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71 Long live the queen: Dominant females possess increased reproductive capacity and 72 immune competence in social African mole-rat species

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Declaration

I, Kyra Mary Elizabeth Wallace, declare that this thesis, 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 by me for a degree at this university or any other tertiary institution.



31 / 08 / 2022

Kyra Mary Elizabeth Wallace

Date

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Disclaimer

96 Chapter 1, 2, 3 and 4 of this thesis have been written and formatted as separate research articles
97 for submission to General and Comparative Endocrinology, Biology Letters, Journal of
98 Zoology (London) and Philosophical Transactions of the Royal Society, London respectively.
99 Overlap of content may therefore occur throughout this thesis. However, the same referencing
100 style (Journal of Zoology) has been used throughout to maintain cohesion in the thesis.

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112 “For I know the plans I have for you,” declares the Lord, “plans to prosper you and not to
113 harm you, plans to give you a hope and a future. Then you will call on me and pray to me,
114 and I will listen. You will seek me and find me when you seek me with all your heart.”

115 **Jeremiah 29:11-13**

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Acknowledgements

Firstly, I would like to thank my outstanding supervisors for their mentorship, support, encouragement and troubleshooting sessions throughout the course of this Masters. I have grown so much, not just a scientist, but as a person, under your guidance. To Professor Nigel Bennett and Dr Daniel Hart, thank you for your seemingly never-ending patience with me and your excitement throughout this study, especially when things were not going the way we planned. Thank you for treating me like a colleague more often than not, rather than a student, thus building me up and giving me the confidence to produce this work as it stands. To Professor Stephanus Venter, thank you so kindly for taking time out of your busy schedule and being willing to take on a Zoology student to patiently teach her all the microbial skills and knowledge needed to practically complete large portions of this work. Additionally, a big thank you to Philip Greef for always being willing to help and patiently explain microbial laboratory protocol and terminology, I am deeply appreciative for your guidance and assistance.

Secondly, I would like to express my gratitude to Dr Nicole Hagenah-Schrader at the Endocrine Laboratory at the University of Pretoria for assisting with endocrine analyses. Similarly, a big thank you to Carien Muller and her team at the Clinical Pathology unit at the Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria for assisting me with the haematological analyses. Further, this Masters would not have been possible without the funding provided by the DST-NRF SARCHI Chair of Mammal Behavioral Ecology and Physiology and the NRF, in terms of bursary support.

To my family and friends, thank you for your continued love and support through all of the ups and downs. Thank you for reminding me to look up and smell the roses when everything got extremely crazy. I appreciate every single moment shared. Mom, Dad, Adrienne – thank you for everything you have sacrificed to get me here, I hope this does you proud. Auntie Juliet and Uncle John, thank you for the home you provided and always being willing to listen. To my soon-to-be in-laws, I cannot wait to be a part of your family and thank you so much for every bit of excitement, calm, love, and motivation you have given me on this journey.

To my fiancé, Keegan, goodness you have kept me sane. Your quiet, steady strength, motivation and inspiration through all instances has made you my absolute rock. Thank you for making me laugh and distracting me when you could see I needed to take a breather. I cannot wait to do life with you.

159 Most importantly, all praise to Jesus for His plans and provision as they are what got
160 me to where I am today. Thank you, Lord.

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Summary

The Damaraland mole-rat is a eusocial, subterranean African bathyergid that exhibits both an extreme reproductive skew and a reproductive division of labour, whereby the breeding female has the highest survivorship. Reproduction is monopolised by a single breeding female (BF), the queen, and a few of the most dominant, non-related males (breeding males; BM's). The remainder of the colony are reproductively quiescent through physiological suppression and inbreeding avoidance. As an induced ovulator, the absence of copulation as an effect of inbreeding avoidance in non-breeding females (NBF's) ensures that the reproductive system is physiologically downregulated, in terms of anovulation and a pituitary with reduced sensitivity. Comparatively, non-breeding males (NBMs) are only behaviourally prevented from breeding due to their obligate outbreeding status. However, recent studies have found neuroendocrine divergences between the breeding and non-breeding males that may affect the presence or absence of mating behaviours. As such, the degree of reproductive activity and readiness in each of the reproductive classes (BF, BM, NBF and NBM) has engendered unique endocrine and metabolic profiles.

Unfortunately, previous studies that have elucidated these endocrine profiles in Damaraland mole-rats have used different sampling periods whereby season, diet and colony dynamics were not standardised. This study therefore set out to rectify any possible bias by using a synchronous single sampling event, obtained at a single point in time to clarify the testosterone, cortisol, progesterone, and prolactin concentrations in all Damaraland mole-rat reproductive classes. It was found that breeding individuals have higher progesterone and testosterone concentrations than their non-breeding counterparts, in addition to higher testosterone: cortisol ratios, indicating their hierarchical dominance. This is a novel finding specifically in male Damaraland mole-rats as this is the first set of work that has identified a significant difference in the testosterone concentrations between breeding and non-breeding males. Furthermore, this is the first study that has characterised the progesterone concentrations in male Damaraland mole-rats and has thus found that breeding males have similar progesterone concentrations to those recorded in non-breeding females, despite the anatomical differences.

The marked reproductive skew in Damaraland mole-rats has resulted in a distinct division of labour between the reproductive classes. Due to their extensive cooperative care duties in the form of pup rearing, foraging, burrow maintenance and colony defence, non-

breeding members of the colony perform 95% of the annual labour in Damaraland mole-rat colonies. Previous studies have calculated this activity budget through behavioural, metabolic, and thermoregulatory experiments, whereby the daily energetic expenditure of breeders is 20-30% less than that of the non-breeding individuals. In this study, we thus set out to further clarify the physiological mechanisms underlying the reproductive division of labour experienced in Damaraland mole-rats through determining the erythropoietic indices and thus oxygen-carrying capacity of the blood of each breeding class and sex. Erythropoiesis is the production of mature red blood cells and is characterised by high haemoglobin, haematocrit and red cell count volumes. High endurance levels and performance of activities is both enabled by, and in turn stimulates the synthesis of erythrocytes. Correspondingly, adrenal and sex steroid hormones are capable of alternately enhancing and/or suppressing red blood cell production. Therefore, through the use of complete blood counts and erythropoietic indices, this study demonstrated that non-breeding Damaraland mole-rats that are intrinsically more active, have higher erythrocytic oxygen-carrying capacities. All individuals used in this study were captive-bred and thus had never performed digging activities; as such, the physiological differences observed have likely resulted from inherent biological differences. Additionally, there was a significant relationship between erythropoietic indices and progesterone, whereby the higher progesterone concentrations expressed in breeding Damaraland mole-rats was reflected by lower haemoglobin, haematocrit, and red cell count volumes.

The innate immune response is the first line of defence against pathogenic invasions and relies on both physical and chemical barriers, in addition to leukocytic constituents such as neutrophils, monocytes, lymphocytes, basophils, and natural killer cells that phagocytise and destroy foreign bodies. As the endocrine and immune axes are closely linked, leukocyte synthesis, maturation and differentiation can be influenced by adrenal- and reproductively relevant hormones. Previous studies have indicated immune-suppressing roles of testosterone and progesterone on immunity, while prolactin and oestrogen have stimulatory effects on the immune response. Additively, biomedical research on the closely related, only other eusocial mammal, the Naked mole-rat (*Hetercephalus glaber*), has indicated specific resistance to tumorigenesis and infection as an enabling factor contributing to the elongated lifespan of naked mole-rat queens. This, in addition to the clear physiological and behavioural distinctions between reproductive classes, making Damaraland mole-rats an optimal model to further clarify the inter-related role of reproduction and its resultant hormones on the innate immunity of an immuno-divergent species.

505 This study therefore set out to characterise the innate immunity of Damaraland mole-
506 rats and whether their reproductive state, and thus their respective endocrine profiles influence
507 their individual immunity and resultant survival. Complete blood cell counts were used to
508 quantify the white blood cell components of each Damaraland mole-rat reproductive class,
509 while microbial killing assays were used to determine the bacteriostatic and/or microbiocidal
510 action of their blood. Despite the heavy role of testosterone in common-place immune
511 responses, there were no immunological differences between the breeding and non-breeding
512 Damaraland mole-rat males. There was, however, a negative correlation between the platelet:
513 white cell count ratio (PWR) and both the testosterone concentration in males and their T/C
514 ratio. Comparatively, despite their extremely low concentrations of prolactin, there was a
515 significant correlation between prolactin levels in male Damaraland mole-rats and their
516 immunologically related leukocytic parameters. These findings are in opposition to the central
517 tenets of both the life history theory and the immunocompetence handicap hypothesis (ICHH).

518 Contrastingly, while Damaraland mole-rat queens have been shown to have extended
519 longevity despite the energetic cost of reproduction, very little is known about the
520 immunological mechanisms that enable this extended survival. Through the same
521 immunological measurements as the males above, Damaraland mole-rat breeding females had
522 stronger immune responses than non-breeding females in terms of the haematological
523 antimicrobial capacity and leukocytic ratios. Similarly, Damaraland mole-rat queens had lower
524 body mass indexes (BMI's) and Lee indexes, indicating they were in a healthier state and had
525 higher survivorship ability than the subordinate non-breeding counterparts. Interestingly,
526 testosterone, progesterone, BMI and the Lee index were each significantly correlated to
527 microbial killing ability of female Damaraland mole-rat blood. This therefore indicates that the
528 individuals with the highest testosterone and progesterone concentrations, the queens, were
529 both the healthiest in terms of BMI and Lee's index, but also had the highest haematological
530 antimicrobial capacity

531 This study has therefore further elucidated the physiological divergences and
532 underlying mechanisms between reproductive and labour classes in Damaraland mole-rats with
533 regards to their endocrine, haematological, and immunological profiles.

General Introduction

Reproduction and Survival

The persistence of an organism requires the optimal functioning and homeostatic balance between the internal and external environments (Perlman, 2000). This mutually synergistic-antagonistic relationship requires complex interactions between multiple interfacing systems to efficiently maintain, react, respond, and adapt to altered physiological and environmental states (Johnson *et al.*, 1997; Romo, Pérez-Martínez & Ferrer, 2016; Oyola & Handa, 2017; Brüggemann *et al.*, 2018). The fitness of an individual can thus be delineated to their direct survival and the survival of their genes in future generations, thus their reproductive success (Graham *et al.*, 2011). For survival to occur, the individual must have sufficient immunocompetence to endure pathogenic infection, disease, and injury. Whereby “immunocompetence” refers to an individual’s ability to launch an effective immune response following pathogenic challenge and is therefore a crucial component of disease resistance and survival (French, Moore & Demas, 2009; Graham *et al.*, 2011). However, an individual’s immune state is constantly fluctuating in response to their environmental and physiological condition (Goldsby, Kindt & Osborne, 2000; Zuk & Stoehr, 2002; Lee, 2006; Rubenstein *et al.*, 2008; Gameiro, Romao & Castelo-Branco, 2010; Foo *et al.*, 2017; Schoenle, Downs & Martin, 2018). As a consequence, the reproductive success of an individual is shaped by the sociality and mating strategies operational in the species (Johnson *et al.*, 1997; Klein & Nelson, 1999; Cichon, Dubiec & Chadzinska, 2001; McKean & Nunnery, 2005).

Reproduction is energetically expensive, and as such, along the evolutionary pathway many mammalian species have adapted various behavioural and physiological reproductive strategies that best enhance their chance of reproductive success (Maynard Smith & Price, 1973; Moshkin, Gerlinskaya & Evsikov, 2000; Davies, Krebs & West, 2012). For example, some species such as the Cape mole-rat (*Georychus capensis*) only reproduce when resources are most abundant and are thus seasonal breeders, whereas other species such as the Mahali mole-rat (*Cryptomys hottentotus mahali*) that reproduce year-round and mate opportunistically are aseasonal breeders (Bennett & Jarvis, 1988; Hart *et al.*, 2020b). However, the year-round anatomical and physiological maintenance of the reproductive system is energetically expensive and for species living in more arid environments where resources are sparse, the energy they preserve by being seasonal breeders may determine the outcome of their survival (Spinks, Horst & Bennett, 1997; Klein & Nelson, 1999). This follows the immunity-fertility

tenet set out by the life history theory (LHT) that implores energetic trade-offs between energetically expensive processes such as immunity, reproduction, growth, and thermoregulation (Lochmiller & Deerenberg, 2000; Zuk & Stoehr, 2002; Graham *et al.*, 2011; Naim *et al.*, 2020). Hormones play a large role in this as testosterone significantly influences resource allocation away from immunological processes and rather augments anabolic growth of active muscle through enhanced protein synthesis and glucose uptake (Muehlenbein & Bribiescas, 2005). Similarly, oestrogen is an important biochemical molecule as it aids fat metabolism, glycogen storage and glucose uptake in skeletal muscles, while progesterone has anti-oestrogenic effects and thus supports protein catabolism (McNulty *et al.*, 2020).

Mammalian haematology

As the medium responsible for the distribution of metabolic-, respirometric-, immune- and endocrine- molecules, blood is crucial for maintaining the homeostatic balance between intrinsic and extrinsic environments (Price, 2008). The function of blood can be separated by its majority constituents; white blood cells (lymphocytes) which support immunity, red blood cells (erythrocytes) which enable oxygen transport, and platelets (thrombocytes) that aid in the clotting of blood.

Red blood cells (RBCs) make up 99% of whole blood and are responsible for the distribution of respiratory gases such as oxygen and carbon dioxide, and the circulation of metabolic resources and by-products such as nutrients and lactic acid, to and from active tissues (Powers *et al.*, 2014; Gjevestad, Holven & Ulven, 2015; Koç *et al.*, 2018). Increased levels of RBCs and haemoglobin (Hgb) enable greater oxygen-carrying capacity of blood, improved tissue oxygenation, elevated haemostasis and, ultimately, improved endurance and ability to be active (Roberson & Bennett-Guerrero, 2012). The regulation of oxygen transport by red blood cells is therefore characterised by two main processes: erythropoiesis and Hgb-O₂ binding affinity (Mairbaur, 2013). Erythropoiesis concerns the production of mature red blood cells and is often characterised by high Hgb, RBC and haematocrit (Hct) volumes, while Hgb-O₂ binding affinity is characterised by the effects of CO₂, temperature, pH and 2,3-DPG on the chemical structure of red blood cells (Dzierzak & Philipsen, 2013; Mairbaur, 2013; Moretti *et al.*, 2018). Furthermore, erythropoiesis can be affected by external stimuli such as exercise, altitude, and hypoxia, while internal hormones and growth factors such as erythropoietin (EPO), glucocorticoids, and sex steroids influence erythrocyte synthesis (Mirand, E.A. and

Gordon, 1966; Gordon *et al.*, 1968; Piliero, Medici & Haber, 1968; Casazza *et al.*, 2002; Ingley, Tilbrook & Klinken, 2004; Hu & Lin, 2012; Mairbaur, 2013). Due to the biochemical crosstalk between endocrine and erythropoietic pathways, there have been numerous studies delineating the role of adrenal- and sex steroid hormones on erythroblast differentiation and maturation, in addition to Hgb production. While cooperative regulation has been suggested in a few studies, the generally accepted trend is that androgens (testosterone), glucocorticoids (cortisol) and prolactin have additive effects on RBC production, while progesterone and oestradiol have suppressive functions on erythrocytic elaboration (Jepson & Lowenstein, 1964; Mirand, E.A. and Gordon, 1966; Piliero *et al.*, 1968; Lavrijsen & Verwilghen, 1984; Kolbus *et al.*, 2003; Ingley *et al.*, 2004; Leberhauer *et al.*, 2005; Price, 2008).

The endocrine system is capable of influencing the production, maturation, and differentiation of leukocytes, and thus the quantity and type of immune cells present at any stage of time (Piliero *et al.*, 1968; Johnson *et al.*, 1997; Brüggemann *et al.*, 2018). Leukocytes are differentiated into monocytes, lymphocytes, and granulocytes; neutrophils, eosinophils, and basophils, whereby they perform their immune responses through phagocytic-, lytic-, or cytokine signalling functions (Romo *et al.*, 2016). The expression of nuclear oestrogen receptors has been identified in lymphocytes, macrophages, monocytes, and natural killer cells, while membrane bound progesterone receptors have only been identified in monocytes (Vrachnis *et al.*, 2014). Further, lymphocytes have also expressed prolactin receptors, therefore allowing lymphocytes to be directly modulated by both oestrogen and prolactin (Gameiro *et al.*, 2010). As a consequence, cortisol, progesterone and testosterone are each capable of suppressing leukocyte maturation, differentiation and proliferation, compared to the stimulatory role of prolactin and oestrogen on immune cells (Muehlenbein & Bribiescas, 2005; Gameiro *et al.*, 2010). These modulatory effects can thus be both synergistic and antagonistic in nature as well as bidirectional whereby immune cells are concomitantly able to bind to steroid hormones and alter their production and action (Daynes *et al.*, 1995; Gameiro *et al.*, 2010).

Mammalian Immunity

The interrelationship between endocrine and immune systems can first be evidenced at ontogeny in many mammalian species, soon followed by the elaboration of afferent-efferent

pathways between immune and neuroendocrine structures (Besedovsky & Sorkin, 1977). The immune system is classified by its innate and adaptive/acquired components. As the first line of defence against infection, the innate immune system is non-specific and composed of physical and chemical barriers, i.e. skin and mucous layers, followed by physiological, phagocytic and inflammatory cell-mediated and humoral components such as cytokines and leukocytes (Romo *et al.*, 2016; Naim *et al.*, 2020). Leukocytic constituents such as monocytes, macrophages, dendritic cells, natural killer (NK) cells and granulocytes provide antigen-presenting, phagocytic and cell-lytic impediment to pathogenic invasions (Pulendran *et al.*, 2001; Zuk and Stoehr, 2002; Gamiero *et al.*, 2010). As antigen-presenting cells (APC's), one of the functions of dendritic cells, monocytes and macrophages is to consume, process and express foreign antigen peptides on their surface membrane and aid the specificity of the stimulated adaptive/acquired immune response (Denkers *et al.*, 2012; Grayfer, Hodgkinson & Belosevic, 2014). As part of the innate immune system, the complement cascade and production of cytokines support and magnify specific- and non-specific immune responses by promoting inflammation and antibody action, thus activating the adaptive/acquired response system (Sheldon & Verhulst, 1996; Lochmiller & Deerenberg, 2000; Pulendran, Palucka & Banchereau, 2001b; Zuk & Stoehr, 2002; Klein, 2004; Romo *et al.*, 2016). As such, the innate immune response is vital in maintaining individual homeostasis and immunocompetence.

The bidirectional neuroendocrine-immune network is interrelated through a common chemical language of shared ligands and receptors (Gaillard, 2001). Equally, immune-neuroendocrine products can be found in both systems whereby endocrine substrates are able to modulate immune responses and immune structures are able to influence endocrine production through gene expression (Gameiro *et al.*, 2010). As such, a wide variety of studies have indicated the immune-suppressive roles of testosterone, cortisol, and progesterone, whereas oestrogen and prolactin have stimulatory effects on the immune response (Daynes *et al.*, 1995; Muehlenbein & Bribiescas, 2005; Gameiro *et al.*, 2010). Due to this, females often have greater immune strength than males (Nunn *et al.*, 2009; Kelly *et al.*, 2018). Immuno-senescence occurs as an individual increases in age, there is a gradual decline in immunological activity in terms of cellular and serological immune responses (Vrachnis *et al.*, 2014; Naim *et al.*, 2020). This is most obvious in post-menopausal women as the reduction in oestrogen results in the removal of the immuno-enhancing effects, therefore producing an immune system that is equal, if not less to that of a male's immunity (Vrachnis *et al.*, 2014).

Study species: Damaraland mole-rats (*Fukomys damarensis*)

The eusocial Damaraland mole-rat (*Fukomys damarensis*; DMR) is a hypoxia-tolerant, subterranean bathyergid, that lives in colonies ranging from two to 41 members (Bennett, 1990; Jacobs *et al.*, 1991; Faulkes *et al.*, 1997; Bennett & Faulkes, 2000a). They live in an elaborate burrow system with extensive foraging tunnels 10-100cm below the surface and nest chambers approximately 2.5m deep (Bennett & Jarvis, 2004; Bennett, 2009). Damaraland mole-rats have become physiologically, morphologically and behaviourally specialised to their thermally buffered (20°C-30°C, >95% humidity), hypoxic (19.9- 20.4% O₂) and hypercapnic (0.4-6.0% CO₂) microclimates (Bennett, 1990, 2009; Bennett & Jarvis, 2004). Hypoxia plays an important role in the innate immune system as hypoxia-inducible factors (HIF's) which mediate hypoxia tolerance also function as regulators moderating immunological responses such as antigen presentation, cytokine production, macrophage phagocytosis and cellular metabolic reprogramming (Krzywinska & Stockmann, 2018).

As a product of being on the extreme end of sociality, Damaraland mole-rats exhibit a severe reproductive skew and division of labour. This is the result of the despotic monopolisation of reproduction by a single breeding female, the queen, and few of the most dominant males, her consorts (Jacobs *et al.*, 1991; Jarvis & Bennett, 1993; Cooney & Bennett, 2000). The remainder of the colonial cohort are reproductively downregulated through physiological and behavioural mechanisms (Bennett, Faulkes & Molteno, 1996; Clarke, Miethe & Bennett, 2001; Bennett, 2009). Damaraland mole-rats are obligate outbreeders and as such the high relatedness and familiarity of colony members translates into inbreeding avoidance being a strong factor dissuading the reproduction of subordinate individuals (Burland *et al.*, 2002; Kelley *et al.*, 2019). Additionally, Damaraland mole-rats are induced ovulators whereby the prevention of copulation and reproductive behaviour in non-breeding females ensures their physiological suppression and anovulatory status (Cooney & Bennett, 2000; Molteno & Bennett, 2000; Clarke *et al.*, 2001a; Voigt, Medger & Bennett, 2021). While recent studies have distinguished neuroendocrine receptor divergences between the male Damaraland mole-rats (Voigt, Leitner & Bennett, 2016; Voigt & Bennett, 2021), there is very little physiological or anatomical evidence, beyond reproductive activity and dominance, that distinguishes between breeding- and non-breeding males (Jacobs *et al.*, 1991; Jarvis & Bennett, 1993). Therefore, while reproductively quiescent males are anatomically and physiologically similar to breeding males, they fail to reproduce and do not display reproductive behaviour. Non-breeding male Damaraland mole-rats have similar circulating testosterone and cortisol

concentrations, as well as similar sized testes and spermatogenesis to breeding males (Jarvis & Bennett, 1993; Voigt *et al.*, 2016). Alternatively, non-breeding female Damaraland mole-rats are physiologically and anatomically distinct from their reproductive counterparts. The breeding female is reproductively active, enabled by complete ovulatory cyclicity, high progesterone and oestrogen concentrations, while the ovulation of non-breeding females is blocked at the follicle maturation stage, producing low progesterone and oestrogen profiles (Jarvis & Bennett, 1993; Bennett *et al.*, 1996; Molteno & Bennett, 2000; Clarke, Miethe & Bennett, 2001b; Voigt *et al.*, 2014, 2021).

Evidence from metabolic experiments indicate a clear energetic distinction between breeders and non-breeders, who are further delineated into frequent and infrequent workers (Scantlebury *et al.*, 2006b). As the most subordinate in the colony, and often the smallest in body size, young non-breeding females are the most frequent workers with regards to foraging, burrow maintenance and alloparental care (Bennett, 1990; Scantlebury *et al.*, 2006b). Infrequent workers, largely the males preparing for dispersion, have lower energetic output and higher fat accumulation (Bennett, 1990; Scantlebury *et al.*, 2006b). Breeders have the least energy expenditure and activity levels.

Correspondingly, breeders, especially the breeding females, have lifespans and reproductive success rates that far surpass the non-reproductive individuals (Schmidt, Jarvis & Bennett, 2015). Schmidt *et al.* (2015) found breeding females to live on average five times longer than her non-breeding counterparts. This contradicts a central tenet of the life history theory (LHT) which dictates that individuals which exhibit early and frequent reproduction, as with Damaraland mole-rat queens, should have shorter lives due to compromised resource partitioning and immune function (Chown & Gaston, 1997; Lutermann & Bennett, 2008). However, several social and physiological factors, such as an abundance of food due to cooperative foraging, involvement of HIF's and reduced oxidative damage, have been suggested to mitigate this deterioration in Damaraland mole-rat queens (Schmidt *et al.*, 2015; Schmidt, Arbi & Bennett, 2020).

Aim of thesis

The aim of this thesis was to address the dearth of knowledge in evolutionary-orientated haematological and immunological components regarding the reproductively distinct Damaraland mole-rat. An additional aim was to look at a multitude of endocrine parameters at

a single sampling stage in a large number of mole-rats of known age and reproductive status. While many studies have focussed on clarifying the role of individual hormones on the reproductive profile of Damaraland mole-rats, they have done so separately, using different samples over a course of time. This study therefore set out to comprehensively characterise the hormone profiles of Damaraland mole-rats from a single sample, obtained at a single point in time, therefore accounting for seasonal, dietary, and social variations within the colony. Additionally, this study set out to clarify the haematological profile of Damaraland mole-rat by conducting complete blood counts. Red blood cell quantifications were used to explore the activity divergences produced by the reproductive division of labour in Damaraland mole-rats. White blood cell measurements were used in conjunction with microbial killing assays to characterise the innate immunity of Damaraland mole-rats.

Research objectives

Chapter 1

1. Provide a comprehensive profile of reproductively relevant hormones from a single sample, at a single point in time for Damaraland mole-rats
2. Determine the previously unknown role of progesterone in male Damaraland mole-rats
3. Introduce the testosterone: cortisol ratio (T/C) as a dominance determinant in Damaraland mole-rats

Chapter 2

1. Determine if the reproductive division of labour in Damaraland mole-rats is mirrored in the haematological oxygen-carrying capacity of each reproductive class
2. Determine if reproductively relevant hormones play a role in haematological divergences of activity in Damaraland mole-rats

Chapter 3

1. Characterise the innate immunity of male Damaraland mole-rats
2. Determine if reproductive status (actively breeding vs non-breeding) influences the innate immunity of male Damaraland mole-rats
3. Determine if there is a correlation between reproductive hormones and innate immunity in male Damaraland mole-rats

4. Determine if Damaraland mole-rat males follow the posits set out by the immunocompetence handicap hypothesis (ICHH) or the stress-linked ICHH

Chapter 4

1. Characterise the innate immunity of female Damaraland mole-rats as a function of survivorship in longevity
2. Determine if physiological suppression and therefore reproductive state (actively breeding vs non-breeding) affects female Damaraland mole-rat innate immunity
3. Determine if there is a correlation between the different endocrine profiles of female Damaraland mole-rats and their innate immunity
4. Determine if there is evolutionary inequality between female Damaraland mole-rats regarding their reproductive and lifespan divergences

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Chapter 1

A comprehensive profile of reproductive hormones in eusocial Damaraland mole-rats (*Fukomys damarensis*)

Abstract

In species where sociality and group cohesion are primarily determined by the maintenance of a reproductive division of labour and cooperative behaviours, the eusocial Damaraland mole-rat (*Fukomys damarensis*) presents a model which provides behavioural and endocrine distinctions between sex (males and females) and reproductive class (breeders and non-breeders). Although previous studies have demonstrated the endocrine aspects of reproductive suppression and behaviour in Damaraland mole-rats, they have focused on one hormone separately and on different conspecifics and samples across time. Unfortunately, this could introduce extrinsic biases when using these studies to compile complete hormonal profiles for comparisons. This study, therefore, set out to obtain a profile of the reproductive hormones from breeding and non-breeding male and female Damaraland mole-rats at a single point in time, from which circulating plasma prolactin and urinary progesterone, testosterone, and cortisol correlates can be compiled to form an accumulative reproductive hormone profile. Furthermore, the testosterone-cortisol (T/C) ratio, an endocrine proxy of socially dominant behaviour, has been investigated. As expected, plasma prolactin and urinary cortisol did not differ between the breeders and non-breeders. However, breeders (both male and female) possessed increased urinary testosterone, T/C and progesterone concentrations compared to their non-breeding counterparts. These results, in conjunction with the variation in the expression of the respective hormonal receptors within the brains of breeders and non-breeders suggest that elevated testosterone and progesterone in breeders establish a neural dominance phenotype, which ultimately aids in controlling breeding activities. This study has emphasised the need for holistic, comprehensive profiling of reproductive endocrine systems.

