Endocrine control of reproductive suppression in three *Cryptomys hottentotus* subspecies

By

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Declaration

I, **Andries Koch Janse van Vuuren**, declare that this thesis/dissertation, which I hereby submit for the degree Master of Science (Zoology) at the University of Pretoria, is my own work and has not been submitted for a degree at this or any other tertiary institution.

31/01/2022

Andries Koch Janse van Vuuren

Date

Disclaimer

The two chapters of this thesis have been prepared and formatted for publication as two separate articles. The first chapter was submitted to General and Comparative Endocrinology and the second chapter to Hormones and Behaviour. Some overlap in content and differences in formatting and references may therefore occur between the two chapters to ensure publishable entities.

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Summary

How social group cohesion is maintained in cooperatively breeding species has been the subject of a significant body of research. In many cooperatively breeding species, subordinate individuals are prevented from breeding through reproductive suppression either by behavioural or physiological means, or a combination of both mechanisms. Although much is known about this phenomenon, the underlying mechanisms that control reproductive suppression are still unclear. The African mole-rats (Bathyergidae) provide an ideal model for studies on reproductive suppression, as they display a wide range of social organisation and cooperative breeding strategies. Many mole-rat species are still understudied with respect to how their reproductive skew is maintained. Several endocrine correlates have been implicated in the maintenance of their reproductive skew. Prolactin was recently proposed to play an essential role in the physiological suppression of subordinate naked mole-rats (*Heterocephalus glaber*), as prolactin is known to inhibit the reproductive axis and promote cooperative behaviour. Stress hormones (glucocorticoids) are known to inhibit reproductive ability and have been shown to inhibit reproduction and promote cooperative behaviours of subordinates of some social mammals. Furthermore, testosterone (androgens) is associated with aggressive, dominant behaviour and is thought to be important in maintaining behavioural means of suppression in the form of aggressive interactions. In this study, I attempt to address gaps in our knowledge by investigating reproductive suppression in three understudied subspecies of the social mole-rat, *Cryptomys hottentotus*, namely *C. h. natalensis* (CHN)*, C. h. pretoriae* (CHP) and *C. h. mahali* (CHM). To do this, endocrine correlates associated with behavioural and physiological reproductive suppression (prolactin, glucocorticoids and androgens) were investigated in the three subspecies. Previous work on the differences in pituitary sensitivity to GnRH and gonad metrics of these species have hinted at the possible contrasting mechanisms controlling reproduction in the three subspecies, but clear findings are yet to be published. Individuals of the three subspecies were captured at three sites for blood and faecal sampling. Plasma prolactin, faecal glucocorticoid metabolites

(fGCMs) and faecal androgen metabolites (fAMs) were determined and compared between breeding and non-breeding males and females of the three subspecies. Comparing CHN and CHP revealed stark differences in reproductive suppression, as CHP showed evidence of physiological suppression through increased levels of circulating prolactin while CHN did not. A possible interactive role of prolactin and fGCMs were also highlighted from this comparison. The difference between these two closely related species led to the investigation of the same mechanisms in more detail in CHM, which revealed that this species also uses physiological suppression, again indicated through increased levels of circulating prolactin and fGCMs. To further investigate the link between prolactin and glucocorticoids, I tested the adrenal sensitivity of both non-breeding male and female CHM to adrenocorticotropic hormone (ACTH). I observed that non-breeding females, that possess higher prolactin levels than their male counterparts, are more sensitive to ACTH, reinforcing the link between prolactin, glucocorticoids, and physiological suppression.

This thesis demonstrates how reproductive suppression differs between closely related subspecies and paves the way for similar studies to be done on other cooperatively breeding species. Furthermore, physiological reproductive suppression controlled by elevated prolactin may be important in many other taxa, which could help us understand how social group cohesion and reproductive skew is maintained.

General Introduction

Reproduction in mammals: Proximate and ultimate factors

Reproduction in mammals is a complex process that must occur in harmony with available dietary, physical and social conditions (Bronson, 1985). Reproduction requires a significant investment in resources and energy, both of which are dependent on the local environmental conditions and includes food availability, ambient temperature, rainfall, the day/night cycle and various social cues (Bronson, 1985). Resource availability restricts the available energy for reproductive investment, and when resources become limited, reproduction becomes more challenging. Intra- and interspecific competition increases the energy requirements needed for reproduction. Competition for mates, resources, and territory and other factors such as predation make breeding attempts expensive. The costs associated with reproduction often outweigh the benefits of avoiding reproduction altogether, which causes many individuals never to reproduce successfully during their lifetime. Some mammals overcome some of these challenges by living in groups.

Sociality of mammals

The evolution of sociality is a long-standing puzzle in evolutionary biology due to the variation of individual benefits incurred between and within species (Safran et al., 2007). Sociality can broadly be described as related or unrelated individuals of the same species that occupy the same territory simultaneously (Emlen, 1995). By living in groups, food is easier to obtain (Wyman, 1967, Packer and Ruttan, 1988), and resources are more easily defended (Kruuk, 1972). Reproductive benefits of group-living include increased rates of infant growth (Mitani and Watts, 1997), increased juvenile survival (König, 1994, Canestrari et al., 2008) and reduced infanticide (Packer et al., 1990). By living in groups, there is a reduction in individual predation risk (dilution effect) (Hamilton, 1971). Vigilance is greater in visually acute groups (Underwood, 1982), and group defence provides additional protection against predators and rival groups (Freedman and Wolkowicz, 1986). Even with the associated costs of group living, such as increased visibility to predators and increased intra-sexual competition, fitness benefits are still high for group members (Clutton-Brock et al., 2006).

Different types of social groups are characterised by the degree of social interactions between individuals and by which individuals in the group reproduce (Vehrencamp, 1983a, Clutton-Brock, 1998). Social behaviour involves various interactions that range from temporary breeding groups (Reynolds and Gross, 1990) to multigenerational family groups with cooperative brood care (Jarvis, 1981).

Cooperative breeding in mammals

Cooperative breeding refers to any breeding system where alloparental care occurs (Saltzman, 2010). Alloparental care is classified as parental care of any form by an individual towards a non-descendant young (Wilson, 2000). Therefore, offspring in such groups receive care from their parents and other individuals, including related individuals such as grandchildren and siblings (Wisenden, 1999). Individuals participating in alloparental care are known as alloparents, helpers or subordinates and help raise young in various ways ranging from direct feeding of offspring to nest/colony defence and maintenance (Cooney, 2002, Nishida, 1983).

Cooperative breeding societies are often characterised by having a reproductive division of labour (Sherman et al., 1995b). In these groups, a small number of individuals usually monopolise the majority of the reproduction, which leads to a considerable variation in reproductive skew: the lifetime reproductive success of the group members (Faulkes and Bennett, 2001). Groups in which a few individuals breed are classified as having high skew, whereas groups with a large proportion of breeding members are known to have a low skew

(Clutton-Brock, 1998). Groups with high reproductive skew are often characterised by having delayed dispersal from the natal group and delayed or even suppressed reproduction in subordinates (Saltzman, 2010).

Subordinates of cooperative breeders are often reproductively inactive adult or sub-adult offspring of the breeding pair (Saltzman, 2010). The occurrence of non-breeding individuals of cooperative species to forego their own chances of reproducing in order to raise offspring that are not their own, has been the subject of extensive research (Hamilton, 1964, Lukas and Clutton-Brock, 2012, König, 1997). This behaviour is often explained by the potential costs involved in dispersing from the natal group in an attempt to join or establish a new group versus the potential fitness benefits received by remaining in the natal colony (Lukas and Clutton-Brock, 2012). Due to high relatedness in these groups, members of the colony help raise relatives that share their own genes and therefore increase their own fitness indirectly (inclusive fitness) (Hamilton, 1964, Emlen, 1995). Along with inclusive fitness, helpers benefit through increased foraging efficiency, protection against predation and possible inheritance of breeding positions (Clutton-Brock, 2009). Individuals that disperse from their natal group often do not succeed in surviving and establishing their own breeding groups, which is why the long-term fitness benefits for helpers that remain in their natal colonies are likely to be higher than for dispersers (Clutton-Brock, 2009).

Cooperative breeding includes a variety of social structures, ranging from groups consisting of a single breeding pair with helpers born in previous seasons (Dickinson et al., 1996) to groups/colonies consisting of multiple breeding males and females with helpers not related to all of the breeders in the group (Haydock et al., 2001). Cooperative breeding systems have been observed in the Canidae (dogs), the Callitrichidae (New World monkeys), the Herpestidae (mongooses), as well as several rodent families including Muridae (mice and rats), Castoridae (beavers), terrestrial Sciuridae (squirrels), Hystrichidae (porcupines) and Bathyergidae (mole-rats) (Lukas and Clutton-Brock, 2012).

Eusociality describes the extreme end of social organisation. This advanced form of cooperative breeding is characterised by individuals of the same species cooperating in caring for young, behavioural division of labour where non-breeding individuals form working groups (castes) and an overlap of at least two generations in life stages capable of contributing to colony labour (Crespi and Yanega, 1995, Jarvis, 1981). Only two mammalian species have been recognised to be eusocial, the Naked Mole-rat (*Heterocephalus glaber*) and the Damaraland Mole-rat (*Fukomys damarensis*) (Jarvis and Bennett, 1993, Jarvis, 1981). Social systems are considered to fall along the sociality continuum, with low skew societies ranked close to zero and high skew societies close to one (Sherman et al., 1995b).

Maintenance of reproductive skew in cooperative breeders

To maintain a high reproductive skew within a group, subordinate reproduction must be inhibited or restricted to some degree. There are conflicting ideas about how reproductive skew is maintained in co-operatively breeding societies, and various models have been developed to explain variation in skew in animal societies (see Hager and Jones, 2009). The two main types of skew models, transactional and compromise models, have different assumptions of how skew is maintained. Transactional models assume that one dominant individual has complete control over reproduction in the group, but may have to allow some subordinates to breed in order to prevent individuals from dispersing from the group (concession theory) (Clutton-Brock, 1998, Vehrencamp, 1983b, Johnstone and Cant, 1999). Compromise models argue that skew is maintained through incomplete control over reproduction within the group and assumes that each individual can act selfishly to claim a share of the reproduction, at the cost of the productivity of the group (Reeve, 1998, Clutton-Brock, 1998). In these models, dominant individuals obtain the majority of breeding opportunities due to having greater competitive abilities (Johnstone and Cant, 2009).

Reproductive suppression in adult individuals may be mediated by behavioural means, physiological means or a combination of both (Bennett et al., 1999, Bennett et al., 1996, Bennett et al., 1997). Behavioural suppression means may include inbreeding avoidance and aggressive behaviour from dominants (Hager and Jones, 2009). In most societies, skew is maintained predominantly or partially through the natural avoidance of mating between relatives (Koenig and Haydock, 2004). Incest avoidance is an innate response present in most sexually reproducing animals that prevents the deleterious effects of inbreeding (Jimenez et al., 1994, Koenig and Haydock, 2004, Haydock et al., 2001).

Physiological reproductive suppression in mammals is frequently brought about by the aggressive behaviour of dominants toward subordinates. Aggressive behaviour of dominant individuals results in stress response in subordinates (Faulkes and Abbott, 1997, Saltzman, 2010). This involves the activation of the hypothalamic-pituitary-adrenal axis (HPA axis), which releases glucocorticoids (Creel, 2001). Glucocorticoids and other hormones enable the body to respond to stressors by increasing the immediate availability of energy for important activities while inhibiting physiological responses not required for immediate survival (Creel, 2005, Saltzman, 2010). Although short-term effects are adaptive and important, prolonged effects of high glucocorticoid levels can be detrimental as it can negatively affect immune function, spatial learning, and the reproductive axis (Pottinger, 1999, Creel, 2001). By facing constant aggressive behaviour from dominant individuals, subordinates endure high levels of stress hormones for long periods, which leads to the dysfunction of the hypothalamic-pituitary-gonadal axis (HPG axis) (Pottinger, 1999). The HPG axis or reproductive axis consists of three endocrine glands that control the release of different hormones to regulate numerous bodily systems, including immune and reproductive functions (Saltzman, 2010, Pottinger, 1999). In breeding adults, the hypothalamus secretes gonadotropin-releasing hormone (GnRH), which stimulates the anterior pituitary gland to secrete luteinising hormone (Malherbe et al.) (Malherbe et al., 2004) and follicle-stimulating hormone (FSH). LH and FSH, in turn, stimulate the gonads in each sex, promoting gametogenesis and the production of steroid hormones (primarily testosterone in males and progesterone and oestradiol in females) (Saltzman, 2010, Feldhamer et al., 2015). Therefore, the HPG axis essentially controls most reproductive activity in mammals and the disruption of this system can lead to suppression of reproduction through, for example, interferences with reproductive hormone functioning, pregnancy maintenance, and gametogenesis (Pottinger, 1999, Saltzman, 2010).

African mole-rats

The species of the African mole-rats (Bathyergidae) family are subterranean, hystricomorph rodents endemic to sub-Saharan Africa (Bennett and Faulkes, 2000). All species are highly adapted to a subterranean lifestyle allowing them to inhabit a variety of soil types and can be found in areas varying in rainfall, altitude and vegetation type (Faulkes et al., 2004, Bennett and Faulkes, 2000). The Bathyergidae consists of 30 or more species comprising six genera (Faulkes et al., 2004, Faulkes and Bennett, 2013). Three of the six genera (*Heliophobius, Bathyergus, Georychus*) are solitary breeders. These species tend to be larger than social mole-rats and usually inhabit mesic environments with predictable rainfall patterns (Faulkes and Bennett, 2013). The social/eusocial genera, *Cryptomys* and *Fukomys*, are found in both xeric and mesic environments, while the eusocial *Heterocephalus* is found exclusively in the xeric regions of East Africa (Faulkes and Bennett, 2013, Bennett and Faulkes, 2000).

Mole-rats have evolved anatomical, physiological, behavioural and morphological adaptations to overcome the unique challenges of subterranean living (Bennett and Faulkes, 2000, Bennett, 2009). Mole-rats live in complex burrow systems made up of a network of tunnels and different compartments serving as nest chambers, foraging galleries, toilet chambers and food stores (Jarvis and Bennett, 1990a, Le Comber et al., 2002, Herbst and Bennett, 2006). The burrow system offers protection against predators and environmental extremes but provides a challenging environment to live in. The burrow system provides a thermally stable environment (Bennett et al., 1988) that is both low in oxygen (hypoxic) and high in carbon dioxide (hypercapnic), with little to no light entering the burrow (Roper et al., 2001, Shams et al., 2005). The resting metabolic rate, as well as the core body temperatures of African mole-rats, are substantially lower than surface-dwelling rodents of equal size (Bennett et al., 1994a, Bennett et al., 1992, Lovegrove, 1986, Bennett, 2009), which is believed to prevent overheating in a closed burrow system (McNab, 1966). The haemoglobin in mole-rat red-blood cells have a higher affinity for oxygen than other rodents, allowing them to live in such a challenging environment (Broekman et al., 2006, Van Aardt et al., 2007, Weber et al., 2017). Bathyergids are herbivorous and feed predominantly on storage organs of geophytes (plants with underground tubers, bulbs, or rhizomes), not only as a source of nutrients, but also as a source of water, as mole-rats do not drink free water (Jarvis, 1981). Most species remain underground to forage, feeding as they encounter food sources randomly (Bennett and Faulkes, 2000). They have extrabuccal incisors that enable them to burrow and feed efficiently (Genelly, 1965). Their bodies are streamlined, with skin loosely connected to their trunk, minimising friction, allowing them to move easily along their tunnels backwards and forwards with equal dexterity (Genelly, 1965, Bennett and Faulkes, 2000). Mole-rats possess functional circadian clocks that integrate locomotor activity and core body temperature with external temperature and light cues (Riccio and Goldman, 2000, Van Jaarsveld et al., 2019).

Sociality of African mole-rats

The African mole-rat family provides an ideal model for studying cooperative breeding and social interactions. The study of these animals has contributed significantly to this topic as this family encompasses a broad spectrum of cooperative breeding strategies that range from solitary species to species with small social groups (social) to eusocial species with extremely large groups (Jarvis and Bennett, 1990b, Bennett and Faulkes, 2000).

Solitary mole-rats only pair up briefly during the mating season to court and breed, and offspring subsequently disperse from the natal burrow after weaning (Bennett and Jarvis, 1988, Bennett et al., 1991). Cooperative breeding strategies and social interactions differ significantly between the social/eusocial mole-rat species (Faulkes and Bennett, 2001, Bennett and Faulkes, 2000). A characteristic feature of social/eusocial mole-rats is a reproductive division of labour between group members, resulting in a high reproductive skew and considerable variation in lifetime reproductive success between individuals (Faulkes and Bennett, 2001, Bennett and Faulkes, 2000). Social/eusocial mole-rat species form colonies that comprise of a small number of breeding individuals (usually one female and one to three males) and differing numbers of non-reproductive subordinates, including related family members and unrelated immigrants (Bishop et al., 2004, Burland et al., 2002, Burland et al., 2004, Jarvis and Bennett, 1990b). Colony growth occurs when offspring become non-reproductive subordinates and remain in their natal groups or immigrants join the group (Jarvis and Bennett, 1990b, Bennett and Faulkes, 2000). Colony size differs greatly between the social mole-rat species. *Cryptomys* species tend to live in small groups, with colony sizes of 5-8 members on average (Oosthuizen et al., 2008, Bennett and Faulkes, 2000). The two eusocial species live in larger groups. Damaraland mole-rat colonies average 12 members, but can consist of up to 40, whereas naked mole-rats live in groups of 80 on average, with some groups exceeding 250 individuals (Jarvis and Bennett, 1993).

