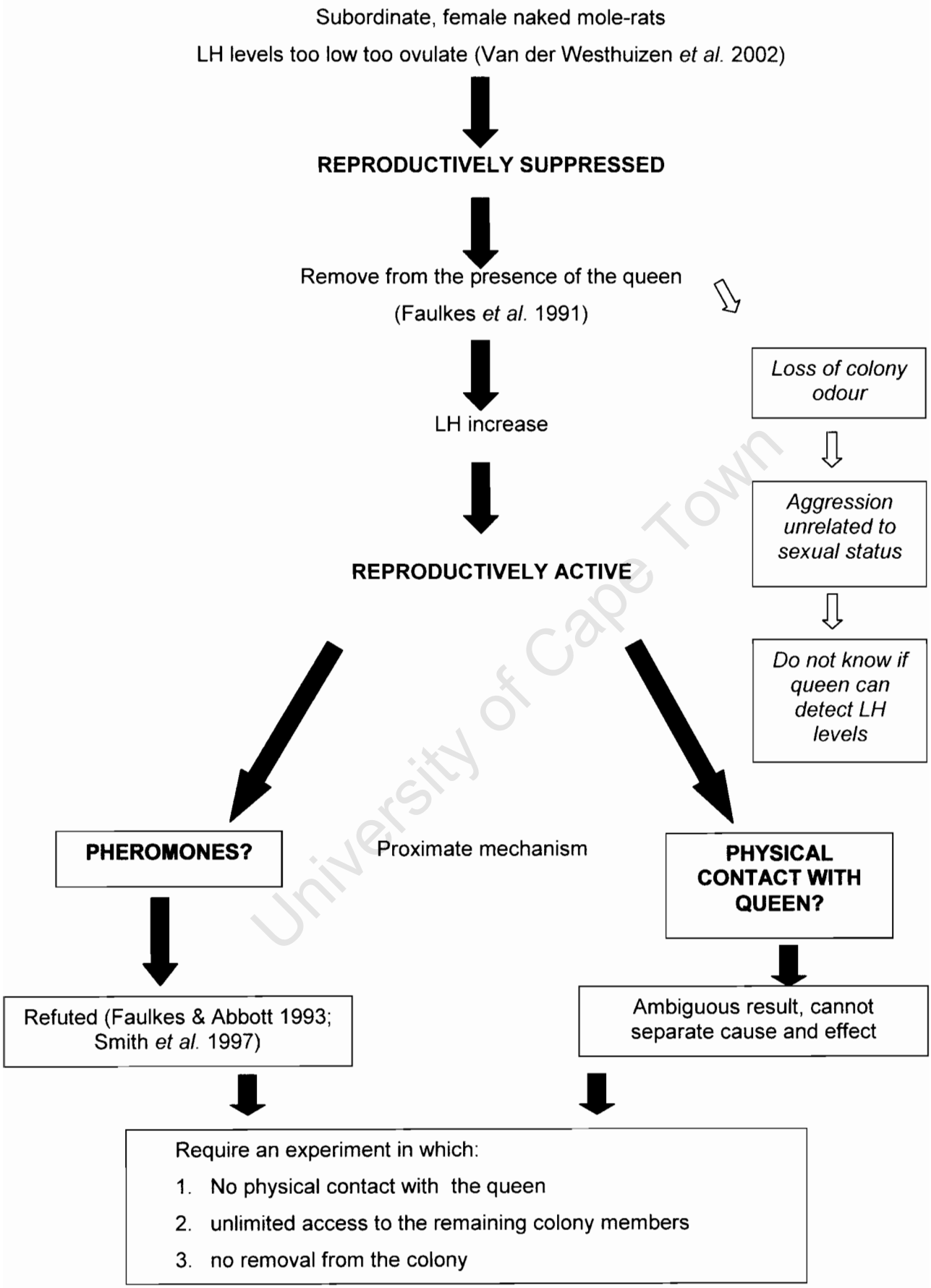

Figure 1: Summary of subordinate reproduction suppression in the naked mole-rat. Where we are now and where to from here?