Introduction

The reproductive system is a complex amalgamation of interacting hormonal axes modulated by the differential expression of neuroendocrine receptor cells and extrinsic environmental interactions (Brüggemann et al., 2018; Burland et al., 2002; Toufexis et al., 2014; Voigt et al., 2014). The hypothalamic-pituitary-gonadal (HPG) axis is responsible for the production of steroid hormones; testosterone, progesterone, and oestrogen, through the activation and stimulatory roles of GnRH (gonadotropin-releasing hormone), LH (luteinising hormone) and FSH (follicle-stimulating hormone) (Brüggemann et al., 2018; Oyola and Handa, 2017; Toufexis et al., 2014). Increases in reproductive steroid hormone concentrations, such as testosterone and progesterone, induce physiological and morphological changes in the animal; however, they also promote several behaviours, such as mating activities and aggression for mate guarding and dominance maintenance (Clarke and Faulkes, 1998; Margulis et al., 1995). However, the HPG axis can be disrupted by several other hormones, such as prolactin and glucocorticoids (released in response to a perceived stressor). For example, prolactin is closely linked to the HPG axis; with the hypothalamus as one of its production sites. Prolactin can naturally suppress steroid hormones, such as progesterone and testosterone, during lactation, often causing infertility at high concentrations (hyperprolactinaemia) (Bennett et al., 2018; Brown et al., 2014; Kaupila et al., 1988; Ziegler, 2000). In addition, prolactin inhibits the release of FSH and LH through a reduction in GnRH release from the hypothalamus and/or reduced sensitivity of the pituitary gland to GnRH (Bennett et al., 2018; Brown et al., 2014; Kaupila et al., 1988; Ziegler, 2000).

Glucocorticoids (such as cortisol), produced by the hypothalamic-pituitary-adrenal (HPA) axis, have been linked to HPG function as a GnRH inhibitor (Toufexis et al., 2014). The HPG and HPA axes are thus interconnected and can be influenced by joint synergistic and antagonistic feedback mechanisms as an effect of the hypothalamus and anterior pituitary being communal sites for reception and synthesis (Toufexis et al., 2014). The activation of the HPA axis can often lead to the reduction of the HPG axis during periods of acute or chronic stress. Similar to reproductive steroid hormones, prolactin and glucocorticoids induce physiological, morphological, and behavioural changes in the animal (Carlson et al., 2006). These behavioural changes include cooperative care behaviours, which can be characterised as non-breeding group members executing alloparental behaviour along with additional burrow/nest maintenance and foraging duties (Montgomery et al., 2018; Schradin et al., 2019). Previous studies have indicated species-, sex- and reproductive status-specific variations in the

endocrine patterns of these reproduction-related hormones (Bens et al., 2018; Carlson et al., 2006; Clarke and Faulkes, 1998; Davies et al., 2016; Gray, 1978; Hart et al., 2022a; Lutermann et al., 2013; Matas et al., 2020; Sapolsky, 1982; Swift-Gallant et al., 2015; Voigt et al., 2021, 2016).

In species whose sociality and group cohesion are primarily determined by their maintenance of a reproductive division of labour, the eusocial Damaraland mole-rat (*Fukomys damarensis*) presents a model which provides behavioural and endocrine distinctions between sex and reproductive class (Bennett, 2009; Cooney and Bennett, 2000; Faulkes et al., 1997; Lovegrove, 1986). The despotic monopolisation of breeding characterises the severe reproductive skew by a single dominant female, the breeding female (BF), and one to three of the most dominant breeding males (BMs) (Bennett, 1990; Jarvis & Bennett, 1993). The remaining non-breeding males (NBMs) and females (NBFs) display cooperative care behaviour and are sexually quiescent due to the suppression of their reproductive system at various stages (Clarke et al., 2001; Molteno and Bennett, 2000).

The downregulation of reproduction in non-breeding Damaraland mole-rats results from the suppression of both behavioural and physiological reproductive characteristics (Bennett *et al.*, 1993, 1996; Jarvis & Bennett, 1993; Kelley *et al.*, 2019; Voigt *et al.*, 2021). As Damaraland mole-rats are obligate outbreeders, inbreeding avoidance is a strong behavioural driver in preventing reproduction in NBFs and NBMs (Burland et al., 2002; Clarke et al., 2001; Kelley et al., 2019; Molteno and Bennett, 2000). Non-breeding males are anatomically and physiologically similar to BMs, yet they fail to reproduce and do not display sexual behaviours. Non-breeding males have similar circulating testosterone and cortisol concentrations and similar-sized testes and spermatogenesis to BMs (Jarvis & Bennett, 1993; Faulkes *et al.*, 1994; Voigt *et al.*, 2016). In contrast, NBF Damaraland mole-rats are physiologically and anatomically distinct from their reproductively dominant counterparts (Jarvis & Bennett, 1993; Bennett, 2011; Lutermann, Young & Bennett, 2013; Voigt *et al.*, 2021). The BF is reproductively active, exhibiting an ovulatory cyclicity and high progesterone and oestrogen concentrations, while ovulation in NBFs is blocked at the follicle maturation stage, resulting in low progesterone and oestrogen profiles (Jarvis & Bennett, 1993; Bennett, 1994; Bennett *et al.*, 1994, 1996; Molteno & Bennett, 2000; Clarke *et al.*, 2001a; Voigt *et al.*, 2014, 2021). These differential profiles have been suggested to stem from lowered pituitary sensitivity to GnRH (Bennett *et al.*, 1993; Voigt *et al.*, 2014; Voigt & Bennett, 2021). As induced ovulators,

reproductively inactive females are thus anovulatory and incapable of breeding in the presence of the BF (Molteno & Bennett, 2000; Kelley *et al.*, 2019; Voigt *et al.*, 2021)

In other cooperative breeding species, such as meerkats (*Suricata suricata*) and Mahali mole-rats (*Cryptomys hottentotus mahali*), both prolactin and glucocorticoids influence cooperative care and reproductive suppression (Carlson *et al.*, 2006; Dantzer *et al.*, 2017; Hart *et al.*, 2022a). Interestingly, only prolactin has been highlighted as a potential driving force behind reproductive suppression and cooperative care in naked mole-rats (*Heterocephalus glaber*) (Bennett *et al.*, 2018; Medger *et al.*, 2019; Edwards *et al.*, 2020). Yet, in the Damaraland mole-rat, there is no evidence indicating that prolactin, glucocorticoids, or testosterone drives reproductive suppression or cooperative care (Bennett *et al.*, 2018; Vullioud *et al.*, 2021; Zöttl *et al.*, 2018).

While previous studies have demonstrated the endocrine aspects of reproductive suppression and cooperative care in Damaraland mole-rats, they have done so by focussing on one hormone separately and on different conspecifics and samples across time. Unfortunately, this could introduce extrinsic biases when using these studies to compile complete hormonal profiles for comparison. Accordingly, now that the endocrine reproductive base has been laid, we can afford to look at the synergistic effects of the reproductive hormones together (Toor *et al.*, 2022). This study, therefore, set out to obtain a profile of reproductive hormones from breeding and non-breeding male and female Damaraland mole-rats at a single point in time, from which circulating plasma prolactin and urinary progesterone, testosterone, and cortisol correlates can be compiled, to form an accumulative reproductive hormone profile. This, therefore, ensures uniformity and eliminates the possible effect of variation in individuals (wild vs captive) (Medger *et al.*, 2018), season (Lutermann *et al.*, 2013), diet (Medger *et al.*, 2018), health (Klein, 2004), or influential dynamic social interactions (Kelley *et al.*, 2019; Medger *et al.*, 2019) within the colony across studies that may alter endocrine correlate values. This study, therefore, predicts that male and female Damaraland mole-rats will have similar prolactin and cortisol profiles, but different progesterone and testosterone profiles. Based on previous studies (Lutermann *et al.*, 2013; Zöttl *et al.*, 2018; Voigt *et al.*, 2021), it is expected that the female colony members will have higher concentrations of progesterone but lower concentrations of testosterone than their male counterparts. Further, it is expected that breeding Damaraland mole-rats will have higher testosterone and progesterone concentrations than non-breeders (Clarke *et al.*, 2001). Additionally, this study will incorporate the testosterone-cortisol (T/C) ratio, an endocrine proxy of socially dominant behaviour (Terburg *et al.*, 2009), to further

clarify the reproductive differences in dominance between breeding and non-breeding individuals (Montoya et al., 2012; Terburg et al., 2009). Based on previous behavioural (Jacobs *et al.*, 1991) and endocrine-orientated hierarchal studies (Kelley *et al.*, 2019) we predict that the dominance of breeding Damaraland mole-rats will be reflected in their low T/C ratio's.

This study is also the first to investigate differences in progesterone concentrations between BMs and NBMs and how these relate to BFs and NBFs. This is surprising as an increased expression of progesterone receptors has been found in BMs, compared to NBMs, in most brain regions examined (medial preoptic area, the bed nucleus of the stria terminalis, the ventromedial nucleus of the hypothalamus, the arcuate nucleus and the medial amygdale) (Voigt et al., 2016). This study therefore predicts that breeding male Damaraland mole-rats will have higher progesterone concentrations than their non-breeding counterparts. Therefore, suggesting the role of progesterone in activating sexual behaviour in Damaraland mole-rat males (Voigt et al., 2016) as found in other mammals (Andersen and Tufik, 2006).

Methods

Ethical note

The University of Pretoria animal ethics committee approved the experimental procedures of this study (NAS017-2021 and NAS022/2021).

Study species

Forty-nine Damaraland mole-rats (10 BF, 8 BM, 16 NBF, 15 NBM) from ten captive colonies housed at the University of Pretoria were used. All individuals used in this experiment were considered adults (Bennett and Faulkes, 2000). The animals were bred from a population caught in the Northern Cape of South Africa, and in Namibia; subsequent colonies were created from this stock. Due to Damaraland mole-rats low inbreeding tolerance the likelihood of relatedness is low between these individuals, hence why inbreeding will not be accounted for in this study. Breeding females were identified by the presence of prominent axillary and inguinal teats, well-developed external genitalia with a perforate vagina, and/or pregnancy-related changes in girth/body size as well as a history of producing young. Breeding males were identified based on observations of copulation with the BF, a dark stain around the

periphery of the mouth, and bulging testes which project from abdominal pockets. Long-term observational records were used to confirm the identity of the socially dominant males and females and the age of non-breeding individuals. All animals have been part of long-term (>20 years) monitoring and breeding projects at the University of Pretoria. Therefore, age data (accurate to 1 day) was available for non-breeding Damaraland mole-rats, while less accurate age data was available for the breeding Damaraland mole-rat colony members and therefore not included in this study.

Animal housing

Damaraland mole-rats were housed in their natal colonies in large plastic crates (1 m × 0.5 m × 0.5 m), with wood shavings and paper towelling provided as nesting material. Housing room temperatures ranged between 26.5 and 27.5 °C, with relative humidity around 50–60%. Animal rooms were maintained on a 12L:12D photoperiod. Photoperiod has not been shown to affect Damaraland mole-rat behaviour (Oosthuizen et al., 2003). Animals were fed *ad libitum* on a variety of chopped vegetables and drank no free water (Hart et al., 2022b).

Urine collection

Urine was collected from all Damaraland mole-rats over two days (approximately 25 animals a day). Each animal was kept in a cylindrical plastic cage with a wire-mesh base on top of the urine collection tray. The wire-mesh base prevented the contamination of the urine sample with faecal matter. Apple and sweet potato were provided. Urine was collected between the hours of 0900 and 1300, and as soon as urine had been voided (usually within 15 minutes), the animal was placed back into its natal colony. Urine was collected with a single-use plastic pipette and immediately frozen at –40 °C.

Blood sampling

Blood samples were collected three days after urine collection between 09h00 and 13h00. The mole-rats were handheld, and venous blood samples collected from the hind foot after sterilisation of the bleed site. Approximately 0.3 – 0.5ml of blood was collected into

heparinised micro-haematocrit tubes, depending on the body mass of the animal. The blood was centrifuged at 1500 g, and the resulting plasma decanted and stored at – 80 °C until further analysis. Only 1% of the total body mass of the individual blood was collected, as permitted by the University of Pretoria Animal Ethics Committee.

Hormone analysis

Plasma prolactin

Plasma prolactin concentrations were quantified using an Elabscience Guinea pig Prolactin ELISA kit (Elabscience Biotechnology Inc., Wuhan, China), as described by Bennett et al. (2018). The sensitivity of the assay was 0.09 ng/mL plasma, and intra-assay precision and repeatability are <10%, according to the manufacturer's guidelines.

Urinary steroid hormones

Urine samples were analysed for testosterone, progesterone and cortisol concentrations using coat-a-count kits (Diagnostic Products Corporation, Los Angeles, California, USA). All assays were conducted according to the manufacturer's protocol. Assays were validated by testing for parallelism between serial dilutions of mole-rat urine (obtained from an individual with high hormone concentrations) and the standard curve (Chard, 1988). The aforementioned hormone kits, including cross-reactivity, sensitivity, and protocols, have been described in previous studies (Hart et al., 2021, 2020; Medger et al., 2018). Only a sub-set of males (5 BMs and 7 NBMs) urinary progesterone was analysed.

There was no significant difference between the serial dilution curve of urinary testosterone of a BM and the calibration curve (ANCOVA: $F_{[1,5]} = 1.1$, $p = 0.53$). The intra-assay coefficient of variation was 4.7%. Furthermore, a serial dilution of urinary cortisol concentration from an individual with high cortisol paralleled the reference preparation; thus, the slopes did not differ significantly (ANCOVA: $F_{[1,5]} = 15.2$, $p = 0.22$). The intra-assay coefficient of variation was 5.5%. Similarly, no significant difference was observed between a BF with high urinary progesterone serial dilution curve and the calibration curve (ANCOVA: $F_{[1,5]} = 2.9$, $p = 0.39$). The intra-assay coefficient of variation for repeated determination of quality control was 7.9%.

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1194 *Creatinine determination*

1195 Urine hormone concentration varied due to variable fluid intake; thus, the concentrations of
1196 steroid hormones (testosterone, progesterone, and cortisol) had to be corrected. Correction was
1197 accomplished by analysing each urine sample for creatinine concentration, as creatinine is
1198 excreted at a relatively constant rate. The creatinine concentration of each urine sample was
1199 determined using a modified Jaffe reaction (Folin, 1914) Final standardised results are
1200 presented as steroid hormone [testosterone (ng), progesterone (ng) and cortisol (µg)] per
1201 milligram of creatinine (mg creatinine).

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1204 ***Data analysis***

1205 Statistical analyses were performed on R 2022.02.0, Microsoft Excel (Version 2205) and
1206 Graphpad Prism 8.4.3. Statistical significance was denoted by $p \leq 0.05$, and data is presented
1207 as mean $\pm$ standard error (SEM). Corrected urinary testosterone concentrations (ng/mg
1208 creatinine) were divided by corrected urinary cortisol concentrations (ng/mg creatinine) to
1209 create the testosterone: cortisol (T/C) ratio (a proxy of socially dominant behaviour).

1210 The normality and homogeneity of the dependent variables (testosterone (ng/mg
1211 creatinine), cortisol (µg/mg creatinine), progesterone (ng/mg creatinine), prolactin (ng/ml) and
1212 the T/C ratio) were determined using Shapiro-Wilk and Levene's tests, respectively. Data that
1213 were not normally distributed were log-transformed. General linear models (GLM's) were used
1214 to analyse normally distributed dependant variables, while generalised linear models
1215 (GLZM's) with gamma distributions and link-identity functions from the *lme4* package were
1216 used to analyse non-normal dependant variables. Urinary testosterone, cortisol, progesterone
1217 concentrations, and T/C ratio data had to be log-transformed into a normal distribution, while
1218 the plasma prolactin data failed to become normally distributed even after log transformation.

1219 Initial models possessed colony size and body mass as covariates and sex (male and
1220 female), status (breeding and non-breeding) and sex*status interactions as the predictor
1221 variables. Additional models were conducted for testosterone, T/C ratio and progesterone
1222 models; BMs were excluded from the second testosterone and T/C models (thus, only BF, NBF

and NBM were included), and BFs were excluded from the progesterone model (thus, only BM, NBF and NBM were included). These models included colony size and body mass as covariates and reproductive grouping (BF, NBF and NBM – testosterone and T/C ratio models or BM, NBF and NBM - progesterone model). *Post-hoc* comparisons to obtain *emmeans* of significant interactions were obtained by Tukey's HSD (honestly significant difference) tests. Due to insufficient data on the breeding individuals, age analyses were only conducted for non-breeders (NBF and NBM). Linear regressions were conducted between urinary testosterone, cortisol, progesterone, prolactin and the T/C ratio and age, respectively, separately for NBMs and NBFs. Lastly, Pearson correlations were conducted between log-transformed urinary testosterone and cortisol and progesterone concentrations, respectively. At the same time, Spearman-rank correlations were performed to determine the occurrence of covariance between prolactin and urinary testosterone, cortisol, and progesterone concentrations, respectively.

Results

Testosterone

Both sex and status significantly affected urinary testosterone concentrations in the Damaraland mole-rat (Table 1, Figure 1a). Colony size and sex*status did not significantly affect urinary testosterone concentrations (Table 1). There was, however, a significant positive relationship between urinary testosterone and body mass, whereby larger animals possessed higher urinary testosterone concentrations (Figure 2a, Table 1). Males (breeding and non-breeding individuals combined; 418.48 ± 102.73 ng/mg creatinine) possessed significantly higher urinary testosterone levels than females (93.68 ± 33.9 ng/mg creatinine; Table 1, Figure 1a). Similarly, breeders (males and females combined; 372.67 ± 98.16 ng/mg creatinine) possessed a greater urinary concentration of testosterone than non-breeders (172.67 ± 65.34 ng/mg creatinine; Table 1, Figure 1a). When BMs were removed from the model, colony size still did not affect urinary testosterone concentrations, but reproductive grouping (BF, NBM and NBF) and body mass did influence urinary testosterone concentrations (Figure 1.b and Figure 2b, Table 2, respectively). Both NBMs ($p = 0.01$, $t = 3.29$) and BFs ($p = 0.03$, $t = 2.68$) possessed significantly higher urinary testosterone concentrations than NBFs (Figure 1b). No

difference was noted between BF's and NBM's urinary testosterone levels ($p=0.90$; Figure 1b). Body mass showed an inverse relationship with urinary testosterone concentrations when BMs were removed (Figure 2b).

Cortisol

There were no significant effects of sex, status, or sex*status interactions on the urinary cortisol concentrations ($\mu\text{g}/\text{mg}$ creatinine) of Damaraland mole-rats (Table 1 and Figure 3). Furthermore, colony size did not significantly affect urinary cortisol concentrations (Table 1). However, there was a significant negative relationship between cortisol concentrations and body mass in Damaraland mole-rats (Figure 4, Table 1).

Testosterone: Cortisol ratio

There was a significant effect of sex and reproductive status on the testosterone-cortisol (T/C) ratio of Damaraland mole-rats (Figure 5a, Table 1). Both colony size and body mass had no significant effect on the T/C ratio (Table 1). Males (BM and NBM) (13.69 ± 4.39) possessed significantly higher T/C ratio's than females (BF and NBF) (2.72 ± 1.02), while breeders (males and females) (17.04 ± 5.39) exhibited the highest T/C ratio compared to non-breeders (males and females) (2.53 ± 0.80 , accordingly; Figure 5a). When BMs were removed, colony size and body mass still did not affect the T/C ratio of the remaining Damaraland mole-rats (Table 2), but reproductive grouping (BF, NBF and NBM) did significantly affect the T/C ratio (Figure 5b and Table 2). Breeding females (4.17 ± 1.71) and NBMs (3.32 ± 0.99) had similar T/C ratios ($p = 0.99$), while both reproductive groups held significantly higher T/C ratios in comparison to NBFs (1.79 ± 1.25 ; $p = 0.02$ and 0.01 , respectively, Figure 5b).

Progesterone

Both sex and status significantly affected urinary progesterone concentration in the Damaraland mole-rat (Table 1, Figure 6a). In contrast, sex*status, body mass and colony size did not significantly affect urinary progesterone concentrations (Table 1). Females (BFs and

NBFs) (85.64 ± 20.5 ng/mg creatinine) possessed significantly higher urinary progesterone concentration than males (BMs and NBMs) (10.79 ± 3.36 ng/mg creatinine, respectively, Figure 6a). Breeding (males and females) individuals (121.68 ± 29.77 ng/mg creatinine) possessed a higher level of urinary progesterone concentration than non-breeding (males and females) colony members (18.26 ± 4.44 ng/mg creatinine, Figure 6a). Once BF's were removed, neither colony size nor body mass had a significant effect on urinary progesterone concentrations (Table 2), but reproductive grouping (BM, NBF and NBM) did have a significant impact on urinary progesterone concentrations (Figure 6b, Table 2.). Non-breeding males (5.92 ± 3.45 ng/mg creatinine) had significantly lower progesterone concentrations compared to NBFs (23.67 ± 5.75 ng/mg creatinine; $p = 0.02$) and BMs (16.47 ± 5.49 ng/mg creatinine; $p = 0.02$) (Figure 6b). Additionally, BMs and NBFs possessed similar urinary progesterone concentrations ($p = 0.99$; Figure 6b).

Prolactin

Neither sex, status, nor sex*status interactions significantly affected plasma prolactin concentrations (ng/ml) in Damaraland mole-rats (Table 1 and Figure 7). Neither did colony size, nor body mass significantly affect plasma prolactin concentrations (ng/ml, Table 1).

Effect of age in non-breeders

A significant inverse relationship between age and urinary testosterone concentrations was present in NBMs but not present in NBFs (Table 3). This implies a decrease in urinary testosterone concentrations in NBMs as they get older. There are no further significant interactions between cortisol, progesterone, prolactin, or the T/C ratio, and age in either NBMs or NBFs (Table 3).

Correlations between hormones

No correlations were found between prolactin and urinary testosterone, cortisol, and progesterone ($r \leq 0.11$, $p \geq 0.25$). In contrast, significant correlations were observed between

urinary cortisol and progesterone ($r = 0.52$, $p \leq 0.001$) and urinary cortisol and urinary testosterone concentrations ($r = 0.36$, $p \leq 0.01$). Similarly, a significant correlation was observed between urinary testosterone and progesterone ($r = 0.36$, $p \leq 0.02$).

Discussion

In contrast to their counterparts, female Damaraland mole-rats and the breeding individuals possessed the highest urinary progesterone concentrations. This was most likely driven by BFs possessing the highest urinary progesterone concentration compared to the other reproductive groupings, including NBFs, BMs and NBMs. As BFs have access to unrelated males (the BM), they ovulate and fall pregnant regularly, having three to four litters throughout the year (Bennett and Faulkes, 2000). Through this and the increased pituitary gland sensitivity to GnRH (Bennett et al., 1993), along with an increased expression pattern of oestrogen receptor α and aromatase (androgen-converting enzyme), the circulating progesterone in BFs is greater than in NBFs and males (Voigt et al., 2014). Interestingly, BMs possess similar urinary progesterone concentration to NBFs, possibly due to two factors: reduced circulating progesterone levels in NBFs and increased circulating progesterone levels in BMs. As NBMs have the lowest urinary progesterone concentration measured, this implies that BMs inherently possess higher circulating progesterone levels. Variation between breeding and non-breeding Damaraland mole-rat males has been found in the differential neuroendocrine expression of androgen-, progesterone- and Rfrp-receptors (AR, PGR, and RFRP-3, respectively), which directly regulate GnRH activity in the hypothalamus (Matas et al., 2020; Swift-Gallant et al., 2015; Voigt et al., 2016, 2014; Voigt and Bennett, 2021). Breeding males have a greater expression of AR and PGR than NBMs, both of which, when stimulated by their respective hormones, activate GnRH neurons to release excess gonadotropins, therefore enabling heightened reproductive behaviour (Voigt et al., 2016). Conversely, NBMs have lower AR and PGR distributions, but elevated RFRP-3, which enacts an inhibitory function on GnRH neurons and subsequent reproductive phenotypes (Voigt et al., 2016; Voigt and Bennett, 2021). The expression of these receptor cells thus alludes to and permits the endocrine variations that facilitates the reproductive state of each individual. Progesterone is a steroid required for reproduction, whereby previous studies have implicated the synergistic activity of progesterone and testosterone via PGR and AR activity on GnRH stimulation and thus reproductive ability and behaviour (Voigt et al., 2016). Further implications have been made regarding the role of

progesterone in reproductive success and spermatozoa morphology, that while breeding and non-breeding male Damaraland mole-rats have comparable spermatozoa production (Jarvis and Bennett, 1993), the spermatozoans of NBMs have more dysmorphologies in terms of double heads, multiple and/or shorted tails and thus a possible additive cause of failure in breeding (N.C. Bennett, personal observation and communication). Additively, NBMs have been reported to be oligospermic and even azoospermic compared to their breeding counterparts (Maswanganye et al., 1999).

Similar to urinary progesterone, higher urinary testosterone concentrations were seen in breeders compared to non-breeders, but also when comparing males with females. This was most likely driven by BMs possessing the highest urinary testosterone concentration, as a somewhat expected trend in mole-rat species (Hart et al., 2022a; Hart et al., 2021), but unexpected in Damaraland mole-rats. Interestingly, other studies investigating the relationship between testosterone concentrations in Damaraland mole-rat males, did not report BM's having significantly higher testosterone concentrations than NBM's (Bennett, 1988; Medger et al., 2018). Likewise, the majority of previous studies on testosterone in Damaraland mole-rats females indicated no significant differences between reproductive states (Bennett, 1994; Bennett *et al.*, 1994; Clarke *et al.*, 2001a; Medger *et al.*, 2018), which contrasts with the present study that found BF's possessing higher urinary testosterone concentrations than NBF's and similar urinary testosterone concentrations to NBMs. Breeding males not only maintain increased testosterone concentrations (this study) but also increased expression of AR in their brains compared to NBMs; in conjunction with increased progesterone concentrations and expression of PGR, this would establish a neural dominance phenotype, which ultimately aids in controlling breeding activities (Voigt et al., 2014). Even though the BF's urinary testosterone concentrations were lower than BM's in this study, the increased expression of AR in BF's compared to NBF's can exploit the lower circulating testosterone concentrations in order to establish this neural dominance phenotype in the BF's (Voigt et al., 2014). Upon observing increased concentrations of testosterone in BF's compared to NBF's in Damaraland and Natal mole-rats (*C. h. natalensis*), a pattern also seen in the other mole-rat species (Clarke and Faulkes, 1998; Hart et al., 2022a; Spinks et al., 1999), Lutermann et al. (2013) linked elevated testosterone concentrations to the ability of an individual to attain and defend the breeding monopoly. This, therefore, re-enforces the hypothesis that female intra-sexual competition exerts selective pressures on testosterone-mediated traits thought to enhance reproductive success (Clutton-Brock, 2007; Lutermann et al., 2013; Medger et al., 2019).

