The relationship between immunocompetence, parasite load and reproductive effort in the highveld mole-rat (*Cryptomys hottentotus pretoriae*) Roberts 1913.

By

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I, Hermien Viljoen (ID: 8206020019084), declare that the thesis/dissertation, which I hereby submit for the degree M.Sc Zoology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Summary

Life-history theory is based on the notion that resources are generally limited in nature, and that organisms have to allocate these resources amongst different physiological systems to maximise their survival and fitness. Therefore life-history traits such as maintenance, growth and fecundity are very often negatively associated with each other. Since the immune system plays a prominent role in fitness and survival of an organism it often features in life-history trade-offs particularly with respect to reproductive effort. This thesis focused predominantly on the interactions between immune function and reproductive effort in the highveld mole-rat, *Cryptomys hottentotus hottentotus* as well as other factors that may influence these two vital systems.

In the first chapter interactions between reproductive effort and immune function in 22 female highveld mole-rats (5 reproductive females and 17 non-reproductive females) is investigated by stimulating reproductive competition amongst females within colonies and monitoring hormonal and haematological parameters. Behavioural observations focusing on agonistic, grooming and sexual behaviour is also included in this chapter mostly as tool to identify reproductive competition amongst females.

The second chapter deals specifically with the effect of the season, gender and reproductive status on a febrile response to an exogenously administered pyrogen. Lipopolysaccharide, a protein produced by gram negative bacteria, was used to induce fever in 37 mole-rats during both winter and summer months.

The final chapter explored the relationships between parasite infestation and host gender and reproductive status while concurrently identifying some of the parasites associated with the highveld mole-rat. A total of 46 mole-rats were assessed for intensity of gastrointestinal parasites, 131 for prevelance of cestodes by faecal examination and 85 for intensity and prevalence of ectoparasites. A possible relationship between host group size and intensity of parasitism is explored. Seasonal patterns of the parasites found is also investigated and related to possible seasonal variation in immune function.

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General Introduction

Life history theory and physiological trade-offs

In the last few decades interest in evolutionary biology has focused on the interactions between various life-history components and in particular how investments into some life-history components may affect others. Since resources are generally limited in nature, organisms have to allocate these resources amongst different physiological systems so that survival and fitness can be maximised (Stearns 1989). The immune system, being an essential component in the survival of organisms, often features in life-history trade-off studies (Martin *et al.* 2008). Since mounting an immune response is energetically expensive one can expect trade-offs to occur with other expensive physiological processes such as reproduction, growth and thermoregulation (Lochmiller & Deerenberg 2000). Along with immunocompetence, the costs of reproductive effort in particular have also received a great deal of attention, since reproduction not only infers survival costs, but also costs to an organism's future reproductive success (Stearns 1989). It is therefore not surprising that evolutionary ecologists have turned their focus to studying the nature of trade-offs between reproduction and the immune function. For example, many studies have shown that increased reproductive effort negatively influences an organism's ability to produce immunologically active cells and substances (Ardia *et al.* 2003, Nordling *et al.* 1998). Furthermore, some studies have found that reproductively active animals generally have lower defences against parasitism and harbour higher intensities of parasites than their non-reproductive counterparts (Allander 1997, Nordling *et al.* 1998, Norris *et al.* 1994). While numerous studies

have found that immune function decreases with increasing reproductive effort, other studies have not found evidence for such interactions (Williams *et al.* 1999) or even that some aspects of immunity may improve with increased reproductive effort (Lutermann & Bennett 2008). This suggests that the nature of the apparent antagonism between immune function and reproduction may be far more complicated than was previously believed.

Variation of immune function

Changes and variation in immune function are likely to be mediated by hormones (Martin *et al.* 2008, Zera & Harshman 2001). Androgens, specifically testosterone, have been associated with inhibition of immune function (Folstad & Karter 1992, Klein 2000). Furthermore, male animals tend to exhibit lower immune responses than their female counterparts and are more susceptible to parasites and pathogens (Folstad & Karter 1992, Klein 2000, Martin *et al.* 2008). It has been suggested that this is because females gain fitness through longevity (Rolff 2002) and therefore invest more energy in humoral and cell-mediated immunity (Martin 2000). Males, on the other hand gain fitness by increasing their mating success and would rather invest energy in reproductive mechanisms such as secondary sexual characteristics (Rolff 2002, Folstad & Karter 1992). However, the exact mechanism of how testosterone (along with other hormones) affects the immune system is still largely unknown and the immunosuppressive effect of testosterone has been debated recently (Braude *et al.* 1999, Evans *et al.* 2000, Klein 2000, Martin *et al.* 2008, Roberts *et al.* 2004). Studies on the immunosuppressive effect of testosterone in females specifically are lacking.