Ideally one would want to remove an individual from the queen's physical presence, but still keep that individual in physical contact with the rest of the colony and olfactory contact with the queen. This has been partially achieved by Smith *et al.* (1997) who removed a subordinate female from a colony, but maintained constant odour contact with her parent colony through soiled bedding transfer. Furthermore, subsets of subordinates from the parent colony were regularly placed in the removed female's burrow, allowing social contact with other colony members. Removed females became reproductively active and thus it was suggested that direct contact with the breeding female was the major suppressive influence in reproductive suppression. However, the study did not unambiguously prove that physical contact with the queen causes reproductive suppression. The study only examined limited access to varying subordinates under potentially stressful conditions (i.e. removal from the burrow) and used the transfer of soiled bedding to simulate odour. We do not know how volatile these pheromones (if any) are or how long they remain biologically active for. Thus, while this represents the best attempt to date to control for the effects of pheromones, it is still not an ideal control.

The idea of an "intelligent" burrow system was thus devised. This burrow system, through a series of microchip-activated doors, was designed to prevent all physical interactions between the queen and a selected subordinate female, while still allowing both test subjects access to all other odour cues i.e. colony members, the communal toilet, the nest and the food chamber. This system will be the first attempt to demonstrate unambiguously that direct physical interactions with the queen suppress subordinate LH levels and thus serve as the proximate mechanism for reproductive suppression in naked mole-rats. This experiment effectively controls for the potential affect of pheromones on a subordinate's hormone level and does not

involve the handling and removal of animals from their natal burrow system (*sensu* Smith *et al.* 1997).

With this system it is possible to directly test the hypothesis that reproductive suppression in subordinate females is mediated through direct physical interaction with the queen.

The following predictions would provide support for the hypothesis.

I predicted that an experimental individual (i.e. one that is removed from the queen's physical presence) would exhibit the following changes:

Become reproductively active with:

i) A perforate vagina

ii) Increased sexual behaviours (anogenital nuzzling, copulation)

iii) Increased aggression behaviour towards other colony members (shoving, incisor fencing)

iv) Increased luteinising hormone levels (LH)

I predicted that the control individuals (i.e. those with access to the queen):

Should remain reproductively inactive with:

i) Imperforate vagina

ii) No increase in sexual or aggressive behaviours

iii) Depressed LH levels

I predicted the queen, when exposed to both control and experimental individuals after a period of separation would:

i) Increase shove rate towards an experimental individual

ii) No increase in shove rate to control individuals

I predicted that following reintroduction to each other, there will be immediate and serious fighting between the queen and experimental individual. If fighting does not prove fatal for either individual, then I further predicted that the experimental individual would become reproductively inactive and exhibit the following changes over time:

Become reproductively inactive with:

i) Imperforate vagina

ii) Decreased sexual and aggressive behaviours

iii) Decreased LH levels

For the behavioural protocol see the Appendix in chapter 5.

4.2 Materials and Methods

4.2.1 The artificial burrow system and the control program

The artificial burrow was designed in collaboration with Aaron Wetzler to physically separate the queen and selected subordinate female through the opening and shutting of mechanical doors located a various points throughout the colony. The tunnels were constructed from Perspex® with glass lids. The gate mechanisms were controlled by a central microprocessor linked to sensors located at various positions along the tunnels. The sensors responded to microchip transponders implanted subcutaneously into the queen and selected subordinate. The DSP microprocessor was programmed to close a door when two sensors, one sensor apart, are trigged by animals carrying microchips. The control program was a simple computer simulation of animals walking around in random configurations so as to generate maximally unpredictable behaviour. From this a set of gate operating rules were created that

was dependent of which of the sensors had been triggered. Continuous testing produced more robust rules, effectively dealing with problems such as random sensor noise and the possibility of a sensor missing a mole-rat with an implanted microchip.