The increase in the testosterone concentration of breeders may suggest increased aggression or dominance displayed by these males toward their non-breeding colony members in breeding monopoly maintenance, which has been suggested to cause an increase in glucocorticoids, namely cortisol (Hart et al., 2022a). However, to date, including in this study, no significant difference in glucocorticoid concentrations between breeding and non-breeding Damaraland mole-rats (from stable colonies) has been observed (Medger et al., 2018), thus indicating no physiological stress in non-breeders. Admittedly, in some studies, including this current study, slightly elevated cortisol concentrations have been found in non-breeding Damaraland mole-rats (Vulliou et al., 2021). Cortisol is released as a biological response to unpredictable stress conditions, but it is also essential under non-stressful conditions to support energetically demanding behaviours, which are often associated with cooperative behaviours, such as digging (Vulliou et al., 2021). Experimentally increasing circulating cortisol in non-breeding Damaraland mole-rats increased their time spent digging but did not affect other behaviours (Vulliou et al., 2021). Even though no physiological indicators of stress have been found in non-breeding Damaraland mole-rats, a difference in socially dominant and sexual behaviour between breeders and non-breeders may still be observed.

Expectedly, as with urinary testosterone, males and breeders had the highest T/C ratio - an endocrine proxy of socially dominant behaviour (Terburg et al., 2009). The BMs (33.13 ± 9.36) possessed the highest T/C, followed by BFs (4.17 ± 1.71), then NBMs (3.32 ± 0.99) and finally NBFs (1.79 ± 1.25). This supports pre-existing literature stating that breeding individuals were the most dominant, or highest in the hierarchy, and the reproductively blocked females were the lowest ranked (Jacobs et al., 1991; Jarvis and Bennett, 1993). The T/C ratio can thus be used as an indicator of dominance as it accounts for how well dominant individuals can maintain their position through high testosterone concentrations and well-adjusted low cortisol concentrations. Additionally, this ratio accounts for the effects that body mass may have on cortisol and testosterone concentrations (this study). Previous hierarchal studies on DMR used a complex and time-consuming combination of behavioural observations, morphological distinctions, and endocrine classifications to determine colony rank (Jacobs et al., 1991; Jarvis and Bennett, 1993). This process can now be shortened by including the clarity the T/C ratio will provide.

The role of prolactin in cooperative breeding and reproduction suppression in African mole-rats is still unclear as each species appears to have its own unique pattern (Bennett et al., 2018; Hart et al., 2022a). This study confirms the findings of Bennett et al. (2018), which found

almost undetectable concentrations of prolactin in Damaraland mole-rats, suggesting it plays no role in reproductive suppression or cooperative care species.

This study has aided in elucidating inconsistencies in prior reproductive endocrinological work and has highlighted a gap in the knowledge base, largely the role of progesterone in male reproductive systems and the introduction of the T/C ratio as a dominance determinant. Furthermore, this study has emphasised the need for holistic, comprehensive profiling of reproductive endocrine systems.

Acknowledgements

The project was funded by the SARChI chair for Mammal Behavioural Ecology and Physiology, South Africa, # 64796, for the DST-NRF awarded to NCB

Author Contributions

N.C.B, D.W.H and K.M.E.W designed the study. K.M.E.W., N.C.B. and D.W.H. collected the data. N.C.B, D.W.H, K.M.E.W and N.H. conducted hormonal analysis. K.M.E.W. compiled the data. D.W.H and K.M.E.W analysed the data. All authors contributed to the writing of the first draft of the manuscript.

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Figures

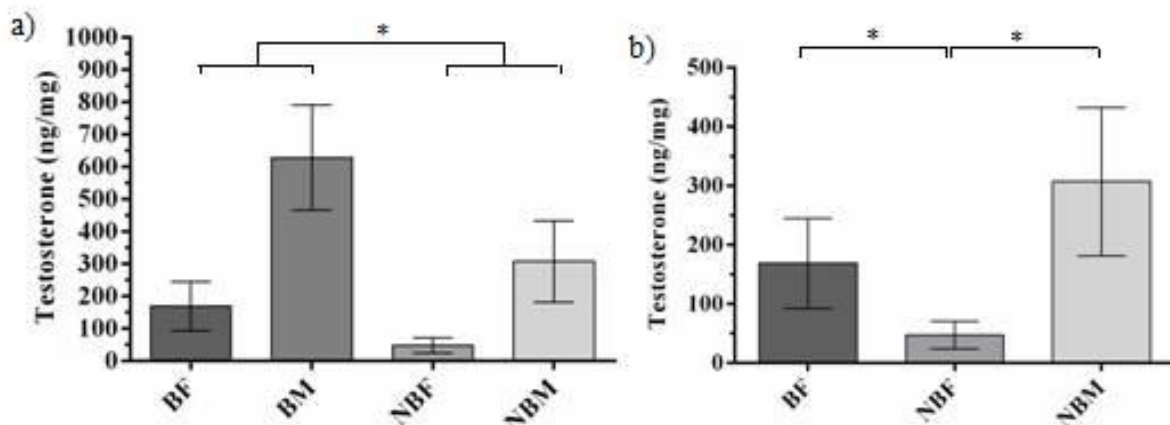


Figure 1. Bar graphs displaying sex and status differences in urinary testosterone (ng/mg creatinine) concentrations in 49 Damaraland mole-rats for (a) all reproductive classes (BF: breeding female, $n = 10$; BM: breeding male, $n = 8$; NBF: non-breeding female, $n = 16$; NBM: non-breeding male, $n = 15$) and (b) after all breeding males were removed. Data presented as mean $\pm$ SEM, with significant differences ($p < 0.05$) indicated by ‘*’.

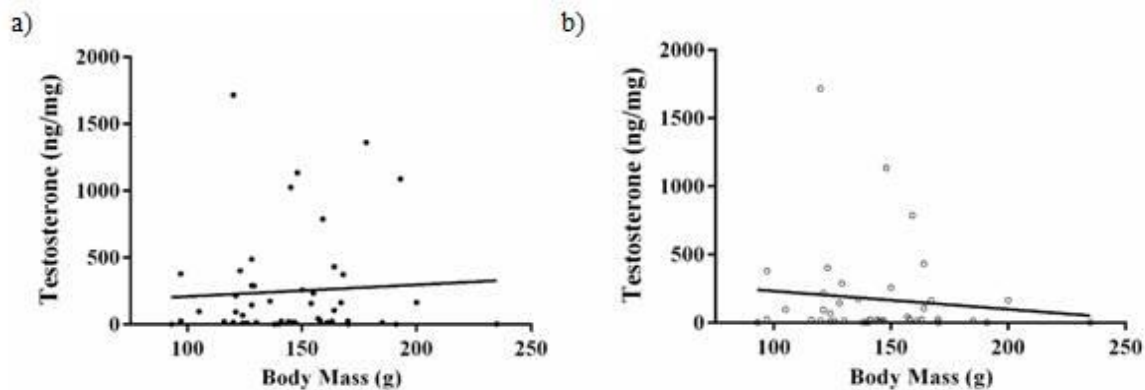


Figure 2. The relationship between urinary testosterone (ng/mg creatinine) and body mass (g) was significant in Damaraland mole-rats for (a) all individuals and (b) breeding females (BF), non-breeding males (NBM) and non-breeding females combined (breeding males –BM– excluded).

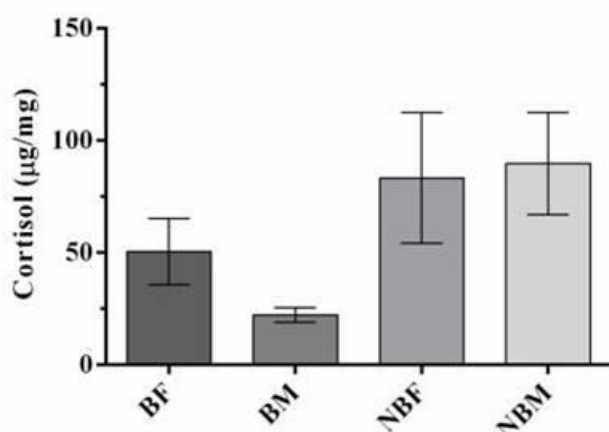


Figure 3. Bar graph displaying the sex and status differences in urinary cortisol (ng/mg creatinine) concentrations in Damaraland mole-rats for all reproductive classes (BF: breeding

female; BM: breeding male; NBF: non-breeding female; NBM: non-breeding male). Data presented as mean $\pm$ SEM.

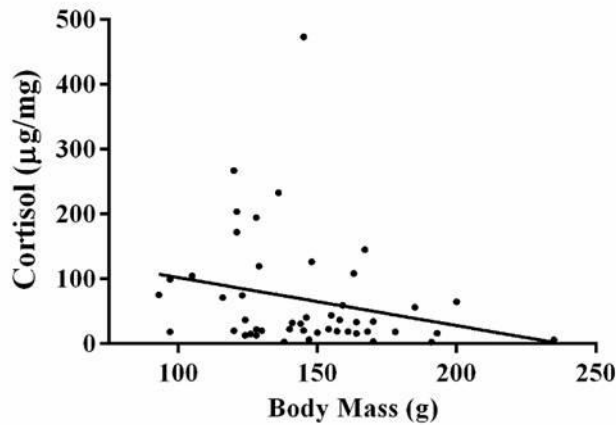


Figure 4. Linear regression indicating a negative, significant relationship between body mass (g) and urinary cortisol concentrations ($\mu\text{g}/\text{mg}$ creatinine) in Damaraland mole-rats.

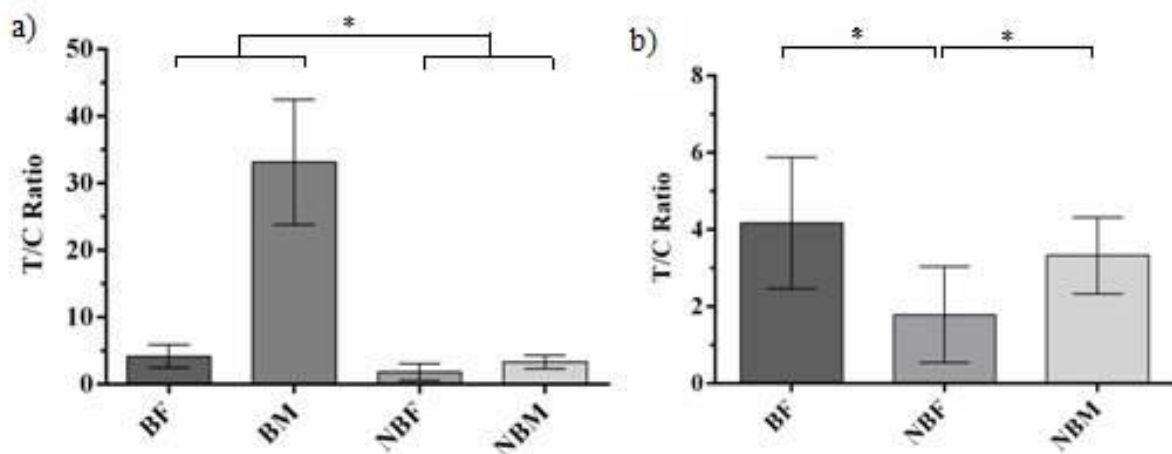


Figure 5. Bar graphs portraying sex and status differences in the urinary testosterone-cortisol ratio in Damaraland mole-rats for (a) all reproductive classes (BF: breeding female; BM: breeding male; NBF: non-breeding female; NBM: non-breeding male) and (b) after all breeding males were removed. Data presented as mean $\pm$ SEM, with significant differences ($p < 0.05$) indicated by ‘*’.

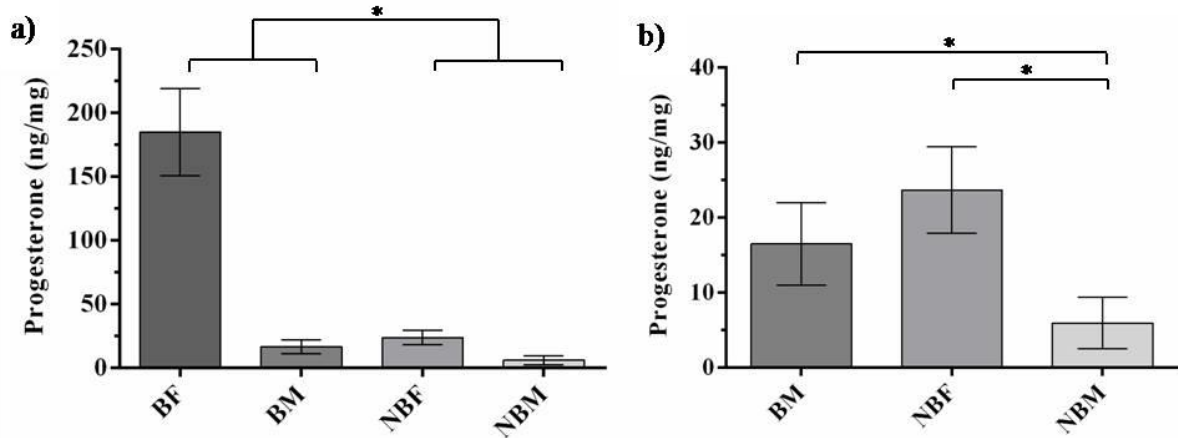


Figure 6. Bar graphs displaying the sex and status differences in urinary progesterone (ng/mg creatinine) concentrations in Damaraland mole-rats for (a) all reproductive classes (BF: breeding female; BM: breeding male; NBF: non-breeding female; NBM: non-breeding male) and (b) after all breeding females were removed. Data presented as mean $\pm$ SEM, with significant differences ($p < 0.05$) indicated by ‘*’.

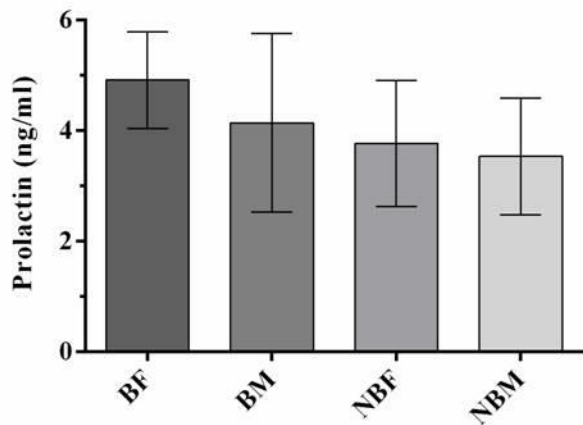


Figure 7. Bar graph displaying sex and status differences in plasma prolactin (ng/ml) concentrations in Damaraland mole-rats for all reproductive classes (BF: breeding female; BM: breeding male; NBF: non-breeding female; NBM: non-breeding male). Data presented as mean $\pm$ SEM.

Table 1. The statistical outputs from the models investigating the effects of body mass, colony size, breeding status ('Status': reproductive vs. non-reproductive) and sex (male vs. female) and their two-way interactions on the urinary testosterone (T; ng/mg creatinine), cortisol (C; µg/mg creatine), testosterone-cortisol ratio (T/C), progesterone (P; ng/mg creatinine) and plasma prolactin (PRL; ng/ml) of Damaraland mole-rats (*Fukomys damarensis*)

	T		C		T/C		PRL		P	
	t	p	t	p	t	p	t	p	t	p
n	49		49		49		49		39	
Colony Size	-0.66	0.51	-0.94	0.35	0.05	0.96	-0.45	0.65	-0.67	0.50
Body Mass	-2.06	0.04*	-2.25	0.03*	-0.45	0.65	0.74	0.45	-0.44	0.65
Sex	2.83	0.01*	-0.82	0.41	0.60	<0.01*	-0.51	0.60	-3.84	<0.01*
Status	-2.66	0.01*	-0.36	0.71	0.57	0.01*	-0.57	0.57	-4.61	<0.01*
Sex*Status	-0.11	0.91	1.23	0.22	0.90	0.23	0.12	0.90	0.19	0.84

Significant relationships (p -value ≤ 0.05) are indicated with '*'.

Table 2. The statistical outputs from the models investigating the effects of body mass, colony size and caste (testosterone: breeding female, non-breeding male and female; testosterone-cortisol ratio: breeding female, non-breeding male and female; progesterone: breeding male, non-breeding male and female) on the urinary testosterone (T; ng/mg creatinine), testosterone-cortisol ratio (T/C) and progesterone (P; ng/mg creatinine) of Damaraland mole-rats (*Fukomys damarensis*).

	T		T/C		P	
	t	p	t	p	t	p
n	41		41		29	
Colony Size	-0.87	0.39	-0.21	0.84	-1.09	0.28
Body Mass	-2.30	0.03*	-0.66	0.51	-0.66	0.51
Caste	-2.68	0.01*	-2.76	0.01*	-2.83	0.01*

Table 3. Linear equations and regression statistical outputs for the relationships between age of a non-breeding male (NBM) Damaraland mole-rats and non-breeding female (NBF) Damaraland mole-rats with body mass and the endocrinal correlates; urinary testosterone (T;

1669 ng/mg creatinine), cortisol (C; $\mu\text{g/mg}$ creatinine), progesterone (P; ng/mg creatinine),
1670 testosterone-cortisol ratio (T/C) and plasma prolactin (PRL; ng/ml).

	NBM				NBF			
	Slope	y-intercept	F	p	Slope	y-intercept	F	p
T	-0.74	1526.0	5.45	0.04*	0.04	-18.76	0.73	0.41
C	-0.00	96.59	0.00	0.96	-0.04	144.0	0.39	0.54
T/C	-0.00	10.01	2.11	0.17	0.00	-2.72	1.24	0.28
P	0.02	22.15	1.77	0.25	0.02	-6.16	2.82	0.12
PRL	0.00	-0.95	0.74	0.40	0.00	1.51	0.35	0.56
Body Mass	-0.01	171.30	0.11	0.74	0.01	119.70	0.50	0.49

1671 Significant relationships ($p\text{-value} \leq 0.05$) are indicated with ‘*’.

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Chapter 2

Erythrocytic divergences in helper Damaraland mole-rats enhances their performance of cooperative behaviour: A function of reproductive suppression

Abstract

Eusocial mammals, such as the Damaraland mole-rat (*Fukomys damarensis*), are characterised by a clear reproductive division of labour, whereby breeding is monopolised by one female and 1-2 males. The remaining colony members are reproductively suppressed and perform 95% of the colony labour. However, the physiological drivers behind this division of labour are unclear. In this study, we show that helper Damaraland mole-rats show erythropoietic specialisations, namely an increase in blood oxygen-carrying capacity through increased haemoglobin concentrations and red blood cell counts, which encourage the performance of cooperative tasks. However, these erythropoietic specialisations are lost once helpers are removed from the colony or attain reproductive dominance. These results indicate that reproductive suppression may be a cause of erythropoietic specialisation. We attempted to identify whether reproductive suppression and associated endocrine profiles are the underlying mechanisms of the observed haematological variations. We suggest that progesterone might be the main predictor of the oxygen-carrying capacity of the blood, whereby animals with lower progesterone concentrations (helpers) possess a higher oxygen-carrying capacity. This study proposes the oxygen-carrying capacity of the blood as the physiological driver behind the apparent division of labour in eusocial Damaraland mole-rats, which itself is driven by reproductive suppression-induced hormonal changes.

Introduction

A pronounced reproductive skew and division of labour are present in organisms that fall on the eusocial end of the social spectrum (Sherman *et al.*, 1995). Damaraland mole-rats (*Fukomys damarensis*) are subterranean African rodents extensively studied for their uniquely eusocial behavioural and physiological characteristics (Jarvis & Bennett, 1993). The monopolisation of reproduction by a single breeding female (BF) and one to three breeding males (BMs) has produced a reproductive division of labour within each Damaraland mole-rat colony (Bennett & Jarvis, 1988; Jarvis & Bennett, 1993). The remaining colony members are reproductively quiescent through physiological and behavioural suppression, including inbreeding avoidance and anovulation in non-breeding females (NBFs) (Bennett *et al.*, 1996; Molteno & Bennett, 2000; Kelley *et al.*, 2019; Voigt *et al.*, 2021). This suppression has resulted in breeding status-specific endocrine profiles, with breeders (males and females) often possessing increased levels of reproductive hormones, specifically progesterone and oestrogen but not testosterone, compared to their non-breeding counterparts (Jarvis & Bennett, 1993; Bennett, 1994; Bennett *et al.*, 1994; Clarke *et al.*, 2001a; Lutermann *et al.*, 2013; Voigt *et al.*, 2014, 2021; Wallace *et al.*, 2022b). Yet, once removed from this suppression, reproductive readiness and subsequent reproductive hormones of removed non-breeders increase to levels greater than those of non-breeders still within the colony but less than an active breeder's levels (Molteno & Bennett, 2000).

Non-breeding individuals are often termed 'helpers' or 'workers' due to their elevated cooperative care involving pup rearing, foraging, tunnel maintenance and colony defence (Jarvis, Bennett & Spinks, 2006; Scantlebury *et al.*, 2006b). Subsequently, in captive and wild Damaraland mole-rats, a marked division of labour occurs, with non-breeders spending approximately double the amount of time being active and performing 95% of annual colony labour compared to their breeding counterparts (Lacey & Sherman, 1997; Scantlebury *et al.*, 2006b; Oosthuizen & Bennett, 2015; Francioli *et al.*, 2020a; Houslay *et al.*, 2020). However, the physiological drivers behind this division of labour are still unclear (Zöttl *et al.*, 2018; Vulllioud *et al.*, 2021). For example, glucocorticoids and prolactin, hormones often linked to cooperative behaviours, are similar between breeding and non-breeding colony members (Bennett *et al.*, 2018; Medger *et al.*, 2018; Vulllioud *et al.*, 2021).

An individual's degree of activity is largely determined by the oxygen (O₂) carrying capacity and O₂ delivery of their blood to respirometric tissues (Dzierzak & Philipsen, 2013; Mairbaur, 2013). Red blood cells (RBCs) transport respiratory gases that support activity and

metabolism, while haemoglobin (Hgb) is the molecule that carries oxygen from the lungs to tissues, enabling energy production during endeavours such as digging (Gjevestad *et al.*, 2015) and running (Hu & Lin, 2012; Moretti *et al.*, 2018). Individuals that show increased physical activity often show increased erythropoiesis (production of RBCs) which results in increased Hgb, RBC, and haematocrit (Hct) levels (Hu & Lin, 2012; Moretti *et al.*, 2018). Additionally, hormones influence erythropoiesis alternatively through the direct modulation of bone marrow RBC differentiation and indirect regulation of renal erythropoietin (EPO), the primary RBC growth hormone (Yamamoto, Hayashi & Yamada, 1974). Numerous studies indicate a stimulatory role of testosterone and prolactin on RBC maturation and Hgb abundance, while progesterone and oestradiol downregulate erythropoietic elaboration (Jepson & Lowenstein, 1964; Mirand, E.A. and Gordon, 1966; Gordon *et al.*, 1968; Piliero *et al.*, 1968; Lavrijsen & Verwilghen, 1984). Congruently, glucocorticoids, prolactin and oestrogen have been implicated in elevated performance, while testosterone and progesterone have been negatively associated with endurance performance (Carlson *et al.*, 2006; Oosthuyse & Bosch, 2010; Bennett *et al.*, 2018; Hooper, Tenforde & Hackney, 2018; Vullioud *et al.*, 2021).

Interestingly, while Damaraland mole-rats show a pronounced difference in activity levels and hormone profiles between breeders and helpers, no study has investigated the differences between haematological profiles and erythropoiesis in breeding and helper colony members. Subsequently, we assessed the oxygen-carrying capacity of blood between breeding and helper captive Damaraland mole-rats through the measurement of haematological parameters. These include haemoglobin concentration (Hgb), red cell count (RCC), haematocrit (Hct) and red blood cell morphology, using mean corpuscular volume (MPV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and red blood cell distribution width (RDW) as proxies. Furthermore, we attempted to identify if reproductive suppression, through the measure of testosterone, progesterone, glucocorticoids (cortisol) and prolactin, is an underlying mechanism in the haematological (and possible activity level) variations between breeders and helpers. Lastly, the haematological parameters of helpers that were removed from their natal colony were measured to investigate if loss of reproductive suppression would affect their blood oxygen-carrying ability and thus activity. We therefore predict that the elevated activity levels of non-breeding helper individuals will be reflected in their enhanced haematological oxygen-carrying capacities and unique endocrine profiles.

Captive populations are exposed to less harsh conditions, as they are provided with *ad libitum* food and are not required to dig or maintain tunnel systems compared to their wild

counterparts. All individuals used in this study have never performed digging activities (as they were born and raised in captivity); any physiological differences between breeders and helpers likely observed would result from inherent biological differences, such as hormonal differences. Consequently, these predisposed physiological differences between breeders and helpers should enable the helpers to expend more energy in burrow maintenance and foraging activity, whereas breeders who perform these tasks less would be relieved of such requirements.

Methods

Animal housing

Forty-six Damaraland mole-rats (9 BF_s, 9 BM_s, 13 NBF_s, 15 NBM_s (non-breeding males) from 10 separate long-term captive colonies were kept and maintained in large polyurethane crates (1m x 0.5m x 0.5m) at the University of Pretoria. All individuals used for this experiment were adults (> 500 days old). Anatomical and behavioural observations and extensive historical data were used to distinguish between BF_s, BM_s, NBF_s and NBM_s (non-breeding males) (Jacobs, Hart & Bennett, 2021). Additionally, a subset (n = 5) of captive helper Damaraland mole-rats were removed from their natal colony and housed alone for several months ($\pm$ 3 months) (Molteno & Bennett, 2000). Wood shavings and paper towelling substituted nesting material and thus restricted digging and work behaviours. Experimental procedures were approved by the University of Pretoria animal ethics committee (NAS017-2021 and NAS022/2021). Further details are provided in the supplementary materials.

Urine collection and analysis

Urine was collected from 33 Damaraland mole-rats (9 BF_s, 5 BF_s, 13 NBF_s, 6 NBM_s) three days prior to blood sampling. Urine samples were analysed for testosterone, progesterone and cortisol using a coat-a-count kit (Diagnostic Products Corporation, Los Angeles, California, USA). All assays were conducted according to the manufacturer's protocol. Urine concentration varied due to variable fluid intake; thus, the concentrations of steroid hormones (namely testosterone, progesterone, and cortisol) had to be corrected. See supplementary material for details.

Blood sampling and analysis

Blood samples were collected from all 51 Damaraland mole-rats (18 breeders, 28 non-breeders, 5 removed) between 09h00 and 13h00. Each mole-rat was weighed prior to bleeding. The

dorsal tarsal vein was pierced using a sterile 23G needle. An EDTA-coated microhematocrit capillary tube was used to collect approximately 300 - 500µl of whole blood into a sterile EDTA tube. Complete blood counts were performed on whole blood samples in an automated haematological analyser (ADVIA 21209(i). Siemens), designed for *in vitro* use at the Clinical Pathology unit at the Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria. Manual differential cell counts were conducted and the following haematological parameters were quantified for each whole blood sample: Hgb (g/L), RCC ($\times 10^{12}/L$), Hct (L/L), MCV (fL), MCH (pg), MCHC; (g/dL) and RDW (%).