The ultimate cause for this range of sociality in this rodent family has been linked with the different ecological constraints each species experiences, which has led to the formulation of the Aridity Food Distribution Hypothesis (AFDH) (Jarvis, 1978, Jarvis et al., 1994, Lovegrove, 1991, Spinks, 1998). The AFDH proposes that cooperative breeding and eusociality in African mole-rats evolved in response to rainfall patterns and their effects on food availability and associated risks and costs of dispersal and foraging (Jarvis et al., 1994). In mesic environments, the food available to solitary species are generally smaller in size but more abundant and evenly dispersed (Jarvis and Bennett, 1990a). Over much of the arid regions that the eusocial and most social species inhabit, food is concentrated into widely dispersed small patches of bulbs and tubers or large single tubers (Lovegrove and Wissel, 1988, Spinks, 1998). Living in groups makes good quality food patches more easily located and defended from other groups (Jarvis et al., 1994, Lovegrove and Wissel, 1988). Therefore, it is believed that the patchy distribution of food and not its absolute abundance promotes group living because of social foraging benefits (Alexander, 1974, Lovegrove and Wissel, 1988). In addition, there are limits to the absolute distance an individual can dig each day, which includes associated energetic costs, maximal incisor growth rate and heat load (Jarvis and Bennett, 1990a, McNab, 1966, Vleck, 1979, Lovegrove, 1991). Because of the harder soil found in arid regions and the patchy distribution of food, the amount of burrowing required to locate rich patches of tubers is extremely high and may only be achieved by the collective burrowing of a group of individuals (Jarvis and Bennett, 1990a, Lovegrove, 1991, Vleck, 1979). The majority of burrowing occurs after rainfall when the soil is more friable and easily workable for both solitary and social species, as this is when the energetic costs associated with burrowing are at a minimum (Jarvis et al., 1994, Vleck, 1979). Due to sporadic rainfall, eusocial mole-rat species need to dig ferociously after rainfall events in order to locate rich food patches sufficient for feeding the colony for a long period of time, which is more easily accomplished by living in large cooperative groups (Jarvis et al., 1994). It is thus suggested that under the constraints of the ecological conditions, the selective pressures related to high energetic costs and the low probability of locating widely dispersed food sources may have led to the evolution of group living in the Bathyergidae in species that inhabit more arid environments (Jarvis and Bennett, 1990a, Jarvis, 1978, Lovegrove and Wissel, 1988, Bennett, 1988). Alternatively, eusociality in African mole-rats has been hypothesised to have evolved as a result of cooperative monogamy, which was reinforced by the subterranean lifestyle. Burda et al. (2000) proposed that dispersal and subsequent colony genesis is restricted due to the subterranean lifestyle of mole-rats causing colony formation and subsequent cooperation, which allowed for continuous breeding throughout the year rather than seasonal breeding. This is thought to have led to the rapid overlapping of generations (Burda et al., 2000). The different levels of cooperation in the different African mole-rat species is therefore proposed to be a by-product of the different rates of evolution between the solitary and social species and not the environments they live in

Maintenance of reproductive skew in African mole-rats

The three social genera fall along the eusociality continuum and comprise of the social *Cryptomys*, the social/eusocial *Fukomys* and the eusocial *Heterocephalus* (Bennett and Faulkes, 2000). High reproductive skew is evident in the eusocial mole-rat species, which occur in large groups with few breeding individuals (Jarvis, 1981, Jarvis and Bennett, 1993). The Damaraland mole-rat and the naked mole-rat fit the classical definition of eusociality (Jarvis and Bennett, 1993, Jarvis, 1981, Bennett and Faulkes, 2000, Sherman et al., 1995a). Unlike other mole-rat species, a behavioural division of labour occurs among non-breeding subordinates of these two species (Faulkes and Bennett, 2001). Eusociality has evolved independently in these species and remains the only two mammalian species recorded eusociality (Jarvis and Bennett, 1993, Faulkes and Bennett, 2013). A lower reproductive skew and a greater breeding opportunity, often through dispersal, is evident in the social mole-rat species.

Nonetheless, all social mole-rat species studied to date have skewed reproduction, although, in terms of lifetime reproductive success, skew differs considerably between even closely related species (Faulkes and Bennett, 2001). Snowdon (1996) proposed two models that explain reproductive skew in cooperatively breeding animals, which is often used to explain skew in African mole- rats. The models are 1) self-restraint models and 2) dominant control models.

The function of the self-restraint model is to limit reproduction until a non-related male is present, adequate parental care is acquired, or sufficient resources are available (Snowdon, 1996). In the self-restraint model, incest avoidance alone is sufficient for maintaining reproductive skew (Snowdon, 1996). While, the dominant control model argues that dominant individuals exert some reproductive control over the less dominant subordinates of the group, which often involves aggressive behaviour and related stress effects (Snowdon, 1996). Dominants often control reproduction by imposing reproductive suppression on subordinates.

Reproductive suppression varies considerably between species but can be divided into two primary forms. Firstly, a behavioural mechanism is subordinates avoide breeding with close relatives (incest avoidance) and/or behaviour of dominants that interrupt subordinate breeding efforts (Faulkes and Bennett, 2009, Saltzman, 2010). Such behaviour often involves aggressive interactions such as interference with subordinate mating attempts and subordinate offspring infanticide; this is commonly observed in naked mole-rats (Faulkes and Abbott, 1997). The second form is physiological, which involves the interference with subordinates reproductive capacities, which can lead to delayed puberty, reduced spermatogenesis, anovulation and blocks to implantation (Saltzman, 2010, Faulkes and Abbott, 1997). However, it is often difficult to discern between physiological and behavioural suppression, as physiological stress responses are triggered by being subjected to aggressive behaviour (Wingfield et al., 1990, Bennett, 2009).

Most mole-rats are obligate outbreeders, and therefore, like most mammals, they avoid mating with relatives (Faulkes and Bennett, 2009). This is important, as many species of mole-rats maintain reproductive skew through incest avoidance alone rather than physiological suppression (Spinks et al., 1999, Oosthuizen et al., 2008, Faulkes and Bennett, 2001, Faulkes and Bennett, 2009). For example, studies have shown that in the common mole-rat, *Cryptomys hottentotus*

hottentotus, neither male nor female subordinates have a physiological block to reproduction and that incest avoidance appears to be sufficient in maintaining reproductive skew in closely related colonies (Spinks et al., 2000). The same situation has been observed in several *Fukomys* species, including the Mashona mole-rat, *Fukomys darlingi,* (Bennett et al., 1997), the giant mole-rat, *Fukomys mechowi* (Bennett et al., 2000) and the Zambian mole-rat, *Fukomys anselli* (Burda, 1995).

Naked mole-rats are unlike other mole-rat and mammal species as they can inbreed to a high degree, which was initially argued to be an essential factor in explaining their eusociality (Reeve et al., 1990). Consequently, it is believed that to ensure that the dominant breeding female maintains her reproductive monopoly and control over her colony in the absence of incest avoidance, the breeding female imposes extreme control over the reproductive physiology of subordinates of both sexes (Faulkes and Abbott, 1997, Jarvis, 1981, Bennett and Faulkes, 2000). In the other eusocial mole-rat species, the Damaraland mole-rat, incest avoidance is present, as this species shows no tolerance to inbreeding, but is not the only mechanism for maintaining reproductive health skew (Bennett et al., 1996, Faulkes and Bennett, 2009, Molteno and Bennett, 2000). In non-reproductive females Damaraland mole-rats, severe physiological suppression prevents them from breeding (Bennett et al., 1994b). This, however, is not the case for non-reproductive males, in which incest avoidance rather than physiological suppression seems to inhibit their reproduction (Bennett, 2011, Bennett, 1994).

Endocrine control of reproductive suppression in social African mole-rats

Naked mole-rats represent the pinnacle of cooperative breeding and socially induced infertility. Both male and female subordinates are physiologically suppressed (Faulkes and Abbott, 1997, Jarvis, 1981, Bennett and Faulkes, 2000). Non-breeding males have been shown to have significantly smaller testes relative to body mass compared to their breeding counterparts (Faulkes et al., 1994). Even though non-breeding males produce sperm, they are significantly fewer in number than breeders, and the sperm they produce are non-motile and often non-viable with defects (Faulkes *et al.*, 1994). Endocrine studies have shown that non-breeders have significantly reduced urinary testosterone levels and that their pituitary glands are less responsive to the administration of exogenous gonadotropin releasing hormone (GnRH) than breeders (Faulkes et al., 1991). This suggests socially induced impairments to the hypothalamic-pituitary-gonadal axis, likely controlled by an induced stress response in non-breeders (Faulkes and Abbott 1991). In non-breeding female naked mole-rats, pituitary sensitivity is also reduced, suggesting that a GnRH release disruption controls reproductive suppression (Faulkes et al. 1990). Anatomical studies have suggested that female subordinate ovaries are functionally quiescent and prepubescent (Kayanja and Jarvis 1971). Furthermore, the non-breeding females have lower progesterone and oestrogen concentrations and a lack of progesterone peaks, which would be suggestive of luteal phases over time (Faulkes et al. 1990). Reduced secretion of LH from the pituitary is also evident and results from social contact with the dominant breeding female in the form of shoving (Faulkes and Abbott, 1997). The importance of physiological suppression is highlighted as female naked mole-rat is a spontaneous ovulator, which would mean if suppression were absent non-breeding females would begin to ovulate within the confines of the colony, which would cause multiply breeders to occur and the possible collapse of the colony hierarchy and structure (Faulkes et al., 1990).

In Damaraland Mole-rats, endocrine control of reproduction is only present in non-breeding females (Bennett et al., 1993). In this species, non-breeding females show anovulation while in the confines of the colony (Bennett, 1994) and reduced pituitary sensitivity to GnRH compared to breeding females (Molteno and Bennett, 2000, Molteno et al., 2004). Subordinate Damaraland mole-rats also show reduced progesterone concentrations when compared to breeding females, suggesting a block to ovulation (Bennett 1994). Unlike the naked mole-rat, the Damaraland mole-rats females are induced ovulators; thus, a female needs to be penetrated by a male to achieve ovulation (Voigt et al., 2021). Therefore, it is likely that both physiological (reduced pituitary sensitivity) and behavioural (incest avoidance and/or mating interruption) suppression mechanisms are at play in keeping subordinate females in a state of anovulation (suppression of reproduction).

Recent work has highlighted the possible role of prolactin in the control of physiological reproductive suppression in some mole-rat species (Bennett et al., 2018). In mammals, prolactin is involved in regulating several processes, including the control of milk production and the activation of parental care (Ziegler, 2000). Prolactin is well known to suppress reproduction naturally during lactation, and elevated levels can disrupt reproductive functions in both sexes (Brown et al., 2014). In naked-mole rats, non-breeding female and male prolactin concentrations are as high as that or more elevated than in breeding females. Prolactin levels in these individuals often exceed levels considered hyperprolactinemic in humans, which suggests that this hormone may play an essential role in suppressing reproduction in the female and male naked mole-rats. The same patterns were not shown in Damaraland mole-rats (Bennett et al., 2018). More research is required to determine the role of prolactin in physiological suppression in these two species and other social Bathyergids.

In other species of the genera, *Cryptomys* and *Fukomys,* physiological suppression of reproduction has not yet been detected, and incest avoidance appears to be the main form of reproductive suppression (Oosthuizen et al., 2008, Janse van Rensburg et al., 2003, Lutermann et al., 2013, Bennett et al., 1997, Bennett et al., 2000). However, extensive endocrinological studies have not yet been conducted in all the species, and more research is required to determine the role of hormones in their maintenance of reproductive skew.

Study species

Recent systematic/phylogeographical studies have provided new insight into the contentious classification of the Bathyergidae (Visser et al., 2019, Faulkes et al., 2004). The genus *Cryptomys* contains one species, *Cryptomys hottentotus*, distributed widely across Southern Africa. However, the species *C. hottentotus* has been split into five closely related sub-species (*Cryptomys hottentotus hottentotus, C. h. mahali, C. h. natalensis, C. h. nimrodi, C. h. pretoriae*), and these may merit individual-specific status (Visser et al., 2019). Overall, the sub-species of *Cryptomys* have been studied extensively, but aspects of their reproductive biology are not yet fully understood. This includes the proximate and ultimate factors that maintain reproductive skew in the different sub-species.

Cryptomys hottentotus mahali - Mahali mole-rat (Roberts, 1913)

The Mahali Mole-rat is distributed across the relatively arid regions of the Northern Cape, the North West and Gauteng provinces north of the Magaliesberg mountain range in Southern Africa and is typically found in clays and sandy soils (Bennett, 2016). This species exhibits aseasonal breeding and exhibit induced ovulation (Hart et al., 2020). They live in colonies containing around eight individuals, with some groups of up to 20 individuals recorded (Hart, 2018). The basic reproductive biology of the Mahali mole-rat has been determined (Hart et al., 2020), but their mechanisms for maintaining reproductive skew remains uncertain.

Cryptomys hottentotus natalensis - Natal mole-rat (Roberts, 1913)

The Natal mole-rat is restricted to the eastern highland parts of Southern Africa, specifically along the Great Escarpment and is distributed across Kwa-Zulu Natal, Mpumalanga, Lesotho and Swaziland (Monadjem et al., 2015). This species reproduces aseasonally and also undergoes induced ovulation (Jackson and Bennett, 2005). They live in colonies of up to 16 individuals, with a mean size of seven (Oosthuizen et al., 2008). Present findings suggest that skew is maintained behaviourally in this species, but is not yet fully understood (Oosthuizen et al., 2008).

Cryptomys hottentotus pretoriae - Highveld mole-rat (Roberts 1913)

The Highveld Mole-rat occurs in the Gauteng and North West provinces (Monadjem et al., 2015) of South Africa and is separated from the Mahali Mole-rat by the Magaliesberg mountain range (Hart, 2018). This species inhabits hard soils of the Southern-African savanna highlands, and they are seasonal breeders and induced ovulators (Malherbe et al., 2004, Janse van Rensburg et al., 2002). This species lives in colonies of about five individuals but can contain up to 12 members in some groups (Janse van Rensburg et al., 2002). Although the reproductive biology of this species has been well studied, the mode of reproductive suppression is not yet fully understood, with previous studies suggesting that incest avoidance mainly controls reproductive skew (Janse van Rensburg et al., 2003).

Research objectives

This thesis aimed to improve our understanding of the physiological mechanisms that drive social cohesion and behaviour in cooperatively breeding species by determining how endocrine correlates associated with reproductive suppression differs between breeding and non-breeding individuals of both sexes within and between the three study species, namely the Mahali, Natal and Highveld mole-rat. What mechanisms control reproductive suppression and social cohesion in these subspecies? Do these mechanisms differ between closely related taxa? Are there links between endocrine correlates across multiple mole-rat taxa? Is prolactin an important hormone in reproductive suppression of these subspecies? Individuals were captured at three sites in Southern Africa, where blood and faecal samples were collected for endocrine analysis.

The first chapter describes a comparison of endocrine correlates associated with physiological suppression between *Cryptomys hottentotus natalensis* and *Cryptomys hottentotus pretoriae* and shows how these hormones differ between even closely related species. In the second chapter *Cryptomys hottentotus mahali* is investigated in detail showing how these hormones differ seasonally between breeding and non-breeding individuals of the same species. This manuscript provides deeper insights into the maintenance of reproductive skew through physiological suppression in the genus *Cryptomys*, highlighting the importance of hormones associated with endocrine suppression in maintaining cooperative behaviour in social species and paves the way for similar studies in the future to enhance our understanding further.

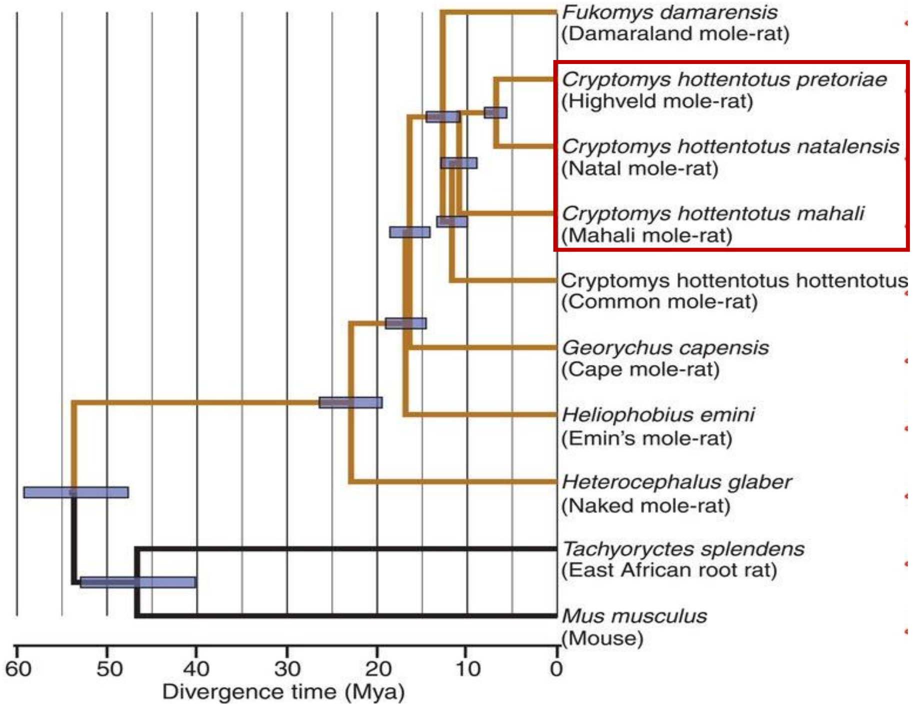

Figure 1. Phylogenetic tree of selected Bathyergids as calculated on the basis of transcriptomic data (modified from Eigenbrod et al., 2019). The species investigated in this study are highlighted by the red box.

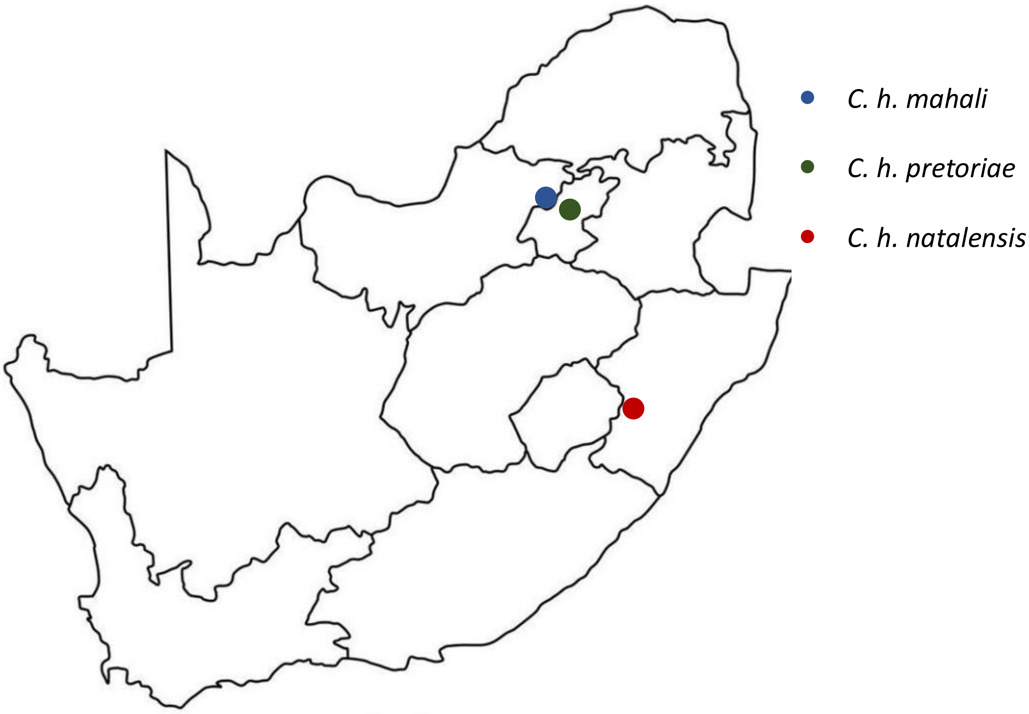

Figure 2. Map of South Africa showing the relative area where trapping of the three study species, *C. h. Mahali*, *C. h. pretoriae* and *C. h. natalensis*, took place.