The sixteen sensors were divided into four quadrants (Figure 2), with a gate between each section. The program was designed to keep the implanted mole-rats separate under all conditions, while simultaneously minimising obstruction of movement of all the remaining colony members. The system accomplished this by tracking the implanted mole-rats movements as they passed the various sensors. As each sensor was triggered the computer updated the position of each animal. The sensors were polled continuously, up to 3000 times per second. If an activated sensor was not directly adjacent to a previously known mole-rat position, then an error condition was activated. If a sensor was activated adjacent to an original position, but if there was a closed gate between the two adjacent sensors, then once again the error condition was activated. In all instances when an error condition occurred, all the doors were shut and the position of the implanted mole-rats was then considered unknown by the microprocessor. Reactivation of the system required either of the two implanted mole-rats to move passed a sensor, where after this mole-rat was assigned to that specific sector of the burrow system. Any other signal received from outside this sector was then automatically assumed to be the "other" mole-rat and the system returned to normal functioning.

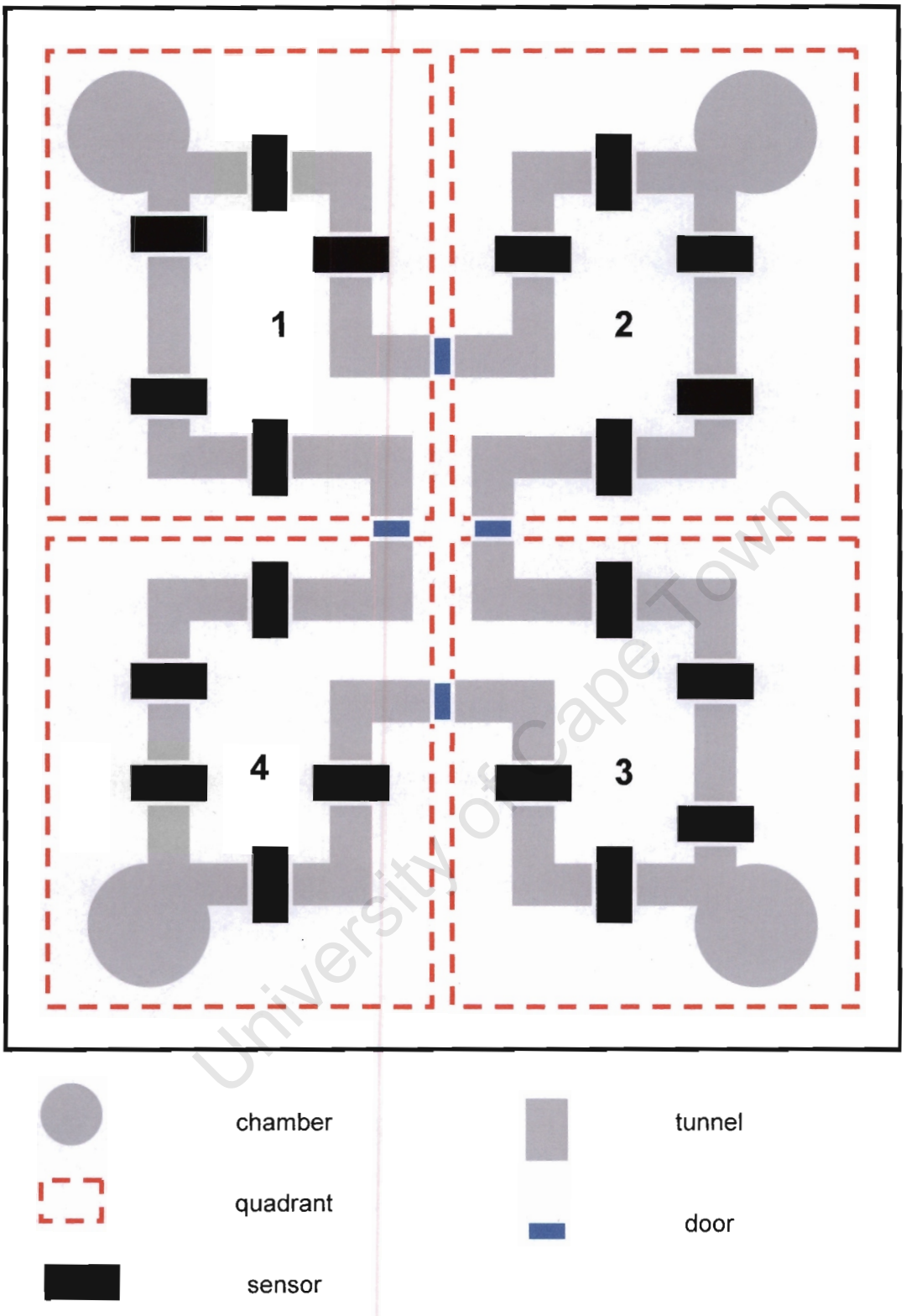

Figure 2: Schematic illustration of the "intelligent" burrow system, showing the position of the sensors, doors and four quadrants.

4.2.2 Door mechanism