Immune defences of vertebrates display seasonal cycles, with immune systems generally becoming weaker during winter (Nelson 2004, Nelson & Demas 1996). However, when immunocompetence is measured under laboratory conditions, where factors such as food intake and temperature are controlled, the pattern reverses and animals exhibit stronger immune reactions under short day conditions (Nelson 2004). It is proposed that animals vary their immune systems in anticipation of challenges, but that the decreased availability of energy during winter ultimately results in a reduction in the immune system's defences (Nelson 2004). The seasonal patterns of abundance that many parasite species exhibit are also believed to play a major role in seasonal immunomodulation of the host (Martin *et al.* 2008). Therefore, by investigating how the intensity and prevalence of parasites vary on a seasonal basis, predictions can be made as to how the immune function of the host, in particular anti-parasitic defences such as inflammation, is altered throughout the year in order to counteract and control parasite infestations.

Another factor that influences immune function is the social structure of a species. For example, an increased risk of parasitism is considered to be a cost of sociality resulting from the increased risk of transmission when animals live in close proximity to each other (Altizer *et al*. 2003, Whiteman & Parker 2004). One would therefore expect social animals to invest more energy in immune defences against parasites than solitary species. Recent studies have shown however, that the intensity of infestation by mobile parasites such as ticks and mites may decrease with increasing host group size (Stanko *et al*. 2002, Snaith *et al*. 2008). A host's social behaviour can play a prominent role in controlling and eliminating parasite infestations. Ectoparasites are often very effectively removed by auto-grooming (Stanko *et al*. 2002) and in social groups where animals groom each another, there

is the added benefit of being able to reach and groom areas inaccessible to the individual itself (Hart & Hart 1988). Mating systems and social organization of groups can also impact immune function by affecting parasite and disease susceptibility (Altizer *et al.* 2003, Ezenwa 2004). For example, promiscuous species run a higher risk of being infected by parasites and pathogens compared to monogamous species due to the higher contact rates between individuals (Altizer *et al*. 2003).

Study animal: the highveld mole-rat

The highveld mole-rat, *Cryptomys hottentotus pretoriae,* is a social subterranean rodent in the family Bathyergidae. In the wild, highveld mole-rats may occur either solitary or in colonies of up to 14 individuals (Malherbe & Bennett 2007). Colonies comprise of reproductively active individuals that monopolize breeding within the confines of the colony as well as non-reproductive individuals (Malherbe & Bennett 2007, Moolman *et al*. 1998). While reproductive suppression in subordinate highveld mole-rat males is probably simply the result of these individuals not having access to unrelated mates (Janse van Rensburg *et al*. 2003), subordinate females are suppressed physiologically from breeding (most likely by reproductive suppression on the hypothalamo-pituitary axis) whilst in the confines of the colony (Van der Walt *et al.* 2001). The highveld mole-rat is a seasonal breeder with the majority of litters being born in late winter to early summer (Janse van Rensburg *et al.* 2002). It has been suggested that sociality in mole-rats evolved because of unpredictable rainfall patterns that limit the time during which burrowing, and therefore the time food harvesting can take place (Faulkes *et al.* 1997). In these conditions larger groups benefit though a division of labour and sharing of food

resources (Moolman *et al*. 1998). The highveld mole-rat however, occurs in a summer-rainfall region in the highveld of South Africa - a region where the food resources (geophytes) are relatively abundant throughout the year. This excludes the possibility that unpredictable availability of food resources being the driving force behind the formation of social groups in this species, but opens the field to exploring other possibilities such as ectoparasite control. The highveld mole-rat, being a social mammal with a reproductive labour division and a seasonal cycle of reproduction is an ideal subject to 1) identify life history trade-off's between immunocompetence and reproduction, 2) examine seasonal variation in immune function and 3) study general interactions between immune function, parasitism and sociality.