An additional 200µl of whole blood was collected into an Eppendorf tube from the 33 Damaraland mole-rats whose urine was collected. This blood was centrifugation at 1500g. The resulting plasma was analysed for prolactin as described by Bennett et al. (2018).

Data analysis

All statistical analyses were performed in R 3.5.2 [38]. The normality and homogeneity of the response variables (Hgb, RCC, Hct, MCV, MCH, MCHC, RCW) were determined using Shapiro Wilk tests and Levene's test, respectively. Log-transformation failed to normalise all non-normal data. Normally distributed dependent variables were analysed using linear models(LMs), whereas non-normally distributed dependent variables were analysed by generalised linear models (GLMs) fitted with link-log function using the *lme4* package (Bates *et al.*, 2015). Each model contained a single haematological parameter as the response variable and sex (male or female), breeding status (breeding or helper), and the two-way interactions included as predictors. Body mass and colony size were included as a covariant in all models. Additionally, further models were conducted containing a haematological parameter as the response variable and reproductive treatment (breeding or helper or removed) as predictors, with body mass included as a covariant. Post-hoc comparisons were conducted using Tukey HSD pairwise comparisons with the *emmeans* package (Lenth *et al.*, 2018). Lastly, to evaluate the effects of hormones (plasma prolactin, urinary testosterone, progesterone and cortisol) on the haematological parameters, separate GLMs fitted with link-log function were conducted. A linear regression further investigated any significant relationships between a hormone and a haematological parameter. Data are presented as mean $\pm$ standard error (S.E.M).

Results

Helper Damaraland mole-rats possessed increased oxygen-carrying capacity of the blood through increased levels of Hgb, namely due to an increased RCC, which resulted in a

higher Hct, in comparison to breeding Damaraland mole-rats ($p \leq 0.05$; Fig.1; Supplementary table 1, 2, 3). However, the observed increase in Hgb and oxygen carry capacity in helpers was not due to a change in RBC structure (represented by MPV, MCH, MCHC and RDW) ($p > 0.05$; Table 1; Supplementary table 5, 6, 7, 8), but rather due to an increase in production of RBCs. Sex or sex* breeding status did not affect Hgb, RCC, Hct, MPV, MCH or MCHC (Supplementary table 1, 2, 3, 4, 5, 6, 7). However, sex differences were seen in RDWs, with females ($16.2 \pm 0.39\%$) possessing a greater RDW than males ($15.74 \pm 0.24\%$) (Supplementary table 8); however, this is a common trend seen among mammals (Alis *et al.*, 2015). Haematological parameters were independent of body mass and colony size (Supplementary tables), which is unsurprising as both these variables do not play a role in activity displayed by breeders and non-breeders in the wild (Francioli *et al.*, 2020a).

When helpers were removed from the colony and housed individually, the oxygen-carrying capacity of the blood was reduced, through lowered levels of Hgb, RBCs and Hct in comparison to helpers within the colony ($p \leq 0.05$, Fig 1; Supplementary table 9, 10, 11, 15). While, levels of Hgb, RBCs and Hct of removed individuals were similar to breeding individuals ($p > 0.05$, Fig 1; Supplementary table 9, 10, 11, 15). Again, RBC structure did not change between breeders, helpers and removed individuals ($p > 0.05$; Table 1; Supplementary table 12, 13, 14, 15).

Only urinary progesterone was found to affect oxygen carry capacity (RCC, Hgb, Hct) (Table 2) of Damaraland mole-rats; mole-rats with higher urinary progesterone showing reduced oxygen carry capacity (RCC, Hgb, Hct) (Fig. 2, Supplementary table 16). Red blood cell morphology (namely MPV, MCH and RDW) was unaffected by relative hormone concentrations (Table 2), barring MCHC which showed a negative relationship with urinary progesterone (Table 2, Fig. 2, Supplementary table 16).

Discussion

This study indicates that helper Damaraland mole-rats possess elevated blood oxygen carrying capacities, which would support their increased cooperative behaviour labour demands, such as digging or foraging. The variation between blood oxygen carrying capacities of breeders and helpers is likely not due to RBC structural differences (as shown by MPV, MCH, MCHC and RDW), but rather a difference in rates of erythropoiesis (as shown by Hgb, RCC and Hct). Increased erythropoiesis can be caused by increased activity (Hu & Lin, 2012; Moretti *et al.*, 2018); however, as the individuals in this study had been bred in captivity for the entirety of

their lives, the metabolic cost of burrowing in wood shaving whilst having access to *ad libitum* food should have been negligible on erythropoietic production, compared to the higher energetic demand that wild colonies experience with regards to foraging, excavation, and food provision in sands. Furthermore, upon removal, the helpers show reduced Hgb, RCC and Hct levels, indicating a reduced rate of erythropoiesis, similar to that of breeders. This suggests that the dissimilar erythrocytic oxygen carrying capacity between the breeders and helpers may be a preceding physiological condition that aids helper mole-rats in their pre-assigned laborious duties and is likely related to reproductive suppression (Bennett *et al.*, 2018; Zöttl *et al.*, 2018; Vullioud *et al.*, 2021).

Removing a Damaraland mole-rat helper from their natal colony effectively released them from reproductive suppression resulting in increased levels of circulating hormones, including progesterone (Molteno & Bennett, 2000). Unsurprisingly, when comparing breeders and helpers still within their natal colony, individuals with higher progesterone levels (breeders) possessed lower Hgb, RCC and Hct levels than those possessing low concentrations of progesterone (Wallace *et al.*, 2022b). Progesterone and associated oestradiol, which are lower in helpers (Jarvis & Bennett, 1993; Voigt *et al.*, 2014, 2021), have been linked to a reduced rate of erythropoiesis (Lavrijsen & Verwilghen, 1984). Concurrently, similar erythropoietic-endocrine profiles have been observed in rats, whereby enhanced estrogen administration induced lower red blood cell parameters (Pilliero *et al.*, 1968). Recently, progesterone and oestrogen have been highlighted as critical hormonal drivers of reproductive division of labour and social behaviours in eusocial African mole-rats (Peragine *et al.*, 2017).

This study therefore furthers the understanding of the physiological mechanisms controlling the expression of cooperative behaviours. Whereby the imposed suppression of reproduction and reproductive hormones by the Damaraland mole-rats breeders on their colony non-breeding counterparts may, in part, drive increased physiological capacity for increased strenuous activity, such as digging, thus predisposing these helpers to perform cooperative behaviours. Such determinations may thus provide further clarification into the physiological drivers supporting the behavioural distinctions between reproductive classes amongst obligate cooperative breeding species such as African wild dogs (*Lycaon pictus*) and meerkats (*Suricata suricata*) (Montgomery *et al.*, 2018). Both African wild dogs and meerkats enforce reproductive suppression mechanisms and largely rely on the subordinate helper individuals that exhibit muted gonadal endocrine concentrations to rear the offspring and thus perform laborious cooperative behaviours (Montgomery *et al.*, 2018). Erythropoietic studies, in conjunction with

endocrine profile characterizations, may thus elaborate the current understanding of what shapes cooperative breeding and the associated behavioural distinctions in social animals.

Acknowledgements

The project was funded by the SARChI chair for Mammal Behavioural Ecology and Physiology, South Africa, # 64796, for the DST-NRF awarded to NCB. A special thanks to Dr Yolandi Rautenbach, Carien Muller and the rest of the Clinical Pathology team at the Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria for conducting the haematological analysis for this project.

Author Contributions

N.C.B and D.W.H designed the study. K.M.E.W., N.C.B. and D.W.H. collected the data. K.M.E.W. compiled the data. D.W.H and K.M.E.W analysed the data. K.M.E.W., N.C.B. and D.W.H. wrote the manuscript.

Competing Interests statement

No competing interests are declared.

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2035 **Figures**

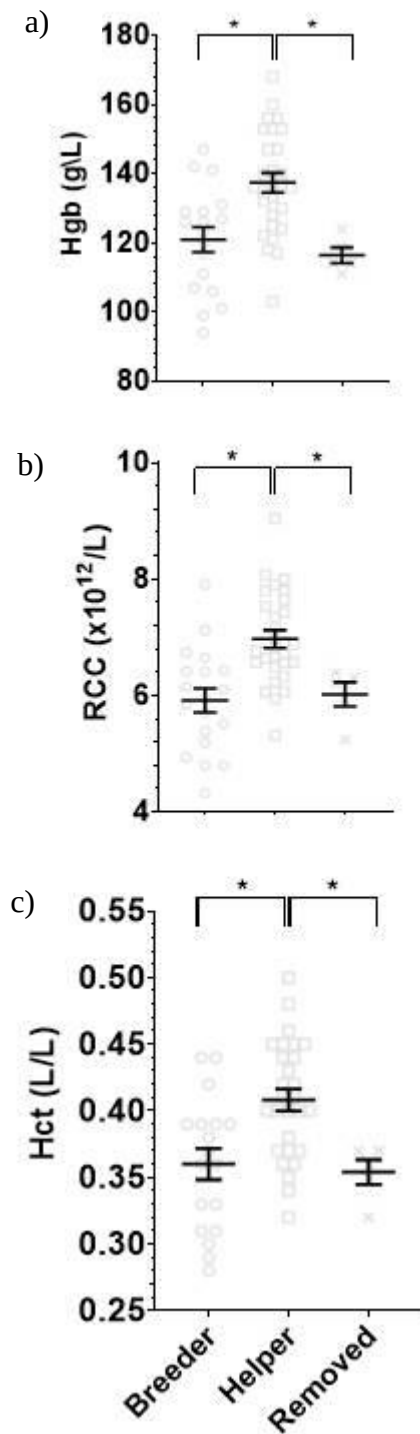
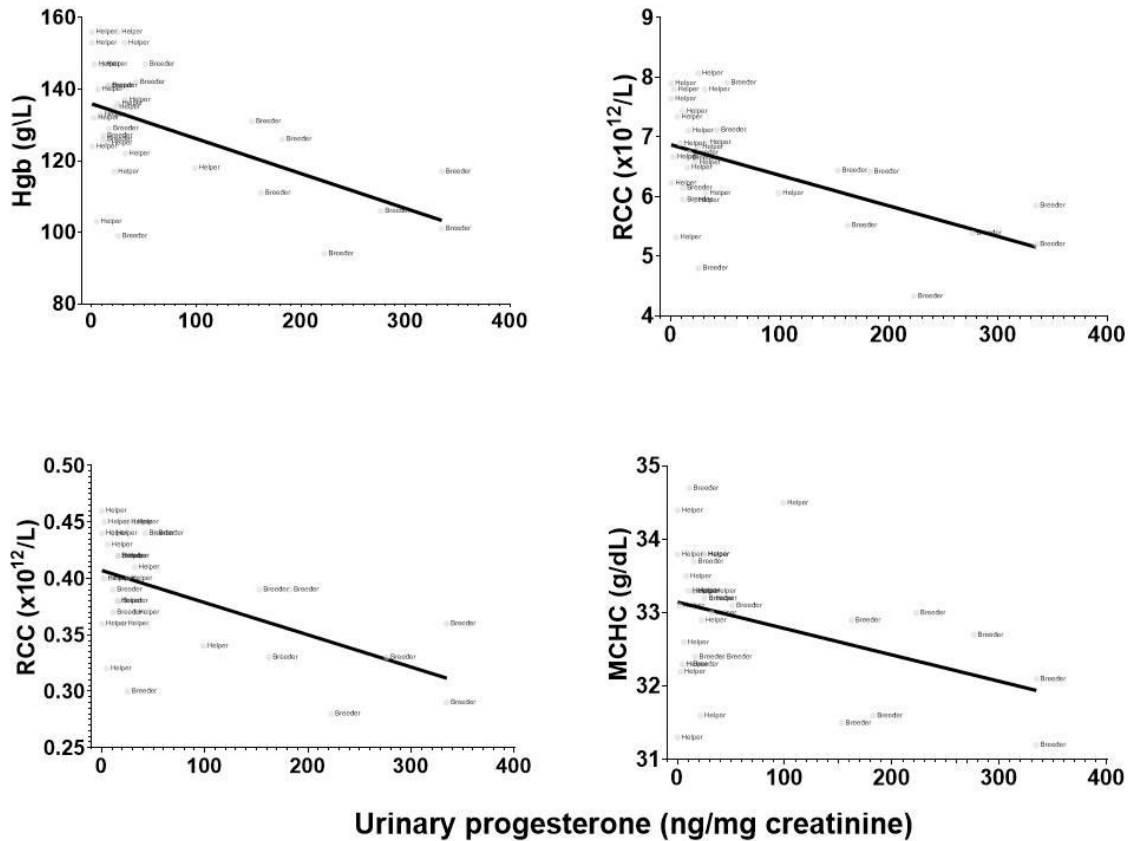


Figure 1. Haematological parameters indicating variation in oxygen-carrying capacity through a) haemoglobin (Hgb - g/L), b) red cell count (RCC - x10¹²/L) and c) haematocrit (Hct - L/L) between Damaraland mole-rats separated by breeding treatment (breeder, helper or removed). Data are presented as mean ± standard error (S.E.M.). Note: An asterisk (*) indicates significance ($p \leq 0.05$).

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2044 **Figure 2.** Linear regressions between urinary progesterone (ng/mg creatinine) and
 2045 haemoglobin (Hgb; g/L; $F = 14.3$, $p = 0.001$); red cell count (RCC; $\times 10^{12}/L$; $F = 12.4$, $p =$
 2046 0.001); haematocrit (Hct; L/L; $F = 14.4$, $p = 0.001$) and mean corpuscular haemoglobin
 2047 concentration (MCHC; g/dL; $F = 5.71$, $p = 0.02$) for 33 breeder and helper Damaraland mole-
 2048 rats.

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2051 Tables

2052

2053 **Table 1:** Haematological parameters [namely mean corpuscular volume (MPV- fL), mean
 2054 corpuscular haemoglobin (MCH - pg), mean corpuscular haemoglobin concentration (MCHC
 2055 - g/dL) and red blood cell distribution width (RDW - %)] for 51 Damaraland mole-rats,
 2056 separated by breeding treatment (breeder, helper or removed). Data are presented as mean $\pm$
 2057 standard error (S.E.M).

	Breeder	Helper	Removed
MCV (fL)	60.5±0.72	58.8±0.37	62.0±0.60
MCH (pg)	20.4±0.32	19.8±0.11	20.6±0.29
MCHC (g/dL)	33.0±0.48	33.2±0.17	33.2±0.43
RDW (%)	16.0±0.27	15.9±0.22	15.6±0.36

Table 2. Statistical output of dredge models indicating significance between hormones (testosterone: Test, ng/mg creatinine, cortisol: Cort, ng/mg creatinine, prolactin: PRL, ng/ml and progesterone: P, ng/mg creatinine) and haematological indices; haemoglobin (Hgb), haematocrit (Hct), red cell count (RCC) and mean corpuscular haemoglobin volume (g/dL) for 33 Damaraland mole-rats.

	Hgb		Hct		RCC		MCHC	
	t	p	t	p	t	p	t	p
Test	0.43	0.67	0.53	0.60	0.23	0.82	-1.17	0.25
Cort	0.62	0.54	0.54	0.59	0.65	0.52	-0.75	0.46
PRL	0.59	0.56	0.21	0.83	0.38	0.71	-0.45	0.66
P	-3.93	<0.01*	-3.87	<0.01*	-3.65	<0.01*	-2.17	0.04*

Note: An asterisk (*) indicates significance ($p \leq 0.05$)

Supplementary Material

Methods

Animal housing

The mole-rats were fed apples, sweet potatoes and carrots *ad libitum*, from which they obtained their water (Bennett & Faulkes, 2000a). They were kept in climate-controlled rooms (relative humidity:40-60%; ambient temperature: 24-26°C; 12h light:12h dark cycle).

Urine Collection

Each animal was kept in a cylindrical plastic cage with a wire-mesh base on top of the urine collection tray. The wire-mesh base prevented the contamination of the urine sample with faecal matter. Urine was unable to be collected from the five removed individuals. Apple and sweet potato slices were provided. Urine was collected with a single-use plastic pipette and frozen at -40°C .

Urinary steroid hormones

Urine samples were analysed for testosterone, progesterone and cortisol using a coat-a-count kit (Diagnostic Products Corporation, Los Angeles, California, USA). All assays were conducted according to the manufacturer's protocol. Assays were validated by testing for parallelism between serial dilutions of mole-rat plasma (obtained from an individual with high hormone concentrations) and the standard curve (Chard, 1988). The aforementioned hormone kits, including cross-reactivity, sensitivity, and protocols, have been recorded in previous studies (Hart, 2019; Hart *et al.*, 2020a, 2021).

There was no significant difference between the serial dilution curve of urinary testosterone of a BM and the calibration curve (ANCOVA: $F_{[1,5]} = 1.1$, $p = 0.53$). The intra-assay coefficient of variation was 4.7%. Furthermore, a serial dilution of urinary cortisol concentration from an individual with high cortisol paralleled the reference preparation; thus, the slopes did not differ significantly (ANCOVA: $F_{[1,5]} = 15.2$, $p = 0.22$). The intra-assay coefficient of variation was 5.5%. Similarly, no significant difference between a pregnant BF's serial dilution curve of urinary progesterone and that of the calibration curve was observed (ANCOVA: $F_{[1,5]} = 2.9$, $p = 0.39$). The intra-assay coefficient of variation for repeated determination of quality control was 7.9%.

Creatinine determination

Urine concentration varied due to variable fluid intake; thus, the concentrations of steroid hormones (namely testosterone, progesterone, and cortisol) had to be corrected. Correction was accomplished by analysing each urine sample for creatinine concentration, as creatinine is excreted at a relatively constant rate. The creatinine concentration of each urine sample was determined using a modified Jaffe reaction (Folin, 1914). Final standardised results are

presented as steroid hormone [testosterone (ng), progesterone (ng) and cortisol (µg)] per milligram of creatinine (mg creatinine).

Plasma prolactin

Plasma prolactin was quantified using an Elabscience Guinea pig Prolactin (PRL) ELISA kit (Elabscience Biotechnology Inc., Wuhan, China) (Bennett *et al.*, 2018). The sensitivity of the assay was 0.09 ng/mL plasma, and intra-assay precision and repeatability are <10%, according to the manufacturers guidelines.

Supplementary Table 1. The statistical outputs from the linear model analysis of the effects of sex (male vs. female) and breeding status (breeder vs. helper), their two-way interactions and colony size and body mass on haemoglobin (g/L) measured for 46 Damaraland mole-rats.

Variable	t-value	p-value
Sex	1.78	0.08
Breeding status	2.95	0.01*
Sex*breeding status	-0.59	0.56
Body mass	0.55	0.59
Colony size	0.68	0.50

Note: An asterisk (*) indicates significance ($p \leq 0.05$).

Supplementary Table 2. The statistical outputs from the linear model analysis of the effects of sex (male vs. female) and breeding status (breeder vs. helper), their two-way interactions and colony size and body mass on the red blood cell count ($\times 10^{12}/L$) measured for 46 Damaraland mole-rats.

Variable	t-value	p-value
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Sex	1.10	0.28
Breeding status	2.98	0.01*
Sex*breeding status	-0.38	0.71
Body mass	0.47	0.64
Colony size	0.97	0.34

Note: An asterisk (*) indicates significance ($p \leq 0.05$).

Supplementary Table 3. The statistical outputs from the linear model analysis of the effects of sex (male vs. female) and breeding status (breeder vs. helper), their two-way interactions and colony size and body mass on the haematocrit (L/L) measured for 46 Damaraland mole-rats.

Variable	t-value	p-value
Sex	1.31	0.20
Breeding status	2.81	0.01*
Sex*breeding status	-0.63	0.53
Body mass	0.52	0.61
Colony size	0.78	0.44

Note: An asterisk (*) indicates significance ($p \leq 0.05$).

Supplementary Table 4. The statistical outputs from the linear model analysis of the effects of sex (male vs. female) and breeding status (breeder vs. helper), their two-way interactions and colony size and body mass on the mean corpuscular volume (fL) measured for 46 Damaraland mole-rats.

Variable	t-value	p-value
Sex	-0.24	0.81

Breeding status	-1.35	0.18
Sex*breeding status	-0.22	0.82
Body mass	-0.19	0.85
Colony size	-0.47	0.64

Note: An asterisk (*) indicates significance ($p \leq 0.05$).

Supplementary Table 5. The statistical outputs from the generalised linear model analysis of the effects of sex (male vs. female) and breeding status (breeder vs. helper), their two-way interactions and colony size and body mass on the mean corpuscular haemoglobin (pg) measured for 46 Damaraland mole-rats.

Variable	t-value	p-value
Sex	1.50	0.14
Breeding status	-1.37	0.90
Sex*breeding status	-0.29	0.78
Body mass	0.30	0.77
Colony size	-1.20	0.24

Note: An asterisk (*) indicates significance ($p \leq 0.05$).

Supplementary Table 6. The statistical outputs from the generalised linear model analysis of the effects of sex (male vs. female) and breeding status (breeder vs. helper), their two-way interactions and colony size and body mass on the mean corpuscular haemoglobin concentration (g/dL) measured for 46 Damaraland mole-rats.

Variable	t-value	p-value
Sex	2.00	0.052

Breeding status	0.95	0.35
Sex*breeding status	-1.06	0.29
Body mass	-0.49	0.63
Colony size	-0.58	0.57

Note: An asterisk (*) indicates significance ($p \leq 0.05$).

Supplementary Table 7. The statistical outputs from the generalised linear model analysis of the effects of sex (male vs. female) and breeding status (breeder vs. helper), their two-way interactions and colony size and body mass on the red blood cell distribution width (%) measured for 46 Damaraland mole-rats.

Variable	t-value	p-value
Sex	-2.32	0.03*
Breeding status	-0.70	0.50
Sex*breeding status	1.00	0.33
Body mass	0.67	0.51
Colony size	0.30	0.77

Note: An asterisk (*) indicates significance ($p \leq 0.05$).

Supplementary Table 8. The statistical outputs from the linear model analysis of the effects of breeding treatment (breeder vs. helper vs. removed) and body mass on haemoglobin (g/L) measured for 51 Damaraland mole-rats.

Variable	t-value	p-value
Breeding treatment	3.76	0.0018*

Body mass	1.10	0.28
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Note: An asterisk (*) indicates significance ($p \leq 0.05$).

Supplementary Table 9. The statistical outputs from the linear model analysis of the effects of breeding treatment (breeder vs. helper vs. removed) and body mass on the red blood cell count ($\times 10^{12}/L$) measured for 51 Damaraland mole-rats.

Variable	t-value	p-value
Breeding treatment	4.04	0.00028*
Body mass	0.78	0.44

Note: An asterisk (*) indicates significance ($p \leq 0.05$).

Supplementary Table 10. The statistical outputs from the linear model analysis of the effects of breeding treatment (breeder vs. helper vs. removed) and body mass on the haematocrit (L/L) measured for 51 Damaraland mole-rats.

Variable	t-value	p-value
Breeding treatment	3.56	0.0018*
Body mass	0.88	0.38

Note: An asterisk (*) indicates significance ($p \leq 0.05$).

Supplementary Table 11. The statistical outputs from the linear model analysis of the effects of breeding treatment (breeder vs. helper vs. removed) and body mass on the mean corpuscular volume (fL) measured for 51 Damaraland mole-rats.

Variable	t-value	p-value
Breeding treatment	-2.24	0.03*
Body mass	-0.17	0.86

Note: An asterisk (*) indicates significance ($p \leq 0.05$).

Supplementary Table 12. The statistical outputs from the generalised linear model analysis of the effects of breeding treatment (breeder vs. helper vs. removed) and body mass on the mean corpuscular haemoglobin (pg) measured for 51 Damaraland mole-rats.

Variable	t-value	p-value
Breeding treatment	2.02	0.04*
Body mass	-0.92	0.36

Note: An asterisk (*) indicates significance ($p \leq 0.05$).

Supplementary Table 13. The statistical outputs from the generalised linear model analysis of the effects of breeding treatment (breeder vs. helper vs. removed) and body mass on the mean corpuscular haemoglobin concentration (g/dL) measured for 51 Damaraland mole-rats.

Variable	t-value	p-value
Breeding treatment	-0.36	0.72
Body mass	0.27	0.79

Note: An asterisk (*) indicates significance ($p \leq 0.05$).

Supplementary Table 14. The statistical outputs from the generalised linear model analysis of the effects of breeding treatment (breeder vs. helper vs. removed) and body mass on the red blood cell distribution width (%) measured for 51 Damaraland mole-rats.

Variable	t-value	p-value
Breeding treatment	-2.82	0.007*
Body mass	-0.42	0.68

Note: An asterisk (*) indicates significance ($p \leq 0.05$).

Supplementary Table 15. The statistical outputs from Tukey HSD pairwise comparisons of the effects of breeding treatment (Breeder [BR] vs. helper [HE] vs. removed [RE]) on the haemoglobin (Hgb: g/L), red cell count (RCC: $\times 10^{12}/L$), haematocrit (Hct: L/L), mean corpuscular volume (MCV: fL), mean corpuscular haemoglobin (MCH: pg), mean corpuscular haemoglobin concentration (MCHC: g/dL) and red blood cell distribution width (RDW-%) measured for 51 Damaraland mole-rats.

	Hgb	RCC	Hct	MCV	MCH	MCHC	RDW
BR vs HE	0.001*	0.001*	0.002*	0.08	0.0501	0.11	0.06
BR vs. RE	0.80	0.99	0.95	0.34	0.051	0.09	0.06
HE vs RE	0.01*	0.04*	0.049*	0.99	0.054	0.62	0.06

Note: An asterisk (*) indicates significance ($p \leq 0.05$).

Supplementary Table 16. The statistical outputs, using linear regressions between urinary progesterone concentration and haemoglobin (Hgb-g/L), red blood cell count (RCC - $\times 10^{12}/L$), haematocrit (Hct-L/L) and mean corpuscular haemoglobin concentration (MCHC - g/dL) respectively, 33 Damaraland mole-rats.

	Slope	y-intercept	F	p
Hgb	-0.10	135.9	14.2	0.001*
RCC	-0.005	6.87	12.36	0.001*
Hct	-0.0003	0.41	14.4	0.001*
MCHC	-0.003	33.2	5.72	0.02*

Note: An asterisk (*) indicates significance ($p \leq 0.05$).

2210 **Supplementary Table 17.** Animal details and haemoglobin (Hgb: g/L), red cell count (RCC:
2211 $\times 10^{12}/L$), haematocrit (Hct: L/L), mean corpuscular volume (MCV: fL), mean corpuscular
2212 haemoglobin (MCH: pg), mean corpuscular haemoglobin concentration (MCHC: g/dL) and
2213 red blood cell distribution width (RDW: %) values for each Damaraland mole-rat ($n = 51$). BF:
2214 breeding female, BM: breeding male, NBF: non-breeding female, NBM: non-breeding male
2215 and removed: animal removed from their natal colony and housed alone.