Doors and their associated opening and closing mechanisms were designed in collaboration with electrical engineering student Aaron Wetzler. Doors were made of clear Perspex, see Figure 3(a). Each door was run on a low power dc motor (b), which was used to drive a gear which in turn drove a toothed rail (c) on the roof of the door. The doors slid shut, in approximately half a second, along two Perspex pieces that acted as a smooth railing to minimize friction. Light sensors (d) on either end of the door indicated whether the door was open or closed or in the process of changing between the two states. The door motor drive boards were designed to accept the door direction control signal as well as the door position signal. The motors draw around 600mA, under normal operating conditions.

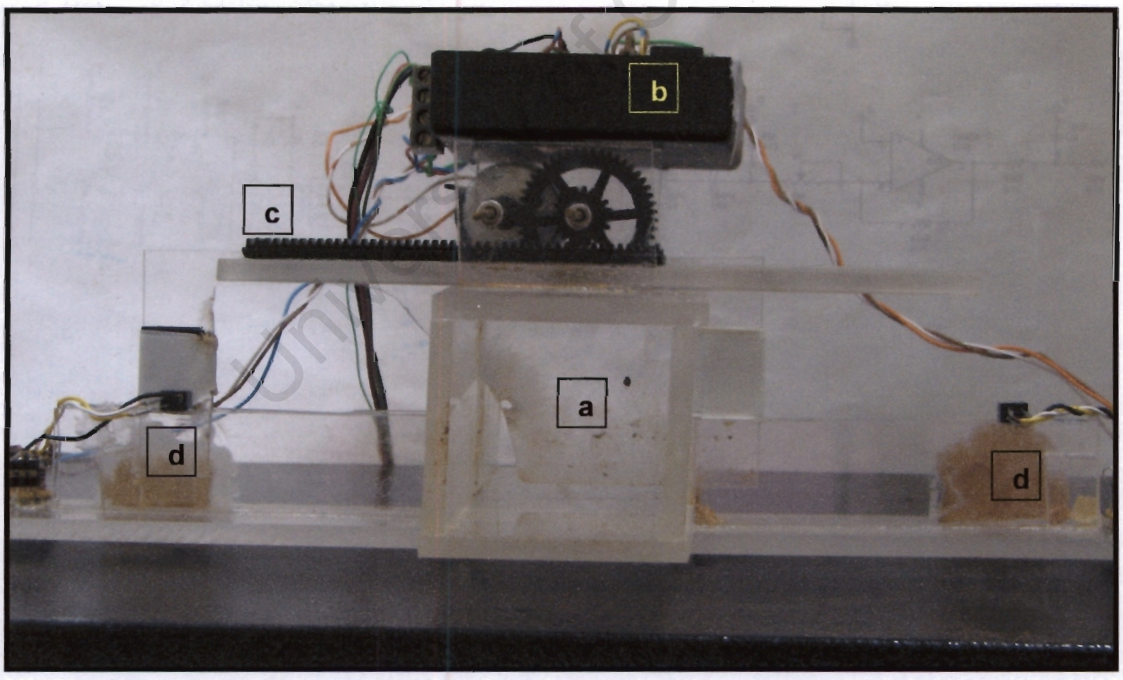

Figure 3: Side view of a door mechanism with its various components a) door, b) motor, c) toothed railing and light sensor (d)

When the control board received a command signal it started the motor and the door moved in the required direction, until the light sensor was activated. The door remained in that position until the next command signal. The programme had to accommodate the possibility of a mole-rat becoming stuck in the door when closing. If, when trying to close a door, the programme registered the door was still open after half a second, possibly indicating an animal was preventing a door from closing, the door was re-opened and attempted to shut again after a half second delay. This allowed the animal to move away from the door after being temporarily trapped. The system was programmed to continue in this manner until the light sensor indicated the door had shut completely.