Thesis structure and content

The aim of chapter 1 is to investigate the effect of reproductive effort and competition between female highveld mole-rats by monitoring hormonal and haematological parameters. More specifically it was determined whether a life-history trade-off exists between reproduction and the immune system. This experiment allowed for comparisons to be made between reproductive and non-reproductive females to determine if reproductive status does indeed affect the immune system of female highveld mole-rats. Reproductive competition between females may be stimulated by removing all males from colonies and replacing them with unfamiliar breeding males (Cooney & Bennett 2000). Behavioural observations were undertaken to confirm that reproductive competition amongst females did in fact occur. There is some support for the notion that changes in immune function is mediated by hormones (Martin *et al.* 2008, Zera & Harshman 2001). Some studies

suggest negative correlations between immune function and the hormones testosterone and cortisol in males (Martin *et al*. 2008, Roberts *et al.* 2004). Urine samples were collected from the female mole-rats between changes in colony composition and were analysed for testosterone and cortisol concentrations to explore similar mechanisms in females. Blood samples were analysed for various blood cell counts so that immunological changes that occurred concurrently with changes in colony composition could be examined. The analysis of the blood cell counts and composition provided insight into the state of an animal's immune function (Beldomenico *et al.* 2008, Male *et al.* 2006). For example, lymphocytes indicate an investment in the adaptive arm of the immune system, while white blood cells of the innate immune system.

Chapter 2 examines the effect of the season as well as how gender and reproductive status influence the febrile response to an exogenously administered pyrogen. Lipopolysaccharide (LPS) is a component of gram-negative bacteria cell wall and is routinely used to induce fever in animals for research purposes (Dinarello 2004, Martin *et al.* 2008). In this chapter LPS was used to induce fever in 37 mole-rats during winter and summer. By analysing the seasonal patterns of the magnitude of fever-responses by monitoring body temperature, deductions can be made about how animals invest in their energy in immune responses. Furthermore, by comparing fever-responses between the reproductive and non-reproductive animals as well as between the sexes, the possible trade-offs between reproduction and immune-responses can be identified.

Finally, chapter 3 explores the relationships between parasitism and host gender and reproductive status with seasonal patterns of parasite infestation of the highveld mole-rat. This can be directly related to the hypothesised life-history trade-

offs between immunity and reproduction as one can expect individuals that invest more in reproduction to have lower defences against parasites and therefore be more likely to be infested and to harbour higher parasite loads. In addition, effects of group size on parasite load were evaluated. Although it is generally assumed that increased parasitism is a cost of sociality (Altizer *et al*. 2003, Bordes *et al*. 2007) some studies show that larger groups may, in some cases, have beneficial effects on the level of parasitism in group living animals (Snaith *et al*. 2008, Stanko *et al*. 2002). Ectoparasites and gastrointestinal parasites were collected from mole-rats throughout the year. Data on the prevalence and intensity of parasites could then be analysed for the effects of various factors hypothesised to affect parasitism, while concurrently identifying some of the parasites associated with the highveld mole-rat.

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Chapter 1

Reproductive competition and immune defence in the female highveld mole-rat

Abstract

Animals distribute available resources in life-history traits so that their fitness is maximised. Because resources are often limited, trade-offs between life-history components may occur. The trade-off between reproductive effort and the immune system is one that has received much attention lately. In this study, reproductive competition was stimulated in a laboratory setting between females of the highveld mole-rat by firstly removing the familiar reproductive male and subsequently introducing an unfamiliar reproductive male into colonies. Behavioural observations focusing on agonistic, grooming and sexual behaviour indicated that reproductive competition was indeed induced between females upon the introduction of an unfamiliar male. Urinary testosterone and cortisol concentrations were measured and blood samples were analysed for blood cell numbers between changes in colony composition. Although cortisol levels were not affected by reproductive status or stages, urinary testosterone of reproductive females increased after the removal of males from colonies and remained higher than baseline levels after the introduction of unfamiliar males. The most important behavioural change that occurred was a significant increase in agonistic behaviour amongst females after the introduction of unfamiliar males. During this stage introduced males were also groomed more than other individuals in the colony. White blood cell number decreased significantly after males were removed from colonies and reproductive females had lower lymphocyte

and higher blood platelet numbers than non-reproductive females. This indicates that, instead of a trade-off between immunocompetence and reproductive effort, reproduction possibly triggers a re-distribution of immune cells to areas where they will be most useful during reproduction. Because reproductive competition was induced it opens the field to future studies to test and measure other possible trade-offs.