Mole-rat ID	Sex/status	Body mass (g)	Colony	Colony size	Hgb	RCC	Hct	MCV	MCH	MCHC	RWC
DMR1	BF	120	1	12	99.0	4.80	0.30	62.10	20.60	33.20	18.1
DMR2	BF	159	3	17	131.0	6.44	0.39	60.60	20.40	31.50	17.3
DMR3	BF	129	7	8	126.0	6.42	0.39	61.60	19.40	31.60	16.0
DMR5	BF	141	10	9	147.0	7.91	0.44	56.20	18.60	33.10	17.7
DMR6	BF	150	12	11	106.0	5.39	0.33	60.30	19.80	32.70	16.7
DMR7	BF	167	13	13	101.0	5.20	0.29	55.80	20.00	31.20	16.9
DMR8	BF	130	15	11	117.0	5.85	0.36	62.90	20.20	32.10	14.6
DMR9	BF	124	19	9	94.0	4.33	0.28	65.80	21.70	33.00	17.2
DMR10	BF	157	20	26	111.0	5.52	0.33	60.50	19.90	32.90	14.8
DMR12	BM	168	3	17	126.0	6.15	0.39	63.90	20.60	32.30	16.7
DMR13	BM	136	7	8	129.0	6.41	0.39	61.50	20.00	32.60	15.2
DMR14	BM	128	8	9	127.0	5.95	0.37	61.40	21.30	34.70	15.1
DMR15	BM	154	10	9	141.0	6.65	0.42	62.90	21.20	33.70	16.2
DMR16	BM	145	12	11	129.0	6.74	0.38	56.40	19.80	32.40	15.0
DMR17	BM	193	13	13	142.0	7.12	0.44	61.60	20.00	32.40	15.2

DMR18	BM	128	15	11	107.0	5.71	0.31	54.30	19.30	31.60	14.4
DMR19	BM	155	19	9	123.0	4.94	0.31	61.80	25.00	40.40	14.9
DMR20	BM	106	20	26	120.0	6.10	0.36	59.40	19.70	33.20	15.7
DMRK21	NBF	105	1	12	137.0	6.91	0.41	59.70	19.80	33.20	16.4
DMRK22	NBF	144	3	17	153.0	7.80	0.45	58.50	19.50	33.30	15.8
DMRK23	NBF	97	3	17	117.0	5.94	0.36	62.10	19.60	31.60	19.7
DMRK24	NBF	97	7	8	133.0	6.89	0.40	57.60	19.30	33.50	14.8
DMRK26	NBF	146	8	9	156.0	8.07	0.45	57.10	19.30	33.80	14.6
DMRK27	NBF	161	15	11	122.0	6.06	0.37	61.00	20.20	33.00	16.8
DMRK28	NBF	163	13	13	135.0	6.57	0.42	62.80	20.60	32.90	15.5
DMRK29	NBF	124	10	9	141.0	7.11	0.42	59.40	19.80	33.30	15.4
DMRK31	NBF	116	12	11	118.0	6.06	0.34	56.70	19.50	34.50	16.3
DMRK32	NBF	93	12	11	125.0	6.49	0.38	57.90	19.30	33.30	15.7
DMRK33	NBF	147	7	8	103.0	5.32	0.32	60.00	19.40	32.30	18.0
DMRK35	NBF	140	15	11	140.0	7.34	0.43	58.60	19.10	32.60	14.9
DMRK36	NBF	170	19	9	147.0	7.80	0.45	58.30	18.80	32.20	16.3
DMRK37	NBM	191	1	12	153.0	7.90	0.44	55.70	20.10	31.30	15.9
DMRK38	NBM	138	3	17	156.0	7.64	0.46	60.40	20.40	33.80	16.2
DMRK39	NBM	235	3	17	124.0	6.23	0.36	58.00	20.00	34.40	17.0
DMRK41	NBM	121	8	9	147.0	7.43	0.44	59.60	19.80	33.30	15.2
DMRK42	NBM	164	10	9	132.0	6.67	0.40	59.60	19.70	33.10	15.7
DMRK43	NBM	200	10	9	136.0	6.83	0.40	59.00	20.00	33.80	15.0

DMRK44	NBM	136	12	11	128.0	6.64	0.37	56.30	19.20	34.20	16.8
DMRK45	NBM	123	13	13	136.0	6.56	0.40	61.70	20.70	33.50	15.2
DMRK46	NBM	148	13	13	130.0	6.57	0.37	56.30	21.30	30.90	16.9
DMRK47	NBM	158	15	11	160.0	8.00	0.48	59.90	20.00	33.40	15.7
DMRK48	NBM	128	15	11	140.0	6.76	0.41	60.80	20.60	33.90	14.9
DMRK49	NBM	164	19	9	121.0	6.32	0.35	55.40	19.70	32.30	17.3
DMRK50	NBM	120	19	9	136.0	6.72	0.40	59.20	20.30	34.30	14.4
DMRK51	NBM	129	20	26	168.0	9.04	0.50	54.90	18.60	33.90	14.6
DMRK52	NBM	170	15	11	153.0	7.53	0.45	59.90	20.20	33.70	15.3
FDA	Removed	177	-	1	114.0	5.95	0.36	59.9	19.1	31.9	18.8
FDB	Removed	116	-	1	114.0	6.31	0.35	54.8	18	32.8	16.4
FDC	Removed	143	-	1	111.0	5.24	0.32	61.7	21.2	34.4	20
FDD	Removed	152	-	1	124.0	6.39	0.37	57.4	19.4	33.8	16.6
FDE	Removed	141	-	1	119.0	6.21	0.37	59.6	19.2	32.2	16.9

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2218 **Supplementary Table 18.** Animal details and urinary testosterone (ng/mg creatinine), cortisol
 2219 (ng/mg creatinine) and progesterone (ng/mg creatinine) and plasma prolactin (ng/ml)
 2220 concentrations for each Damaraland mole-rat (n = 33).

Mole-rat ID	Testosterone	Cortisol	Progesterone	Prolactin
DMR1	15,16	20,21	25,19	5,46
DMR2	789,93	59,55	152,96	4,17
DMR3	289,71	119,68	182,27	3,29
DMR5	24,16	32,21	51,25	2,55
DMR6	258,93	17,46	276,26	7,93
DMR7	163,80	145,34	334,44	2,51
DMR8	15,16	20,21	334,51	3,85
DMR9	68,58	13,09	222,48	6,81
DMR10	44,63	19,49	161,89	2,03
DMR12	374,47	19,12	10,78	13,03
DMR14	488,59	22,46	10,96	8,24
DMR15	157,66	22,83	15,49	0,10
DMR16	1026,48	20,81	16,60	2,84
DMR17	1088,41	16,56	42,11	0,10
DMRK21	96,92	104,93	31,97	0,10
DMRK22	21,45	31,07	31,27	1,03
DMRK23	25,66	99,39	21,45	3,04
DMRK24	379,31	18,61	8,23	1,17
DMRK26	14,34	40,52	24,78	16,10
DMRK27	14,15	18,86	31,83	7,95
DMRK28	24,58	108,37	22,39	1,08

DMRK29	10,82	36,90	16,02	2,37
DMRK31	20,43	71,25	98,67	3,76
DMRK32	3,15	75,56	15,03	2,43
DMRK33	14,55	6,57	4,77	0,52
DMRK35	3,40	22,61	5,88	1,27
DMRK36	2,76	3,68	2,67	0,10
DMRK37	1,90	2,53	0,18	16,54
DMRK38	2,30	3,07	0,18	4,83
DMRK39	4,67	6,22	0,37	3,50
DMRK41	215,40	172,32	10,34	5,84
DMRK42	432,24	33,59	2,02	1,37
DMRK43	165,13	64,62	24,93	1,08

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Chapter 3

Debunking the immunocompetence handicap hypothesis in male Damaraland mole-rats (*Fukomys damarensis*)

Abstract

In a subterranean, eusocial species where reproductive states (breeder vs non-breeder) are naturally delineated within each sex, Damaraland mole-rat (*Fukomys damarensis*) males provide the optimal model to explore the expected immunosuppressive effect of testosterone and cortisol. To characterise the innate immunity of each animal, blood samples were taken to determine the leukocytic constituents, their ratios and antimicrobial capacity of the blood through complete blood count haematological analyses and microbial killing assays (MKA's), respectively. Additionally, urinary testosterone and cortisol concentration profiles (ng/mg creatinine and µg/mg creatinine, respectively), and testosterone: cortisol (T/C) ratio were determined for the Damaraland mole-rat breeding- and non-breeding males. The two distinct reproductive states test the LHT, while testosterone concentrations alone directly test the ICHH, followed by the T/C ratio testing the stress-linked ICHH. The results indicate that despite breeding males (BMs) having significantly higher testosterone concentrations (BM: 628.06±162.72, NBM: 148.54±54.7 ng/mg creatinine) and T/C ratio's (BM: 33.13±9.36, NBM: 3.23±1.4), there was no difference in the male's immunological strength. This study therefore did not support either the immunocompetence handicap hypothesis (ICHH) or the stress-linked ICHH in male Damaraland mole-rats. However, males with the lowest testosterone concentrations, and thus T/C ratio's, had the highest platelet: white blood cell count ratio (PWR) indicating lower probability of sepsis and mortality risk, which could be an effect of their enhanced cooperative behaviour. This study has therefore provided an immunological basis from which the immune system of socially- and reproductively distinct mammals, such as the Damaraland mole-rats, can be explored.

Introduction

Under the life-history theory (LHT), the costly activation and maintenance of immune defence (“survival”) demands a trade-off with fellow energetic- and nutrient-necessitating functions such as growth, reproduction and thermoregulation (Zuk & Stoehr, 2002; Romo *et al.*, 2016; Schoenle *et al.*, 2018; Naim *et al.*, 2020). Accordingly, there is an inverse relationship between reproductive investment and immunological competence, whereby individual immunocompetence is determined by the organism's innate and adaptive/acquired immune responses (Sheldon & Verhulst, 1996; Romo *et al.*, 2016). Furthermore, a general pattern of sex bias in immunity has been observed, with females generally more immunocompetent than males (Folstad & Karter, 1992; Nunn *et al.*, 2009; Kelly *et al.*, 2018). The cause of this apparent sex bias in immunity has been a point of great interest for several decades, yet the underlying cause is still unclear.

The various hypotheses that address the causes of sexual dimorphism in immunocompetency highlights the apparent differences between male and female endocrine profiles, particularly the sex differences in testosterone. As such, the immunocompetence handicap hypothesis (ICHH), proposes a trade-off between the positive effects of increased testosterone (a reproductive hormone) on a male's reproductive potential and the suppressive effects on the immune system (Folstad & Karter, 1992; Foo *et al.*, 2017; O'Brien *et al.*, 2018). However, the endocrine system is a complex amalgamation of interacting hormones, and therefore the ICHH was revised to the stress-linked ICHH, which includes the interaction between testosterone and stress-related hormones, namely glucocorticoids (Folstad & Karter, 1992; Evans, Goldsmith & Norris, 2000; Roberts *et al.*, 2007). As circulating testosterone increases, levels of glucocorticoids also increase, whereby according to the stress-linked ICHH, testosterone affects the immune system via glucocorticoids, such as cortisol (mammals) or corticosterone (birds) (Folstad & Karter, 1992; Zhang & He, 2014). Short-term exposure to glucocorticoids (acute stress) enhances immunocompetence (Martin, 2009), but chronic exposure to glucocorticoids (chronic stress) has been observed to suppress the immune system (Martin, 2009; Demas & Nelson, 2012; Schoenle *et al.*, 2018). Furthering our knowledge of these two hypotheses (ICHH and the stress-linked ICHH) is critical in understanding the links between the endocrine and immune systems, and thus further aid in unravelling the sexual dimorphism in longevity seen in most mammalian species, including humans.

Recently, O'Brien et al. (2018) experimentally tested both of these hypotheses in free-roaming male Cape ground squirrels (*Xerus inauris*). O'Brien et al. (2018) artificially increased circulating testosterone and subsequently measured changes in cortisol and several innate immune proxies, namely *in-vitro* bacterial growth inhibition (through microbial killing assays; MKAs) and the measurement of white blood cell (WBC) differentials. O'Brien et al. (2018) concluded that their study did not support either the ICHH or stress-linked ICHH, since exogenous testosterone did not alter any immune proxy and did not affect the cortisol levels of the males. However, possible reasons for these results may be due to shortcomings within the experimental setup. As this was a field study, a number of unavoidable and possibly unknown factors, including interaction with other animals, pathogens and stressors, may have influenced the results. Furthermore, artificially manipulating reproductive effort via exogenous testosterone may not represent natural processes. Thus a more controlled and natural study is needed to support or challenge the finding of O'Brien et al. (2018).

This study proposes the eusocial African mole-rat species, the Damaraland mole-rat (*Fukomys damarensis*), as the perfect model species to evaluate the ICHH and stress-linked ICHH. As reproduction is naturally controlled within the species, no experimental manipulation is required for individuals who do not breed. In Damaraland mole-rats, a few dominant animals monopolise reproduction, with one female and one to three larger males (breeding males; BMs) being solely responsible for reproduction (Jarvis & Bennett, 1993; Cooney & Bennett, 2000). The remaining colony members (non-breeding females and non-breeding males; NBFs and NBMs, respectively) are reproductively quiescent (Bennett & Faulkes, 2000a), where both sexes are able to reproduce, but are naturally suppressed through behavioural and physiological mechanisms (Bennett *et al.*, 1996; Bennett & Faulkes, 2000a; Clarke *et al.*, 2001a; Bennett & Jarvis, 2004). Damaraland mole-rats are obligate outbreeders, and as such, the high relatedness and familiarity of colony members due to overlapping generations translates into inbreeding avoidance as a strong factor dissuading the reproduction of non-breeding males (Burland *et al.*, 2002; Kelley *et al.*, 2019). While recent studies have distinguished neuroendocrine receptor divergences between the male Damaraland mole-rats (Voigt *et al.*, 2016; Voigt & Bennett, 2021), there is very little physiological or anatomical evidence, beyond reproductive activity and dominance hierarchies, that distinguish between breeding- and non-breeding males (Jacobs *et al.*, 1991; Jarvis & Bennett, 1993). However, a recent study on Damaraland mole-rats indicated elevated testosterone concentrations in BMs and a higher testosterone-cortisol ratio demonstrating higher dominance levels in the breeders

(Wallace *et al.*, 2022b). Furthermore, Damaraland mole-rats are often successfully housed and bred in sterile laboratory conditions, thus eliminating the possibility of unavoidable and unknown factors affecting their immune system. Therefore, the captive-bred male Damaraland mole-rats are the ideal model to test both the ICHH and the stress-linked ICHH.

This study, using captive-bred male Damaraland mole-rats, attempted to evaluate the ICHH and stress-linked ICHH by comparing various innate immune proxies between BMs and NBMs. Such proxies include *in-vitro* bacterial growth inhibition experiments (through MKA's), the measurement of white blood cell (WBC) differentials and leukocytic ratios. The prognostic use of leukocytic ratios in immunologic studies has become increasingly prolific due to the ease of its inexpensive and non-invasive testing. The neutrophil-to-lymphocyte ratio (NLR), monocyte-to-lymphocyte ratio (MLR), neutrophil-to-monocyte ratio (NMR) and platelet-to-lymphocyte ratio (PLR) has each been reported as indicators of inflammation, disease severity, mortality, and tumorigenesis (Fang *et al.*, 2019; Gao *et al.*, 2019; Liu *et al.*, 2020; Stefaniuk, Szymczyk & Podhorecka, 2020; Seyit *et al.*, 2021; Zhang, Hu & Hu, 2021a). Immunocompromised individuals will often exhibit high NLR, MLR and PLR (Gao *et al.*, 2019; Russell *et al.*, 2019; Liu *et al.*, 2020). In contrast, a low platelet count (PC) to white blood cell count (WCC) ratio (PWR) has been associated with infection and higher severities of liver failure and ischemic strokes (Jie *et al.*, 2018; Amalia & Dalimonthe, 2020). As such, these leukocytic ratios are being increasingly utilised as haematological diagnostic indices for immune activity across multiple disciplines. Furthermore, urinary testosterone and cortisol was measured just prior to the measurement of the innate immune proxies giving this study the ability to link these hormones and immune strength. Based on the principles set by the ICHH and stress-linked ICHH, this study therefore predicts that the male Damaraland mole-rats with the highest testosterone concentrations, the breeders, will have the highest immunocompromised parameters.

Materials and Methods

Study species

This study used 26 adult male Damaraland mole-rats, 10 BMs and 16 NBMs, from 10 separate captive-bred colonies in the mole-rat laboratory at the University of Pretoria. All animals were fed *ad libitum* and housed in large polyurethane crates (1m x 0.5m x 0.5m), stocked with wood shavings and paper towelling for nesting material in climate-controlled rooms (humidity: 40-

60%; ambient temperature: 24-26°C). Anatomical, morphological, behavioural, and historical records were used to distinguish between reproductive classes. Experimental procedures were approved by the animal ethics committee of the University of Pretoria (NAS017-2021 and NAS022/2021).

Sample collection

Blood and urine from all 26 individuals were sent for haematological and endocrinological analyses, while the MKA's used blood samples from a subset of 20 individuals (10 BMs and 10 NBMs). Each mole-rat was weighed and identified through subcutaneous PIT tags upon separation for sample collection.

Urine samples were collected by separating all 26 Damaraland mole-rats into plastic, cylindrical chambers that had an elevated mesh-floor above the collection tray. Animals were given apple and sweet potato while separated. Upon excretion (usually 15 minutes), urine was pipetted into 1.5mL Eppendorf tubes and stored at -80°C until hormonal analyses were conducted, and the male was returned to their colony. All samples were collected in the morning between 09:00 and 12:00 to avoid daily rhythms of hormone secretion.

Three days post urine collection, whole blood was collected from all males. Before blood collection, a sterile bleed site was ensured by thoroughly swabbing the individual's hindfoot with Biotaine (0.5% Chlorhexidine gluconate, 70% ethanol; Braun Medical PTY Ltd, Reg. no. 33/13.1/0526) and allowing it to dry. The dorsal tarsal vein was pricked using a sterile 23G needle, and a microhaematocrit capillary tube was used to collect the blood into sterile, 1.5mL Eppendorf tubes or a heparinised collection tube. Blood sample volumes ranged from 400 - 600µl of whole blood, depending on the animal's body size, whereby 300 - 400µl of whole blood was dedicated to haematological analyses, approximately 100µl was committed to MKA's. Only 1% of the total body mass of the individual blood was collected, as permitted by the University of Pretoria Animal Ethics Committee. Difficulty bleeding some animals was combatted by prioritising blood for MKAs and diluting whole blood (1:3) for haematological analyses. Samples were kept at 4°C and used within 2 hours of collection for the antimicrobial assays.

2398

2399 ***Microbial killing assay procedure***

2400 Due to the small volume of blood available per mole-rat, the MKA procedure utilised in this
2401 study followed the protocol demonstrated by Millet et al (2007) and DeRogatis et al (2020),
2402 with modifications (Millet *et al.*, 2007; DeRogatis *et al.*, 2020). Blood was found to have a
2403 high inherent bacterial load (K. Wallace, unpublished results), thus necessitating the use of
2404 antibiotic infused media to be used in the ensuing MKA's. The restrictive nature of microbiota
2405 in blood and the resultant use of antibiotic-infused media prevented the use of fellow "golden
2406 standard" microbes, *Candida albicans* and *Staphylococcus aureus*, thus ensuring *Escherichia*
2407 *coli* as the microbial challenge to enable comparison between similar studies. Each antibiotic
2408 evaluated had specific antibiotic-resistant *E. coli* strains associated with it. A tetracycline
2409 working concentration of 0.075g/5mL of nuclease-free water proved the most effective in
2410 deterring blood microbial growth, thus indicating the use of SURE competent tetracycline-
2411 resistant *E. coli* (Agilent, #200238) as the microbe used in the killing assays. SURE *E. coli* was
2412 reconstituted from a stock culture, which was grown and re-streaked on tetracycline-infused
2413 tryptic soy agar (TTSA) every week to ensure optimal growth, resistance, and activity of
2414 bacteria. The working solution concentration was pre-determined through optimal growth
2415 experiments in conjunction with the optical density under specific conditions. A bacterial
2416 working solution of 10⁴ colony forming units per millilitre (CFU/mL) of tryptic soy broth was
2417 made fresh each day before blood collection and serially diluted ten-fold before use in MKA.

2418 Whole blood was diluted 1:4 with L-glutamine and CO₂-independent media (Sigma-
2419 Aldrich NO. G3126 and #18045; Gibco-Invitrogen, CA respectively; L&C, 4mM) to aid in
2420 leukocyte maintenance, therefore aliquots of 15µl whole blood: 60µl L&C. The amount of
2421 bacterial working solution to incorporate was determined as one-tenth of the sample volume,
2422 therefore, 7.5 µl diluted bacterial working solution was pipetted into 75µl diluted blood and
2423 vortexed. Once combined, the inoculated samples were incubated in a water bath at 41°C for
2424 30 minutes. The total volume of this inoculum was pipetted and spread onto TTSA and
2425 incubated at 37°C for 24 hours following inversion, after which, the amount of CFU were
2426 quantified for each sample. Samples were run in duplicate. Positive controls constituted
2427 definite growth of 7.5 µl diluted SURE *E.coli* working solution on TTSA plates following
2428 incubation. Two negative controls were done for each individual; (i) 15µl of whole blood was
2429 spread onto TTSA and (ii) a combination of 15µl whole blood and 60µl of L-glutamine and

CO₂-independent media (4mM) was spread onto a TTSA plate. All procedural pipette- and plating techniques were conducted within sterile laminar flow hoods under aseptic conditions.

Haematological analyses (Complete blood counts)

The Clinical Pathology unit (Department of Companion Animal Clinical studies) at the Veterinary Science Faculty, University of Pretoria performed the automated haematological analyses and manual differential cell counts. An *in-vitro* automated haematological analyser (ADVIA 21209(i). Siemens) was used to determine the complete blood cell counts of each Damaraland mole-rat. Quantification of white cell count (WCC; $\times 10^9/L$), segmented neutrophil (N; $\times 10^9/L$), lymphocyte (L; $\times 10^9/L$), eosinophil (E; $\times 10^9/L$), basophil (B; $\times 10^9/L$), monocyte (M; $\times 10^9/L$) and platelet count (PC; $\times 10^9/L$) was conducted on each whole blood sample. From these results, ratios of neutrophil-to-lymphocyte (NLR), monocyte-to-lymphocyte (MLR), platelet-to-lymphocyte (PLR), neutrophil-to-monocyte (NMR), and complete platelet-to-white cell count (PWR) were determined.

Endocrinological Analyses (Hormone profiles)

Urinary steroid hormones

Urine samples were analysed for testosterone and cortisol using a coat-a-count kit (Diagnostic Products Corporation, Los Angeles, California, USA). All assays were conducted according to the manufacturer's protocol. Assays were validated by testing for parallelism between serial dilutions of mole-rat urine (obtained from an individual with high hormone concentrations) and the standard curve (Chard, 1988). The aforementioned hormone kits, including cross-reactivity, sensitivity, and protocols, have been recorded in previous studies (Hart, 2019; Hart *et al.*, 2020a, 2021).

There was no significant difference between the serial dilution curve of urinary testosterone of a BM and the calibration curve (ANCOVA: $F_{[1,5]} = 1.1$, $p = 0.53$). The intra-assay coefficient of variation was 4.7%. Furthermore, a serial dilution of urinary cortisol concentration from an individual with high cortisol paralleled the reference preparation; thus, the slopes did not differ significantly (ANCOVA: $F_{[1,5]} = 15.2$, $p = 0.22$). The intra-assay coefficient of variation was 5.5%.

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2462

2463 *Creatinine determination*

2464 Urine concentration varied due to variable fluid intake; thus, the concentrations of steroid
 2465 hormones (namely testosterone and cortisol) had to be corrected. Correction was accomplished
 2466 by analysing each urine sample for creatinine concentration, as creatinine is excreted at a
 2467 relatively constant rate. The creatinine concentration of each urine sample was determined
 2468 using a modified Jaffe reaction (Folin, 1914). Final standardised results are presented as steroid
 2469 hormone [testosterone (ng) and cortisol (µg)] per milligram of creatinine (mg creatinine).

2470

2471 **Data analysis**

2472 Statistical analyses were performed on R 2022.02.0, Microsoft Excel (Version 2205) and
 2473 Graphpad Prism 8.4.3. Statistical significance was denoted by $p \leq 0.05$, and data is presented
 2474 as mean $\pm$ standard error (S.E.M). Antimicrobial capacity from the MKAs was determined as
 2475 the proportion (%) of the *E.coli* inoculum that was killed, calculated as $[1 - (\text{Positive control}$
 2476 $\text{CFU} - \text{Inoculated sample CFU})/\text{Positive control CFU}]$, where the positive control CFU
 2477 indicated the quantity of bacteria before the inclusion and interaction of blood. Segmented
 2478 neutrophil, lymphocyte, eosinophil, basophil, and monocyte data was presented as percentage
 2479 (%) of the total number of WCC. The division of segmented neutrophils ($\times 10^9/\text{L}$) and
 2480 monocytes ($\times 10^9/\text{L}$) by lymphocytes ($\times 10^9/\text{L}$) produced the neutrophil: lymphocyte and
 2481 monocyte: lymphocyte ratio's (NLR and MLR, respectively). Division of the platelet count
 2482 ($\times 10^9/\text{L}$) by the lymphocyte count ($\times 10^9/\text{L}$) and white cell count ($\times 10^9/\text{L}$) create the platelet:
 2483 lymphocyte and platelet: white cell count ratio's (PLR and PWR, respectively). Segmented
 2484 neutrophil ($\times 10^9/\text{L}$) was divided by monocyte ($\times 10^9/\text{L}$) to create the neutrophil: monocyte ratio
 2485 (NMR). Corrected urinary testosterone concentrations (ng/mg creatinine) were divided by
 2486 corrected urinary cortisol concentrations (µg/mg creatinine) to create the testosterone: cortisol
 2487 (T/C) ratio.

2488 The normality and homogeneity of the immunological and endocrine dependant
 2489 variables (antimicrobial capacity (%), white cell count ($\times 10^9/\text{L}$), segmented neutrophil (%),
 2490 monocyte (%), lymphocyte (%), eosinophil (%), basophil (%), platelet count ($\times 10^9/\text{L}$), NLR,
 2491 MLR, PLR, NMR, PWR, urinary testosterone (ng/mg creatinine) and cortisol (µg/mg

creatinine) and the T/C ratio) were determined using Shapiro-Wilk and Levene's tests, respectively. The NLR, MLR, and urinary cortisol data had to be log-transformed into a normal distribution, while the monocyte, eosinophil, basophil, NMR, urinary testosterone, and T/C ratio data failed to become normally distributed even after log transformation.

Linear models were used to analyse normally distributed dependant variables, while generalised linear models with gamma distributions and link-identity functions from the *lme4* package were used to analyse non-normal dependant variables. All models possessed colony size and body mass as covariates and status (breeding and non-breeding) as the predictor variables. Further linear regression analysis were conducted on any significant covariate (body mass and/or colony size). Lastly, Spearman rank correlations were conducted to determine the occurrence of covariance between urinary testosterone and cortisol and the T/C ratio and all immunological dependants.

Results

Effect of reproductive status on endocrine profiles

Breeding males possessed higher urinary testosterone concentrations and T/C ratios compared to their non-breeding counterparts (Table 1, Fig. 1.a,b.). Comparatively, colony size and body mass did not significantly affect urinary testosterone concentrations and T/C ratios (Table 1). In contrast, the breeding status had no effect on urinary cortisol levels (Table 1, Fig 1.c.), however, both colony size (Slope = -0.12, $F = 6.68$, $p = 0.02$) and body mass (Slope = -0.01, $F = 6.80$, $p = 0.02$) affect urinary cortisol levels (Table 1). Males that were heavier had lower urinary cortisol levels, similarly, males from larger colonies possessed lower urinary cortisol concentrations.