4.2.3 Sensors

The sensors (Figure 4) were designed and constructed in collaboration with electrical engineer, J.P. Kloppers. Sensors were designed to detect a metal object no smaller than 3mm in diameter. The sensors enabled the position of animals (implanted with microchips) to be constantly monitored within the burrow system. By linking the sensors to the microprocessor the system was able to ensure the doors restricted physical access between selected individuals.

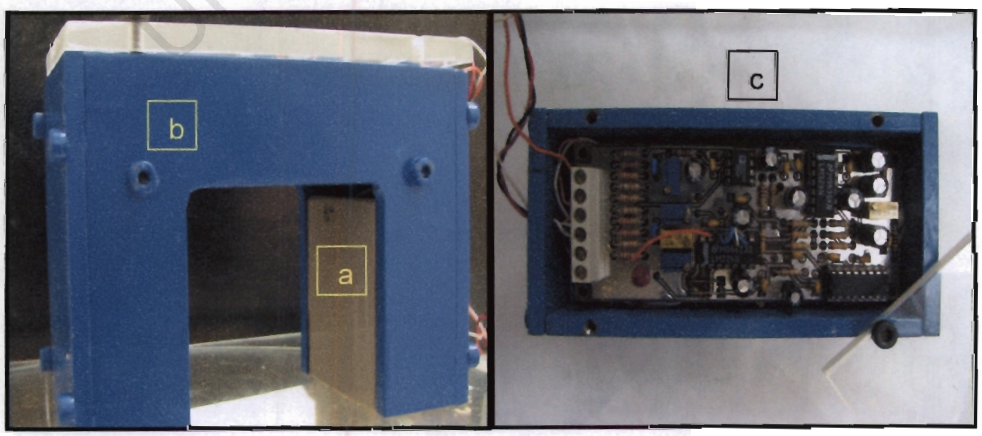

Figure 4: Side view of a sensor with the magnet (a) within the metal housing (b) to prevent interference from surrounding metal objects. (c) Provides a top view of the sensor showing the circuit linked to the microprocessor.

The sensor worked by using search coils to pick up slight disturbances in a magnetic field, created using strong earth magnets (see figure 4). A disturbance in the field occurred when a metallic object was moved through the magnetic field. The metallic object used in the mole-rats was a copper-based microchip. When a metallic object (e.g. the microchip) moved through the field, it created a low frequency disturbance in the signal that the search coils, wound around the magnets, detected as a disturbance coming from within the magnetic field. Through a process of analogue filtering the low frequency disturbance was isolated from the rest of the random noise (e.g. electrical noise) picked up by the search coil. A threshold was then set which determined the sensitivity of the sensor. Once this threshold was crossed (i.e., when an animal carrying a microchip passed through the field), an alarm signal pulse was generated and sent to the microprocessor indicating that an implanted naked mole-rat had passed through the sensor. To minimise the affect of outside electrical noise, and therefore to enhance its sensitivity, the sensor was housed in a 5mm thick metal box (see Figure 4). This allowed the sensor to be paced in a room containing other metals without them interfering with the sensors.

4.2.4 Microchips

Selected individuals were implanted subcutaneously with two Identipet® microchips (sealed tubes containing copper coils), on the surface of the dorsal cervical-thoracic region, posterior to the scapula, of the selected individuals (see figures 5; 6). Animals were allowed six weeks to recover before being introduced to the experimental burrow system.