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Chapter 1

Contrasting patterns of socially induced reproductive suppression in two closely related subspecies of *Cryptomys hottentotus*

Abstract

The physiological and behavioural mechanisms that allow for the maintenance of reproductive suppression and subsequent group living in cooperatively breeding species have been extensively studied. Nevertheless, much is yet to be understood regarding how social group cohesion is maintained. The African mole-rats (Bathyergidae) have provided an ideal model to address such gaps in our knowledge however, some key social species have received little to no attention. In this study, we attempt to fill the gaps in our understanding of how social groups are maintained by investigating the endocrine correlates implicated in the control of subordinate reproduction; namely the concentrations of prolactin, glucocorticoids (cortisol) and the androgen (testosterone), in two sub-species of the understudied social mole-rat genus, *Cryptomys* (*Cryptomys hottentotus pretoriae* - CHP and *Cryptomys hottentotus natalensis* - CHN). Faecal glucocorticoid and androgen metabolites (fGCM and fAM) and plasma prolactin concentrations were determined and compared between reproductive classes (breeding and non-breeding males and females) within and between the two subspecies. Our results reveal that physiological reproductive suppression (through the interaction between glucocorticoids and prolactin) is essential in maintaining reproductive skew in CHP colonies. At the same time, behavioural suppression (through testosterone mediated aggression and incest avoidance) is the dominant mechanism in CHN. This study reveals stark differences in the reproductive biology of two closely related taxa and paves the way for further research to elucidate the pathways and mechanisms controlling reproductive skew and cooperative behaviour in these and other species.

Introduction

The concept of cooperative behaviour and cooperative breeding in a wide array of taxa has been the subject of a significant interest for close on five decades of research (Wilson, 2000). Cooperative breeding is defined as a socially breeding system in which alloparental care arises, where non-breeding individuals of the group care for young that are not their own (Solomon and French, 1997). This form of social behaviour has evolved in many species over a diverse array of taxa and differs considerably with group size, the number of helpers or subordinate members and the mechanisms that are used to maintain social organisation (Solomon and French, 1997). In many species, individuals forego their own reproduction to help raise the young of breeding individuals that are usually related to themselves (Reyer, 1986, Clutton-Brock et al., 2001, Hamilton, 1964). This behaviour can often be explained by the indirect fitness benefits that the helpers receive, as they are often related to the breeders and therefore share common genes with those of the offspring (Hamilton, 1964).

Usually for a cooperatively breeding system to be successful, reproduction by subordinates is limited or more often prevented (Solomon and French, 1997). To achieve this, breeding individuals impose strict control over the reproductive function of the subordinates (Hager and Jones, 2009). In some cases, subordinates are suppressed from reproduction either through behavioural or physiological means; or a combination of both (Bennett et al., 1999, Bennett et al., 1996, Bennett et al., 1997, Young, 2009, Medger et al., 2019). However, the mechanisms by which reproductive suppression is controlled differs between species, and many of the mechanisms operating on these non-breeding individuals are not yet fully understood (Hager and Jones, 2009).

A large body of work has focussed on the physiological control of reproductive suppression, which involves the down-regulation of one or more components of the reproductive system in subordinates (Creel et al., 1992, Arnold and Dittami, 1997, Faulkes and Bennett, 2013, Carlson et al., 2004, Young, 2009). In some species, female subordinates appear to be unable to conceive and show reduced levels of luteinising hormone, oestrogen, progesterone and/or testosterone compared to the dominant females (O'Riain et al., 2000, Carlson et al., 2004, Bennett et al., 1993, Bennett, 1994, Bennett et al., 1994, Faulkes et al., 1990, Faulkes et al., 1991). This suggests that the secretion of gonadotropin-releasing hormone (GnRH), controlling LH production, is low, or that the pituitary in non-breeders is less sensitive to GnRH (Faulkes and Abbott, 1991, Faulkes et al., 1990, Faulkes et al., 1991). The disruption of the hypothalamic-pituitary-gonadal (HPG) axis can lead to impairments in physical reproductive ability, a situation that has been documented in non-breeding subordinate individuals of several cooperatively breeding species (Faulkes et al., 1991, O'Riain et al., 2000, Faulkes and Abbott, 1997).

The disruption of the HPG axis and the corresponding suppression of subordinate reproduction has been attributed to stress in some taxa (Hackländer et al., 2003, Young et al., 2006). Chronic stress is well known to affect the reproductive system of mammals (Pottinger, 1999) and induced stress appears to be an essential factor in maintaining reproductive skew within groups of some species (Beehner and Lu, 2013, Reyer, 1986). Dominant individuals in cooperatively breeding societies often behave aggressively towards subordinates to assert dominance (Hau, 2007). In many species, this behaviour can be attributed to higher testosterone levels in the dominant breeding individuals than their non-breeding subordinate counterparts (Wingfield et al., 1990, Clarke and Faulkes, 1998). Higher testosterone and associated aggressive behaviour may be linked to induced stress and suppression in some cooperatively breeding species (Young et al., 2006, Clarke and Faulkes, 1998). However, the relative importance of stress in reproductive suppression is still debated and differs between species (Creel et al., 2013, Young, 2009). Dominant individuals of some cooperatively breeding species show equal or higher glucocorticoid (the physiological measure of stress) levels than subordinates, suggesting that induced stress may not be directly involved in controlling subordinate reproduction (Creel et al., 2013, Creel and Creel, 2002). However, increased glucocorticoid levels are also crucial in enhancing cooperative behaviour in some social species, suggesting that stress may have a dual function in maintaining cooperative behaviour and reproduction in cooperatively breeding species (Vullioud et al., 2021).

Lastly, prolactin plays a crucial role in cooperative breeding and social behaviour in mammals (Carlson et al., 2006, Snowdon and Ziegler, 2015). Recently, the possible importance of prolactin in reproductive suppression operational in the highly social naked mole-rat, *Heterocephalus glaber*, has also been highlighted (Bennett et al., 2018). Along with promoting milk production and cooperative care of the young, elevated prolactin levels mediate the natural suppression of reproduction in lactating females. Elevated levels of prolactin (hyperprolactinaemia) are well known to cause infertility in males and females (Kauppila et al., 1988), as prolactin inhibits the secretion of GnRH from the hypothalamus, leading to a reduction in circulating levels of LH (Matsuzaki et al., 1994). Prolactin levels in subordinate females are expected to be extremely low as they rarely lactate; however, in cooperatively breeding eusocial naked mole-rats (*Heterocephalus glaber*), female subordinates show incidences of hyperprolactinaemia with levels of prolactin, potentially impeding their reproductive physiology (Bennett et al., 2018). The role of prolactin has been largely overlooked in the past and could prove vital in suppressing reproduction in many cooperative breeders.

The study of hormones, such as testosterone, glucocorticoids and prolactin, are pivotal in understanding how cooperative behaviour is maintained and reproductive suppression is orchestrated in mammals. The African mole-rats of the family Bathyergidae, provide a unique model for studies to unravel the patterns of reproductive suppression that occur in cooperatively breeding mammals as they display a wide range of social behaviour and vary significantly in their reproductive physiology. African mole-rats differ considerably in their degree of sociality, ranging from solitary species to social species through to truly eusocial ones that live in groups of up to 300 (Bennett and Faulkes, 2000, Jarvis and Bennett, 1990b, Jarvis and Bennett, 1990a). In social and eusocial mole-rats, breeding is monopolised by one breeding female and one to three breeding males (Bennett and Faulkes, 2000, Bennett et al., 1999). The remaining colony members are reproductively inactive subordinates and juveniles (Bennett and Faulkes, 2000). In many species of social and eusocial mole-rats, subordinate breeding is believed to be controlled behaviourally through incest avoidance and aggressive behaviour of dominant individuals towards subordinates (Bennett and Faulkes, 2000, Burda, 1995). While in others, physiological control of suppression is more evident (Faulkes and Bennett, 2009, Faulkes and Bennett, 2001, Medger et al., 2019, Bennett, 1994, Bennett et al., 1993, Bennett et al., 1994, Faulkes and Abbott, 1991, Faulkes et al., 1990). In the Damaraland mole-rat (*Fukomys damarensis*),subordinate females and naked mole-rat subordinates of both sexes show a reduced response to an exogenous GnRH challenge compared to dominant breeding colony members (Bennett et al., 1993, Faulkes and Abbott, 1997, Faulkes and Abbott, 1991, Faulkes et al., 1990). This suggests a reduction in pituitary sensitivity of subordinates to GnRH, which is supported by lower concentrations of circulating LH reported in subordinates compared to dominant breeding colony members (Bennett et al., 2007, Bennett et al., 1993). Reduced levels of testosterone, oestrogen and/or progesterone have been recorded in subordinates of both these species leading to documented physical impairments to reproduction, including sperm that are both reduced in number and non-motile in naked mole-rat male subordinates and a block to ovulation in subordinate females of both species (Faulkes et al., 1990, Faulkes et al., 1994, Faulkes et al., 1991, Bennett et al., 2007, Bennett, 1994, Bennett et al., 1994, Molteno and Bennett, 2000).

Recently, it has been suggested that prolactin could play a key role in mole-rat reproductive suppression (Bennett et al., 2018). Prolactin levels recorded in naked mole-rat female and male subordinates are as high as in dominant lactating breeding females and, in some cases, exceeds levels considered to be hyperprolactinaemic in humans (Bennett et al., 2018). This hormone could also control cooperative behaviour by enhancing parental care behaviour in non-breeding females and males. Levels of prolactin measured in Damaraland mole-rats are, however, considerably lower and detected only in lactating and pregnant females only, indicating a possibly different mechanism of socially induded infertility at play (Bennett et al., 2018).

To better understand reproductive suppression in cooperatively breeding species, androgens (testosterone), glucocorticoids (cortisol), and prolactin must be investigated in the understudied social mole-rat species, such as the genus *Cryptomys*. Therefore, in this study, I compared the levels of these hormones between dominant breeders (breeding female – BF and male – BM) and non-breeders (non-breeding female – NBF and male – NBM) of both sexes in two social mole-rat subspecies: the Highveld mole-rat (*Cryptomys hottentotus pretoriae* - CHP) and the Natal mole-rat (*Cryptomys hottentotus natalensis* - CHN).

Past studies have investigated pituitary sensitivity to GnRH in both subspecies and found that in CHP non-breeders of both sexes are less sensitive to the administration of GnRH compared to their breeding counterparts, suggesting that a physiological mechanism of suppression may be partly responsible for their reproductive inhibition (Van der Walt et al., 2001). While in CHN, however, breeders and non-breeders are equally sensitive to GnRH administration, pointing to a more behavioural means of suppression as the primary mechanism of regulating subordinate reproduction, incest taboos (Oosthuizen et al., 2008a). We, therefore, expect hormones associated with physiological suppression (glucocorticoids and prolactin) to differ between breeders and non-breeders in CHP, but to be similar between CHN individuals. Furthermore, we predict hormones associated with behavioural suppression (androgens) to differ between breeders and non-breeders in both CHN and CHP.

Materials and methods

Animal capture

Cryptomys hottentotus natalensis and *Cryptomys hottenotus pretoriae* were captured between January and December 2020. Animals were caught using Hickman live traps (Hickman, 1979) baited with sweet potato. Traps were placed at the entrances of tunnels and checked once every two hours. A colony was deemed to be completely captured when no trap activity was observed for five consecutive days (Jarvis and Bennett, 1993). Upon capture, individual body mass was recorded to the nearest 0.1 g (Scout Pro SPU123, Ohaus Corporation, Pine Brook, New Jersey, U.S.A).

A total of 119 CHN individuals (BFs: n=24; NBFs: n=36; BMs: n=19; NBMs: n=40) were captured at Glengarry Holiday Farm in the Kamberg valley in Kwa-Zulu Natal, South Africa (29°19'24.3"S 29°42'32.8"E). Once captured, mole-rats were transported to a remote laboratory. While a total of 107 CHP individuals (BF: n=17, NBF: n=38, BM: n=32, NBM: n=20) were captured in the National Botanical Gardens of Pretoria (25°44'13.92"S 28°16'24.24"E) in Gauteng, South Africa. Captured individuals were transported to the mole-rat laboratory at the Department of Zoology and Entomology at the University of Pretoria.

Permission to capture was obtained from all landowners, and a collecting permit was obtained from the relevant nature conservation authorities (Permit number: CPF6-0127- Gauteng & OP1545/2021- Kwa-Zulu Natal). The Animal Use and Care Committee of the University of Pretoria evaluated and approved the experimental protocol (ethics clearance number: NAS128-2020) and DALRRD section 20 approval (SDAH-Epi-20070806200) and SDAH-Epi-12/11/1/1/8.

Animal housing

Entire colonies were housed together in plastic containers (49.5 × 28.0 cm). Mole-rats were fed daily on sweet potato and apples, while nesting material in the form of wood shavings and paper towelling were supplied to each colony. The food provided satisfied all water requirements of the animals, as mole-rats obtain all their water from their food (Bennett and Jarvis, 1995). Animals were kept until all colony members were captured, which took about seven days on average. Upon the capture of entire colonies, blood samples were collected. After samples were collected, individuals were returned to their burrows.

Blood collection

First, the dorsal pedal tarsal vein of the hind foot was pricked using a sterile 23G needle. Next, a heparin-coated microhematocrit capillary tube was used to collect the blood into a sterile heparin tube. Whole blood sample volumes ranged from 300 - 500µl of whole blood, depending on their body size. After obtaining the samples, the blood collection site was stemmed with tissue paper before the animal was returned to its colony. Whole blood was subsequently centrifuged at 3000 rpm for 15 min. The plasma fraction was separated from blood cells and stored at -20°C until hormone analysis.

Determination of reproductive status

All individuals with a dark coat colour weighing less than 40 g were considered to be juveniles. Furthermore, individuals captured alone in tunnel systems after a considerable time of no activity in traps were classified as dispersing individuals. Juveniles and dispersers were not considered for this study. BMs were easily distinguishable from NBMs by their large, descended inguinal testes, as well as their typical yellow staining around their mouths. Additionally, BMs were distinguished by usually being the largest male in their respective colonies, as this is most often the case (Bennett and Faulkes, 2000). The BFs were easily identified within each group, as these individuals display prominent auxiliary teats and perforated vaginas, traits that are absent in NBFs and dispersers (Bennett and Faulkes, 2000)

Hormonal analysis

Faecal sample collection

Once captured, individuals were kept separate for faecal samples to be collected. To ensure that faecal glucocorticoid metabolite (fGCM) and faecal androgen metabolite (fAM) concentrations recorded were not affected by the stress from being captured and handled, samples were collected within a 20-hour period after capture. Samples were collected using sterilised tweezers and stored and frozen in Eppendorf tubes at -20 ^{0} C. Frozen samples were analysed at the Endocrine Research Laboratory at the University of Pretoria.

Faecal hormone metabolite extraction and analysis

Faecal samples were lyophilised, pulverised, and sieved through a wire-mesh strainer (Medger et al., 2018). Between 0.05 and 0.06 g of faecal powder was weighed out per sample and extracted using 1.5 ml of 80% ethanol (Medger et al., 2018). The suspension was shaken for 15 minutes on a multi-vortex, after which it was centrifuged for 10 minutes at 1500 g (Medger et al., 2018). The supernatant was transferred into a clean Eppendorf tube and stored at -20 ^{0}C until analysis.

A cortisol enzyme immunoassay (EIA) was used to analyse the faecal glucocorticoid metabolite (fGCM); cortisol is the most dominant glucocorticoid found within African mole-rats (Ganem and Bennett, 2004). The fGCM concentrations are given as ng/g faecal dry weight. A testosterone EIA was used to measure fAM concentrations of samples (detailed assay characteristics are given by (Palme, 1993). FAM concentrations are given as µg/g faecal dry weight (please see supplementary material for further detials). All EIAs were performed at the

Endocrine Research Laboratory of the University of Pretoria, as described by (Ganswindt et al., 2012, Ganswindt et al., 2014).

Plasma prolactin

Plasma prolactin was analysed using an EIA as described by Bennett et al. (2018) and Medger et al. (2018). The sensitivity of the assays was 0.1 ng/ml for the prolactin EIA. Kits were performed according to the manufacturer guidelines; both the intra-assay precision and repeatability are < 10% for this EIA. See supplementary material for the standard curve (Figure S1 and Figure S2).

Statistical analysis

All statistical analyses were performed in R 3.5.2, and statistical significance was assumed at the $p \leq 0.05$ level. All data are presented as mean ± standard error (SE). The normality of dependent variables (plasma prolactin concentration and *fGCM* and f*AM*) was determined using Shapiro-Wilk tests (S-W). Non-normally distributed dependent variables were transformed (either log or square-root) where possible to obtain normal distributions. The homogeneity of normally distributed data was confirmed using Levene's tests. Normally distributed dependent variables were analysed using General linear models (GLMs). All non-normal dependent variables were analysed using Generalised linear models (GLZMs) fitted with gamma distributions and link-identity functions using the *lme4* package (Bates et al., 2015). The prolactin GLZM was fitted with a tweedie distribution using the *tweedie* package (Dunn and Smyth, 2005) due to the high number of zero values found in CHN. Post-hoc comparisons were made using Tukey's honesty significant difference (Tukey's HSD) tests using the *emmeans* package (Lenth et al., 2018).

All models contained hormone concentrations (plasma prolacin, fAM or fGCM) as response variables and reproductive status (breeding vs non-breeding), sex, subspecies, with two-way and three-way interactions, and body mass (co-variate) as predictors.

Results

Prolactin

Mean plasma prolactin concentration for all animals was significantly higher in CHP (14.75 ± 1.64 ng/nl) than in CHN (1.88 ± 0.30 ng/ml) throughout the sampling period (Figure 1, Table 1). In CHN, 17 out of 43 (40%) breeders of both sexes had undetectable levels of prolactin, with none of the individuals (n=43) exceeding levels of 25 ng/ml, which is considered to be hyperprolactinaemic (Bennett et al., 2018). In non-breeding CHN individuals, 38 out of 76 (50 %) had undetectable levels of prolactin, with only one individual exceeding 25 ng/ml plasma. Contrastingly, in CHP, one out of 49 (2%) breeders had undetectable prolactin levels, with 11 individuals (2 BFs and 9 BMs) having concentrations exceeding 25 ng/ml. In non-breeders, all individuals analysed (n=58) had detectable prolactin levels, with 11 (19%) individuals (9 NBFs and 2 NBMs) having levels greater than 25 ng/ml plasma. Reproductive status did not affect plasma prolactin levels as concentrations were similar between breeders and non-breeders (Table 1). Neither the sex or body mass of individuals or the interactions between sex, subspecies, and reproductive status significantly affected prolactin concentrations in the two species (Figure. 1., Table 1).

fGCMs

FGCMs differed significantly between individuals of CHN and CHP (Figure 2, Table 2). Mean fGCM concentrations were higher in CHP (160.8 ± 6.68 ng/ml) than in CHN (55.11 ± 1.48 ng/ml) throughout the sampling period (Figure 2). However, fGCM concentration did not differ significantly between individuals of different reproductive statuses or sexes (Table 2). Body mass, along with two and three-way interactions between sex, reproductive status and season, did not have significant effects on fGCM levels (Table 2).

fAMs

All statistical results of the GLZM are presented in Table 3. When considering individuals from both species as a whole, it was shown that fAM concentrations did not differ between individuals when considering their sex or species respectively (Figure 3, Table 3). Similarly, the body mass of an individual did not affect fAM levels (Table 3). Reproductive status was a significant predictor of fAM concentration, as breeders (0.437 ± 0.047 µg/ml) had significantly higher levels of testosterone than their non-breeding conspecifics 0.28 ± 0.03 µg/ml) ($p = 0.009$) when both species were combined (Figure 3).

All two-way and three-way interactions were significant predictors of fAM concentrations of individual CHN and CHP (Table 3.). However, upon further investigation of the interaction between sex*subspecies, it was revealed that there were no significant differences between individuals ($p \geq 0.085$ for all, Table S1).