Effect of reproductive status on innate immunity

There was no significant difference between breeding and non-breeding Damaraland mole-rat males for all quantitative innate immunity parameters: white cell count (WCC; $\times 10^9/L$), segmented neutrophils (N; %), lymphocytes (L; %), monocytes (M; %), eosinophils (E; %), basophils (B; %), platelet count (PC; $\times 10^9/L$) and their corresponding ratios (NLR, MLR, PLR, NMR and PWR; Fig. 2, Table 2., Table 3. and Table 4). Neither was there a difference in the

antimicrobial capacity between the males ($t = -0.78, p = 0.45$), whereby neither colony size nor body mass were influential in affecting microbial killing ability ($t = 1.07, p = 0.30$ and $t = 2.02, p = 0.06$, respectively; Fig. 3). As a consequence, colony size and body mass did not affect either of the immunological parameters or leukocytic ratio's (Table 2., Table 3.).

Effect of reproductive-related hormones on innate immunity

The platelet count-white cell count ratio (PWR) had a significant, negative correlation to both urinary testosterone concentrations (ng/mg creatinine) and the T/C ratio (Table 5., Fig.4.a. and Fig.4.b.). The antimicrobial capacity (%) of the Damaraland mole-rat males was not correlated to any of the reproductive-related hormones (Table 5.).

Discussion

Similar to the findings of O'Brien et al. (2018), this current study did not support either the ICHH, or the stress-linked ICHH. Breeding and non-breeding males possessed similar innate immune proxies, even though breeders possessed higher testosterone levels, therefore calling into question the ICHH. Previously, controlling for stress-related hormones (glucocorticoids) resulted in a significant effect of testosterone on the immune system (see Evans *et al.*, 2000). However, despite correcting for differences in cortisol in this study, via the T/C ratio, breeding and non-breeding males still possessed similar innate immune proxies. Additionally, if cortisol alone was affecting the immune system, similar negative patterns between the innate immune proxies and colony size and body mass, respectively, would have been seen in urinary cortisol; this was not the case.

O'Brien et al. (2018) suggested that low circulating (plasma) levels of testosterone (1.88ng/ml) in the Cape ground squirrels allow them to avoid the trade-off between testosterone and immunity. This could be the case for Damaraland mole-rats as well. Maswanganye et al. recorded plasma testosterone of only 0.2ng/l in both breeding and non-breeding Damaraland mole-rats (Maswanganye *et al.*, 1999). As in the Cape ground squirrel, all male Damaraland mole-rats, non-breeders have similar-sized testes and degree of spermatogenesis to breeders (Jarvis & Bennett, 1993; Faulkes *et al.*, 1994; Voigt *et al.*, 2016), to maintain reproductive readiness year round. Therefore, the lower levels of testosterone in this species could be a means to cope with continually having to be reproductively active and thus avoiding the high

costs of testosterone production and the trade-offs between testosterone and immune competence (O'Brien *et al.*, 2018). Variation between endocrine profiles between breeding and non-breeding male Damaraland mole-rats are therefore likely related to social dominance and sexual behaviour rather than reproductive readiness and investment (Lutermann *et al.*, 2013; Wallace *et al.*, 2022b).

Interestingly, there was a significant correlation between the PWR and testosterone, whereby individuals with the highest testosterone concentrations had the lowest PWR. Similarly, the more dominant individuals, those with higher T/C ratios (breeders), also had the lowest PWR. Low PWR has recently been identified as a prognostic tool for indicating sepsis and elevated mortality risk in post-operative and liver failure patients (Lathouras *et al.*, 2019; Zhang *et al.*, 2020). Non-breeding individuals, are often termed 'helpers' or 'workers' due to their elevated cooperative care involving pup rearing, foraging, tunnel maintenance and colony defence (Bennett, 1990; Scantlebury *et al.*, 2006b). Furthermore, non-breeders have been observed to be 50% more active than their breeding counterparts (Scantlebury *et al.*, 2006b; Kotze, Bennett & Scantlebury, 2008). Therefore, the likelihood of a non-breeding male (with low testosterone and T/C ratio) encountering an intruder and thus resulting in an aggressive altercation is higher than the breeding male. As such, an increased PWR would be beneficial in decreasing the risk of mortality after an aggressive altercation.

This study serves as a foundation from which the immune system of reproductively distinct mammals, such as the Damaraland mole-rat, can be used to further elucidate the effect of endocrine and reproductive systems on the survival and persistence of a species. However, further research studies on the Damaraland mole-rat, including manipulative studies, are needed to investigate other hypotheses within the life history theory, including the immune suppression hypothesis.

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Figures

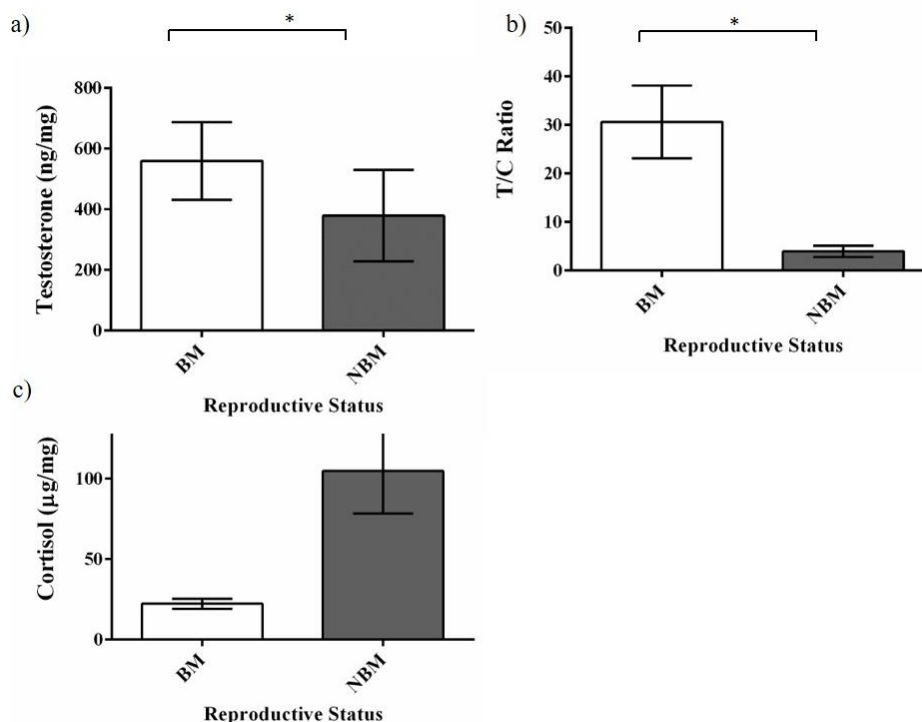


Figure 1. Bar graphs showing the difference in a) urinary testosterone concentrations (ng/mg creatinine), b) the urinary testosterone: cortisol ratio (T/C) and c) the urinary cortisol concentrations ($\mu\text{g}/\text{mg}$ creatinine) between Damaraland mole-rat breeding males (BM) and non-breeding males (NBM), after colony size and body mass have been accounted for. Significance is denoted by an asterisk ‘*’

Figure 2. Bar graph displaying the difference in leukocytic composition (%) between breeding and non-breeding Damaraland mole-rat males in terms of segmented neutrophils, lymphocytes, monocytes, and eosinophils as immunological indicators. No significant differences were found. Data are presented as mean $\pm$ S.E.M.

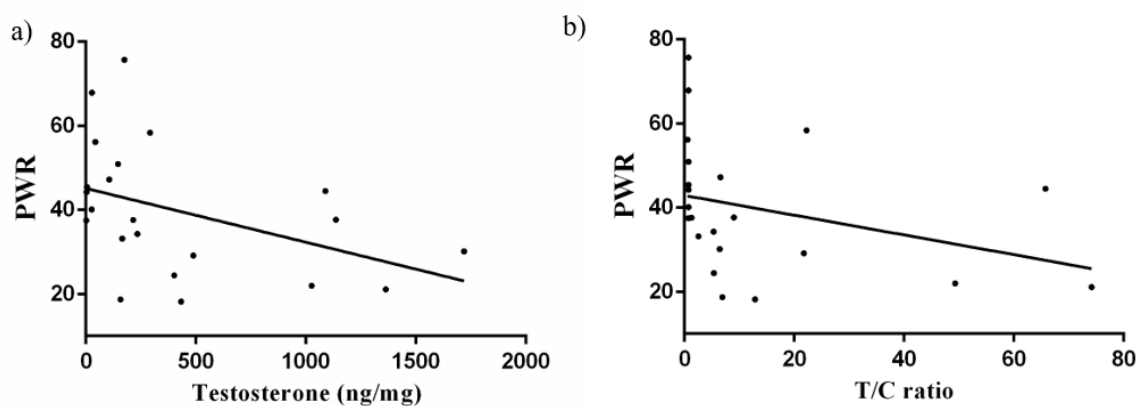


Figure 3. Microbial killing capacity (%) of breeding and non-breeding male Damaraland mole-rat blood. Data are presented as mean $\pm$ S.E.M.

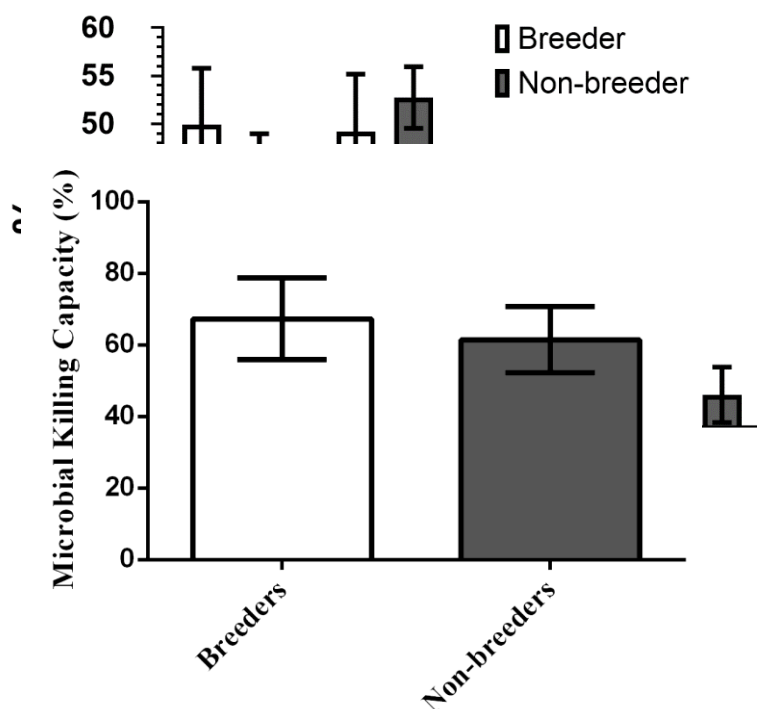


Figure 4. Linear regression lines demonstrating the negative, significant relationships between the platelet count-white cell count ratio (PWC) and a) testosterone concentrations (ng/mg creatinine) and b) the urinary testosterone-cortisol ratio (T/C) in male Damaraland mole-rats.

Tables

Table 1. Statistical output of linear regression indicating effect of colony size, body mass, and breeding status (breeder vs non-breeder) on the urinary testosterone concentrations (ng/mg creatinine), cortisol concentrations ($\mu\text{g}/\text{mg}$ creatinine) and the testosterone: cortisol ratio (T/C) in male Damaraland mole-rats.

	Testosterone		Cortisol		T/C	
	t-value	p-value	t-value	p-value	t-value	p-value
Colony size	0.03	0.87	5.63	0.03*	0.20	0.66
Body mass	3.27	0.07	4.65	0.05*	0.001	0.98
Breeding status	4.23	0.04*	3.90	0.06	26.2	0.001*

Significance is denoted by an asterisk '*’.

Table 2. Linear model statistical output indicating the role of body mass (BM), colony size (CS) and reproductive state (Status) on the innate immunological parameters: white cell count (WCC; $\times 10^9/\text{L}$), segmented neutrophils (N; %), lymphocytes (L; %), monocytes (M; %), eosinophils (E; %), basophils (B; %), platelet count (PC; $\times 10^9/\text{L}$).

	WCC		N		L		M		E		B		PC	
	t	p	t	p	t	p	t	p	t	p	t	p	t	p
BM	-0.53	0.60	-1.01	0.33	0.99	0.34	0.22	0.83	-0.33	0.75	-0.53	0.60	-0.21	0.84
CS	0.18	0.86	1.83	0.08	-1.93	0.07	0.40	0.69	0.29	0.78	0.06	0.95	0.87	0.40
Status	0.11	0.91	-0.77	0.45	0.68	0.50	0.70	0.49	0.01	0.10	1.10	0.28	1.84	0.08

Significance ($p \leq 0.05$) is indicated with an asterisk '*’.

Table 3. Linear model statistical output indicating the role of body mass (BM), colony size (CS) and reproductive state (Status) on the innate immunological leukocytic ratio's: segmented neutrophil-to-lymphocyte ratio (NLR), segmented neutrophil-to-monocyte ratio (NMR), monocyte-to-lymphocyte ratio (MLR), platelet-to-lymphocyte ratio (PLR) and platelet-to-white cell count ratio (PWR).

	NLR		NMR		MLR		PLR		PWR	
	t	p	t	p	t	p	t	p	t	p
BM	-1.42	0.17	-0.04	0.97	0.06	0.95	-0.77	0.45	-0.05	0.96
CS	1.96	0.06	1.28	0.22	0.48	0.64	1.15	0.26	0.34	0.74
Status	-1.09	0.29	-0.47	0.65	0.45	0.66	0.32	0.75	1.75	0.10

Significance ($p \leq 0.05$) is indicated with an asterisk '*'.

Table 4. Mean $\pm$ S.E.M. of breeding (BM) and non-breeding (NBMs) male Damaraland mole-rats immunological parameters: white cell count (WCC; $\times 10^9/L$) and platelet count (PC; $\times 10^9/L$) and the leukocytic ratios: segmented neutrophil-to-lymphocyte ratio (NLR), segmented neutrophil-to-monocyte ratio (NMR), monocyte-to-lymphocyte ratio (MLR), platelet-to-lymphocyte ratio (PLR) and platelet-to-white cell count ratio (PWR).

	WCC	PC	NLR	NMR	MLR	PLR	PWR
BM	8.45 $\pm$ 1.00	255 $\pm$ 36.5	1.26 $\pm$ 0.26	24.9 $\pm$ 10.6	0.02 $\pm$ 0.01	78.3 $\pm$ 19.4	31.0 $\pm$ 4.37
NBM	8.44 $\pm$ 0.60	342 $\pm$ 23.5	0.99 $\pm$ 0.13	17.97 $\pm$ 5.88	0.03 $\pm$ 0.01	86.8 $\pm$ 9.22	43.0 $\pm$ 3.69

Table 5. Correlation statistical output indicating the relationships between microbial killing ability (MKA), the segmented neutrophil-to-lymphocyte ratio (NLR), segmented neutrophil-to-monocyte ratio (NMR), monocyte-to-lymphocyte ratio (MLR), platelet-to-lymphocyte ratio (PLR), platelet-to-white cell count ratio (PWR) and reproductive-related hormones: urinary testosterone (ng/mg creatinine), cortisol (μ g/mg creatinine), progesterone (ng/mg creatinine) and plasma prolactin (ng/ml).

	T		C		T/C	
	r	p	r	p	r	p
NLR	0.03	0.90	-0.17	0.46	0.03	0.91

NMR	-0.33	0.14	-0.25	0.27	-0.29	0.19
MLR	-0.14	0.52	-0.03	0.91	-0.25	0.26
PLR	-0.31	0.15	-0.10	0.64	-0.31	0.16
PWC	-0.42	0.05*	0.00	0.99	-0.42	0.05*
MKA	0.19	0.46	-0.34	0.18	0.31	0.22

2761 Spearman's rho ('r) indicates the correlative strength and statistical significance ($p \leq 0.05$) is
 2762 indicated by an asterisk '*'.
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Chapter 4

Long live the queen: Dominant females possess increased reproductive capacity and immune competence in social African mole-rat species

Abstract

The trade-off between self-maintenance and reproduction is exaggerated in group-living species. Breeding individuals are responsible for offspring and are usually the more dominant group members but show reduced investment into self-maintenance. Within the social species of the family Bathyergidae, some of the most extreme social hierarchies and degrees of reproductive skew occur with reproduction monopolised by one breeding female (the queen) per colony. The remaining subordinate female members are reproductively suppressed in their natal family colony. This study investigated the inequality in female reproductive readiness and dominance (using endocrinological proxies- progesterone: cortisol (Prog/Cort) and testosterone: cortisol ratio (Test/Cort), respectively) and immune competency in the Damaraland mole-rat. As predicted, queens possessed higher reproductive readiness and dominance (higher Prog/Cort and Test/Cort levels) than subordinate females. Surprisingly, several immune strength biomarkers indicated that queens possessed increased immune competency, including neutrophil: lymphocyte and normal levels of monocytes. This was confirmed by a heightened ability of the queen to clear bacterial infection, measured through a bacteria-killing assay when compared to subordinate counterparts. Another social species, the common mole-rat exhibited increased queen immune competency. Social African mole-rats show some of the most extreme life-history inequalities, with dominant females having a higher rate of reproductive success and an increased likelihood of survival.

1. Introduction

Widespread evidence has indicated reduced fertility in individuals under pathogenic challenges, while an increase in reproduction induces a drop in immune strength (Naim *et al.*, 2020). Accordingly, as per the evolutionary ageing-orientated disposable soma theory, the immunity-fertility axis infers an inverse relationship between reproductive investment and immunocompetence-related aging (Sheldon & Verhulst, 1996). Succinctly, biological reproduction involves significant nutrition investments that could have been invested in somatic maintenance for survival (Kirkwood & Rose, 1991; Hsu *et al.*, 2021). Therefore, higher fertility, reproductive capacity and output come with the costs of lower lifespan or higher late-life mortality (Hsu *et al.*, 2021).

However, in extreme animal societies where breeding dominance hierarchies form, the dominant members are often more fertile than their reproductively suppressed, subordinate counterparts (Jarvis *et al.*, 1994; Sherman *et al.*, 1995; Faulkes *et al.*, 1997). According to the somatic evolutionary theory of ageing, these dominant breeders should have a reduced longevity (Kirkwood & Rose, 1991). Yet, this is not the case for some social African mole-rat species, particularly those that fall on the eusocial end of the sociality spectrum, such as the naked (*Heterocephalus glaber*) and Damaraland (*Fukomys damarensis*) mole-rats (Jarvis, 1981; Jarvis *et al.*, 1994). Several species of African mole-rat exhibit cooperative breeding and possess an extreme reproductive skew, with the naked mole-rat having an estimated 99% of individuals in the population never attaining a breeding role (Bennett & Faulkes, 2000b). Consequently, a single breeding female, the queen, physiologically monopolises breeding with 2-3 male consorts, but also has the longest lifespan (Horvath *et al.*, 2022). This is uncommon among vertebrates and rather resembles the structure of eusocial insect societies more so than in vertebrates, where reproduction typically shortens lifespan (Schwander *et al.*, 2010). The physiological costs of reproduction often impair the functionality of other physiological processes by reducing resources available for self-maintenance, but also through direct destructive effects resulting from reproduction itself (Harshman & Zera, 2007; Speakman, 2008). Nevertheless, the mechanisms underlying the extraordinarily long lives of breeders and particularly the queen are still unclear.

Immunocompetence has often been considered a proxy for survival and longevity, as an individual's ability to mount an immune response to pathogenic invasion provides vital resistance to disease and infection (Graham *et al.*, 2011; French & Neuman-lee, 2012). The

immune system is thus responsible for maintaining homeostasis and recognising self from non-self through innate and adaptive/acquired mechanisms. The innate immune system provides non-specific protection against pathogens through anatomical, physiological, phagocytic, and inflammatory defences (Gameiro *et al.*, 2010; Vrachnis *et al.*, 2014). On the other hand, the adaptive response is more specific, with a high memory capacity through humoral components such as antibodies and T- and B- lymphocytes (Gameiro *et al.*, 2010; Vrachnis *et al.*, 2014). Innate components primarily consist of cell-mediated and humoral components such as dendritic cells, natural killer (NK) cells, the complement cascade and leukocytic constituents; monocytes, macrophages, neutrophils, basophils, and eosinophils (granulocytes) (Pulendran *et al.*, 2001a; Zuk & Stoeckl, 2002). Ratios of these leukocytic components have proven invaluable as tools for diagnosing the degree of inflammation, disease severity, mortality and tumorigenesis (Fang *et al.*, 2019; Gao *et al.*, 2019; Liu *et al.*, 2020; Stefaniuk *et al.*, 2020; Seyit *et al.*, 2021; Zhang, Hu & Hu, 2021b). Consequently, a high neutrophil-to-lymphocyte ratio (NLR), monocyte-to-lymphocyte ratio (MLR) and platelet-to-lymphocyte ratio (PLR) are incredibly informative in identifying immunocompromised patients due to infection and risk stratification uses (Gao *et al.*, 2019; Russell *et al.*, 2019; Liu *et al.*, 2020). Similarly, a low neutrophil-monocyte ratio (NMR) and platelet count (PC) to white blood cell count (WCC) ratio (PWR) has been used for risk stratification of infection, immune dysfunction, the severity of liver failure and ischemic strokes (Jie *et al.*, 2018; Amalia & Dalimonthe, 2020; Stefaniuk *et al.*, 2020).

The comprehensive bidirectional relatedness of immune and endocrine systems, particularly the reproductive endocrine system, is demonstrated by the localisation of stress and sex hormone receptors on immune cells (lymphocytes, macrophages and granulocytes), thus enabling steroid hormones to modulate immune expression directly (Danel *et al.*, 1983; Cutolo *et al.*, 1996; Miller & Hunt, 1996; McKay & Cidlowski, 1999; Vegeto *et al.*, 1999; Roberts, Walker & Alexander, 2001; Webster, Tonelli & Sternberg, 2002; Klein, 2004). The immunosuppressive effect of testosterone, despite its bipotential fitness in males, has been experimentally demonstrated to decrease female immune function, thus indicating a possible inhibitory mechanism of androgen receptors on immune cells (Benten *et al.*, 1997). Additionally, while there are reports of a stimulatory action of oestrogen on the cytokine-, antibody- and helper T-cell facilitated responses, contradictory analyses have indicated immunosuppressive effects of oestrogen on cell-mediated immune responses (Luster *et al.*, 1984; Satoskar & Alexander, 1995; Miller & Hunt, 1996; Remoue *et al.*, 2001). Moreover,

while some studies have indicated an immuno-enhancing role of progesterone, it is primarily considered to have immunosuppressive functions, particularly on macrophage-, antibody- and NK cell activity (Furukawa *et al.*, 1984; Toder *et al.*, 1984; Baley & Schacter, 1985; Rai, 1998; McKay & Cidlowski, 1999; Diodato *et al.*, 2001). The immunosuppressive effect of progesterone is most apparent during pregnancy, whereby the female has increased susceptibility to infection following elevated progesterone concentrations needed to support pregnancy (Piccinni *et al.*, 1995, 2000; Naim *et al.*, 2020). Furthermore, the ability of progesterone to bind to the prolific glucocorticoid receptors on immune cells may aid this inhibitory pathway (Miller & Hunt, 1996; Klein, 2004). Ultimately, glucocorticoids are responsible for resource distribution and have an enhanced function on immune responses at an acute level (Dhabhar & McEwen, 1999; Dhabhar, 2000). However, chronically high glucocorticoid concentrations suppress the function of innate-, cell-mediated and humoral immune responses by inhibiting the synthesis of the required immunological components (Elenkov & Chrousos, 1999; Sapolsky, Romero & Munck, 2000; Webster *et al.*, 2002; Zuk & Stoehr, 2002).

Other proxies for survival and longevity that have also been observed to be affected by the endocrine system include the degree of body fat in an individual, as measured by the body mass index (BMI) and the Lee index (Lovejoy, 1998; Gaesser, 1999). Increased BMI and Lee index values are an indication of raised body fat, which can negatively affect health and longevity (Gaesser, 1999). Body fatness, and thus the BMI and Lee index of an individual, can be affected by various biological parameters, including circulating levels of steroid hormones, such as testosterone, progesterone, and glucocorticoids (Lovejoy, 1998). Increased glucocorticoids such as cortisol often result in heightened body fatness (Van der Valk, Savas & van Rossum, 2018). At the same time, raised levels of testosterone (Diaz-Arjonilla *et al.*, 2009) and progesterone (Toth *et al.*, 2001) result in reduced, healthier levels of body fatness.

In African social mole-rats, queens and female non-breeding subordinates show vastly different physiological reproductive investments, which result in vastly different endocrine profiles (Wallace *et al.*, 2022b). In some social species, such as the common mole-rat (*Cryptomys hottentotus hottentotus*), incest avoidance and possible increased testosterone-mediated aggression towards female subordinates by the queen results in reproductive suppression in female subordinates (behavioural suppression) (Spinks, Bennett & Jarvis, 1999). This increased aggression is also believed to increase glucocorticoids, such as cortisol, which disrupts the Hypothalamic-Pituitary-Gonadal (HPG) axis, subsequently reducing

progesterone and oestrogen concentrations in female subordinates (Spinks *et al.*, 1999; Hart *et al.*, 2022). While in the eusocial Damaraland mole-rats, a combination of incest avoidance (behavioural suppression) and a reduced pituitary sensitivity to gonadotropin-releasing hormone (GnRH) (physiological suppression), rather than queen aggression, have resulted in reproductive suppression in female subordinates (Bennett *et al.*, 1996; Molteno & Bennett, 2000; Clarke *et al.*, 2001a). Additionally, Damaraland mole-rats are induced ovulators whereby the prevention of copulation and reproductive behaviour in non-breeding females ensures their physiological suppression and condition of anoestrus (Cooney & Bennett, 2000; Molteno & Bennett, 2000; Clarke *et al.*, 2001a; Voigt *et al.*, 2021). The queen is therefore reproductively active, enabled by complete ovulatory cyclicity, high progesterone and oestrogen concentrations, while the ovulation of non-breeding females is blocked at the follicle maturation stage, producing low progesterone and oestrogen profiles (Jarvis & Bennett, 1993; Bennett *et al.*, 1996; Molteno & Bennett, 2000; Clarke *et al.*, 2001a; Voigt *et al.*, 2014, 2021). Additionally, while Damaraland mole-rat queens possess increased testosterone, this does not result in increased queen aggression or elevated levels of circulating glucocorticoids in subordinates (Wallace *et al.*, 2022b). Variations between the levels of testosterone between queens and subordinate female Damaraland mole-rats are therefore likely related to social dominance and sexual behaviour rather than aggression (Wallace *et al.*, 2022b). Interestingly, in species that solely use behavioural suppression, such as the common mole-rat, pituitary sensitivity to an exogenous GnRH challenge is similar between queens and female subordinates (Spinks *et al.*, 2000).