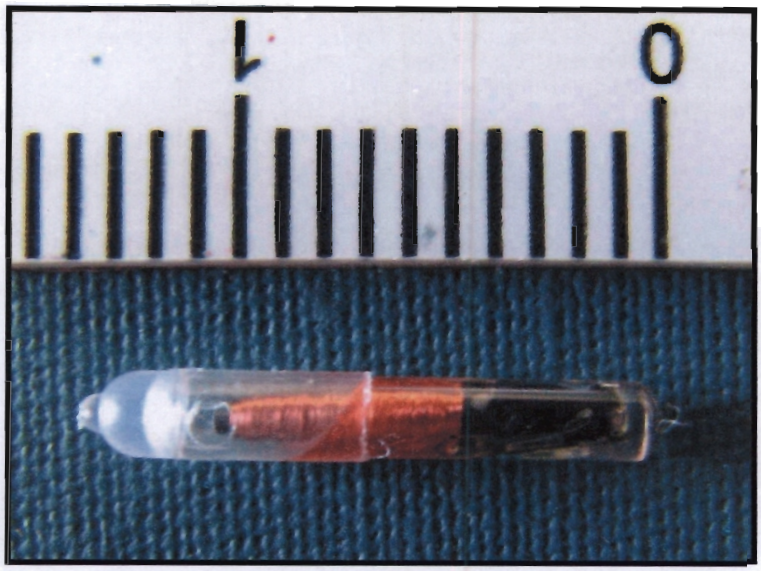

Figure 5: An Identipet® microchip showing copper coil housed within a glass covering.

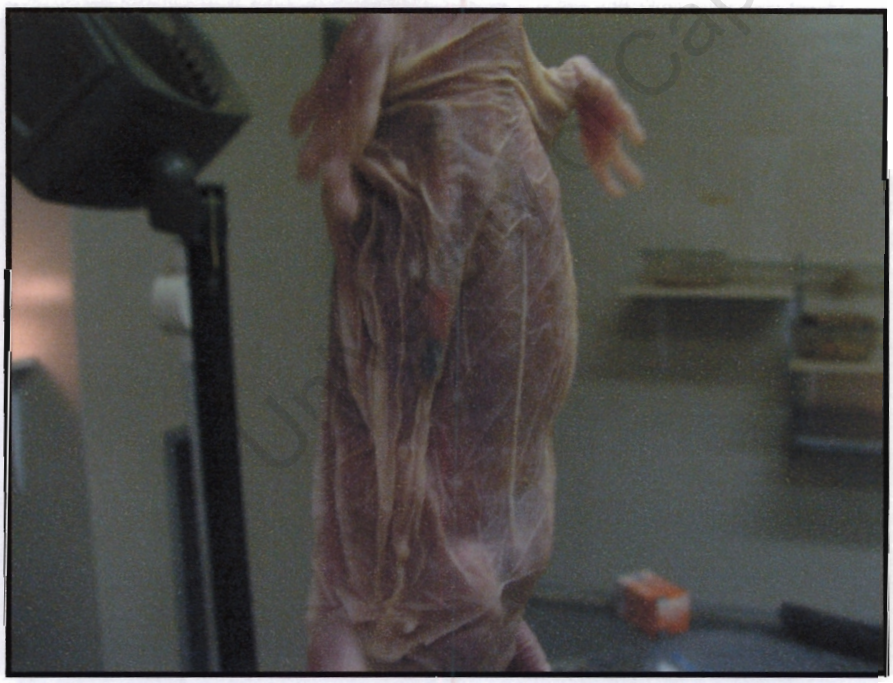

Figure 6: A microchip, under the skin of a female, naked mole-rat. The microchip has moved since implantation and is now located on the abdomen of the animal. This did not affect the ability of sensors to detect the animal's movement.

4.2.5 General

A trial experiment was performed on a single captive colony of naked mole-rats, maintained in the Department of Zoology at the University of Cape Town. The entire burrow system was placed upon a large block of Styrofoam that served to dampen vibrations and provided a suitable medium through which to conduit the many electrical wires (Figure 7). Wood-shavings were supplied as nest material. The toilet chamber was cleaned daily and fresh wood-shavings added.