Further analysis of the sex*reproductive status interaction showed that across the two species BMs (0.46 ± 0.11 µg/ml) had significantly higher levels of fAMs compared to NBFs (0.26 ± 0.04 µg/ml) ($p = 0.033$). The remaining comparisons between individuals for this interaction were all nonsignificant ($p \geq 0.158$ for all, Table S2).

The reproductive status*species interaction revealed that fAM concentrations of CHP breeding individuals (0.48 ± 0.051 µg/ml) were significantly higher than CHN non-breeding individuals (0.25 ± 0.02 µg/ml) ($p = 0.044$). However, the remaining contrasts of this interaction yielded no significant results ($p \geq 0.72$ for all, Table S3).

The three-way interaction between sex*species*reproductive status demonstrated that BF CHN (0.44 ± 0.06 µg/ml) had significantly higher levels of fAMs compared to NBF CHN (0.21 ± 0.02 µg/ml) ($p = 0.044$). While, BM CHP (0.58 ± 0.07 µg/ml) also showed significantly higher levels of the hormone compared to NBF CHN ($p = 0.014$). The other interaction contrasts yielded insignificant results ($p \geq 0.157$ for all, Table S4, Figure 3).

Discussion

As predicted from previous work investigating the basal LH concentrations and the response to a GnRH challenge in the different sexes and reproductive categories of CHP and CHN (Oosthuizen et al., 2008b, Van der Walt et al., 2001), the hormones measured revealed contrasting mechanisms and patterns of suppression operational in the two subspecies of *Cryptomys*. The differences in the levels of the various hormones associated with physiological reproductive suppression mirror the pituitary gland sensitivity to GnRH in both species, confirming that behavioural suppression was operating in CHN probably as a consequence of incest avoidance, whereas a combination of behavioural and physiological suppression occurs in CHP.

The first and most compelling evidence that supports this is the marked contrast of prolactin concentrations in both species. Plasma prolactin levels of CHP individuals were significantly higher than those recorded in CHN individuals, with numerous individuals of the latter species exhibiting low or undetectable levels of the hormone. High plasma prolactin concentrations in NBF CHP may act on the GnRH and neural pulse generator in these individuals, inhibiting the production and release of luteinising hormone and follicle stimulating hormone as seen in naked mole-rats (Bennett et al., 2018). Elevated prolactin levels recorded in the NBF of CHP are as high as those recorded in BFs, with some individuals having levels considered hyperprolactinaemic (Bennett et al., 2018). The plasma prolactin levels detected are extreme for individuals that do not lactate and could quite possibly affect their gametogenesis, since prolactin is a well-known suppressor of reproduction as it inhibits GnRH secretion (Brown et al., 2014, Kauppila et al., 1988). This is in line with previous work that showed reduced pituitary sensitivity to GnRH in NBF CHP (Van der Walt et al., 2001) and provides further support for the endocrine control of suppression in this species. Similarly, high plasma prolactin levels were detected in NBM CHP, but these levels were lower those of BFs, NBFs and BMs. None the less, the level of prolactin may in fact be high enough to result in reproductive suppression and bring about parental care behaviour in NBM CHP that have been found to have pre-pubescent sized gonads and lowered hormones related to reproduction whilst in the confines of their natal colony, as reported in NBF CHP (Janse van Rensburg et al., 2002). Additionally, NBF CHP and NBM CHP show a reduced pituitary sensitivity to GnRH when compared to BM (Van der Walt et al., 2001).

Interestingly, BMs possessed some of the highest plasma prolactin levels. CHP have the smallest colony sizes of all cryptomids (see Hart et al., 2021 for review), and therefore cooperative behaviour, whether it be in the form of protecting the nest or digging tunnels or caring for offspring, is divided between a smaller number of individuals compared to the other sub-species of cryptomids. Breeding male CHP may therefore be required to invest more time in offspring care and other cooperative behaviours when compared to other subspecies of *Cryptomys*, which would explain their higher levels of plasma prolactin. These increased prolactin levels should impair their breeding abilities as they would inhibit GnRh and subsequent FSH and LH secretion (Bennett et al. 2018). In adult sexually, i.e. post- pubescent, mature men (*Homo sapiens*) however, prolactin has been observed to be a pituitary hormone that stimulates testosterone secretion from the Leydig cell of the testes (Rubin et al., 1976). Consequently, once a CHP male attains reproductive dominance in a colony or escapes the social suppression of their colony, circulating prolactin may take on a stimulatory role of both reproduction and parental care. However, additional research is required to unravel this phenomenon in this sub-species.

The patterns of prolactin levels found between CHP and CHN is similar to the pattern that was shown by Bennett et al. (2018), where it was demonstrated that naked mole-rats had extremely high levels of prolactin, whereas Damaraland mole-rats did not. In naked mole-rats, subordinates of both sexes are under strict physiological suppression while in the confines of the colony along with some degree of behavioural suppression (i.e., aggressive interactions) (Faulkes and Abbott, 1997, Faulkes and Bennett, 2009, Reeve et al., 1990), resulting in neo-natal reproductive characteristics of both subordinate males and females (Faulkes et al., 1994, Kayanja and Jarvis, 1971). It is proposed that high prolactin in this species may inhibit subordinate reproductive ability as prolactin exceeded levels considered to be hyperprolactinaemic (Bennett et al. 2018). Naked mole-rats can inbreed to a high degree, which is why it is thought that incest avoidance may not be as important in their reproductive skew maintenance compared to other mole-rat species (Reeve et al., 1990). In Damaraland mole-rats, however, behavioural suppression in the form of incest avoidance and aggressive interactions is vital in maintaining reproductive skew within their colonies (Clarke et al., 2001). Although it seems that behavioural suppression is the most crucial mechanism in this species, female Damaraland mole-rats are physiologically suppressed from reproducing, as NBFs show reduced pituitary sensitivity to GnRH, affecting some aspects of their reproductive capabilities (Bennett, 1994, Bennett et al., 1993, Bennett et al., 1996), resulting in pre-pubescent reproductive characteristics. Prolactin levels in Damaraland mole-rats were demonstrated to be extremely low in breeding and non-breeding individuals of both sexes and it is now believed that physiological suppression in the two species is implemented through two disparate pathways (Bennett et al., 2018).

Studies on the neuroendocrine pathways that control the downregulation of the GnRH system in these two eusocial species, naked and Damaraland mole-rats, suggests that two different mechanisms may be responsible. Kisspeptin (*Kiss-1*) and RFamide-related peptide-3 (*RFRP-3*) are neuropeptides that are well-known regulators of gonadotropin release (Irwig et al., 2004, Kriegsfeld et al., 2006). *Kiss*-1 stimulates GnRH secretion, while *RFRP-3* acts as an inhibitor of GnRH. In Damaraland mole-rats, BFs showed significantly higher levels of *Kiss-1* expressing cells than NBFs, suggesting that this pathway may play a vital role in the suppression of reproduction in this species (Voigt and Bennett, 2018). In naked mole-rats, *Kiss-1* expressing cells also differed between individuals of different reproductive statuses (Zhou et al., 2013), however subsequent research have shown that *RFRP-3* may be important for controlling their reproductive suppression (Peragine et al., 2017). The expression of *RFRP-3* has been shown to be higher in naked mole-rat NBFs than BFs; and the peptide has also been shown to suppress gametogenesis and mating behaviour in this species (Peragine et al., 2017). Additional studies have also found increased expression of genes involved in dopamine metabolism in naked mole-rat BFs and BMs in comparison to NBFs and NBMs, which would result in decreased prolactin in breeders and increased prolactin in non-breeders resulting in hypogonadism (Mulugeta et al., 2017).

The differences in plasma prolactin seen in CHN and CHP may also be the result of different pathways controlling their suppression. CHP non-breeders, for example, may possess higher *RFRP-3* peptide expressing cells and a lower number of genes involved in dopamine metabolism, as occurs in the naked mole-rats, since subordinates also appear to be physiologically suppressed through high prolactin concentrations. CHN on the other hand may use *Kiss-1* peptides as in the Damaraland mole-rats. Whether this is the case or whether other pathways are responsible is still unknown and requires further investigation.

No consistent patterns have been recorded for differences in glucocorticoids between reproductive classes in the various social African mole-rat species (Medger et al., 2019, Medger et al., 2018) and other cooperatively breeding mammalian species (Carlson et al., 2004, Creel, 2005, Hager and Jones, 2009, Young et al., 2006), and findings have often been contradictory. Recently, however, a link in the stress pathway (hypothalamic-pituitary-adrenal axis) and the dopamine-prolactin axis has been established in both humans and other rodents (Zhukov et al., 2021). Zhukov et al. (2021) showed that stress and anxiety-related behaviour was correlated with prolactin levels, with those individuals with higher levels of prolactin exhibiting higher levels of stress and anxiety-related behaviour. Furthermore, in several studies, individuals with increased prolactin levels exhibit adrenocortical cell hypertrophy (Silva et al., 2004) and increased adrenal sensitivity to ACTH stimulation, resulting in increased glucocorticoid release in comparison to those individuals with lower prolactin levels (Jaroenporn et al., 2007). The higher glucocorticoid (stress) levels observed in CHP may be linked to their physiological suppression due to their increased prolactin concentrations, and therefore prolactin and glucocorticoid may work in tandem in the control reproduction and cooperative behaviour in CHP. The low levels of glucocorticoids observed in CHN may be linked to their lack of circulating prolactin and their behavioural suppression, since reproductive skew is mainly believed to be maintained through incest avoidance and aggressive interactions (Oosthuizen et al., 2008b). Further studies are needed to fully understand the link between glucocorticoid and prolactin and how this affects reproductive suppression and cooperative behaviours.

All social mole-rat species show some form of behavioural suppression, which varies in importance between them. In naked mole-rats, for example, behavioural suppression in the form of aggressive interactions is present, despite physiological suppression being the more dominant mode of inhibiting subordinate breeding (Bennett and Faulkes, 2000, Faulkes and Abbott, 1993). Behavioural suppression has been shown to be the dominant mechanism of suppressing subordinate breeding in CHN by studies showing no differences in pituitary sensitivity to GnRH or gonadal function between breeders (Oosthuizen et al., 2008b). Behavioural suppression in CHN is also clearly demonstrated by the results of this study. Apart from low prolactin levels, different concentrations of fAMs between individuals from different reproductive classes support this claim. FAM levels of BF CHN were significantly higher than that of NBFs; and since androgens such as testosterone increase aggressive and dominant behaviour, it is likely that this hormone enhances aggressive behaviour of BFs towards NBFs to assert dominance to prevent them from mating (Lutermann et al., 2013). In CHN males, breeders and non-breeders had similar concentrations of fAMs. It is important to note that BM and NBM CHN are equally capable of breeding since previous work has shown that BMs and NBMs do not differ in sperm count or gonad size and, therefore, similar levels of testosterone can be expected in CHN males (Oosthuizen et al., 2010). It is thus believed that incest avoidance through incest taboos mainly prevents NBM CHN from breeding within the confines of the colony.

In CHP, fAM concentration did not differ between BFs and NBFs. This further supports the argument that NBFs are not behaviourally suppressed from reproduction but are rather physiologically suppressed since increased aggressive behaviour is absent. BMs showed much higher testosterone levels than NBMs, but this is not significant. Non breeding males of this subspecies appear to be both behaviourally and physiologically suppressed (Janse van Rensburg et al., 2003, Van der Walt et al., 2001), which is supported by the lower testosterone levels of NBM compared to BMs but still higher than expected prolactin levels. NBMs may also be less inclined to compete for dominance, since they can easily disperse to other colonies where competition is not intense or pair up with unrelated NBFs. Nevertheless, it is still believed that incest avoidance would still play a role in the reproductive suppression of both male and female CHP.

When considering all three endocrine correlates investigated in this study, it is clear that the mechanism of reproductive suppression differs between CHN and CHP. Our results indicate that subordinate CHN are predominantly suppressed through behavioural means in the form of aggressive behaviour and incest avoidance rather than through physiological means. In CHP, on the other hand, physiological suppression appears to be the main mechanism, although incest avoidance is also likely to contribute to inhibiting reproduction in these individuals. Further studies on other African mole-rat species and other cooperatively breeding mammals should be done to investigate how prolactin concentrations differs between breeding and non-breeding group members. Further research should also focus on neuroendocrine pathways in the mole-rat brains to determine what mechanisms responsible for their physiological reproductive suppression. The link between prolactin and stress is also an exciting new area of research. My findings will contribute to elucidating the mechanisms responsible for this phenomenon.

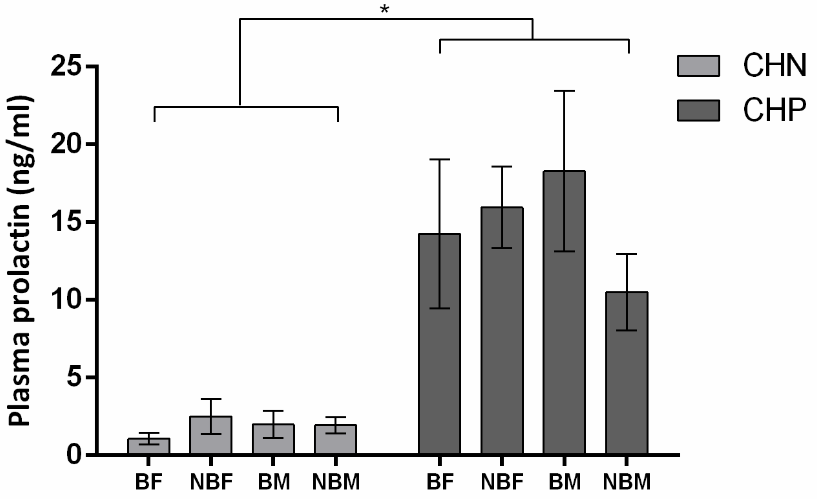

Figure 1. Bar graphs showing differences in plasma prolactin concentrations (ng/ml) between reproductive classes (BF: Breeding female; BM: Breeding male; NBF: Non-breeding female; NBM: non-breeding male) of *C.h. natalensis* and *C. h. pretoriae* (CHP).

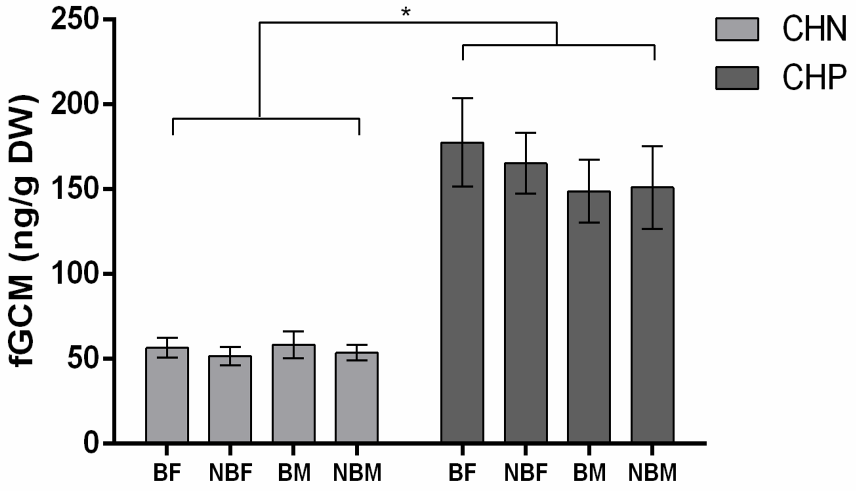

Figure 2. Bar graphs showing differences in faecal glucocorticoid metabolite (fGCM) concentrations (ng/ml dry weight) between reproductive classes (BF: Breeding female; BM: Breeding male; NBF: Non-breeding female; NBM: non-breeding male) of *C.h. natalensis* (CHN) and *C. h. pretoriae* (CHP).

Figures and Tables

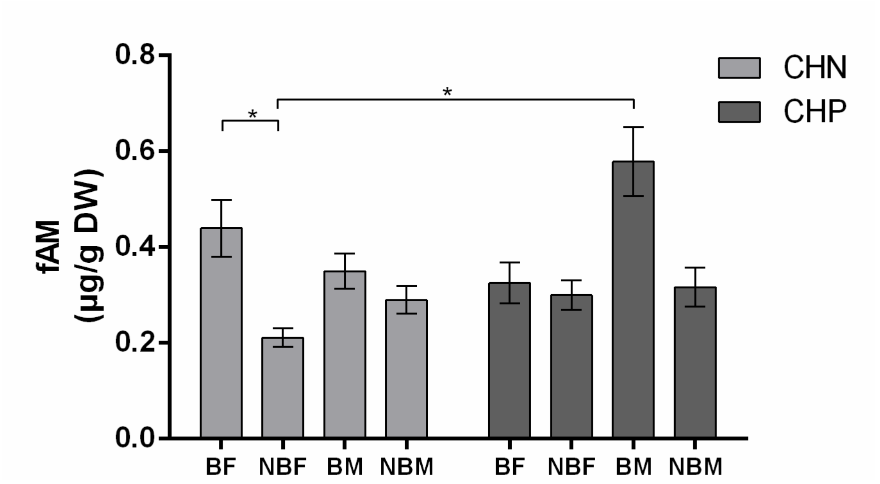

Figure 3. Bar graphs showing differences in faecal androgen metabolite (fAM) concentrations (ng/ml dry weight) between reproductive classes (BF: Breeding female; BM: Breeding male; NBF: Non-breeding female; NBM: non-breeding male) of *C.h. natalensis* (CHN) and *C.h. pretoriae* (CHP).

Table 1. The statistical outputs of a generalised linear model showing the effects of reproductive status, sex and species and their two-way and three-way interactions, along with body mass on plasma prolactin concentrations within and between *C.h. natalensis* and *C.h. pretoriae.*

Variable	**t-value**	**p-value**
Body mass	-0.446	0.656
Reproductive status	1.329	0.185
Sex	0.950	0.343
Subspecies	4.384	<0.001*
Sex*reproductive status	-1.072	0.285
Reproductive status*subspecies	-1.082	0.28
Sex*subspecies	-0.508	0.612
Reproductive status*sex*subspecies	0.229	0.819

*Indicates significance ($p \leq 0.05$)

Table 2. The statistical outputs of a generalised linear model showing the effects of reproductive status, sex and subspecies and their two-way and three-way interactions, along with body mass on faecal glucocorticoid metabolite (fGCM) concentrations within and between *C.h. natalensis* and *C.h. pretoriae.* Individuals

Variable	**t-value**	**p-value**
Body mass	-0.114	0.91
Reproductive status	-0.532	0.595
Sex	0.185	0.853
Subspecies	5.761	<0.001*
Sex*reproductive status	0.012	0.99
Reproductive status*subspecies	0.070	0.944
Sex*species	-0.768	0.444
Reproductive status*sex*subspecies	0.221	0.826

* Indicates significance ($p \leq 0.05$)

Table 3. The statistical outputs of a generalised linear model showing the effects of reproductive status, sex and subspecies and their two-way and three-way interactions, along with body mass on faecal androgen metabolite (fAM) concentrations within and between C.h. natalensis and C.h. pretoriae individuals.