Correspondingly, breeding Damaraland mole-rats, especially the queens, have lifespans and reproductive success rates that far surpass the non-reproductive individuals (Schmidt *et al.*, 2015). Schmidt *et al.* (2015) found breeding females to live, on average, five times longer than her non-breeding counterparts. This contradicts a central tenet of the LHT which insists individuals that exhibit early and frequent reproduction, as with Damaraland mole-rat queens, should have shorter lives due to compromised resource partitioning and immune function (Chown & Gaston, 1997; Lutermann & Bennett, 2008). Similarly, in several phylogenetically related social, subterranean African mole-rats (Bathyergidae, Rodentia), the despotic breeders have demonstrated extended lifespans in comparison to the subordinate non-breeders, despite their respective reproductive investments (Dammann *et al.*, 2011; Begall *et al.*, 2021; Sahm *et al.*, 2021).

Therefore, this study's objective was to better understand the inequality of life-history traits, namely breeding and longevity, in social African mole-rats. This study, therefore, set out to record the likely differences between queen and subordinate female health, immune and endocrine profiles of eusocial Damaraland mole-rats. This was done by determining the progesterone, testosterone, and cortisol concentrations and the progesterone: cortisol (Prog/Cort; reproductive readiness proxy) and testosterone: cortisol ratios (Test/Cort; social dominance phenotype proxy) of Damaraland mole-rats. Next, we attempted to observe if breeding status plays a role in overall health and degree of body fat in female Damaraland mole-rats by measuring the BMI and Lee index of queens and subordinate females. We further attempted to observe if the distinct endocrine profiles of queens and subordinate females affect the BMI and Lee index of individuals. Lastly, we attempted to explain the variation in longevity between queens and subordinate female Damaraland mole-rats by evaluating the immunocompetency of each breeding status, through the measure of the innate immune system (haematological diagnostic analyses such as white blood cell counts and ratios), in addition to the microbial killing capacity of their blood. The *in-vitro* microbiocidal capacity of blood was determined through microbial killing assays (MKAs), which measure the general function of immune components such as phagocytes, opsonising proteins, and antibodies at removing pathogens (French & Neuman-lee, 2012; DeRogatis *et al.*, 2020; Solak *et al.*, 2020). We attempted to confirm if immunocompetency does in fact lead to increased longevity of queens by comparing the immunocompetency of common mole-rat queens and female subordinates. The common mole-rat was used to provide contrast to the physiologically suppressed non-breeding Damaraland mole-rat females, as while the common mole-rat also has a despotic, cooperative breeding system, the subordinate non-breeders are not reproductively downregulated through physiological mechanisms. We thus predict that while it is the opposite of what is dictated by the life history theory, the Damaraland mole-rat queens will have the highest immune competency, as echoed by their extended lifespans and superior body conditions. However, due to the decreased strength of behavioural reproductive suppression compared to physiological suppressive mechanisms, we expect the immune-endocrinal variations between reproductive classes in Common mole-rats to be less than what is determined for the Damaraland mole-rat females.

2. Methods

2.1. Study species

Twenty-four adult female Damaraland mole-rat, comprising nine queens and 15 subordinates from ten captive-bred colonies from the mole-rat laboratory at the University of Pretoria (25.7545° S, 28.2314° E), South Africa, were used in this study. In addition, a further 14, seven queens and seven subordinates, adult female common mole-rat, were captured using Hickman live traps, baited with a small piece of sweet potato (Hickman, 1979) from Steinkopf (29.2602° S, 17.7340° E) and Kamieskroon (30.2195° S, 17.9236° E), Northern Cape, South Africa. 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 for the day and left overnight, being checked first thing in the morning. All animals were group housed in large polyurethane crates (1m x 0.5m x 0.5m) with wood shavings and paper towelling for nesting material. Animals were maintained at ~25°C and 50% humidity with a 12 L:12D light cycle. Animals were fed fresh tubers and vegetables *ad libitum*.

In the wild-caught social common mole-rat, all individuals below 40 g with a dark coat colour were considered juveniles. The juveniles were not considered further for this study. The queens possessed prominent auxiliary teats and a perforated vagina, which was absent in the subordinate females. In Damaraland mole-rat captive colonies, long-term observational records along with prominent auxiliary teats and a perforated vagina were used to confirm the identity of the queens. All Damaraland mole-rat queens had produced at least five litters prior to the onset of sampling. Furthermore, a female subordinate had not bred and was still housed within their natal colony. All individuals used for this experiment were adults (> 500 days old).

All common mole-rats were in captivity for approximately one month before the start of this study. Before sampling, Damaraland mole-rats and common mole-rats were weighed to the nearest 0.01 g using a digital scale (Scout Pro SPU123; Ohaus Corporation, Pine Brook, New Jersey, USA). Furthermore, a standard tape measure was used to measure each *F. damarensis* body length (nose to anus; cm). The body mass index (BMI; g/cm) and the Lee index (g/cm) (Eq. 1 & 2) were calculated for all Damaraland mole-rats as recommended by Novelli et al. (2007) (Novelli *et al.*, 2007).

$$Eq. 1: BMI = \frac{Body\ mass}{(Body\ length)^2}$$

$$Eq. 2: Lee\ index = \frac{\sqrt[3]{Body\ mass}}{Body\ length}$$

Experimental procedures were approved by the animal ethics committee of the University of Pretoria (NAS016/2021, NAS017-2021 and NAS022/2021).

2.2. Urine collection and hormonal analysis

Urine samples were collected from all 24 female Damaraland mole-rats by placing each female into a plastic, cylindrical chamber with an elevated mesh-floor above the collection tray. Each female was placed separately in the collection chamber and given apple and sweet potato. Upon excretion (usually within 15 minutes), urine was pipetted into 1.5mL Eppendorf tubes and stored at -80°C until hormonal analyses were conducted. Then, the females were returned to their colony.

Urine samples were analysed for testosterone, progesterone and cortisol using a coat-a-count kit (Diagnostic Products Corporation, Los Angeles, California, USA). All assays were conducted according to the manufacturer's protocol. Assays were validated by testing for parallelism between serial dilutions of mole-rat plasma (obtained from an individual with high hormone concentrations) and the standard curve (Chard, 1988). The aforementioned hormone kits, including cross-reactivity, sensitivity, and protocols, have been recorded in previous studies (Hart, 2019; Hart *et al.*, 2020b, 2021).

There was no significant difference between the serial dilution curve of an individual with high urinary testosterone and the calibration curve (ANCOVA: $F_{[1,5]} = 1.1$, $p = 0.53$). The intra-assay coefficient of variation was 4.7%. Furthermore, a serial dilution of urinary cortisol concentration from an individual with high cortisol paralleled the reference preparation; thus, the slopes did not differ significantly (ANCOVA: $F_{[1,5]} = 15.2$, $p = 0.22$). The intra-assay coefficient of variation was 5.5%. Similarly, no significant difference between a queen serial dilution curve of urinary progesterone and that of the calibration curve was observed (ANCOVA: $F_{[1,5]} = 2.9$, $p = 0.39$). The intra-assay coefficient of variation for repeated determination of quality control was 7.9%.

Urine concentration varied due to variable fluid intake; thus, the concentrations of steroid hormones (namely testosterone, progesterone, and cortisol) had to be corrected. The correction was accomplished by analysing each urine sample for creatinine concentration, as creatinine is excreted at a relatively constant rate. The creatinine concentration of each urine sample was

determined using a modified Jaffe reaction (Folin, 1914). Final standardised results are presented as steroid hormone [testosterone (ng), progesterone (ng) and cortisol (μ g)] per milligram of creatinine (mg creatinine).

Corrected urinary testosterone concentrations (ng/mg creatinine) were divided by corrected urinary cortisol concentrations (μ g/mg creatinine) to create the testosterone: cortisol (Test/Cort) ratio (a proxy of socially dominant behaviour). While urinary progesterone concentrations (ng/mg creatinine) were divided by corrected urinary cortisol concentrations (μ g/mg creatinine) to create the progesterone: cortisol (Prog/Cort) ratio (a proxy of reproductive readiness).

2.3. Blood collection

Whole blood was collected from all 24 female Damaraland mole-rats, three days after urine collection, and all 14 common mole-rats. Before blood collection, a sterile bleed site was ensured by thoroughly swabbing the female hindfoot with Biotaine (0.5% Chlorhexidine gluconate, 70% ethanol; Braun Medical PTY Ltd, Reg. no. 33/13.1/0526) and allowing it to dry. Next, the dorsal tarsal vein was pierced using a sterile 23G needle. Finally, an EDTA-coated microhematocrit capillary tube was used to collect the whole blood into a sterile EDTA tube.

2.3.1. Plasma prolactin

From all 24 female Damaraland mole-rats a sub-sample of whole blood was centrifuged at 1500g. Plasma prolactin was then quantified using an Elabscience Guinea pig Prolactin (PRL) ELISA kit (Elabscience Biotechnology Inc., Wuhan, China), as described by Bennett et al. (2018). The sensitivity of the assay was 0.09 ng/mL plasma, and intra-assay precision and repeatability are <10%, according to the manufacturers guidelines.

2.3.2. Complete blood count

Complete blood counts were performed on all 24 Damaraland mole-rats and 14 common mole-rats whole blood samples in an automated haematological analyser (ADVIA 21209(i)).

Siemens), designed for *in vitro* use at the Clinical Pathology unit at the Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria. Quantification of white cell count (WCC; $\times 10^9/L$), segmented neutrophil (N; %), lymphocyte (L; %), eosinophil (E; %), basophil (B; %), monocyte (M; %) and platelet count (PC; $\times 10^9/L$) was conducted on each whole blood sample. From these results, ratios of neutrophil-to-lymphocyte (NLR), monocyte-to-lymphocyte (MLR), platelet-to-lymphocyte (PLR), and complete platelet-to-white cell count (PWR) were determined.

2.3.3. Microbial killing assay (MKA)

Whole blood from a subset of 15 female Damaraland mole-rats, six queens and nine subordinates, and all 14 female common mole-rats were used to determine the microbial killing capacity of each species blood.

Due to the small volume of blood available per mole-rat, the microbial killing assay (MKA) procedure used in this study followed the protocol demonstrated by Millet et al (2007) and DeRogatis et al (2020), with modifications. Damaraland mole-rat blood was found to have a high inherent bacterial load (K. Wallace, unpublished results), thus necessitating the use of antibiotic infused media to be used in the ensuing MKAs. The restrictive nature of microbiota in Damaraland mole-rat blood and the resultant use of antibiotic-infused media prevented the use of fellow “golden standard” microbes, *Candida albicans* and *Staphylococcus aureus*, thus ensuring *Escherichia coli* as the microbial challenge to enable comparison between similar studies. Each antibiotic evaluated had specific antibiotic-resistant *E. coli* strains associated with it. A tetracycline working concentration of 0.075g/5mL of nuclease free water proved the most effective in deterring Damaraland mole-rat blood microbial growth, thus indicating the use of SURE competent tetracycline-resistant *E. coli* (Agilent, #200238) as the microbe used in the killing assays. SURE *E. coli* was reconstituted from a stock culture, which was grown and re-streaked on tetracycline-infused tryptic soy agar (TTSA) every week to ensure optimal growth, resistance, and activity of bacteria. The working solution concentration was pre-determined through optimal growth experiments in conjunction to the optical density under specific conditions. A bacterial working solution of 104 colony forming units per millilitre (CFU/mL) of tryptic soy broth was made fresh each day before blood collection and serially diluted ten-fold before use in MKA.

Whole blood was diluted 1:4 with L-glutamine and CO₂-independent media (Sigma-Aldrich NO. G3126 and #18045; Gibco-Invitrogen, CA respectively; L&C, 4mM) to aid in leukocyte maintenance, therefore aliquots of 15µl whole blood: 60µl L&C. The amount of bacterial working solution to incorporate was determined as one tenth of the sample volume, therefore 7.5 µl diluted bacterial working solution was pipetted into 75µl diluted blood and vortexed. Once combined, the inoculated samples were incubated in a water bath at 41°C for 30 minutes. The total volume of this inoculum was pipetted and spread onto TTSA and incubated at 37°C for 24 hours following inversion, after which, the amount of CFU were quantified for each sample. Samples were run in duplicate. Positive controls constituted definite growth of 7.5 µl diluted SURE *E.coli* working solution on TTSA plates following incubation. Two negative controls were done for each individual; (i) 15µl of whole blood was spread onto TTSA and (ii) a combination of 15µl whole blood and 60µl of L-glutamine and CO₂-independent media (4mM) was spread onto a TTSA plate. All procedural pipette- and plating techniques were conducted within sterile laminar flow hoods under aseptic conditions.

All procedures were repeated for common mole-rat blood samples. Data is presented as percentage (%) *E. coli* killed or prevented from growing.

2.4. Data analysis

Statistical analyses were performed with R 2022.02.0 and Graphpad Prism 8.4.3. Statistical significance was denoted by $p \leq 0.05$, and data are presented as mean $\pm$ standard error (S.E.M). In addition, data were checked for normality (Shapiro-Wilk test) and equal variance (Levene's Test). Differences between Damaraland mole-rat queens and subordinates' hormonal parameters (plasma prolactin, urinary testosterone, progesterone and cortisol and Test/Cort and Prog/Cort ratios) and body matrixes (body mass, BMI, and Lee index) were investigated using either unpaired *t*-tests or Mann-Whitney U test. Similarly, differences between innate immune strength proxies (MKA, WCC, N, L, E, B, M, PC, NLR, MLR, PLR and PWR) of queens and subordinates of Damaraland mole-rat and common mole-rat, respectively, were investigated using either unpaired *t*-tests or Mann-Whitney U test. Spearman-rank correlations between all hormonal parameters and body matrixes were conducted for all Damaraland mole-rat females. Likewise, Spearman-rank correlations between all hormonal parameters and innate immune strength proxies were conducted for all Damaraland mole-rat females. Lastly, Spearman-rank

correlations between MKA and BMI and Lee index, separately, were conducted for all Damaraland mole-rat females.

3. Results

3.1. Hormonal parameters

Plasma prolactin, urinary testosterone, and cortisol did not differ between Damaraland mole-rat queens and subordinates (Supplementary table 1). In contrast, queens possessed greater urinary progesterone concentrations in comparison to subordinates (Mann-Whitney $U = 6$, $p = 0.0001$), which led to an increase in reproductive readiness (Prog/Cort) in queens compared to subordinates (Mann-Whitney $U = 4$, $p = 0.0001$, Fig. 1). Similarly, queens showed a higher social dominance phenotype (Test/Cort) compared to their non-breeding counterparts (Mann-Whitney $U = 29$, $p = 0.02$, Fig. 1).

3.2. Body matrixes

Queens and subordinate Damaraland mole-rats did not differ in body mass ($t = 1.32$, $p = 0.20$), but differed in their BMI ($t = 2.34$, $p = 0.03$) and Lee index ($t = 3.36$, $p = 0.002$, Fig. 2). Queens possess lower BMI and Lee indices than the subordinates. A negative relationship between Lee index and urinary progesterone was observed, where individuals with high microbial killing ability scores high urinary progesterone concentrations (namely queens) have a lower Lee index (Supplementary Table 2). No other significant correlations were seen between hormonal parameters and body high microbial killing ability scores matrixes (Supplementary Table 2).

1.1. Innate immune strength proxies

The blood of queens from both Damaraland and common mole-rats possessed greater microbial killing abilities compared to subordinate females (Damaraland mole-rat: Mann-Whitney $U = 10$, $p = 0.05$; common mole-rat: $t = 2.48$, $p = 0.03$, Fig. 3). Damaraland mole-rat queens also possessed lower NLR ($t = 2.08$, $p = 0.05$, Fig. 3) but higher monocyte prevalence ($t = 2.65$, p

= 0.02, Fig. 3) compared to subordinate females. However, no pattern was seen in common mole-rats with respect to the other parameters of immune strength (Fig. 3) (NLR: Mann-Whitney U = 19, p = 0.54; monocyte prevalence: Mann-Whitney U = 20, p = 0.61; Fig. 3). All remaining innate immune strength proxies (WCC, N, L, E, B, M, PC, PLR and PWR) were similar between common mole-rats queens and subordinates (Supplementary table 3). Likewise, WCC, N, L, E, PC, MLR PLR and PWR were similar between Damaraland mole-rat queens and female subordinates (Supplementary table 4). However, Damaraland mole-rat queens possessed a lower percentage of B compared to female subordinates but a higher MLR ratio (Supplementary table 4). No significant correlations were observed between Damaraland mole-rat plasma prolactin, urinary cortisol and progesterone and any innate immune strength proxy (Supplementary table 5). While, Test/Cort showed a significant positive relationship with PC, M and MLR (Supplementary table 5). Individuals with higher Test/Cort (queens) possessed higher PC, M and MLR values. Similarly, a significant positive relationship was observed between Prog/Cort and M and PWR, respectively (Supplementary table 5). Female Damaraland mole-rat with higher Prog/Cort (queens) possessed higher M and PWR values. In contrast, a significant negative relationship between Prog/Cort and B were seen, with individuals with higher Prog/Cort (namely queens) possessing lower B (Supplementary table 5). Interestingly, both urinary testosterone and progesterone concentrations had a positive relationship with MKA (%) of Damaraland mole-rat; with females of higher urinary testosterone and progesterone levels (queens) possessing greater microbial killing abilities (Supplementary table 5). Both BMI ($r = 0.50$, $p = 0.08$) and Lee index ($r = -0.56$, $p = 0.05$) showed a negative relationship with MKA, with females with lower BMI and Lee index (namely queens) possessing higher MKAs.

4. Discussion

As with eusocial insects, the queens of social African mole-rat groups possess not only increased reproductive output, capacity, and fertility, but also possess many traits, including increased immunocompetency and decreased body matrixes, that may explain their increased longevity compared to their subordinates.

Not only are Damaraland mole-rat queens more reproductively active than their subordinate counterparts, but they also have the best chance of survival in terms of health and immune strength indices. Reproductive monopolisation was characterised by the queens'

dominant position in the hierarchy of the queen (high Test/Cort) and their advanced reproductive readiness (high Prog/Cort). Subsequently, reproductively quiescent subordinate females had higher BMI and Lee index values than their queens, therefore indicating that the reproductive females are in better health and may have greater survivorship than the subordinates. This is interesting since the subordinate females are the lowest in the hierarchy; and are kept the most physically active through cooperative behaviours such as burrowing and foraging activity (Bennett, 1990; Scantlebury *et al.*, 2006b; Zöttl *et al.*, 2018). Due to this, subordinate females would thus be expected to be the most physically healthy. It is likely that due to being in captivity, non-breeders are not able to expend as much energy as they would normally in the wild. Since non-breeders are predisposed to increased activity and work, through progesterone, (see Wallace, Bennett & Hart, 2022a) their sedentary lifestyle in captivity has increased their BMI and Lee index values. Wallace *et al.* (n.d) observed that lower levels of progesterone in non-breeding Damaraland mole-rats resulted in increased oxygen-carrying abilities of their blood, thus predisposing these non-breeders to strenuous work. Without the ability to work (due to captivity), but still under the influence of progesterone, subordinates would gain unhealthy fat.

Furthermore, the longevity-related survivorship of Damaraland mole-rat queens is supported by the elevated immune capabilities of their blood, in conjunction with their leukocytic constituent profiles. Damaraland mole-rat queen possessed low neutrophil: lymphocyte ratio that portrays low inflammation within their immune system (Zöttl *et al.*, 2018; Liu *et al.*, 2020; Stefaniuk *et al.*, 2020). Subsequently, Damaraland mole-rat female subordinates showed below average (2-8%) percentage of monocytes, but a comparably high NLR, indicating increased susceptibility to infection – a trait not shared with the common mole-rat. A possible reason for this species-specific difference in leukocyte variation may be due to the use of physiological reproductive suppression in female Damaraland mole-rats versus only behavioural suppression in female subordinate common mole-rats. This difference in suppression strength may result in the variation of immunological parameters. However, it could be additionally suggested that the elevated BMI and Lee index in subordinate Damaraland mole-rat females may be the cause of their impaired and thus divergent leukocytic immunocompetence (Frel *et al.*, 2020). Yet, the common mole-rat was in captivity for less than a month (and thus has not yet become fully habituated to captivity) and they show a similar immunocompetency pattern between queens and subordinates. It is thus unlikely that the diminished immunity of subordinate Damaraland mole-rats is caused by the degree of body fat

and “unhealthiness” but is rather a result of lowered male and female sex hormones (testosterone and progesterone, respectively) simultaneously inducing a heightened BMI and Lee index, while impairing immune proxies.

When progesterone and testosterone concentrations are high, as found in Damaraland mole-rat queens, so is the microbial killing ability of their blood; likely through activated phagocytic-, opsonising protein-, and antibody- components. Further, this study demonstrated that high progesterone levels are correlated to low Lee indexes, which itself is correlated to high microbial killing ability (MKA) scores, therefore reinforcing the health and survivability of Damaraland mole-rat queens through hormonal-interlinked body condition and immunity. Interestingly, both Damaraland and common mole-rat queens possessed increased microbial killing ability, compared to their subordinates. Recently, an apparent trend has been observed in all studied social African mole-rat species, whereby queens possess higher circulating testosterone than non-breeding females (Spinks *et al.*, 2000; Lutermann *et al.*, 2013; Medger *et al.*, 2019; Hart *et al.*, 2022; Wallace *et al.*, 2022b). The immunosuppressive effect of testosterone is well known (Benten *et al.*, 1997), yet recently, females with raised levels of testosterone have an increased protective capacity against infections (Rivero *et al.*, 2002; Marin *et al.*, 2010). However this only seems to be the case in conjunction with other sex hormones, such as oestrogen and progesterone (Rivero *et al.*, 2002). Queens of both species mate with unrelated males and will have increased female reproductive hormones (progesterone and oestrogen), through pregnancy and ovulation, compared to their reproductively suppressed counterparts. As such, the combination of increased progesterone and oestrogen along with increased testosterone is likely to result in increased microbial killing ability of the queens.

Similar endocrine-immunocompetency results have been observed in the only other eusocial mammal, the naked mole-rat (Bégay *et al.*, 2022). Bégay *et al.* (2022) observed increased immunocompetency in non-breeding colony members of a higher rank compared to a lower ranked non-breeding colony member; where these high-ranked colony members often possessed increased female (progesterone and oestrogen) and male (testosterone) reproductive hormones compared to lower ranked non-breeding colony members (Clarke & Faulkes, 1997, 1998). However, even though it has been documented that females that possess increased levels of both female and male reproductive hormones show increased defence against infection (Rivero *et al.*, 2002), further research is needed in this realm.

Social vertebrates and invertebrates often oppose well-established evolutionary theories by exhibiting clear reproductive and lifespan inequalities through the existence of long-lived dominant breeders. In the case of the Damaraland mole-rats, and other social African mole-rats such as the common mole-rat, increased longevity is likely due to the endocrine profiles related to being socially dominant and reproductively active. This study has therefore provided a heightened insight into the physiological mechanisms that aid the extended longevity and reproductive superiority of eusocial Damaraland mole-rat queens. Considering recent findings in humans (*Homo sapiens*), the importance of a person's perceived social standing in a society can directly affect their longevity (Hamlat *et al.*, 2022), health (De Vogli, 2004) and reproductive output (Fieder & Huber, 2007). The physiological underpinning of how social living could cause inequality of evolutionary success, via reproduction and longevity, is currently not fully understood in mammals. But the study of social African mole-rat species may be critical to address this dearth of knowledge and address such naturally occurring inequalities.

5. References

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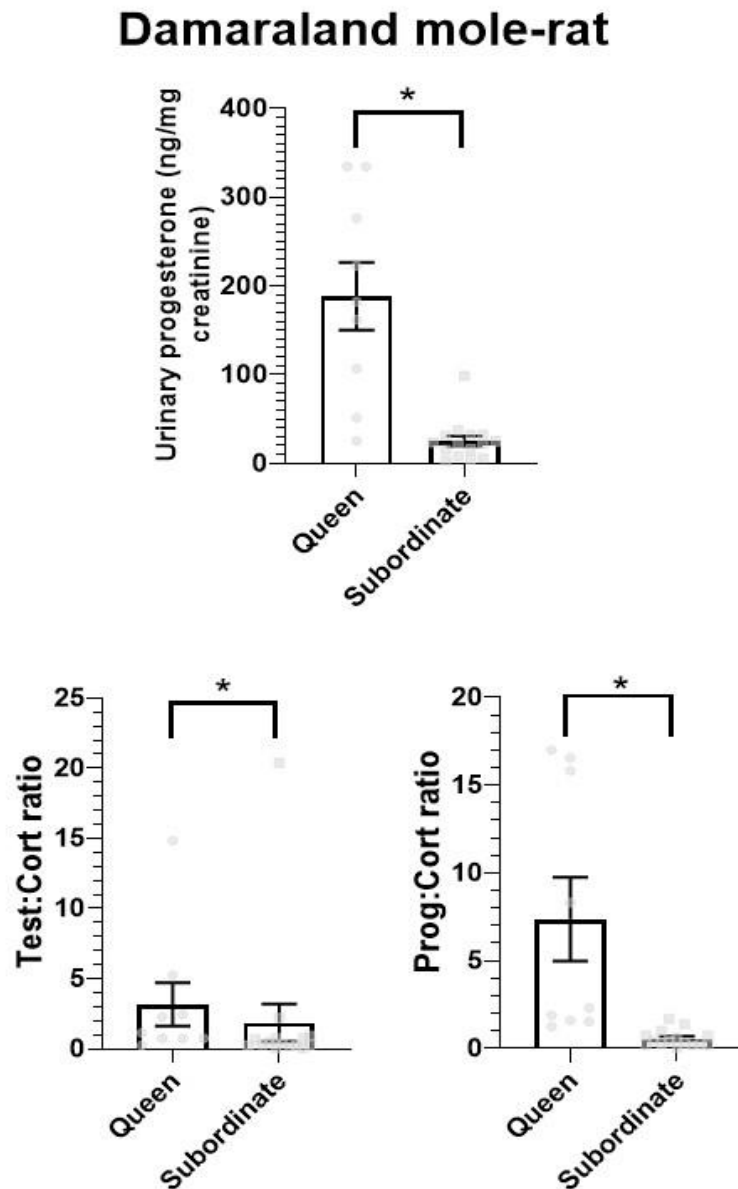
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3523 **Figures**



3524 **Figure 1.** The difference in urinary progesterone concentrations (ng/mg creatinine), the
 3525 testosterone: cortisol ratio (Test: Cort, – proxy for social dominance phenotype), and the
 3526 progesterone: cortisol ratio (Prog: Cort – proxy for reproductive readiness) between queen and
 3527 female subordinate Damaraland mole-rats (*Fukomys damarensis*). Data are presented as mean
 3528 ± standard error (S.E.M.). Note: An asterisk (*) indicates significance ($p \leq 0.05$).