Keywords: Bathyergidae, highveld mole-rat, reproductive competition, allogrooming, aggression, submission, testosterone, cortisol, leukocytes, lymphocytes, platelets

Introduction

Since resources are generally limited in nature, animals are faced with the dilemma of not being able to maximise investment in all life history traits concurrently (Stearns 1992). As a consequence resources tend to be distributed unequally between different physiological systems within an organism in a way that will optimise reproduction and overall survival. Life-history traits such as maintenance, growth and fecundity are therefore often negatively associated with each other (Zera & Harshman 2001). Since the immune system plays a prominent role in fitness and survival of an organism it often features in life-history trade-offs particularly with respect to reproductive effort (Martin *et al.* 2008). Breeding effort has been shown to reduce the ability of memory B-cells to produce antibodies and therefore long-term immune function and survival in tree swallows (*Tachycineta bicolour*) (Ardia *et al.* 2003). Furthermore, Nordling *et al.* (1998) showed that increasing brood size negatively affected the level of Newcastle-disease virus-specific antibodies in

vaccinated collared flycatchers (*Ficedula albicollis*) at the end of the nestling feeding period. Likewise, a higher prevalence of protozoan blood parasites was found in Great tits (*Parus major L*.) with larger broods showing that increased reproductive effort reduced resistance to parasites (Allander 1997). Circulating leukocyte numbers reduced with the onset of the breeding period in male Belding's ground squirrels (*Spermophilus beldingi*) and only returned to normal after the breeding period was over (Bachman 2003). Mounting an immune response is expensive as the production of immune cells and immunologically active substances normally require a substantial amount of energy (Derting & Compton 2003, Lochmiller & Deerenberg 2000, Nelson *et al*. 2002).

Haematological analyses can supply us with valuable information about the state of an animal's immune system. Red blood cell count provides an indication of aerobic capacity and is therefore an indicator of the overall condition of animals, while leukocyte counts provide insights into the different components of the immune system (Beldomenico *et al.* 2008). A measurement of lymphocytes (B and T cells) found in blood indicate immunological investment, as these cells are the main adaptive immune system cells and decrease during immunosuppresion (Beldomenico *et al.* 2008, Male *et al.* 2006). B cells are responsible for reactions against extracellular pathogens (humoral arm of the adaptive immune system), while T cells attack intracellular pathogens such as viruses (cell-mediated arm of adaptive immune system) (Male *et al*. 2006).

The innate immune system is largely mediated by white blood cells (Aderem & Ulevitich 2000). These cells (monocytes, neutrophils, basophils and eosinophils) typically play an important role in the inflammatory response and are important mediators of immune reactions against parasites (Male *et al.* 2006). Neutrophils are

critical effector cells in innate and humoral immunity against bacterial infections and make up the largest proportion of leukocytes in the blood stream (Burg & Pillinger 2001). Basophils have granules that, when released, cause inflammation in surrounding tissues (Male *et al.* 2006). Eosinophils are specialised to attack the membranes of extracellular parasites (Male *et al.* 2006). Blood platelets are cell fragments containing granules that upon release results in increased capillary permeability, attraction of leukocytes and clotting (Male *et al*. 2006). These cells also have phagocytic and bactericidal properties (Promislow 1991).

Any change in immune function caused by increased reproductive effort is likely to be mediated by hormones (Martin *et al.* 2008, Wedekind & Folstad 1994, Zera & Harshman 2001). Androgens, specifically testosterone, have been associated with an inhibition of immune function (Folstad & Karter 1992, Klein 2000, Lutton & Callard 2006, Martin *et al.* 2008). Male vertebrates are generally more susceptible to infections with parasites, fungi, bacteria and viruses and exhibit lower immune responses than their female counterparts (Klein 2000). For example, gonadectomised male rats (*Rattus norvegicus)* have better resistance against *Angiostrongylus malaysiensis* infection, heavier thymic mass and higher circulating leukocyte numbers than gonadoectomised male rats treated with exogenous testosterone propionate (Kamis *et al.* 1992). Besides inhibiting immune responses physiologically, testosterone may also alter behaviour in such a way that the immune system is compromised (Klein 2000). High testosterone levels in males increase competitiveness and aggression, leading to increased mating success (Klein 2000). An increase in aggressive encounters may, however, increase exposure to pathogens and parasites through increased wounding and direct contact with other animals (Glass *et al*. 1988, Klein 2000). Testosterone can also play an important role