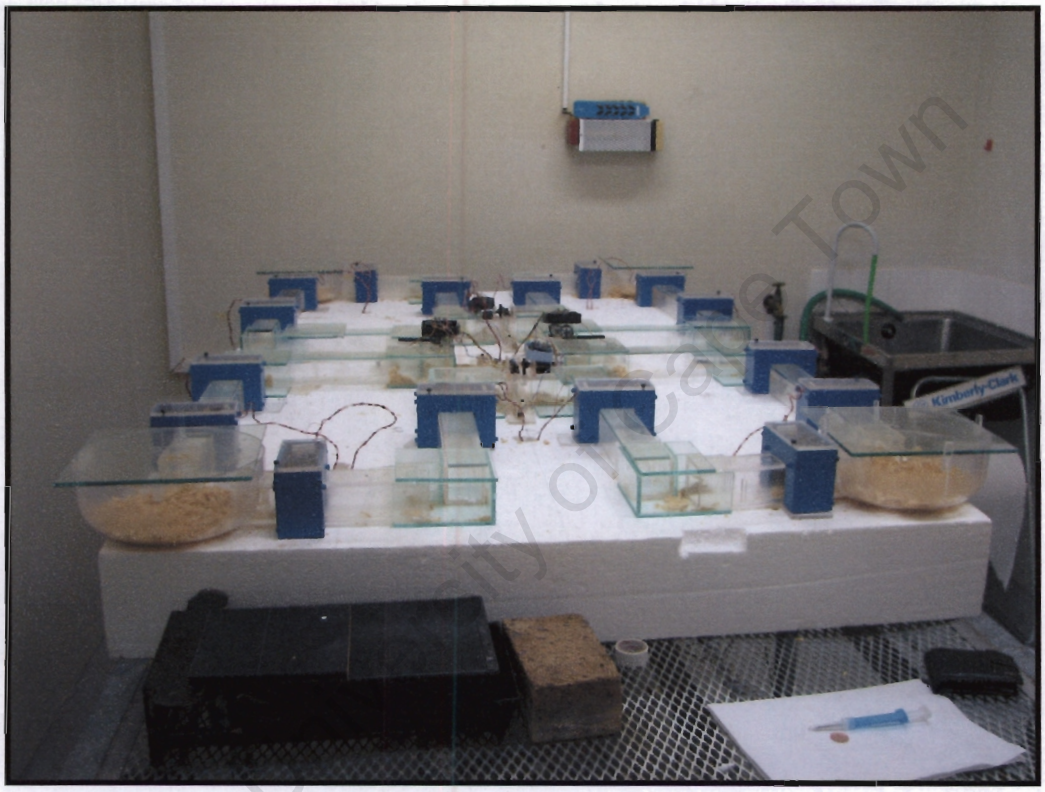

Figure 7: An aerial view of the entire burrow system with sensors (blue), doors at the centre of the system, chambers and interlinking tunnels.

The mole-rats were fed daily on a variety of vegetables and fruit. All food items provided were fairly large (approximately 3cm in diameter), to discourage the animals from removing them from the food chamber and into the tunnel system where they could get jammed in the door.

4.2.6 Marking

Animals were permanently marked by toe-clipping to provide them with a unique ID number. For the purposes of behavioural data collection, each animal's ID number was then written on its back with a non-toxic black marker pen.

4.3 Discussion

A preliminary trial of the burrow system was preformed upon completion. The system worked smoothly for four days, before the programme failed and the queen and subordinate gained access to one another. While this separation was shorter than intended it was interesting to note that the experimental female was observed nuzzling the breeding male's genitals for the first time after just three days of separation, but never prior to separation. She also showed an increase in physical aggression towards other colony members.

The problem encountered while in error mode was that background noise (source unknown) was interpreted by the programme as an implanted mole-rat. The programme thus had incorrect information regarding the exact whereabouts of the two implanted mole-rats. The result of this was that the doors remained open, allowing for the reintroduction of the experimental animals. The actual meeting of the queen and subordinate after four days separation was not witnessed so it is not known what the queen's response to the subordinate was. To correct this flaw, the programme was rewritten to enable the system to clearly distinguish between erroneous noise and a signal from an implanted micro-chip. I fervently believe that the "intelligent burrow" system is the only way forward in the exploration of the mechanism of reproductive suppression in the naked mole-rat. The subordinate female displaying sexual and aggressive behaviour towards the breeding male and other colony members respectively after just three days of separation, provides

strong circumstantial evidence that the system will provide the key to solving the mystery of reproductive suppression in the naked mole-rat. Unfortunately due to personal reasons, realising this objective within the time constraints of a Master's degree was not possible. It will be completed in collaboration with myself and team of colleagues during the course of 2006.