Variable	**t-value**	**p-value**
Body mass	1.888	0.06
Reproductive status	-3.078	0.002*
Sex	-1.566	0.119
Species	-1.628	0.105
Sex*reproductive status	2.198	0.029*
Subpecies*reproductive status	2.403	0.017*
Subpecies*sex	3.033	0.003*
Subspecies*sex*reproductive status	-3.072	0.002*

* Indicates significance ($p \leq 0.05$)

Supplementary material

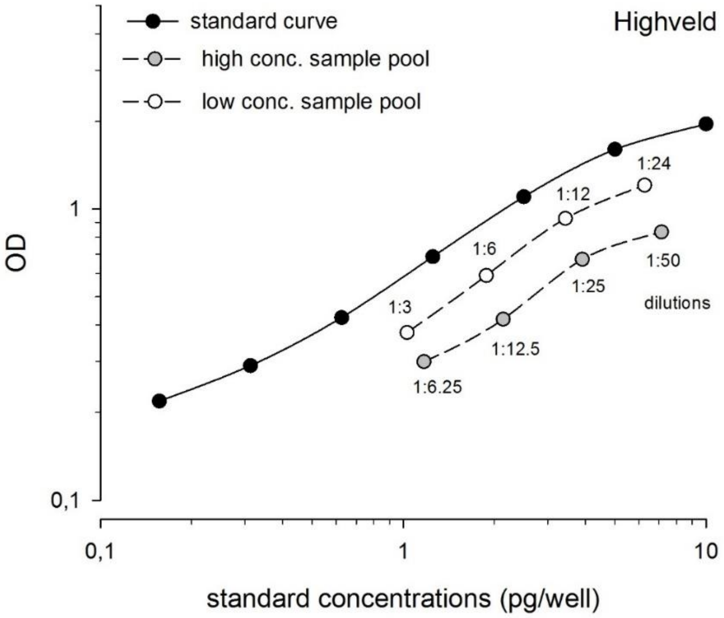

Figure S1. Assay validation revealed by parallelism of serial dilutions of spiked plasma pools for Highveld mole-rats (*Cryptomys hottentotus pretoriae*), with standard for the prolactin ELISA assay. Relative variation (%) of the slope of respective trendlines were <2% for Highveld mole-rat plasma. OD is the optical density measures the ELISA colour change, which is proportional to the concentration of prolactin.

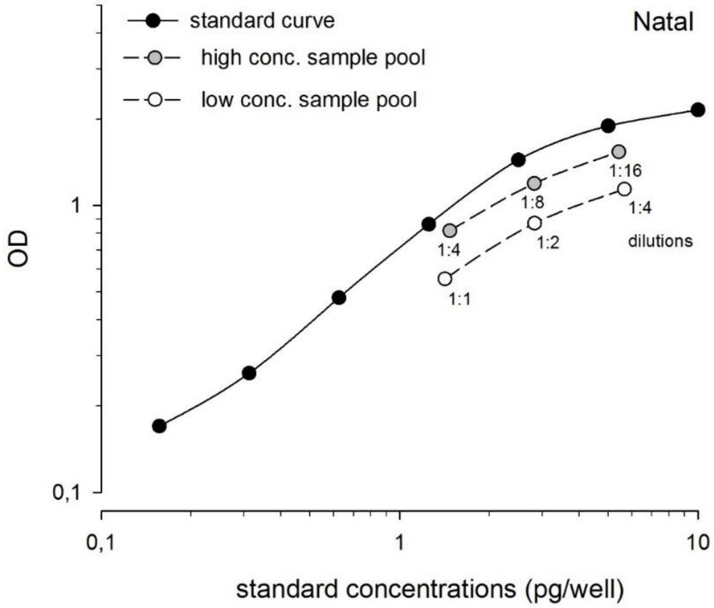

Figure S2. Assay validation revealed by parallelism of serial dilutions of spiked plasma pools for Natal mole-rats (*Cryptomys hottentotus natalensis*), with standard for the prolactin ELISA assay. Relative variation (%) of the slope of respective trendlines were <2% for Natal mole-rat plasma. OD is the optical density measures the ELISA colour change, which is proportional to the concentration of prolactin.

Table S1. Statistical outputs of an *emeans* analysis summarising the linear contrasts for faecal androgen metabolite (fAMs) concentration for sex*subspecies. Contrasts between males (M) and females (F) of the highveld mole-rat (*Cryptomys hottentotus pretoriae* - CHP) and the Natal mole-rat (*Cryptomys hottentotus natalensis* - CHN) are shown.

Contrast	P - value
F, CHN – M, CHN	0.943
F, CHN – F, CHP	0.952
F, CHN – M, CHP	0.329
M, CHN – F, CHP	1.000
M, CHN – M, CHP	0.085
F, CHP – M, CHP	0.104

Table S2. Statistical outputs of an *emeans* analysis summarising the linear contrasts for faecal androgen metabolite (fAM) concentration for sex*reproductive status. Contrasts between breeding (B) and non-breeding (NB) male (M) and female (F) individuals of the highveld mole-rat (*Cryptomys hottentotus pretoriae*) and the Natal mole-rat (*Cryptomys hottentotus natalensis*) are shown.

Contrast	P - value
B, F – NB, F	0.172
B, F – B, M	0.756
B, F – NB, M	0.597
NB, F – B, M	0.033*
NB, F – NB, M	0.687
B, M – NB, M	0.158

Table S3. Statistical outputs of a *emeans* analysis summarising the linear contrasts for faecal androgen metabolite (fAM) concentration for reproductive status*subspecies. Contrasts between breeding (B)and non-breeding (NB) individuals of the highveld mole-rat (*Cryptomys hottentotus pretoriae* - CHP) and the Natal mole-rat (*Cryptomys hottentotus natalensis* - CHN) are shown.

Contrast	P - value
B, CHN – NB, CHN	0.072
B, CHN – B, CHP	0.883
B, CHN – NB, CHP	0.546
NB, CHN – B, CHP	0.047*
NB, CHN – NB, CHP	0.427
B, CHP – NB, CHP	0.275

Table S4. Statistical outputs of a *emeans* analysis summarising the linear contrasts for faecal androgen metabolite (fAM) concentration for sex*subspecies*reproductive status. Contrasts between male (M) and female (F) breeding (B) and non-breeding (NB) individuals of the highveld mole-rat (*Cryptomys hottentotus pretoriae* - CHP) and the Natal mole-rat (*Cryptomys hottentotus natalensis* - CHN) are shown.

Contrast	**P - value**
F, CHN, B – M, CHN, B	0.771
F, CHN, B – F, CHP, B	0.733
F, CHN, B – M, CHP, B	0.967
F, CHN, B – F, CHN, NB	0.043*
F, CHN, B – M, CHN, NB	0.467
F, CHN, B – F, CHP, NB	0.599
F, CHN, B – M, CHP, NB	0.754
M, CHN, B – F, CHP, B	1.000
M, CHN, B – M, CHP, B	0.166
M, CHN, B – F, CHN, NB	0.777
M, CHN, B – M, CHN, NB	0.999
M, CHN, B – F, CHP, NB	1.000
M, CHN, B – M, CHP, NB	1.000
F, CHP, B – M, CHP, B	0.165
F, CHP, B – F, CHN, NB	0.945
F, CHP, B – M, CHN, NB	1.000
F, CHP, B – F, CHP, NB	1.000
F, CHP, B – M, CHP, NB	1.000
M, CHP, B – F, CHN, NB	0.014*
M, CHP, B – M, CHN, NB	0.118
M, CHP, B – F, CHP, NB	0.157
M, CHP, B – M, CHP, NB	0.235
F, CHN, NB – M, CHN, NB	0.717
F, CHN, NB – F, CHP, NB	0.467
F, CHN, NB – M, CHP, NB	0.650
M, CHN, NB – F, CHP, NB	1.000
M, CHN, NB – M, CHP, NB	0.999
F, CHP, NB – M, CHP, NB	1.000

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Chapter 2

The endocrine control of reproductive suppression in an aseasonally breeding social subterranean rodent, the Mahali mole-rat (*Cryptomys hottentotus mahali*)

Abstract

Cooperative behaviour, sociality and reproductive suppression in African mole-rats has been extensively studied. Nevertheless, endocrine correlates of some species of social mole-rats have been neglected, and these species may hold the key to understanding the behavioural and physiological complexity that allows the maintenance of social groups in African mole-rats. In this study, we investigated endocrine correlates implicated in the suppression of reproduction and cooperative behaviours, namely glucocorticoids (a stress-related indicator) through faecal glucocorticoid metabolites (fGCM), plasma testosterone (an indicator of aggression) and plasma prolactin in the Mahali mole-rat (*Cryptomys hottentotus mahali)* across reproductive classes (breeding females and males, non-breeding females and males) and season (wet and dry). Breeders possess higher levels of testosterone than non-breeders. In reproductively suppressed non-breeding females, plasma prolactin and fGCM were significantly higher than breeders. Chronically high levels of prolactin and fGCM are reported to cause reproductive suppression and promote cooperative behaviours. However, during the wet season, a relaxation of suppression occurs through reduced prolactin, but with elevated levels of plasma progesterone in non-breeding females. Prolactin is hypothesised as the primary hormone controlling reproductive suppression and cooperative behaviours in non-breeding females. An adrenocorticotropic hormone stimulation test (ACTH challenge test) on both male and female non-breeders revealed female non-breeders to show a greater response to the ACTH challenge than males. Adrenocortical cell hypertrophy and increased adrenal sensitivity to ACTH stimulation results in increased

glucocorticoid levels in individuals exhibiting increased prolactin levels. This study provides new endocrine findings for the maintenance of social suppression in the genus *Cryptomys*.

Introduction

Through the study of the subterranean family of the Bathyergidae (African mole-rats), many essential findings involving the ultimate and proximate factors that bring about the maintenance of social groups and socially induced infertility in mole-rats specifically and mammals generally have been uncovered. The answer as to how and why (ultimate factors) sociality has evolved in African mole-rats and other mammalian species has been well studied and is relatively well understood (Bennett et al., 1999; Bennett and Faulkes, 2000; Burda et al., 2000; Faulkes and Bennett, 2013; Firman et al., 2020; Jarvis et al., 1994). However, the physiological and/or behavioural mechanisms (proximate factors) that maintain sociality, group cohesion and dominance are less understood (Faulkes and Bennett, 2013).

The social organisation in the various species of African mole-rats ranges from strictly solitary to truly social (colony size range: 2-20) to eusocial (colony size range: 2-300) (Bennett and Faulkes 2000). Social and eusocial African mole-rat species exhibit cooperative breeding, a reproductive division of labour, cooperative care of young (alloparental care), and an overlap of at least two resident litters and often the apparent formation of a hierarchical structure in a colony (Bennett and Faulkes, 2000). Reproduction in these species is often monopolised by a single dominant breeding female (BF) and one or two of the largest dominant breeding males (BMs) within the colony (Bennett and Faulkes, 2000). The subordinate colony members (non-breeding females - NBFs and non-breeding males - NBMs) are reproductively quiescent, but are not permanently sterile. Both NBFs and NBMs are able to reproduce, but are naturally reproductively suppressed within the confines of their natal colony. These subordinate, socially suppressed individuals exhibit reproductive hormone concentrations similar to those of juvenile (sexually immature) individuals, and anovulatory gonads are often possessed by NBFs (Bennett and Faulkes, 2000).

Socially induced infertility is orchestrated through both behavioural and physiological means, by the dominant breeding individuals (Bennett et al., 1996, 1999, 2018; Blecher et al., 2020; Burda, 1995; Lutermann et al., 2013; Medger et al., 2019, 2018). The non-breeding colony members spend a large portion of their time foraging, expanding and defending their burrow system, and caring for the offspring of the BF (alloparental care) (Francioli et al., 2020; Houslay et al., 2020; Lacey and Sherman, 2009; Oosthuizen and Bennett, 2015; Scantlebury et al., 2006; Zöttl et al., 2018).

The two eusocial African mole-rat species, the naked mole-rat (*Heterocephalus glaber*) and the Damaraland mole-rat (*Fukomys damarensis*) have received the greatest attention, and these models have revealed behavioural and physiological control of reproductive inhibition, predominantly through neuroendocrine and endocrine mechanisms (Faulkes and Bennett, 2013). In both species, the reproductively suppressed individuals show a reduced pituitary sensitivity to gonadotropin-releasing hormone (GnRH) compared to their breeding counterparts (Bennett et al., 1993; Faulkes et al., 1991). This finding implies that there are socially induced impairments to the hypothalamic-pituitary-gonadal axis (HPG axis) in suppressed individuals. As a consequence, this leads to reduced androgens, such as testosterone, concentrations detected in NBMs and lower or non-detectable oestrogen and progestogen levels recorded in NBFs (Faulkes et al., 1990; Faulkes and Abbott, 1997; Molteno et al., 2004; Molteno and Bennett, 2000; Voigt and Bennett, 2018). However, the two species show dramatic differences in their mechanism of reproductive suppression.

In the spontaneously ovulating naked mole-rat, both NBFs and NBMs are physiologically suppressed (Faulkes and Abbott, 2009, 1997). Non-breeding males have been shown to have significantly smaller testes, lowered sperm counts, reduced sperm mobility and decreased circulating testosterone in comparison to their breeding counterparts (Faulkes et al., 1994; Faulkes and Abbott, 1997). In NBFs, ovaries are functionally quiescent and prepubescent, and lower or non-detectable concentrations of circulating progesterone and oestrogen are observed compared to BFs (Faulkes et al., 1990; Faulkes and Abbott, 1997). In the naked mole-rat social suppression in subordinates has been suggested to be the result of interaction with the dominant BF who shoves non breeders and exerts aggressive interaction towards them (as a result of increased testosterone). As a consequence increased glucocorticoid levels in the subordinates (a proxy for an increase in both acute and chronic stress) may result (Bennett and Faulkes, 2000; Clarke and Faulkes, 1997; Faulkes and Abbott, 1997). Chronically high levels of glucocorticoids, such as cortisol, have been associated with decreased reproductive fertility and even reproductive suppression (Toufexis et al., 2014). Furthermore, in cooperatively breeding species, increased glucocorticoid concentrations have been linked to cooperative behaviour such as offspring care (alloparental care) in subordinate group members (Carlson et al., 2006a). Recently, breeding and non-breeding naked mole-rats were observed to possess similar levels of glucocorticoids, thus challenging the notion that glucocorticoids and chronic stress play a role in reproductive suppression and alloparental care in naked mole-rats (Edwards et al., 2020; Medger et al., 2019). A more plausible hypothesis for the control of physiological suppression of reproduction in naked mole-rats has come from the peptide hormone prolactin (Bennett et al., 2018). In mammals, prolactin regulates several processes, including the inhibition of the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH), the control of milk production and the activation of parental care (Brown et al., 2014). Prolactin is also well-known for suppressing reproduction during lactation, and elevated levels (hyperprolactinemia) can disrupt reproductive function in both sexes (Kauppila et al., 1988; Ziegler, 2000). In naked mole-rat NBFs and NBMs, prolactin concentrations are as high or even higher than those recorded in BFs, even during lactation (Bennett et al., 2018).

In contrast, hyperprolactinemia is absent in Damaraland mole-rats regardless of breeding status or sex; therefore, it is unlikely that prolactin plays a pivotal role in the reproductive suppression or alloparental care in Damaraland mole-rats (Bennett et al., 2018). Furthermore, in contrast to naked mole-rats, only NBF Damaraland mole-rats are under physiological suppression of reproduction, as NBMs can breed, have similar sized testes and testosterone concentrations compared to the BMs, but lack the opportunity due to an absence of unrelated NBFs in the colony (Faulkes et al., 1994; Jarvis and Bennett, 1993, Bennett, 1994). In this species, NBFs show anovulation and reduced progesterone concentrations while in the confines of their natal colony (Bennett et al., 1994). However, unlike their naked mole-rat counterparts, induced ovulation appears to be present in Damaraland mole-rats (Voigt et al., 2021), and there is a strong aversion to inbreeding in this species (Burland et al., 2002; Cooney and Bennett, 2000). It has been hypothesised that a behavioural mode of suppression through the avoidance of breeding with related individuals in a colony (incest avoidance) or, if unrelated individuals are present in the colony, their reproductive attempts may be interrupted by a breeder of the same sex (Burland et al., 2004; Clarke et al., 2001). As in naked mole-rats, breeding and non-breeding Damaraland mole-rats possess similar levels of glucocorticoids regardless of their sex, implying that chronic stress may not play a role in their reproductive suppression or alloparental care (Molteno, 1999; Medger et al., 2018). Nevertheless, physiological suppression occurs to some extent in NBF Damaraland mole-rats through the mechanism of reduced LH response to a GnRH challenge, as well as reduced basal levels of LH while in the confines of the colony with the BF present (Bennett et al., 1996; Bennett et al., 1993; Rickard and Bennett, 1997).

In contrast to the comparatively well-studied naked and Damaraland mole-rats, little is known regarding the mechanisms of reproductive suppression operating in the social genus *Cryptomys*. Thus, to further understand the potential mechanisms of reproductive suppression and the underlying endocrine control in African mole-rats and other cooperatively breeding mammal species, the genus *Cryptomys* provides an ideal model to unravel the underlying physiological mechanisms for socially induced infertility.

The basic reproductive biology of many sub-species of *Cryptomys*, including the Mahali mole-rat (*Cryptomys hottentotus mahali*), has been described (Janse van Rensburg et al., 2002; Oosthuizen et al., 2008; Spinks et al., 1997, 1999; Hart et al., 2020; Hart et al., 2021). However, the mechanisms of reproductive suppression and its potential endocrine control are unknown for most of the sub-species. To date, it is believed that behavioural interactions (through aggression as a result of increased testosterone in breeders) and/or inbreeding avoidance are the primary strategies underlying the suppression of reproduction in the non-breeding colony members in the genus *Cryptomys* (Lutermann et al., 2013; Oosthuizen et al., 2008). In this study, we investigate this assumption for the Mahali mole-rat by comparing several endocrine correlates implicated in the suppression of reproduction and alloparental care between the reproductive classes (BF, BM, NBF and NBM) and season (wet and dry). The hormone classes investigated include glucocorticoids (a stress-related indicator) through faecal glucocorticoid metabolites (fGCM), plasma testosterone (an indicator of aggression) and plasma prolactin. As a prerequisite, we examine the suitability of enzyme immunoassays for detecting changes in fGCM concentrations of Mahali mole-rats by performing an adrenocorticotropic hormone stimulation test (ACTH challenge test) on both male and female non-breeders.

Materials and Methods

Animal capture

A total of 224 Mahali mole-rats, comprising 109 males and 115 females, were captured within the study period between October 2016 and September 2017; with mole-rat colonies being captured monthly for an entire calendar year (Hart et al., 2021). A total of 118 individuals were captured within the wet season (December – May), and 106 individuals were captured within the dry season (June – November) (see Table 1 for full capture breakdown). Mole-rats were captured using Hickman live traps, baited with a small piece of sweet potato (Hickman, 1979). The traps were positioned at the entrance of excavated burrows where tunnels were open. Traps were monitored for captures or blocking every 2-3 hours over the course of the day and left overnight, being checked first thing in the morning. Entire colonies were caught out, with a colony being deemed to be completely trapped if no trap activity was observed five consecutive days after capture of the last animal (Hart et al. 2020). Capture sites were at smallholdings or at farms in and around the area of Patryshoek, Pretoria (25°40' S, 28°2' E), South Africa. Permission to capture the mole-rats was obtained from all landowners, and a collecting permit was obtained from the relevant nature conservation authorities (Permit number: CPF6-0127). On capture, the sex and body mass (to the nearest 0.1 g) were recorded for each animal (Scout Pro SPU123, Ohaus Corporation, Pine Brook, New Jersey, U.S.A). The Animal Use and Care Committee of the University of Pretoria evaluated and approved the experimental protocol (ethics clearance number EC044-16 and NAS 128/2020) and DAFF section 20 approval (SDAH-Epi- 20070806200).