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Damaraland mole-rat

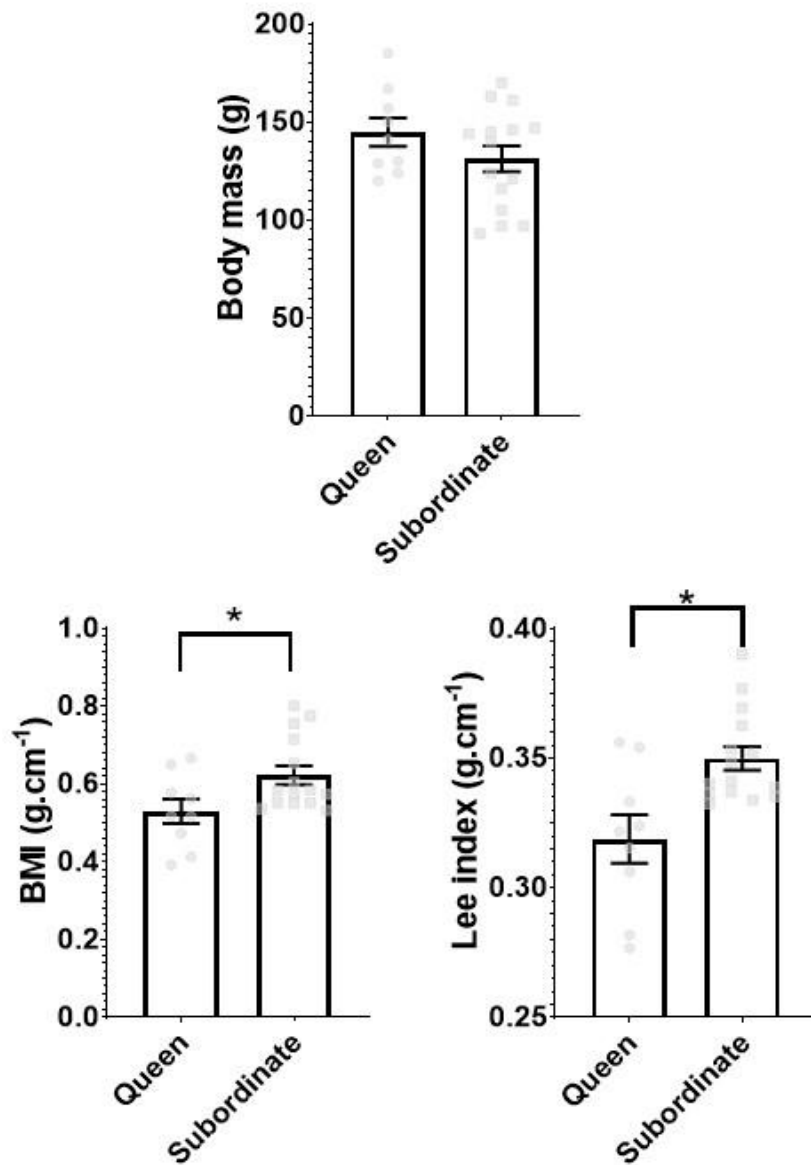


Figure 2. The variation of body matrixes, namely body mass, body mass index (BMI; g/cm) and Lee index (g/cm) between Damaraland mole-rat (*Fukomys damarensis*) breeding females (queens) and subordinate non-breeding females. Data are presented as mean $\pm$ standard error (S.E.M.). Note: An asterisk (*) indicates significance ($p \leq 0.05$).

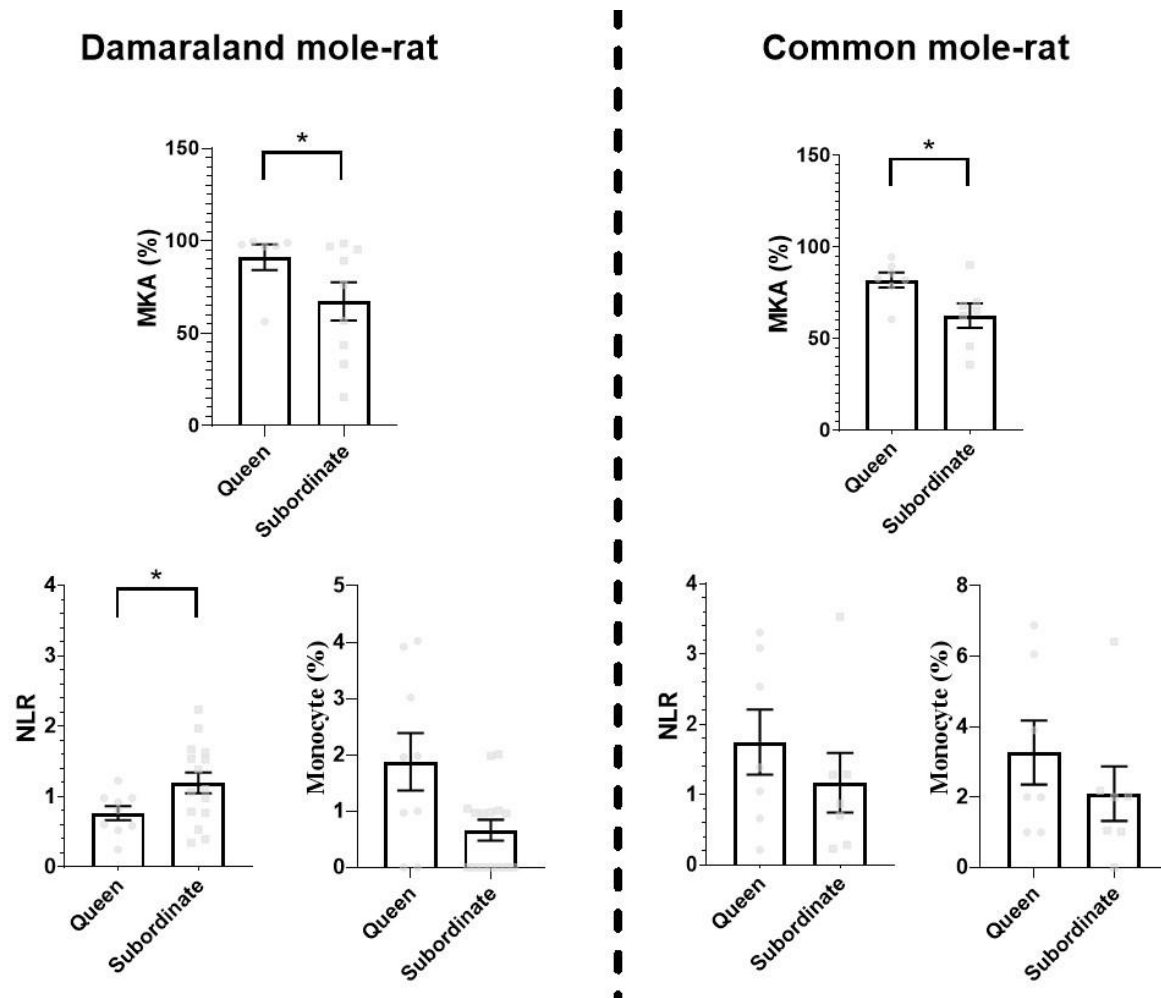


Figure 3. The differences between breeding females (queens) and their non-breeding counterparts (subordinates) blood microbial killing ability (MKA; %), neutrophil: lymphocyte ratio (NLR) and prevalence of monocytes (%) in two social African mole-rat species; the Damaraland mole-rats (*Fukomys damarensis*) and the common mole-rats (*Cryptomys hottentotus hottentotus*). Data are presented as mean $\pm$ standard error (S.E.M.). Note: An asterisk (*) indicates significance ($p \leq 0.05$).

Supplementary material

Supplementary table 1: The difference between breeding (queen) and non-breeding female (subordinate) Damaraland mole-rats (*Fukomys damarensis*) hormonal parameters (plasma prolactin (ng/ml), urinary testosterone concentrations (ng/mg creatinine), urinary cortisol concentrations (μ g/mg creatinine), urinary progesterone concentrations (ng/mg creatinine); testosterone: cortisol (Test/Cort) ratio – a proxy of socially dominant behaviour) and progesterone: cortisol (Prog/Cort) ratio – a proxy of reproductive readiness).

Hormonal parameter	Queen	Subordinate	t-value/ Mann-Whitney U	p-value
Testosterone	99.3±36.7	49.4±24.7	39	0.09
Cortisol	49.4±16.4	87.7±30.7	56	0.51
Prolactin	5.00±0.97	3.79±1.22	39	0.09
Progesterone	188.3±38.1	25.0±5.96	6	0.0001*
Test/Cort	3.16±1.56	1.85±1.33	29	0.02*
Prog/Cort	7.36±2.39	0.56±0.12	4	0.0001*

*: indicates significance ($p < 0.05$); Mean±SE

Supplementary table 2. Correlations between body matrixes (body mass (g), body mass index (BMI – g/cm) and the Lee index (g/cm)) and hormonal parameters (plasma prolactin (ng/ml), urinary testosterone concentrations (ng/mg creatinine), urinary cortisol concentrations (µg/mg creatinine), urinary progesterone concentrations (ng/mg creatinine); testosterone: cortisol (Test/Cort) ratio – a proxy of socially dominant behaviour and progesterone: cortisol (Prog/Cort) ratio – a proxy of reproductive readiness) of female Damaraland mole-rats (*Fukomys damarensis*).

Endocrine parameters	BMI		Lee index		Body mass	
	r	p	r	p	r	p
Testosterone	-0.34	0.10	-0.29	0.17	-0.21	0.33
Cortisol	-0.10	0.65	-0.07	0.74	-0.18	0.40
Progesterone	-0.29	0.17	-0.43	0.04*	0.15	0.48
Test/Cort	-0.24	0.25	-0.27	0.21	0.05	0.83
Prog/Cort	-0.17	0.43	-0.35	0.09	0.36	0.08
Prolactin	0.02	0.92	0.06	0.78	0.02	0.92

*: indicates significance ($p < 0.05$); Mean ± S.E.M.

Supplementary table 3. The difference between breeding (queen) and non-breeding females (subordinate) common mole-rats (*Cryptomys hottentotus hottentotus*) innate immune strength

proxies (white cell count (WCC; $\times 10^9/L$), segmented neutrophil (N; %), lymphocyte (L; %), eosinophil (E; %), basophil (B; %), monocyte (M; %) and platelet count (PC; $\times 10^9/L$), neutrophil-to-lymphocyte (NLR), monocyte-to-lymphocyte (MLR), platelet-to-lymphocyte (PLR), and complete platelet-to-white cell count (PWR) ratios).

Innate immune strength proxies	Queen	Subordinate	t-value/ Mann-Whitney U	p-value
N	53.9 $\pm$ 8.29	42.8 $\pm$ 7.63	0.98	0.35
L	41.9 $\pm$ 7.39	51.9 $\pm$ 8.20	0.91	0.38
E	0.30 $\pm$ 0.30	0.22 $\pm$ 0.14	22	0.99
B	0.70 $\pm$ 0.47	0.09 $\pm$ 0.09	20	0.46
PC	306 $\pm$ 190	359 $\pm$ 56.24	8	0.67
MLR	0.07 $\pm$ 0.01	0.05 $\pm$ 0.03	12	0.13
PLR	173 $\pm$ 94.7	346 $\pm$ 138	9	0.83
PWCCR	77.2 $\pm$ 47.4	144 $\pm$ 54.8	9	0.83
WCC	3.35 $\pm$ 0.62	30.5 $\pm$ 25.1	10	0.99

*: indicates significance ($p < 0.05$); Mean $\pm$ S.E.M.

Supplementary table 4: The difference between breeding (queen) and non-breeding female (subordinate) Damaraland mole-rats (*Fukomys damarensis*) innate immune strength proxies (white cell count (WCC; $\times 10^9/L$), segmented neutrophil (N; %), lymphocyte (L; %), eosinophil (E; %), basophil (B; %), monocyte (M; %) and platelet count (PC; $\times 10^9/L$), neutrophil-to-lymphocyte (NLR), monocyte-to-lymphocyte (MLR), platelet-to-lymphocyte (PLR), and complete platelet-to-white cell count (PWR) ratios).

Innate immune strength proxies	Queen	Subordinate	t-value/ Mann-Whitney U	p-value
N	40.3 $\pm$ 3.50	50.2 $\pm$ 3.4	1.90	0.07
L	56.2 $\pm$ 3.50	47.8 $\pm$ 3.50	1.61	0.12
E	0.50 $\pm$ 0.30	0.20 $\pm$ 0.10	63	0.74
B	0.30 $\pm$ 0.30	1.10 $\pm$ 0.3	30	0.02*
PC	423 $\pm$ 60.4	337 $\pm$ 30.2	1.43	0.17

MLR	0.04±0.01	0.02±0.01	2.09	0.05*
PLR	83.5±12.8	75.8±8.70	0.52	0.61
PWCCR	45.1±6.60	35.0±3.70	1.44	0.17
WCC	10.1±1.80	10.3±0.90	0.11	0.91

3578 *: indicates significance ($p < 0.05$); Mean $\pm$ S.E.M.

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3580 **Supplementary table 5:** Correlations between innate immune strength proxies (Antimicrobial
3581 capacity (MKA; %), white cell count (WCC; $\times 10^9/L$), segmented neutrophil (N; %),
3582 lymphocyte (L; %), eosinophil (E; %), basophil (B; %), monocyte (M; %) and platelet count
3583 (PC; $\times 10^9/L$), neutrophil-to-lymphocyte (NLR), monocyte-to-lymphocyte (MLR), platelet-to-
3584 lymphocyte (PLR), and complete platelet-to-white cell count (PWR) ratios) and hormonal
3585 parameters (plasma prolactin (ng/ml), urinary testosterone concentrations (ng/mg creatinine),
3586 urinary cortisol concentrations ($\mu g/mg$ creatinine), urinary progesterone concentrations (ng/mg
3587 creatinine); testosterone: cortisol (Test/Cort) ratio – a proxy of socially dominant behaviour)
3588 and progesterone: cortisol (Prog/Cort) ratio – a proxy of reproductive readiness) of female
3589 Damaraland mole-rats (*Fukomys damarensis*).

	Testosterone		Cortisol		Progesterone		Test/Cort		Prog/Cort		Prolactin	
	r	p	r	p	r	p	r	p	r	p	r	p
MKA	0.73	0.01*	0.32	0.28	0.73	0.01*	0.43	0.15	0.35	0.24	0.34	0.23
PC	0.35	0.09	-0.16	0.47	0.22	0.29	.406*	0.05	0.34	0.10	-0.28	0.18
WCC	0.20	0.35	0.12	0.58	-0.21	0.33	0.30	0.16	-0.17	0.43	-0.40	0.05
N	-0.08	0.70	0.27	0.20	-0.15	0.48	-0.33	0.12	-0.35	0.10	0.05	0.83
L	0.15	0.48	-0.22	0.31	0.16	0.44	0.33	0.11	0.33	0.11	-0.07	0.75
M	0.32	0.13	-0.22	0.29	0.39	0.06	0.59	0.00*	0.41	0.05*	0.03	0.89
E	0.07	0.75	-0.27	0.21	0.26	0.21	0.26	0.23	0.24	0.25	0.35	0.09
B	-0.17	0.43	0.31	0.14	-0.36	0.09	-0.38	0.07	-0.58	0.00*	-0.21	0.33
NLR	-0.12	0.59	0.26	0.22	-0.14	0.51	-0.35	0.10	-0.34	0.11	0.06	0.77
MLR	0.28	0.18	-0.23	0.28	0.33	0.12	0.50	0.01*	0.37	0.07	0.04	0.85
PLR	0.18	0.41	-0.22	0.29	0.30	0.16	0.06	0.80	0.31	0.14	0.13	0.53
PWCCR	0.22	0.30	-0.36	0.08	0.28	0.18	0.21	0.33	0.41	0.05*	0.05	0.81

3590 *: indicates significance ($p < 0.05$); Mean $\pm$ S.E.M.

General Discussion

The marked reproductive skew in eusocial Damaraland mole-rats (*Fukomys damarensis*) has resulted in clear differences in the endocrine profiles of the two primary physiological castes, namely breeders and non-breeders. Furthermore, due to this distinct variation in endocrine profiles, haematological and immunological divergences between reproductive classes are apparent, which likely leads to the well-reported division of labour and noticeable differences in longevity between the two breeding castes. This study's findings provide further evidence for the Damaraland mole-rat being a eusocial mammal with distinct physiological castes. Reproductive classes are most clearly observed through the endocrine profiles, reproductive behaviour, and daily burrow maintenance activities of each member of the colony.

In somewhat of a surprising find, both breeding males (unexpected) and females (expected) possessed increased progesterone compared to their non-breeding counterparts. Furthermore, this is the first study to identify a significant difference in urinary testosterone concentrations between male and female reproductive classes. While there were no differences in either prolactin or cortisol concentrations amongst reproductive classes, using the testosterone: cortisol (T/C) ratio proved informative in distinguishing dominance hierarchy in Damaraland mole-rats. Finally, the progesterone: cortisol (P/C) ratio was introduced to measure reproductive readiness in Damaraland mole-rats.

These findings are noteworthy, as previous reports on the endocrinology of male Damaraland mole-rats did not reveal physiological differences between the reproductive classes (Bennett, 1994; Faulkes *et al.*, 1994; Medger *et al.*, 2018). Accordingly, only recent work on the neuroendocrinology of the Damaraland mole-rats has provided distinctions between breeding and non-breeding males in terms of the quantitative expression of hypothalamic androgen-, progesterone- and Rfrp-receptors (AR, PGR, and RFRP-3, respectively) (Voigt *et al.*, 2016; Voigt & Bennett, 2021). The heightened upregulation of AR and PGR expression in breeding males reinforces their potential for reproductive behaviour by the elevated secretion of gonadotropins post-GnRH neuron excitation. Similarly, reproductive phenotypes are suppressed in non-breeding males by higher expressions of GnRH-inhibitory RFRP-3 and lower GnRH-stimulatory AR and PGR (Voigt *et al.*, 2016; Voigt & Bennett, 2021). The neuroanatomical findings support the dominant reproductive phenotype of elevated AR and PGR distributions and higher urinary progesterone and testosterone concentrations in breeding male and female Damaraland mole-rats. A higher T/C ratio, as found in breeders,

further supports this dominance phenotype whereby high testosterone levels indicate an individual's ability to attain and defend the breeding monopoly, and a lower cortisol concentration that portrays their coping ability (Lutermann *et al.*, 2013; Vulliou, 2017; Vulliou *et al.*, 2021). Similarly, high P/C ratios found in breeding members indicate their enhanced reproductive readiness due to the role of progesterone in reproduction and the additive effect on the androgen receptor activity. The lack of circulating progesterone in non-breeding males may be due to heightened RFRP-3 receptors (Voigt & Bennett, 2021).

The mechanisms behind several similarities between the eusocial African mole-rats and eusocial insects have eluded researchers for years. This study helps address two, namely, the mechanisms behind the division of labour between breeders and non-breeders and their noticeable differences in longevity. Previous studies have found a division of labour between breeding and non-breeding Damaraland mole-rats with non-reproductive helper individuals performing 95% of the annual colony labour (cooperative behaviours) and expending 20% or more daily energy than the reproductively active members (Lacey & Sherman, 1997; Scantlebury *et al.*, 2006b; Francioli *et al.*, 2020b). Yet, how these non-breeding helpers can perform such tasks is unknown. More so, the driving force behind this possible physiological specialisation for work is unknown. As a novel investigation, this study identified a predisposed haematological condition present in non-breeding Damaraland mole-rats that enables the enhanced cooperative care behaviour of 'helpers'. Captive-bred helper Damaraland mole-rats exhibited higher oxygen carrying capacities due to the erythropoietic properties of the red blood cell, haemoglobin, and haematocrit volumes. In the past, it has been thought that differences in endocrine profiles drive this increased cooperative behaviour in non-breeders. Yet, recent studies have disqualified the possibility of cortisol and testosterone being the primary drivers of this.

Interestingly, in my study the only hormone to be correlated to elevated erythropoietic values was progesterone (a previously un-investigated hormone). Progesterone and oestradiol, which are found at low concentrations in non-breeding helper mole-rats, have recently been associated as hormonal drivers of both erythropoietic (Lavrijsen & Verwilghen, 1984). Breeding individuals with the highest progesterone concentrations had the lowest oxygen carrying capacity, and consequently, they had reduced haematological activity. Furthermore, upon removal of the helpers from the colony and the ensuing removal from physiological reproductive suppression operative on them by the queen, the erythropoietic indices of the removed helpers decreased. This decrease in blood oxygen carrying capacity was accompanied

by an adjustment in burrow maintenance activities, from laborious and energetically demanding cooperative care under colonial and reproductive suppression to dispersal-preparation and reproductive readiness behaviour once free from physiological suppression. Therefore, this suggests the involvement of physiological reproductive suppression mechanisms in moulding the erythropoietic capabilities of non-breeding workers, allowing them to perform cooperative tasks. This also suggests that, like eusocial insects, distinct physiological castes are present in Damaraland mole-rats.

Comparatively, predisposed physiological and morphological specialisations that allow helpers to perform specialised cooperative tasks are usually determined during early development or via environmental and/or genetic mechanisms in eusocial insects (Schwander *et al.*, 2010). However, this seems not to be the case in Damaraland mole-rats as morphological (Young & Bennett, 2010) and physiological (this study) divergence of breeders from helpers appears to occur when helpers are removed from the confines of the colony and on the attainment of social dominance as dispersers. However, reproductive suppression has been observed to result in differences in transcriptomic and genomic signals between breeders and helpers in both naked (*Heterocephalus glaber*) (Bens *et al.*, 2018) and Damaraland mole-rats (Johnston *et al.*, 2021), but not all systems have been investigated, including haematopoiesis. Since helpers are born into this reproductive suppression and never reach sexual maturity whilst in the confines of the group, inherent molecular differences, such as increased erythropoiesis, may allow for an increased ability to perform predisposed strenuous cooperative tasks, such as digging, which is similar to what has been observed in eusocial insects (Miura & Scharf, 2010).

Additionally, the burrow systems' constant hypoxic and hypercapnic microclimatic conditions may play an undiscovered interactive physiological role in the erythropoietic indices obtained in Damaraland mole-rats, in terms of oxygen consumption demand and ventilation rates between and within breeding castes. This is particularly true as breeding individuals huddle together in nesting chambers for a longer period of time than helpers, they maintain a lower metabolic rate and daily energy expenditure, thus requiring lower tissue oxygenation than their more aerobically active counterparts (Lovegrove & Wissel, 1988; Scantlebury *et al.*, 2006a; Kotze *et al.*, 2008; Ivy *et al.*, 2019).

It is likely that reproductive suppression also plays a role in the variation in longevity between breeding females and non-breeding females, namely through increased immunocompetence and body conditions possessed by breeding females. The breeding female

Damaraland mole-rats had the highest reproductive readiness (P/C ratio) and the highest marker of innate immunity proxies, supporting and enabling the extended lifespans and fitness profiles identified in previous Damaraland mole-rat (Schmidt *et al.*, 2015) and naked mole-rat studies (Horvath *et al.*, 2022). This study demonstrated that the Damaraland mole-rat queens' blood has a higher microbial killing capacity than their subordinate non-reproductive counterparts. A trait shared by the social common mole-rat, *Cryptomys hottentotus hottentotus*. Unlike the common mole-rat, queen Damaraland mole-rats also possessed lower neutrophil: lymphocyte ratio (NLR) and normal levels of monocyte prevalence compared to their non-reproductive subordinates. As per previous haematological studies, the low NLR in Damaraland mole-rat queens indicates low inflammatory activation and stress within the immune system (Russell *et al.*, 2019; Liu *et al.*, 2020; Rizo-Tellez *et al.*, 2020; Stefaniuk *et al.*, 2020). Multiple cross-species studies have indicated that increased levels of progesterone and estrogen (like those seen during pregnancy and ovulation) require a female to be in a selective state of immunomodulation, whereby adaptive immune responses are downregulated, and innate immunity components, such as monocyte production, are stimulated (Luppi *et al.*, 2002; Luppi, 2003; Naim *et al.*, 2020). As subordinate female Damaraland mole-rats are physiologically suppressed, they never experience conditions similar to a pregnant or ovulating queen and thus have disparate leukocytic activations.

Comparatively, where there is no physiological suppression (only behavioural suppression) of subordinates in common mole-rats, there was no difference in the leukocytic constituents between breeding or non-breeding females. The physiological suppression of subordinate female Damaraland mole-rats is most evident in their reduced pituitary sensitivity to gonadotropin-releasing hormone (GnRH) and circulating luteinizing hormone (LH) compared to the queens (Molteno & Bennett, 2000; Clarke *et al.*, 2001), while common mole-rat subordinate females are not physiologically suppressed and have similar GnRH sensitivity and LH profiles to their queens (Spinks *et al.*, 1999). Thus, the weaker form of reproductive suppression in common mole-rats may result in reduced differences in leukocytic activations between breeding and non-breeding females.

Similarly, a lack of physiological suppression is observed in non-breeding male Damaraland mole-rats (Maswanganye *et al.*, 1999; Voigt *et al.*, 2016). Identical to female common mole-rats, male Damaraland mole-rats do not demonstrate immunological distinctions across the two reproductive groupings. This suggests that reproductive suppression, through the changes in each caste endocrine profile, may be driving the variation

in immunocompetency. Interestingly this result also contradicts well-established hypotheses, such as the life history theory (LHT) or the more specific immunocompetence handicap hypothesis (ICHH) and stress-linked ICHH which was put forward to explain immunological sex and reproductive dimorphisms (Folstad and Karter, 1992; Foo et al., 2017). Even though breeding males possessed higher testosterone (ICHH) and higher T/C ratio (stress-linked ICHH), breeding and non-breeding males possessed similar immunocompetence proxies.

These findings mirror that found in African ground squirrels, whereby the reproductive-immune axis did not follow the breeding/non-breeding patterns suggested by the ICHH, or the stress-linked ICHH (O'Brien *et al.*, 2018). Similar manipulative studies on the immunocompetence of male zebra finches (*Taeniopygia guttata*) have attempted to further elaborate this elusive immunosuppressive effect of testosterone on males, with regards to the role of leptin in mediating immune-trade-offs with elevated testosterone induced sexual displays (Alonso-Alvarez, Bertrand & Sorci, 2007). Alonso-Alvarez, Bertrand and Sorci (2007) suggest that high levels of leptin, and thus high lipid reserves in males, would decrease the immunological impairment of simultaneously expressed high testosterone concentrations. This may be an additional consideration to account for when determining the innate immune capacity of breeding male Damaraland mole-rats as while they are less active, with higher fat reserves (Scantlebury *et al.*, 2006b), they have higher testosterone concentrations, but do not display downregulated immune responses. Furthermore, as evident by breeding female Damaraland mole-rats, the combined effect of increased testosterone and progesterone (and likely oestrogen) plays a significant role in the increased immunocompetency of breeding females. Even though breeding males show an increase in progesterone compared to non-breeding males, their levels are still similar to that of non-breeding females, therefore, breeding males may lack the necessary levels of progesterone to cause an increase in immunocompetency. However, further studies are needed to investigate this.

Males did, however, demonstrate a significant relationship between the testosterone concentrations and platelet count: white cell count ratio (PWR). The more dominant breeding males had the highest testosterone and T/C ratio's and the lowest PWR, which diagnostically suggests sepsis and a higher risk of mortality (Lathouras *et al.*, 2019; Zhang *et al.*, 2020). In contrast, the low PWR and thus lower mortality risk detected in less dominant, non-breeding males would benefit their daily activity as colony defenders in the instance of agonistic encounters whilst foraging for food resources and investigating breached sections of the tunnel. These similarities and differences in male Damaraland mole-rats suggest a varied reproductive

and immunological investment, influenced by differential degrees of burrowing activity and task specialisation.

The current results give useful insight into the possible mechanisms behind the unique eusocial characteristics seen in Damaraland mole-rats. All current evidence points towards the cause for the apparent division of labour and longevity variation between breeders and non-breeders is caused due to reproductive suppression and the subsequent differences in endocrine profiles. As such, social African mole-rats may hold the key to unravelling long-standing questions on how the endocrine system affects health and longevity in all mammals, but more in-depth studies are needed to build on current knowledge, including this study.

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