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CHAPTER FIVE

5.1 Appendix

5.1.1 Behavioural protocol to be followed when using the "intelligent" burrow system

Two-minute scan samples, for a total of between 10 and 20 non-consecutive hours, should be performed and the behaviour and position of all colony members recorded. In addition a minimum of 45, 20 minute focals should be recorded for the implanted queen, subordinate and control animals. Behavioural observations should begin five days prior to the door mechanism being switched on, to ascertain the baseline behaviour in the colony and of the experimental and control animals. Further observations should be continued after the system is activated (approximately ten days) and again when the doors are switched off and the queen and experimental female re-introduced to each other (five days).

Focal observations will allow for the quantification of behavioural changes in the experimental and control animals, before, during and after physical separation. A list of behaviours to be recorded is provided in Appendix I. In addition, any rare dominance and/or sexual related behaviour should be recorded *ad libitum.* if observed.

Blood sampling

Blood samples should be taken on three separate occasions:

1) *Baseline* (after animals have acclimatised to new burrow)

2) *After 10 days of separation* (non-reproductives take *ca.* 7-10 days to become reproductively active when housed singly – Faulkes *et al.* 1990)

3) *After the experimental individuals have been re-exposed to the queen* (approximately 10 days)

Every animal in the colony must be weighed on the same day blood is taken from the experimental animals.

Blood is to be obtained by the same methods outlined in Chapter one and two.

5.1.2 Ethogram of the naked mole-rat: non-vocal behaviours (adapted from Lacey et al. 1991)

Grooming

Resting

1. yawning
2. dozing (a mole-rat stands motionless with drooped head)
3. huddling singly or in groups (a mole-rat lies on its back or stomach apparently asleep)

Thermoregulation

1. basking (a mole-rat stands at heat source and its head does not droop)
2. crouching (a mole-rat stands with a hunched posture in physical contact with other colony members)

Feeding

Eliminating

1. defecating
2. urinating
3. urinating with crotch dragging
4. urinating with scratching (vigorous scratching after urination)
5. wallowing (rubs shoulders and flanks against bottom or sides of toilet immediately after defecation or urination)

Coprophagy

1. autocoprophagy (consumption of own feces)
2. allocoprophagy (consumption of faeces by another individual)

3. begging (nudging of anal area before allocoprophagy)

Locomotion

1. walking (forward or backward)
2. running (forward or backward)
3. passing (side by side or over and under)

Cleaning

1. sweeping (a mole-rat kicks loose dirt behind itself while moving backward)
2. carrying
3. dragging

Digging

1. gnawing
2. backshovelling
3. foreleg digging

Mating

1. backing (breeding female backs up to male)
2. mounting
3. copulating

Interactive behaviours

1. nose pressing
2. nuzzling (a mole-rat rubs its muzzle against body of another individual)
3. ano-genital nuzzling or sniffing (a mole-rat of one sex sniff or nuzzles the genitalia of a mole-rat of the opposite sex)
4. sniffing

5. head deflecting (a mole-rat turns its head down and to the side)
6. pawing

Agonistic behaviours

1. open-mouth gaping (two mole-rats stand face to face, with open mouths and muzzles touching, hissing sound produced)
2. incisor fencing (two mole-rats lock incisors at right angles, typically shove back and forth)
3. batting (two mole-rats swat at each other's muzzles)
4. biting
5. shoving (two mole-rats stand face to face with muzzles pressed together and one mole-rat moves forward pushing the second backwards)
6. tugging (an individual bites a body part of a second individual and pulls backwards)
7. tetany (when a mole-rat lies still with its feet in the air in response to being shoved)