Animal housing and Dissection procedure

The captured animals were transported to the mole-rat laboratory at the University of Pretoria, Department of Zoology and Entomology. Complete colonies of mole-rats were housed together in plastic crates (49.5 × 28.0 cm). Nesting material, comprising of wood shavings and paper towelling were supplied to each colony. The mole-rats were fed daily on sweet potatoes and apples. All water requirements of the animals were satisfied with the provided food (Bennett and Jarvis, 1995). The animals were maintained in a climate-controlled room at a constant temperature of 25 ± 1°C and a light cycle of 12 hours light and 12 hours dark.

The capture of animals (entire colonies) was finalised by the middle of each month (a total of 31 colonies were captured during the entire study), and all animals were euthanised with an overdose of isoflurane in one day. However, to ensure that post-mortem examination was as accurate as possible, functionally complete colonies were maintained together for approximately 1 week after all individuals in a system had been trapped. Blood was obtained from the mole-rats by exsanguination from the heart and centrifuged at 1500g for 15 minutes. The separated plasma was stored at -70°C until hormone analysis.

Determination of reproductive status

All individuals below 40 g with a dark coat colour were considered to be juveniles (Hart et al., 2021). Additionally, individuals captured alone in a tunnel system after a considerable trapping effort of 5 days were assumed to be dispersing individuals. The juveniles and dispersing individuals were not considered further for this study. The BMs were distinguishable from NBMs by their large, descended inguinal testes and yellow staining around the mouth. The BFs possessed prominent auxiliary teats and a perforated vagina, which was absent in the NBFs or dispersers. During the dissection process, the breeding status of a female was confirmed by the presence of foetuses or placental scars on the uterine horns of queens.

ACTH challenge and faecal collection

Three additional NBMs and three NBFs were captured during May 2018 and maintained in captivity for six months. These captive mole-rats were housed separately throughout the six-month period. The mole-rats were subsequently moved to collection chambers with a wire-mesh bottom and a collection dish underneath for faecal collection. The wire-mesh bottom prevented any contamination of the faecal samples by urine. Collection chambers were checked hourly or bi-daily for faeces, depending on the protocol. Throughout the experiment mole-rats were fed pieces of sweet potato and apple daily. No additional water was provided as the animals received all the water from their food (Bennett and Jarvis, 1995). Animals were placed in the collection chambers for ten days before the administration of ACTH to allow the mole-rats to acclimatise to their new surroundings. The faecal collection started seven days prior to the ACTH challenge to obtain baseline values, and the collection continued for five days post-ACTH challenge. Chambers were checked for faeces every hour for the first 24 hours post-ACTH injection and twice a day for the rest of the experimental days before and after treatment. Synthetic ACTH

(Synacthen ® depot, Novartis, South Africa (Pty) Ltd) was dissolved in a sterile isotonic saline solution, and individuals were injected intramuscularly (0.3 ml) at 10:00 a.m. with a dose of 5–10 IU (50–100 µg) per 100 g body mass (Medger et al. 2018). The handling time during injection was <1 min. Sampling commenced one hour after injection (11:00 a.m.). Faeces were collected in Eppendorf tubes using tweezers. Tweezers were cleaned thoroughly with 70% ethanol between sampling events. All samples were frozen immediately after collection and stored at −20 °C until further preparation. The Animal Use and Care Committee of the University of Pretoria evaluated and approved the experimental protocol (ethics clearance number EC031-18).

Faecal collection of wild animals

The above collection protocol was similarly used for faecal collection from wild-caught individuals. Within the first four hours, only the first faecal sample dropped by the wild-caught individuals was collected, frozen and subsequently analysed for the fCGM. In general, animals usually defecate within two hours of capture.

Faecal hormone metabolite extraction and analysis

Faecal samples were lyophilised, pulverised, and sieved through a wire-mesh strainer (Medger et al. 2018). Between 0.050 and 0.055 g of faecal powder was weighed out per sample and extracted using 1.5 ml of 80% ethanol. The suspension was shaken for 15 minutes on a multi-vortex and subsequently centrifuged for 10 minutes at 1500g (Medger et al. 2018). The supernatant was transferred into a clean Eppendorf tube and stored at −20 °C until analysis.

Faecal steroid extracts (n = 72) from all six ACTH treated animals were measured for immunoreactive fGCM concentrations using five different enzyme immune-assays (EIAs): (i) Cortisol; (ii) 11-oxoaetiocholanolone I (detecting 11,17 dioxoandrostanes); (iii) 11-oxoaetiocholanolone II (detecting fGCMs with a 5β- 3α-ol-11-one structure), (iv) Corticosterone, and (v) 5α-pregnane-3β,11β,21-triol-20-one (detecting fGCMs with a 5α-3β-

11β-diol structure). Detailed assay characteristics, including full descriptions of the assay components and cross-reactivities, have been provided for the 11-oxoaetiocholanolone I, cortisol and corticosterone EIAs by Palme (1997), 11-oxoaetiocholanolone II EIA by Möstl and Palme (2002) and for the 5α-pregnane-3β,11β,21-triol-20-one EIA by Touma et al. (2003). The sensitivities of the EIAs used are 1.8 ng/g faecal dry weight Corticosterone, 0.6 ng/g faecal dry weight for Cortisol, 11-oxoaetiocholanolone I, and 11-oxoaetiocholanolone II, and 2.4 ng/g faecal dry weight for 5α-pregnane-3β,11β,21-triol-20-one, respectively. The coefficients of variance for intra-assay variance were 5.67% and 6.90% for the Cortisol, 5.61% and 7.03% for the Corticosterone, 5.65% and 6.11% for the 11-oxoaetiocholanolone II, 2.21% and 2.15% for the 11-oxoaetiocholanolone I, and 4.27% and 4.34% for the 5α-pregnane-3β,11β,21-triol-20-one EIA, respectively. The coefficients of variance for inter-assay variance were 10.19% and 14.70% for the Cortisol, 12.83% and 13.58% for the Corticosterone, 10.07% and 13.48% for the 11-oxoaetiocholanolone II, 11.69% and 12.36% for the 11-oxoaetiocholanolone I, and 5.30% and 8.09% for the 5α-pregnane-3β,11β,21-triol-20-one EIA, respectively.

The Cortisol EIA discriminated best between pre- and peak ACTH-injection fGCM levels (see 3.2 Results) and was solely used for quantifying fGCMs in the sample extracts of the wild animals. Faecal steroid concentrations are presented as µg/g faecal dry weight (DW). EIAs were performed at the Endocrine Research Laboratory, University of Pretoria, as described by Ganswindt et al. (2012, 2014).

Plasma testosterone, progesterone, and prolaction analyses

Coat-a-count hormone kits (IBL International GmBH, Hamburg, Germany) were used to determine plasma testosterone and progesterone concentrations as described by Hart et al. (2021). All assays were carried out according to the manufacturer's protocol. Serial dilutions of respective plasma samples gave displacement curves that were parallel to the respective standard curves for both testosterone and progesterone. (Testosterone: $F_{[1,5]} = 0.18$; $p = 0.11$;

Progesterone: $F_{[1,5]} = 2.27$; $p = 0.07$). The sensitivity of the testosterone assay was 0.015 ng/dl plasma, and the intra-assay coefficient of variation was 4.7%. The sensitivity of the progesterone assay was 1.48 nmol/L or 0.47 ng/ml, and the intra-assay coefficient of variation was 7.9%.

Plasma prolactin was quantified using an Elabscience Guinea pig PRL (Prolactin) ELISA kit (Elabscience Biotechnology Inc., Wuhan, China) as described by Bennett et al. (2018). The sensitivity of the assay was 0.1 ng/ml plasma and intra-assay precision, and repeatability are <10%, according to the manufacturer's guidelines. Serial dilutions of plasma samples gave displacement curves that were parallel to the respective standard curve with relative variation of the slope of the trend lines < 2%. See supplementary material for the standard curve (Figure S1).

Data analyses

All statistical analyses were performed in R 3.5.2, and statistical significance was assumed at $p \leq 0.05$. All data are presented as mean ± standard error (SE).

Mean individual baseline values for fGCM were calculated from the samples collected seven days prior to ACTH administration. Baseline values were used to calculate the percentage change of fGCM concentration during the ACTH challenge. Average individual fGCM concentrations were calculated every 12 hours from 0 to 24 hours post-treatment and for 24-hour intervals for the rest of the post ACTH challenge period. Faecal GCM concentrations of captive individuals were compared between the sexes and the 13 experimental times before and after the ACTH challenge using generalised mixed models (GLMM) with the individual as a random factor. Sex and sampling time and the interaction of sex and sampling time were included as independent factors in the GLMM.

The normality of dependent variables (body mass and hormone concentrations) was determined using Shapiro-Wilk tests (S-W). Non-normally distributed dependent variables were transformed (either log or square-root) to obtain normal distributions if possible. The homogeneity of normally distributed data was confirmed using Levene's test. Normally distributed dependent variables were analysed using General linear models (GLMs). All non-normal dependent variables were analysed using Generalised linear models (GLZMs) fitted with gamma distributions and link-identity functions using the lme4 package. *Post-hoc* comparisons were made using Tukey's honestly significant difference (Tukey's HSD) tests.

Models contained hormone concentrations (plasma prolactin, plasma testosterone, or fGCM) or body mass as response variables and reproductive class (BF, NBF, BM and NBM) and season (Dry and Wet) as predictors, with two-way interactions included. Model selection was conducted for each model using the *dredge* function of the *Mumin* package (Barton and Barton 2015). Model suitability was assessed using Akaike information criterion values corrected for a small sample size (AICc). Models with ΔAICc < 2 were considered equally parsimonious, the coefficients of which were subsequently averaged to construct a final model. Conditional average values were reported for final models with more than one competing model. The final models selected are presented in the supplementary material.

A total of 180 individuals were assessed for fGCMs (BF: n = 17, NBF: n = 68, BM: n = 29, NBM: n = 66). The fGCM data were log-transformed to obtain a normal distribution. Two separate models were used to analyse plasma testosterone data due to the extremely high values of BMs. The first model retained BMs (213 individuals) (BF: n = 20, NBF: n = 89, BM: n = 30, NBM: n = 74), while the second model possessed only BF, NBF and NBM data with the exclusion of BM data (197 individuals; NBF: n = 89, BM: n = 30, NBM: n = 74. Plasma testosterone data for both models could not be transformed into a normal distribution. A total of 90 individuals were assessed for plasma prolactin (BF: n = 13, NBF: n = 52, BM: n = 10, NBM: n = 15). Plasma prolactin data were log-transformed to obtain a normal distribution. Due to insufficient data, seasonal comparisons of plasma prolactin were made for female individuals only in a separate model (65 females; BF: n = 13, NBF: n = 52). Additionally, 90 females (BF:

20, NBF: 70) were assessed for variation of plasma progesterone concentrations between reproductive class and season (non-normally distributed data). Body mass data of 212 individuals were analysed (BF: n = 20; NBF: n = 88; BM: n = 28; NBM: n = 76), and a normal distribution was obtained after a square-root transformation. See supplementary material for further details.

To investigate if prolactin was linked to reproductive suppression in NBFs, Spearman's rank-order correlations were conducted between plasma prolactin and plasma progesterone. Likewise, to investigate if stress is linked to reproductive suppression in NBFs, Spearman's rank-order correlations were conducted between fGCM and plasma progesterone. Furthermore, the correlation between plasma testosterone and plasma progesterone was investigated in NBFs. Further correlations were conducted between body mass and four variables, respectively, namely fGCM, plasma prolactin, plasma testosterone and plasma progesterone.

To investigate if prolactin or stress (fGCM) were linked to reproductive suppression, Spearman's rank-order correlations were conducted for NBMs between plasma prolactin and plasma testosterone, as well as between fGCMs and plasma testosterone, respectively. Furthermore, Spearman rank correlations were conducted between body mass and three variables respectively, namely plasma prolactin, plasma testosterone and fGCM, respectively.

Results

Body mass

The body mass of the Mahali mole-rat was explained by season and reproductive class (Table S1). Body mass differed significantly between reproductive status throughout the year ($F = 44.3$, $df = 3$, $p < 0.001$, Figure 1a) and was significantly affected by season ($F = 10.65$, $df = 1$, $p \leq 0.002$, Figure 1b). Breeding males were significantly heavier than BFs ($p \leq 0.008$), NBFs ($p < 0.001$) and NBMs ($p < 0.001$) respectively, throughout the year (Figure 1a). BFs were significantly heavier than both NBMs ($p \leq 0.003$) and NBFs ($p < 0.001$), while NBMs were significantly heavier than NBFs throughout the year ($p \leq 0.004$). Furthermore, individuals captured in the dry season were significantly heavier than those captured in the wet season ($F = 13.39$, $df = 1$, $p < 0.001$, Figure 1b).

ACTH Challenge

Faecal GCM concentrations were significantly elevated after the injection of ACTH ($F = 4.54$, $df = 12$, $p \leq 0.001$). At 12 hours post-ACTH administration, fGCM concentrations were 200 ± 113.0% above baseline ($p \leq 0.03$) and remained significantly elevated until 24 hours post-treatment ($p \leq 0.05$). Faecal GCM concentrations returned to baseline levels approximately 48 hours after treatment. Overall mean fGCM concentrations differed significantly between females (0.22 ± 0.03 µg/g DW) and males (0.17 ± 0.02 µg/g DW) ($F = 0.64$, $df = 1$, $p \leq 0.001$, Figure 2a).

The interaction between sex and time was not significant (GLMM: $F = 0.82$, $df = 12$, $p = 0.63$), and as such, the response of fGCM concentrations in the mole-rats to an ACTH challenge appeared to be similar for both females and males (Figure 2a and b). However, the response of males 12 hours (387.0%) and 24 hours (155.6 ± 2.0%) post-ACTH challenge was considerably less than that of the response arising in females 12 hours (2155.0 ± 1957.1%) and 24 hours (649.9 ± 525.8%) post-ACTH challenge (Figure 2 b). This difference, however, is not significant ($F = 0.82$, $df = 12$, $p = 0.63$).

fGCMs

The variation in fGCM concentrations between individuals was different depending on their reproductive status (Table S2). Reproductive status had a significant effect on the fGCM concentrations ($F = 3.2$, $df = 3$, $p \leq 0.03$; Figure 4) with NBFs showing significantly higher levels of fGCMs than BFs ($p \leq 0.05$) throughout the year. Breeding males showed similar fGCM levels to NBMs ($p = 0.66$) and BFs ($p = 0.89$), respectively (Figure 4). Furthermore, NBFs also showed similar concentrations of fGCMs to BMs ($p = 0.12$) and NBMs ($p = 0.51$), respectively (Figure 4).

Plasma testosterone

The variation in plasma testosterone concentration of BM data were explained by reproductive status and season, respectively (Table S3). Reproductive status significantly affected plasma testosterone concentrations ($z = 3.00$, $p < 0.003$, Figure 5a), while season did not have a significant effect throughout the sampling period ($z = 1.01$, $p \geq 0.31$). Breeding males had significantly higher plasma testosterone concentrations compared to BFs, NBFs and NBMs ($p < 0.001$, for all, Figure 5a). Similar concentrations of plasma testosterone concentrations were found in BFs, NBFs and NBMs ($p > 0.05$, for all, Figure 5a).

Once excluding BMs, the variation in plasma testosterone concentrations between NBMs, BFs and NBFs was explained by both reproductive status and season (Table S4). Plasma testosterone concentrations were significantly affected by reproductive status ($z = 3.178$, $p < 0.002$, Figure 5b), but not by season ($z = 1.018$, $p \geq 0.3$). NBMs showed significantly higher levels of plasma testosterone compared to NBFs ($p < 0.001$) and similar levels to BFs ($p = 0.45$, Figure 5b). Additionally, BFs and NBFs possessed similar plasma testosterone concentrations throughout the sampling period (Tukey's HSD: $p = 0.47$).

Plasma progesterone

The variation in female plasma progesterone concentrations was explained by reproductive status and season (Table S4). Both season ($t = 4.57$, $p < 0.001$) and reproductive status ($t = -3.07$, $p < 0.003$) had a significant effect on plasma progesterone concentrations (Figure 7). Breeding female plasma progesterone concentrations were significantly higher than that of NBFs ($p < 0.001$, Figure 7a) throughout the sampling period. Further analysis revealed that plasma progesterone concentrations were found to be significantly higher during the wet season compared with the dry season ($p < 0.001$, Figure 7b).

Plasma prolactin

Plasma prolactin concentrations differed significantly between reproductive status throughout the sampling period ($F = 5.412$, $df = 3$, $p \leq 0.002$, Figure 6a). Breeding females and NBFs showed comparable concentrations of plasma prolactin ($p = 0.98$, Figure 6a). Likewise, BMs and NBMs also showed similar concentrations of plasma prolactin ($p = 0.89$, Figure 6a), while NBFs showed significantly higher plasma prolactin concentrations compared to BMs and NBMs, respectively ($p \leq 0.05$ for both, Figure 6a). Furthermore, BFs had significantly higher plasma prolactin concentrations than BMs (Tukey's HSD: $p \leq 0.03$) but similar levels to NBMs (Tukey's HSD: $p = 0.089$, Figure 6a).

On removing males, season alone explained the variation in plasma prolactin concentrations between breeding and non-breeding females (Table S4). Plasma prolactin levels of both breeding and non-breeding females were observed to be significantly higher in the dry season than in the wet season ($F = 32.8$, $df = 1$, $p < 0.001$, Figure 6b).

Correlational links between hormones and body mass in non-breeders

Non-breeding females with the highest concentration of plasma prolactin also had the lowest concentrations of plasma progesterone ($r = -0.44$, $p = 0.06$). Contrastingly, plasma testosterone and fGCM concentrations did not affect plasma progesterone concentrations in NBFs ($r \leq -0.07$, $p \geq 0.57$). Body mass did not affect fGCM, or plasma prolactin, testosterone, or progesterone concentrations ($r \leq 0.05$, $p \geq 0.63$).

Neither plasma prolactin ($r \leq 0.13$, $p \geq 0.62$) nor fGCM concentrations ($r \leq -0.12$, $p \geq 0.34$) were significantly correlated with plasma testosterone concentrations. Non-breeding males with the highest body mass had the highest testosterone concentrations ($r = 0.39$, $p < 0.001$). Contrastingly, there were no significant correlations between body mass and prolactin ($r \leq -0.42$, $p \geq 0.12$), nor between body mass and fGCM concentrations ($r \leq -0.13$, $p \geq 0.27$).

Discussion

This study suggests that a combination of behavioural and physiological reproductive suppression modes are operational in the Mahali mole-rat. Similar to the Natal (*C. h. natalensis*), Damaraland and naked mole-rats, increased circulating testosterone levels were observed in breeding Mahali mole-rats of both sexes compared to their non-breeding counterparts (Faulkes et al., 1991; Lutermann et al., 2013; Medger et al., 2019; Swift-Gallant et al., 2015). Therefore, the increased testosterone, coupled with increased body mass, would result in the physical dominance of breeders over their non-breeding counterparts. As a result, breeders may interrupt breeding attempts between unrelated colony members, or unrelated NBMs, and the BF. Furthermore, non-breeders may also be the object of aggression that may lead to chronic stress and thus chronic exposure to increased levels of glucocorticoids.