in reproductive competition amongst female animals of many species by inducing male-typical anatomical features and behaviour (Beehner *et al.* 2005, Staub & De Beer 1997, Sandell 2007). For example, elevated levels of testosterone occur in dominant and breeding females of the naked mole-rat (*Heterocephalus glabur*), the meerkat (*Suricata suricatta*) and the spotted hyaena (*Crocuta crocuta*) just to name a few (Beehner *et al*. 2005, Clarke & Faulkes 1997, Clutton-Brock 2007). Studies on the immunosuppressive effects of testosterone in females are lacking however.

The exact mechanism of how testosterone affects the immune system is still unknown and undoubtedly very complex (Martin *et al.* 2008, Roberts *et al.* 2004). Recently the immunosuppressive effect of testosterone has been debated (Braude *et al.* 1999, Evans *et al.* 2000, Greenman *et al*. 2004, Klein 2000, Martin *et al.* 2008, Roberts *et al.* 2004). In a review by Roberts *et al.* 2004 it was found that the link between testosterone and immunosuppression is likely to be a causal one and that evidence for a direct relationship is weak. Braude *et al.* (1999) proposed that testosterone does not necessarily have an inhibitory effect on leukocyte production, but that it merely causes a temporary shift of immune cells to compartments where it may be more useful at the time (immuno-redistribution). The perceived immunosuppressive effect of testosterone in some experiments may be due to the effects of glucocorticoids (Martin *et al*. 2008). For example, experimentally induced increases in testosterone caused a reduction in antibody production of male house sparrows (*Passer domesticus*) but also increased corticosterone levels (Evans *et al.* 2000). After statistically controlling for the effect of corticosterone, testosterone was found to increase antibody production (Evans *et al*. 2000). Glucocorticoids (which include cortisol and corticosterone) are steroidal hormones that are secreted by the adrenal gland in response to stress (Sorrells & Sapolsky 2010). Glucocorticoids have

long been believed to inhibit inflammatory responses and antibody production (Evans *et al*. 2000, Martin *et* al. 2008, Norris 1997). Conversely, recent advances in the field of endocrinology show that glucocorticoids can enhance certain aspects of the immune system (Martin *et al*. 2008, Sapolski *et al*. 2000, Sorrells & Sapolsky 2010, Wilckens & De Rijk 1997). For example, glucocorticoids can increase the production and release of pro-inflammatory cytokines (Sorrells & Sapolsky 2010). Even minor increases of glucocorticoid plasma concentrations can cause considerable reductions in circulating leukocytes (Sapolsky *et al*. 2000). It has been shown however, that this is probably the result of leukocytes leaving the bloodstream and accumulating in peripheral tissues where they might be more useful during stressful situations (Martin *et al*. 2008, Sapolski *et al*. 2000). Stress-induced increases in plasma corticosterone levels in male Sprague-Dawley rats resulted in a rapid reduction in circulating leukocytes and lymphocytes, and an increase in neutrophils (Dhabhar *et al*. 1995). Within three hours of the cessation of stress, leukocytes returned to normal levels, with neutrophils showing further increases (Dhabhar *et al*. 1995). Corticosterone administration to adrenalectomised rats produced similar changes in blood cell numbers (Dhabhar *et al*. 1995). It is therefore more likely that stress-induced changes in leukocytes numbers are as a result of leukocyte-redistribution as opposed to increased/decreased destruction and production of cells (Dhabhar *et al*. 1995, Martin *et al*. 2008, Sapolski *et al*. 2000). Increased brood size caused elevated levels of corticosterone in male pied flycatchers (*Ficedula hypoleuca*) (Ilomen *et al.* 2003). This increase in serum corticosterone was in fact accompanied by significantly increased numbers of peripheral leukocytes (Ilomen *et al.* 2003). Glucocorticoids therefore play an important role in altering the immune system by preventing hypersensitivity and

driving immune-redistribution (Dhabhar *et al*. 1995, Martin *et al*. 2008, Sapolski *et al*. 2000).