Unlike the scenario presented in Damaraland and naked mole-rats, a clear relationship is visible between the hypothalamic-pituitary-adrenal (HPA) axis and the HPG axis, and consequently reproductive hormones such as testosterone and progesterone, in the Mahali mole-rat. This study provides evidence that reproductive suppression and possibly alloparental care may arise from increased fGCM concentrations (glucocorticoids), possibly by chronic stress. Non-breeding females possessed significantly higher fGCMs than BFs throughout the year. It is possible that that this may arise as a result of them being targeted by increased aggression directed towards them by BFs. Chronically high glucocorticoid levels have been linked to reduced reproductive abilities (Toufexis et al., 2014) and increased cooperative behaviours such as offspring care

(Carlson et al., 2006a) in cooperatively breeding meerkats. Consequently, one of the possible mechanisms inhibiting NBF breeding (reproductive suppression) within the colony may be chronically high levels of glucocorticoids, but further research is required to substantiate this statement. In contrast, NBMs, which are not reproductively suppressed within the confines of the colony (Hart et al., 2021), exhibited similar levels of fGCMs to BMs throughout the year. Unrelated NBMs cannot attempt breeding with NBFs, as these NBFs are reproductively suppressed within the colony and, therefore, it is likely that BMs interrupt breeding attempts between unrelated NBMs or the BF (Bennett and Faulkes, 2000). Breeding interruptions would be a short confrontation (acute stress) and therefore would not result in chronic stress, or prolonged levels of elevated glucocorticoids, resulting in no reproductive suppression. Likewise, incest avoidance by the NBMs would also not induce increased levels of fGCMs.

On closer examination of the HPA axis of the Mahali mole-rat, sex differences are evident in the adrenal sensitivity to ACTH. Female Mahali mole-rats show increased sensitivity to administered ACTH when compared to males resulting in females producing higher fGCMs for the same ACTH dose related stimulation. The dominant route of excretion (urine or faeces) of glucocorticoids may vary between the sexes (Teskey-Gerstl et al., 2000); however, it can be hypothesised that the female HPA axis has become more sensitive to stressors to allow for efficient cooperative breeding to occur in this species. In humans (*Homo sapiens*), females produce more ACTH from their pituitary gland than males in response to the same stressor, but have similar adrenal responses to ACTH, resulting in similar glucocorticoid levels (Gallucci, 1991). While in rats (*Rattus norvegicus*), as with the Mahali mole-rat, females typically have a more robust neuroendocrine response to stress, as evidenced by their increased glucocorticoid and ACTH response compared to males (Heck and Handa, 2019). In contrast to the Mahali mole-rat, however, female rats have been shown to have a delayed return to baseline ACTH and glucocorticoid levels after stress (Heck and Handa, 2019). Male Mahali mole-rats show a delayed return in glucocorticoid levels when compared to females, indicating sex differences in the negative feedback regulation of the HPA axis, which is in contrast to rats (Heck and Handa, 2019).

As with fGCMs, plasma prolactin appears to play a critical role in reproductive suppression and cooperative care in the Mahali mole-rat. Similar to naked mole-rats (Bennett et al., 2018), NBFs possessed comparable concentrations of plasma prolactin to BF Mahali mole-rats. However, the plasma prolactin titres of both NBFs and BFs were not at the level of hyperprolactinemia (greater than 20 ng/ml), or the levels of prolactin recorded for the naked mole-rat by Bennett et al. (2018). Plasma prolactin levels for the Mahali mole-rats were, however, higher than that of naked mole-rats reported in Medger et al. (2019) and Damaraland mole-rats in Bennett et al. (2018). Elevated prolactin is well known to suppress fertility and mediate parental and alloparental care in cooperatively breeding mammals and birds (Carlson et al., 2006b; Schoech et al., 1996). The elevated plasma prolactin levels in NBFs may inhibit GnRH release, and as a consequence the downstream levels of LH, FSH and subsequently oestrogen and progesterone, leading to the well-characterized anovulation in NBF Mahali mole-rats (Hart et al., 2021). This conclusion is supported by NBFs possessing lower plasma progesterone concentrations than BFs in addition to the lack of copoea lutea of ovulation (Hart et al. 2021). Furthermore, NBFs that possess higher plasma prolactin levels also possessed lower plasma progesterone levels, suggesting that prolactin acts as an inhibitory factor of progesterone release in the Mahali mole-rat (this study). In addition, we can speculate that increased plasma prolactin, as with the increased glucocorticoids of NBFs, may also play a part in the mechanisms eliciting cooperative behaviour (Bennett et al., 2018). Both BMs and NBMs possessed lower plasma prolactin titres than BFs and NBFs; this is somewhat expected as both NBMs and BMs can breed within the colony, and it is unlikely that they perform many alloparental care tasks (Hart et al. 2021).

Unlike fGCMs, plasma prolactin in female Mahali mole-rats showed a variation between seasons, as females captured in the dry season possessed higher plasma prolactin concentrations than those captured in the wet season. The possible reason for this could be two-fold: firstly, a greater number of pregnant BFs were captured in the dry season (n = 6) compared to the wet season (n = 4) (see Hart et al. 2021). Pregnancy has been observed to increase circulating levels of prolactin (Grattan, 2001). Secondly, there is an apparent relaxation of suppression of reproduction during the wet season in NBF Mahali mole-rats, as illustrated by increases in progesterone levels of females in the wet season, even though there are fewer pregnant BFs (this study; Hart et al. 2021). Hart et al. (2021) reported indications of relaxation of suppression in NBF Mahali mole-rats supported by increased plasma progesterone concentrations and more frequent events of female dispersal during the months of the wet season, whereas increased suppression in NBFs was observed during the months of the dry season. Dispersal from the natal colony occurs under favourable environmental conditions when the soil characteristics are optimal for excavation and digging, such as periods of good rainfall (Jarvis et al., 1994; Kotze et al., 2008). This phenomenon has been recorded in several social and eusocial mole-rats, including the common mole-rat (*C.h hottentotus*) (Spinks et al., 1999, 1997), highveld mole-rat (*C. h. pretoriae*) (Janse van Rensburg et al., 2002), Damaraland mole-rat (Molteno and Bennett, 2002) and the naked mole-rat (Westlin et al., 1994).

The relaxation of suppression in NBFs seen during the wet season may have an effect on fGCM concentrations, as BFs may increase aggression towards NBFs to discourage dispersal (Kutsukake and Clutton-Brock, 2006). This may, in turn, result in similar fGCM levels between the seasons in NBF Mahali mole-rats. Consequently, prolactin may be the primary controlling factor in reproductive suppression in the Mahali mole-rat; as adrenocortical cell hypertrophy (Silva et al., 2004) and increased adrenal sensitivity to ACTH stimulation has been observed to result in increased glucocorticoid levels in individuals exhibiting increased levels of prolactin (Jaroenporn et al., 2007).

Conclusion

This study provides a comprehensive overview of the controlling endocrine mechanisms that possibly mediate both reproductive suppression and alloparental care in the cooperative breeding, social Mahali mole-rat. The study presents a novel understanding of the proximate factors involved in the maintenance of sociality in African mole-rats and possibly other social mammalian species. Furthermore, the results show possible interactions between prolactin, the HPA axis and the HPG axis and highlight the dearth of knowledge regarding these interactions. A better understanding of how natural reproductive suppression occurs may allow for greater strides in hormone therapies and contraceptives. Furthermore, the health benefits of being naturally suppressed have been recently highlighted in African mole-rats, with the causes of the benefits being linked to endocrine mechanisms controlling reproductive suppression (Jacobs et al., 2021a, 2021b). Further research on the understudied species, such as those in the genus *Cryptomys,* will be required to further our knowledge on how social groups are kept together in mammals as well as the potential benefits of reproductive suppression and social living.

Figures and Tables

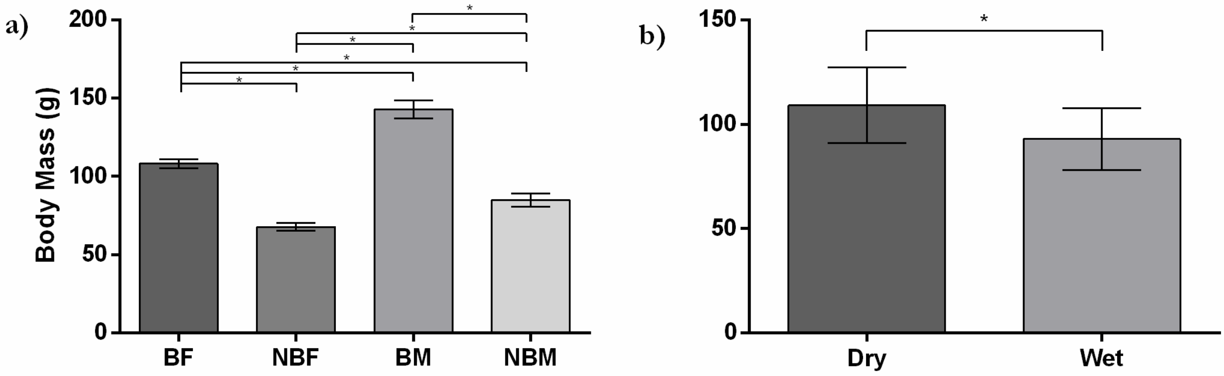


Figure 1. Bar graphs showing differences in body mass (g) of Mahali mole-rats (*C. h. mahali)* by

a) reproductive class (BF: Breeding female; BM: Breeding male; NBF: Non-breeding female; NBM: non-breeding male) and b) season (Wet: December – May; Dry: June – November). * indicates significant difference ($p < 0.05$).

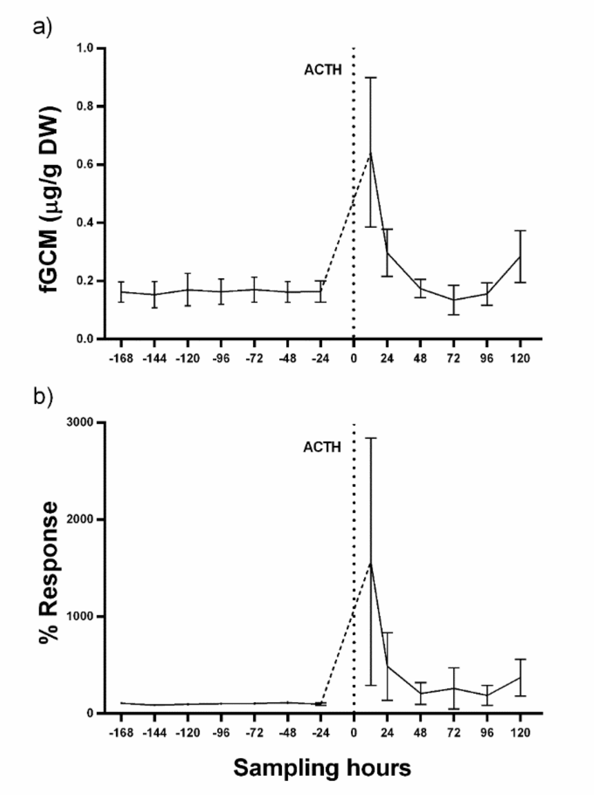


Figure 2. Concentrations (µg/g DW) of a) faecal glucocorticoid metabolites (fGCM) and b) % response to baseline values in Mahali mole-rats (*C. h. mahali*). fGCM and % response values (mean ± standard error) are shown for 168 hours prior to and 120 hours after a challenge with synthetic ACTH.

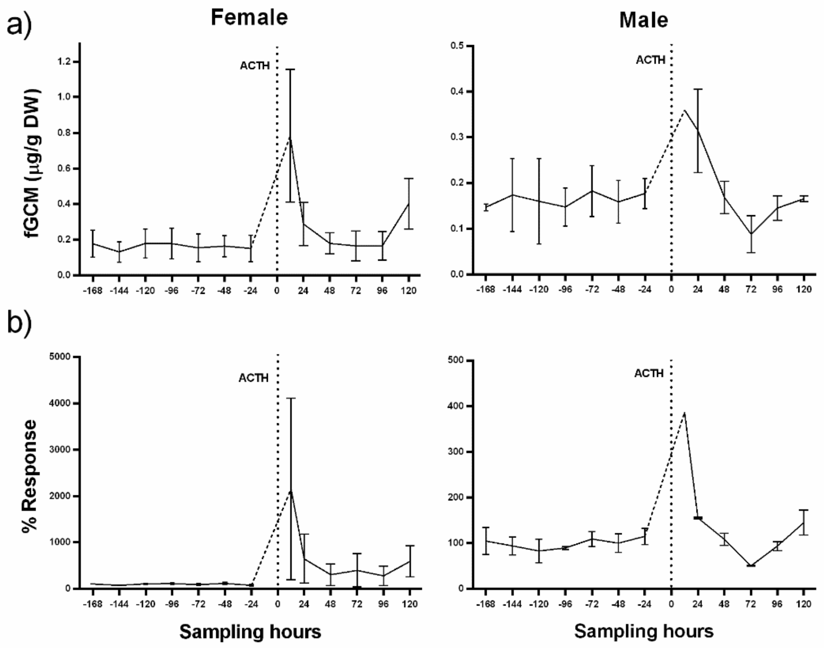


Figure 3. Concentrations (µg/g DW) of a) faecal glucocorticoid metabolites (fGCM) and b) % response to baseline values of male and female Mahali mole-rats (*C. h. mahali)*. fGCM and % response values (mean ± standard error) are shown for 168 hours prior to and 120 hours after a challenge with synthetic ACTH. Note: only one faecal sample was collected for the male cohort at the sampling hour 24.

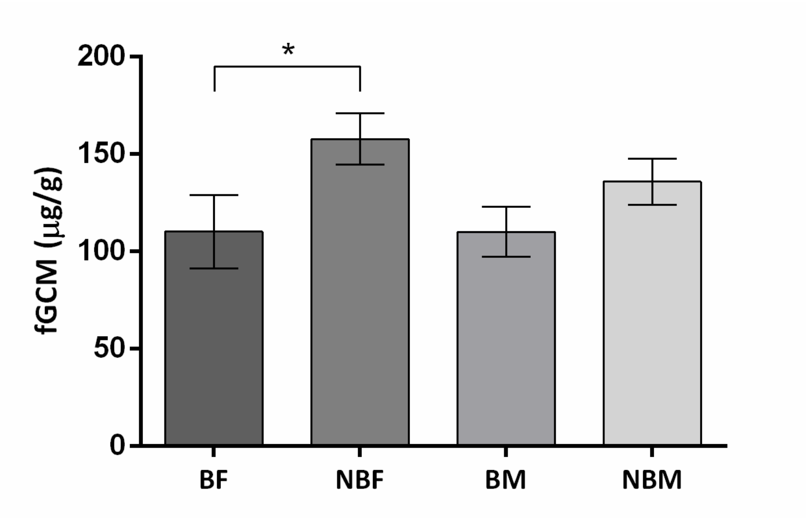
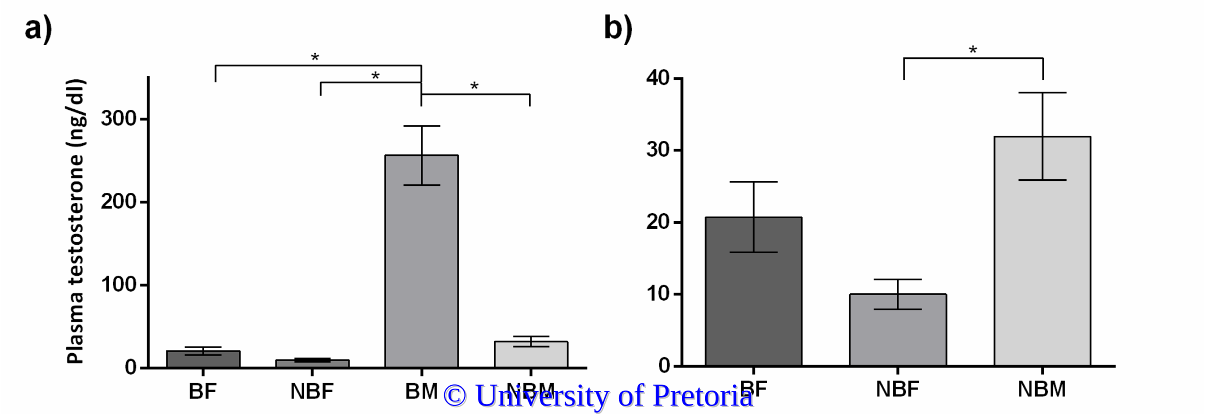


Figure 4. Bar graph showing differences in faecal glucocorticoid metabolite (fGCM) (µg/g DW) concentrations of Mahali mole-rats (*C. h. mahali)* by reproductive status (BF: Breeding female; BM: Breeding male; NBF: Non-breeding female; NBM: non-breeding male). Data shown as



mean ± SEM. * indicates significant difference ($p < 0.05$).

Figure 5. Bar graph showing differences in plasma testosterone concentrations (ng/dl) of Mahali mole-rats (*C. h. mahali)* by a) reproductive class (BF: Breeding female; BM: Breeding male; NBF: Non-breeding female; NBM: non-breeding male) and b) reproductive status, excluding BMs. * indicates significant difference ($p < 0.05$).

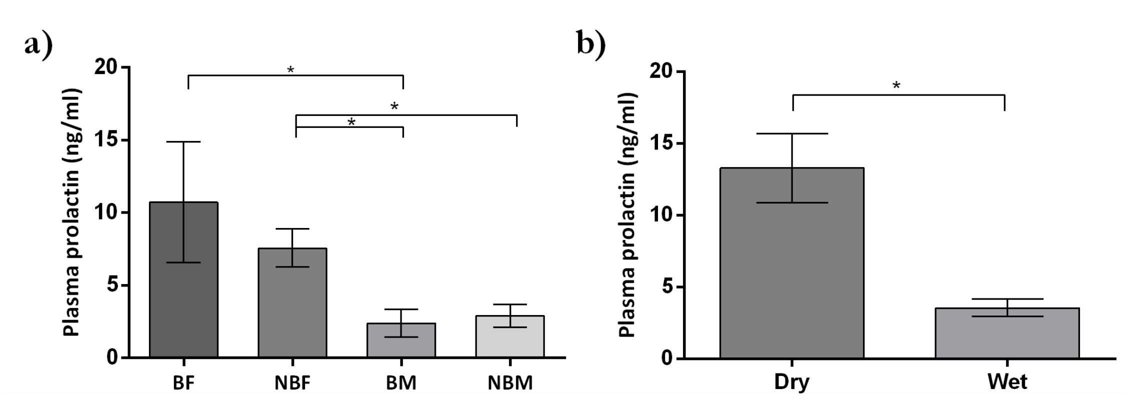


Figure 6. Bar graphs showing differences in plasma prolactin (ng/ml) concentrations of Mahali mole-rat (*C. h. mahali)* females by a) reproductive status (BF: Breeding female; NBF: Non-breeding female) and b) season (Wet: December – May; Dry: June – November). Data shown as mean ± SEM. * indicates significant difference ($p < 0.05$).