In this study, the relationship between reproductive stress, plasma hormone levels and blood parameters in the highveld mole-rat, *Cryptomys hottentotus pretoriae* was investigated. I documented agonistic and reproductive behavioural patterns, measured urinary testosterone and cortisol levels and analysed blood samples for blood counts. Highveld mole-rat colonies are comprised of non-reproductive males and females, up to three breeding males and usually a single reproductive female (Malherbe & Bennett 2007). Subordinate female highveld mole-rats are physiologically suppressed from breeding whilst in the confines of the colony, most likely by reproductive suppression on the hypothalamo-pituitary axis (Van der Walt *et al.* 2001) while reproductive suppression in males is simply the result of subordinate males not having access to unrelated mates (Janse van Rensburg *et al*. 2003). Reproductive competition can be induced in social mole-rats by providing opportunities for reproduction by giving previously non-reproductive females access to unfamiliar males from other colonies in the presence of other non-reproductive and reproductive females (Cooney & Bennett 2000). Therefore two types of reproductive stressors might be involved: firstly, the physiological switch from a non-reproductive to a reproductive female and secondly, reproductive competition amongst females. Cooney and Bennett (2000) showed that aggression between breeding and non-breeding female Damaraland mole-rats (*Fukomys damarensis*) increased dramatically with the introduction of an unrelated male into the colony, and that subordinate females showed a significant increase of reproductive hormones. Similarly, in the naked mole-rat, aggression amongst high-ranking females escalated after the removal of the queen from colonies (Clarke &

Faulkes 1997). It was therefore predicted that, with the introduction of an unfamiliar male, aggressive behaviour amongst females would increase and would be accompanied by increased urinary testosterone and cortisol concentrations. Finally, I predicted that the number of blood cells with immune-related function would decrease upon the introduction of an unfamiliar male as a result of increased reproductive competition as well as increased testosterone and cortisol levels.

Materials and Methods

Capture and housing

Five complete colonies of highveld mole-rats (ranging in size from 5 to 9 animals; mean colony size: 5.8) were captured from the Tygerpoort area (S25°46'35.45" E28°21'37.34") in Tshwane, South Africa during May 2008. Mole-rats were captured using Hickman live-traps (Hickman 1979) baited with sweet potato. Traps were checked thrice a day and the bait was replaced on a daily basis. Mole-rats from the same colony (i.e. animals caught from the same burrows) were housed together in plastic crates (49.5 x 28 cm) and provided with wood shavings and paper towelling for nesting material. Mole-rats were provided with fresh sweet potato and apple daily and wood shavings were replaced weekly. Room temperature was kept constant at 25 ± 2°C and the light cycle was set to 12L:12D. Mole-rats were kept in the laboratory for one year prior to the onset of the trial. Individuals were marked with different fur clippings for easy identification. Markings remained visible on the animals for the duration of the experiment and it was not necessary to re-do fur-clippings. Colonies were named colony A, B, C, D and E and female individuals numbered within their colonies (Ex. A1, A2, etc.). The heaviest breeding male in

each colony was named M(A), M(B), etc. Putative reproductive males were identified on the basis of being the heaviest males in the colony and their prominent inguinal testes (Bennett 1992, Rosenthal *et al*. 1992) and reproductive females were distinguished from non-reproductive females by the presence of axillary teats (only in breeding females), pregnancies and perforate vaginae (Moolman *et al*. 1998). When only referring to "females" in the results section it means that the results for both reproductive and non-reproductive females are lumped together.

Experimental design

The experiment comprised of three stages. In the first stage of the experiment males were removed from the colony, except for the largest breeding male that was left in the colony. The second stage of the experiment involved the removal of the breeding male from the colonies to create all-female colonies so that aggression and competition amongst non-reproductive and reproductive female animals could be monitored in the absence of the reproductive female's mate/s and any other potential breeding mates. After the removal of males, five females were left in one colony (Colony A) and three females left in each of four other colonies (Colony B-E). For the third and final stage of the experiment, an unfamiliar reproductive male (>150g) from one of the other colonies was introduced into each all-female colony to induce reproductive competition between females of either breeding status. All females were used for the behavioural component of this study (n=17), however one small non-reproductive female (E1, 65g) was excluded from the hormone assays and haematology analyses because no viable urine or blood samples could be collected from this individual.