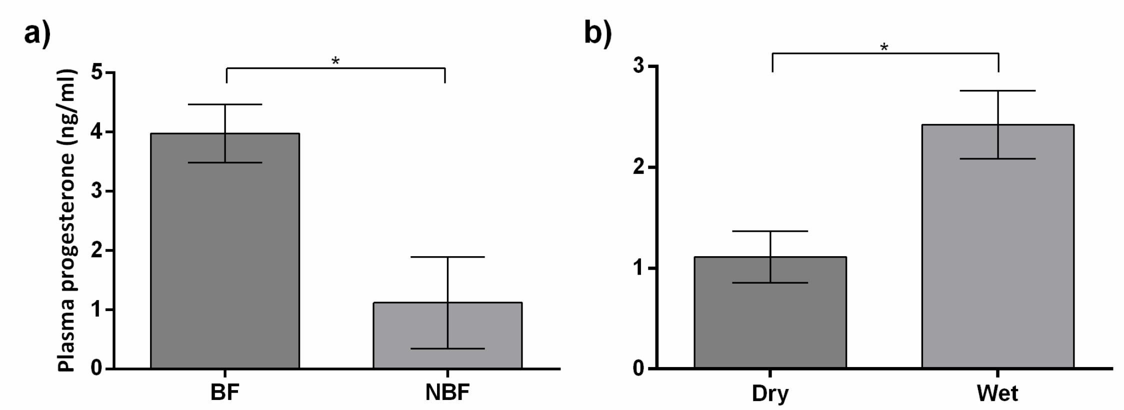


Figure 7. Bar graphs showing differences in plasma progesterone (ng/ml) concentrations of Mahali mole-rats (*C. h. mahali)* by a) reproductive status (BF: Breeding female; BM: Breeding male; NBF: Non-breeding female; NBM: non-breeding male) and b) season for female individuals. Data shown as mean ± SEM. * indicates significant difference ($p < 0.05$).

Supplementary material

Table S1. Comparison of general linear models tested to explain body mass (g) as a function of season and reproductive status in Mahali mole-rats (*Cryptomys hottentotus mahali)*. Best model fits are ranked by AICc, while model weights are estimates across all models and add up to 1.

Model	*df*	*AICc*	*Delta AICc*	*Model weight*	*logLik*
Reproductive status	5	74.6	0.00	0.598	-32.108
Reproductive status + Season	6	76.7	2.14	0.205	-32.107

Null	2	77.7	3.18	0.122	-36.836
Season	3	79.7	5.17	0.045	-36.796
Reproductive status * Season	9	80.5	5.97	0.030	-30.735

Table S2. Comparison of general linear models tested to explain variation in faecal glucocorticoid metabolite (fGCM, µg/g) concentrations as a function of season and reproductive status in Mahali mole-rats (*C. h. mahali)*. Best model fits are ranked by AICc, while model weights are estimates across all models and add up to 1.

Model	***Df***	***AICc***	***Delta AICc***	***Model weight***	***logLik***
Reproductive status + Season	6	2031.8	0.00	0.874	-1009.703
Reproductive status * Season	9	2035.7	3.93	0.123	-1008.428

Table S4. Comparison of general linear models tested to explain plasma progesterone concentrations (ng/ml) as a function of season and reproductive status in Mahali mole-rat (C. h. mahali) females. Best model fits are ranked by AICc, while model weights are estimates across all models and add up to 1.

Model	***df***	***AICc***	***Delta AICc***	***Model weight***	***logLik***

Table S3. Comparison of generalised linear models tested to explain plasma testosterone concentrations (ng/dl) as a function of season and reproductive status in Mahali mole-rats (*C. h. mahali)*. Best model fits are ranked by AICc, while model weights are estimates across all models and add up to 1.

Model	***Df***	***AICc***	***Delta AICc***	***Model weight***	***logLik***
Season	3	176.3	0.00	0.698	-84.935
Reproductive status + Season	4	178.5	2.27	0.225	-84.931
Reproductive status * Season	5	180.7	4.41	0.077	-84.828
Null	2	201.3	25.04	0.000	-98.556
Reproductive status	3	203.4	27.11	0.000	-98.491
Reproductive status	5	2043.0	11.17	0.003	-1016.346
Season	3	2152.3	120.46	0.000	-1073.079
Null	2	2159.8	127.99	0.000	-1077.870

Reproductive status+ Season	4	226.4	0.00	0.747	-108.982
Reproductive status * Season	5	228.6	2.16	0.253	-108.942
Reproductive status	3	256.7	30.26	0.000	-125.210
Season	3	265.4	38.95	0.000	-129.553
Null	2	271.1	44.68	0.000	-133.490

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Table S5. Comparison of general linear models tested to explain plasma prolactin concentrations (ng/ml) as a function of season and reproductive status in Mahali mole-rat (*C. h. mahali)* females. Best model fits are ranked by AICc, while model weights are estimates across all models and add up to 1.

Model	***df***	***AICc***	***Delta AICc***	***Model weight***	***logLik***
Reproductive status	5	1647.7	0.00	0.539	-818.719
Reproductive status+ Season	6	1648.7	0.95	0.335	-818.135
Reproductive status* Season	9	1650.6	2.92	0.125	-815.878
Season	3	1748.9	101.13	0.000	-871.371
Null	2	1755.9	108.15	0.000	-875.913

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Synthesis

In this thesis, I set out to improve our understanding of how cooperatively breeding species control subordinate reproduction using African mole-rats as a model family. It has been well established that the endocrine system plays a vital role in maintaining reproductive suppression of subordinate individuals in cooperatively breeding societies (see Soares et al. 2010 for review). However, often only one endocrine correlate (i.e. a steroid hormone or neuropeptide) is measured and related to cooperative behaviour or reproductive suppression. Even though this has been a successful endeavour that has resulted in drastic leaps in our understanding of how cooperatively breeding groups stay together, this approach still lacks the understanding of the observed complexity of the biological system, namely how the various endocrine correlates may interact with one another to produce cooperatively breeding behaviours and suppression of reproduction.

In this thesis, I attempted to address this problem by measuring three endocrine correlates, namely glucocorticosteriods (an indicator of stress), androgens (a physiological driver of aggressive behaviour) and prolactin in three closely related understudied *Cryptomys* subspecies which exhibit cooperatively breeding behaviour, a reproductive division of labour and reproductive suppression in subordinate group members (Janse Van Rensburg et al., 2002; Oosthuizen et al., 2008; Hart et al., 2021). Furthermore, I attempted to unravel how these three endocrine correlates may interact with one another and how they possibly drive the type (behavioural or physiological) of reproductive suppression observed in each sub-species.

The findings of this thesis revealed that the mode of reproductive suppression differs considerably between the three closely related species (See Chapters 1 and 2). Unsurprisingly, this was mirrored by previous work on pituitary sensitivity to GnRH differences between breeders and non-breeders within the *Cryptomys* sub-species (Van der Walt et al., 2001; Oosthuizen et al., 2008). This link between pituitary sensitivity to GnRH and the three endocrine correlates investigated in this thesis may point to contrasting mechanisms of suppression (both neuro-endocrinological and endocrinological) in these species similar to the hypothesised differences between the Damaraland mole-rat, *Fukomys damarensis* - FD and the naked mole-rat, *Heterocephalus glaber* – HG, which hinges on the implication of increased circulating prolactin levels (Bennett et al., 2018; Voigt and Bennett, 2018).

Prolactin concentrations in African mole-rats differ considerably between the species measured to date, which may hold true for other cooperative breeders (See Figure 1). However, during this research on measuring circulating prolactin concentrations, an exciting trend was discovered. It appears that there is a link between plasma prolactin and faecal glucocorticoid metabolites (fGCMs-stress), which may drive physiological suppression in some African mole-rats. Prolactin and fGCMs have been measured in five species (*Cryptomys hottentotus natalensis* -

CHN, *Cryptomys hottentotus mahali* - CHM, *Cryptomys hottentotus pretoriae* - CHP, FD and HG) (Bennett et al., 2018; Medger et al., 2018; Edwards et al., 2020; ; Chapter 1 and 2). Species with the lowest prolactin levels show the lowest levels of glucocorticoids and vice versa (Figures 1-4). Prolactin and glucocorticoid concentrations measured in the five species in increasing order are CHN, FD, CHM, CHP, and HG (Figures 1-4). Subordinate individuals of HG, with the highest prolactin (Bennett et al., 2018) and fGCMs (Edwards et al., 2020) levels, are well known to be under strict physiological suppression (Faulkes et al., 1990, 1991; Clarke and Faulkes, 1997, 1998; Zhou et al., 2013). CHN, on the other hand, shows very little evidence of physiological suppression (supported by no pituitary sensitivity to GnRH differences between breeders and non-breeders (Oosthuizen et al., 2008)) and has the lowest prolactin and fGCMs levels (Chapter 1).

Prolactin has been shown linked to adrenocortical cell hypertrophy (Silva et al., 2004), and increased adrenal sensitivity to adrenocorticotropic hormone stimulation has been observed to result in increased glucocorticoid levels in individuals exhibiting increased levels of prolactin (Jaroenporn et al., 2007). Furthermore, recent work on humans (*Homo sapiens*) and other rodents have shown that circulating prolactin and anxiety levels are correlated (Zhukov et al., 2021). This is an important finding, as glucocorticoids do not appear to be linked to aggressive interactions and thus androgen levels, as once hypothesised (see Introductory chapter for review). Thus it is hypothesized that in the HG, CHP and CHM, the increased circulating levels of prolactin, which alone can decrease reproductive capacity and increases cooperative care (Bennett et al., 2018), also increase the subordinates sensitivity to stress and thus increases their levels of glucocorticoids, which alone can also decrease reproductive capacity and increases cooperative care (Medger et al., 2018). This would suggest, through the interaction of prolactin and hypothalamic–pituitary–adrenal axis (with glucocorticoids being the final product), strict physiological suppression is being employed on HG male and female subordinates (Faulkes et al., 1990, 1991; Faulkes and Abbott, 2009; Bens et al., 2018) and subordinate female CHP and CHM (Janse Van Rensburg et al., 2002; Hart et al., 2021; Chapter 1 and 2).

However, it cannot be said that behavioural suppression is not evident in these species, in particular incest avoidance (Burda, 1995; Bennett et al., 1996, 1999; Clarke et al., 2001; Burland et al., 2004). Naked mole-rats can inbreed to a high degree, which is why it is thought that incest avoidance may not be as crucial in their reproductive skew maintenance compared to other mole-rat species (Reeve and Sherman, 1991), this is turn may be the reason why the prolactin-glucocorticoids interaction mechanism is so apparent (Figure 1, 4) (Bennett et al. 2018; Edwards et al. 2020). However, as the genus *Cryptomys* is somewhat understudied, the inbreeding level is unknown. Furthermore, even though the work presented in the thesis looked at three endocrine correlates, I cannot confidently say that the prolactin-glucocorticoid interaction mechanism is solely responsible for physiological suppression, namely because subordinate FD females show apparent evidence of physiological suppression (see Bennett (2011) for review) and they possess almost undetectable levels of prolactin and low fGCMs levels (Bennett et al., 2018; Medger et al. 2018).

As this thesis was purely to observe and measure the proximate factors of cooperative breeding and reproductive suppression in three sub-species of African mole-rat species, I did not explore the ultimate factors that might have selected for these vastly different mechanisms of suppression in the family of African mole-rats. However, I would hypothesize that the phylogenetic relationship between the different species does not play a role in how a species maintains its cooperative behaviour, reproductive skew and ultimately reproductive suppression (See Figure 1 in the General Introduction). I believe that even after three decades of research, we are only scratching at the surface of the possible mechanisms behind the maintenance of sociality, cooperative breeding, and reproductive suppression in African mole-rats and other mammals. As we explore the interactions between neuropeptides, such as prolactin, and other endocrine correlates, such as glucocorticoids, in other cooperative breeding mammals, we may then only begin to unravel some of these answers. Further research should also focus on neuroendocrine pathways in the mole-rat and a thorough investigation of the neuroanatomy and neuroendocrinology of the brains of cooperatively breeding species to determine what mechanisms are responsible for their physiological reproductive suppression and cooperative behaviours (Voigt et al. 2014; Voigt et al. 2016; Voigt & Bennett, 2017; Voigt & Bennett, 2018a, b; Voigt & Bennett, 2021).

Figures

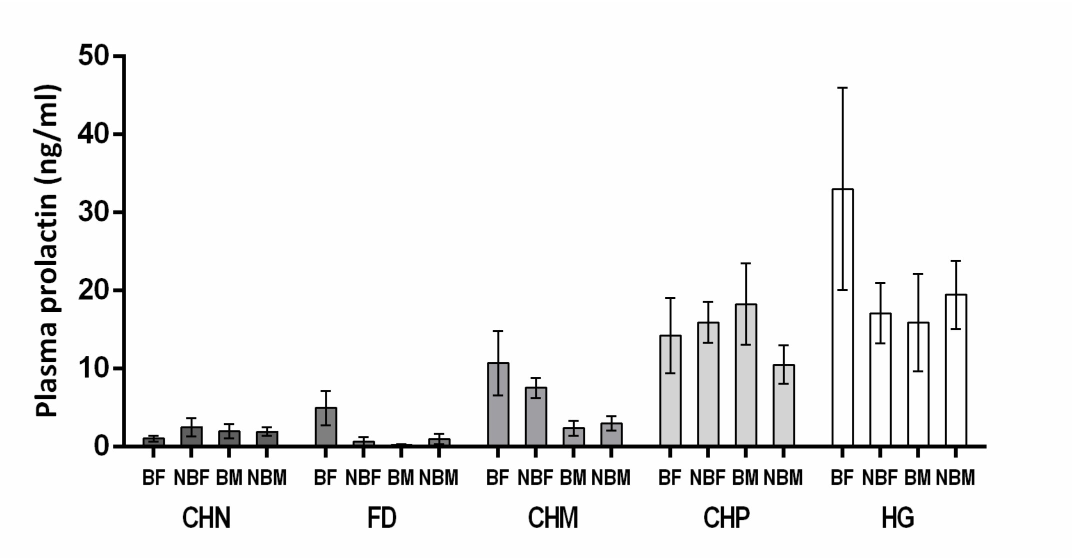

Figure 1. Bar graphs showing the differences in plasma prolactin concentrations between breeding and non-breeding male and female individuals of five African mole-rat species (Cryptomys hottentotus natalensis - CHN, *C. h. mahali* - CHM, *C. h. pretoriae* - CHP, *Fukomys damarensis* - FD and *Heterocephalus glaber* - HG) (Bennett et al. 2018; Chapter 1 and 2).

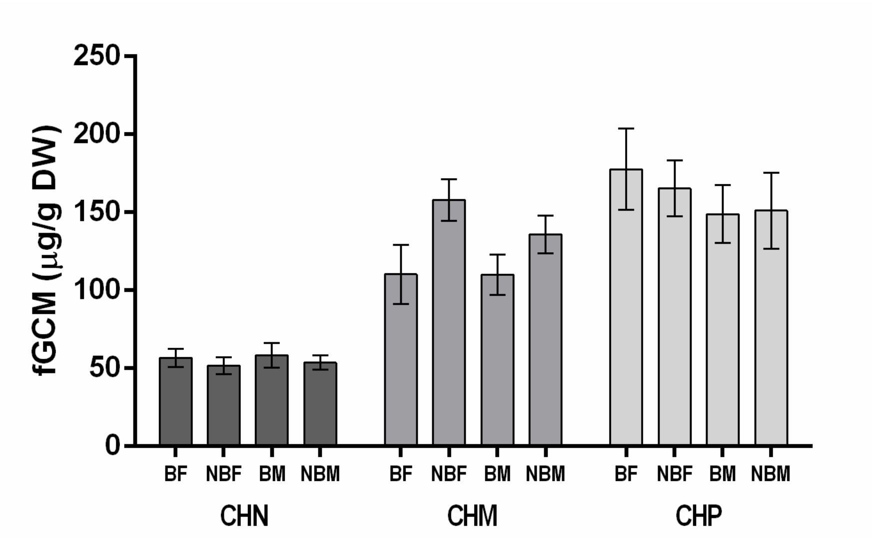

Figure 2. Bar graphs showing differences in faecal glucocorticoid metabolite (fGCM dry weight) concentrations between breeding and non-breeding male and female individuals of three *Cryptomys hottentotus* subspecies (*C. h. natalensis* - CHN, *C. h. mahali* – CHM and *C. h. pretoriae* - CHP) (Chapter 1 and 2).

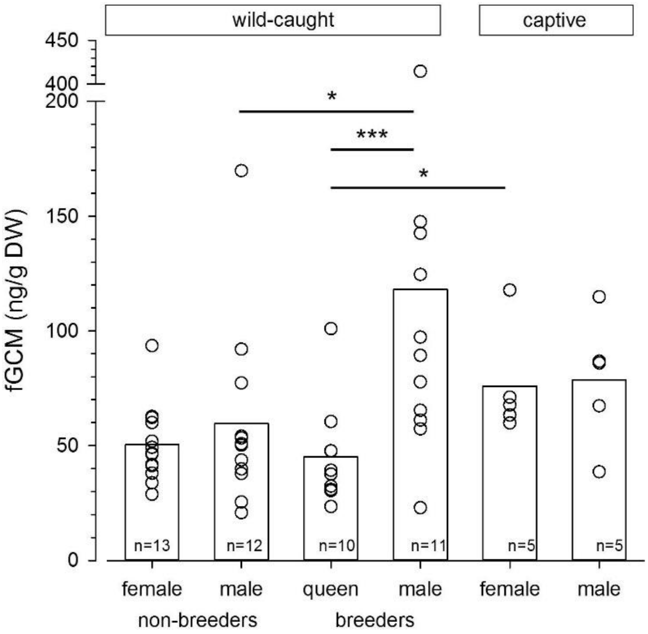

Figure 3. Bar graphs showing differences in faecal glucocorticoid metabolite (fGCM dry weight) concentrations of breeding and non-breeding male and female *Fukomys damarensis* individuals (Medger et al. 2018)

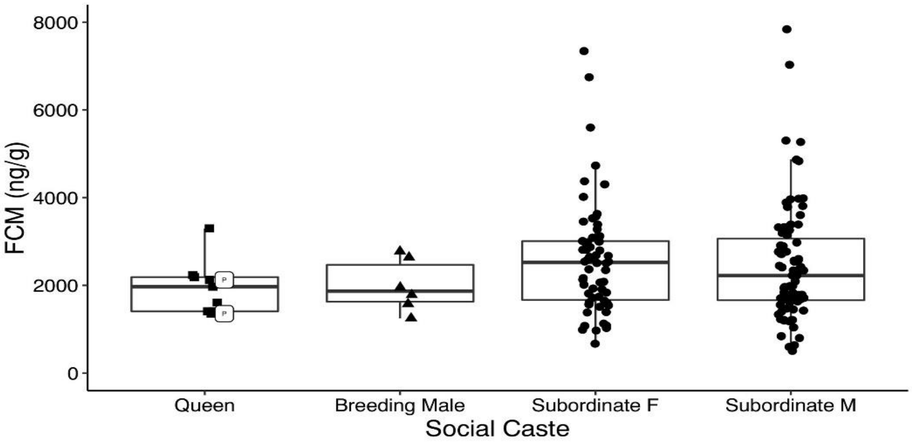

Figure 4. Box plots showing differences in faecal glucocorticoid metabolite (fGCM dry weight) concentrations of breeding and non-breeding male and female *Heterocephalus glaber* individuals (Edwards et al. 2020).

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