Behavioural observations

A study by Oosthuizen *et al.* (2003) revealed that highveld mole-rats are more active during dark cycles. Thus, mole-rats were observed within their respective colonies under red light in the dark phase of the light cycle and behavioural categories were recorded along with the individuals involved in the actions and interactions. The behaviours of all female colony members were observed in parallel for a total of five hours per stage. Interactions with males in the colonies were also recorded. Periods of observation lasted for one hour duration and observation times were spread out evenly in the dark cycle over a time span of two to three weeks. Recording sessions were divided into one-minute sample intervals during which it was noted whether or not aggressive, submissive or grooming behaviour had occurred during the interval (one-zero sampling) (Martin & Bateson 1986). The total score was then expressed as the total minute intervals for the entire observation period per stage. Focus was placed on behavioural acts that may indicate agonistic relationships amongst colony members (aggression, submission and grooming) and reproductive activity (courting and mating). Behaviours that involved two or more mole-rats were recorded from the perspective of both the actor and the receiver of the behaviour. A detailed description of the behaviours recorded is provided in Appendix A. Grooming behaviour was subdivided in autogrooming and allogrooming and both the groomer and the recipient of the grooming were recorded. Sexual behaviour was divided into two categories: courtship and copulation. Courtship displays appeared very similar to submissive behaviour; females lift their tails dorsally providing access to the males to smell their genitalia. This was usually accompanied by high pitch squeals. Unlike commonly observed submissive

behaviour, the female would then reverse, trying to get under the male. Also, as opposed to submissive behaviour, this type of behaviour was only seen once an unrelated male was introduced into the all-female colonies, was never the result of the male or any other female showing aggressive behaviour and was normally followed by copulation. Because of the short duration bouts and low frequency of occurrence of courting and mating, these behavioural acts were counted and expressed as the total number of occurrences for the entire observation period per stage.

Urine collection

After completion of behavioural observations for every experimental stage, urine was collected from females by placing each individual into a cylindrical collection chamber with a mesh floor. Samples were only collected between 06h00 and 12h00 during the day to reduce the possible effect of diurnal fluctuations in hormone levels. The collection chamber was fitted with a plastic tray under the mesh floor where deposited urine was collected. The tray was checked on an hourly basis for urine. The urine was drawn into a Pasteur pipette and transferred to Eppendorf tubes after which samples were frozen (-30°C) for subsequent hormone determination. After approximately 1ml urine was collected, the mole-rats were removed from the collection chambers and placed back into crates with the rest of the colony members.

Testosterone assay

Total testosterone was measured using a coat-a-count testosterone kit (Diagnostic products Corporation). Five urine samples from reproductive females

and eleven samples from non-reproductive females were assayed for testosterone in 1) intact colonies, 2) in all-female colonies and 3) in all-female colonies with an introduced male. The antiserum is highly specific for testosterone, with cross-reactivity for dihydrotestosterone being less than 5%. A series of known calibrators of testosterone were assayed to set up a standard curve. Urine samples (50µl) and ^{125}I-labelled testosterone (1000µl) were added to assay tubes in duplicate and briefly vortexed. Assay tubes were then incubated in a water bath (37ºC) for 3 hours. Bound and free ^{125}I-labelled testosterone was separated by emptying the assay tubes, which were then counted in a gamma counter for one minute. A calibration curve was used to convert the counts into testosterone concentrations. A serial double dilution of a sample containing a high concentration testosterone paralleled the standard curve, validating the assay (ANCOVA, F=2.78, P>0.05) following a log-logit transformation of the data (Chard 1987). The sensitivity of the assay was 0.011 nmol/L and the intra-assay coefficient of variation was 7.25%.

Cortisol assay

Cortisol was measured using a coat-a-count cortisol kit (Diagnostic Products Corporation) using the same procedure as described above for the testosterone assay. The assay tubes were, however, incubated in the waterbath (37ºC) for a shorter period of only 45 minutes. A serial double dilution of sample containing a high concentration cortisol paralleled the standard curve, validating the assay (ANCOVA, F=1.34, *P*<0.05). The sensitivity of the assay was 5.5 nmol/L and the intra-assay coefficient of variation was 13.42%.

Creatinine determination

Subsequent to the cortisol and testosterone assays, urine samples were assayed for creatinine. Creatinine is formed by the breakdown of tissue muscles and is excreted at a relatively constant rate (Schmidt-Nielsen 1998). By expressing hormone concentrations per milligram of creatinine, urine concentration can be standardised for variation in the concentration of the urine (Carrieri *et al*. 2001). Cortisol and testosterone concentrations are therefore expressed as ng per mg creatinine. Creatinine concentrations were determined using a modified Jaffe reaction (Folin 1914). A volume of 10µl of standard or sample was added in duplicate to the wells of a microplate, leaving two wells empty as a duplicate control blank. A further 300µl of picric reagent was added to all the wells, including the blanks. Picric reagent comprised of saturated picric acid solution, alkaline triton and double deionised H_2O (1:1:10). The alkaline triton was composed of 4.2 ml triton X-100, 12.5 ml NaOH 1N (AVS) (10g in 250ml water) and 66.0 ml double deionised H_2O. The microplate was then incubated in the dark at room temperature, for a period of 1.5 hours to allow colour development to occur. A standard curve (R^2 >0.99) was used to determine all sample values.

Blood sampling

The plastic housing crates with mole-rats were placed in an oven (36-38°C) for half an hour to increase blood flow of the individual animals. Blood samples were obtained from veins in the feet of female animals one day after urine collection. A volume of at least 250 µl of blood was collected by capillary action using heparinised microhematocrit tubes and subsequently stored in tubes coated with EDTA. The blood samples were sent to the Clinical Pathology Laboratory at the Onderstepoort

Veterinary Academic Hospital, University of Pretoria where haematological analyses were carried out within 8 hours of collection using an electronic cell counter (Coulter Electronics Inc.). To assess the immunological status of reproductive and non-reproductive females across the three stages the following parameters were documented: red blood cell count (RCC), lymphocytes, white blood cell counts (WCC), monocytes, neutrophils (mature and immature), eosinophils, basophils and platelet counts. Mean corpuscular volume (MCV) was also measured to assess the effect that repetitive blood collection had on the animals. An increase in MCV values indicates macrocytosis that can be associated with recent blood loss (Barger 2003, Carmel 1979). Clotted samples could not be analysed and were discarded (9 out of 48 samples).

Statistical analysis

Statistical analyses were carried out with SPSS (Version 17.0, Chicago, Ill). Behavioural data and datasets from hormone assays were tested for normality using the Shapiro-Wilk test (Shapiro & Wilk 1965). None of the variables were normally distributed and as a consequence non-parametric tests were employed (Siegel 1957). Data (behavioural and hormone) were collected from all females used in the experiment and were compared between stages and between reproductive and non-reproductive females within the stages. Comparisons between stages were made using Friedman ANOVA tests. In case these yielded significant results, post-hoc Wilcoxon matched-paired tests were carried out. The change in frequency and incidence of courtship and copulation prior to the removal of males from colonies (stage 1) and following the introduction of unrelated males (stage 3) were also compared using Wilcoxon matched-pair tests. Data between reproductive and non-

reproductive females were compared within stages using Mann-Whitney U tests. In addition, comparisons were also made between females observed to copulate with unrelated males (new-reproductive females: RF_{new}) and other females (previous RF's and other NRF's) for the third stage (Mann-Whitney U tests) to determine if activation of reproductive activity had an effect on behaviour, urinary hormone concentrations and blood parameters. I tested for correlations between aggressive behaviour and courting (the two main indicators of reproductive competition) and hormone data to establish possible direct relationships using Spearman's correlation coefficient. Because a number of blood samples were lost due to clotting (leading to an incomplete dataset), haematological parameters were analysed for the effect of stage and reproductive status using generalised estimating equations (GEE's) (Zeger & Liang, 1986). GEE's are suitable to analyse datasets with repeated measures (stage) and missing points (Liu *et al*. 2009, Zeger & Liang 1986). The best fitted distribution for each variable was chosen according to Pan's quasi likelihood under the independence model information criterion (QIC) (the lowest QIC score is judged to be the best) (Ballinger 2004). GEE's are robust against the wrong choice of correlation structure, and results remain similar regardless of the choice of distribution (Liu *et al*. 2009, Zeger & Liang 1986). In case the effect of stage on any haematological parameter was significant, post hoc least significant different tests (LSD) were employed to determine the nature of the effect. Full-factorial interactions of parameters were tested for but none was found to be significant and are thus not further discussed in the results section. The total number of intervals for aggression over the complete observation period was added as a covariate in the models to determine if reproductive competition influences haematological parameters. No effects were found for any of these variables and thus these are not discussed in

further detail. I also tested for significant correlations between the number of intervals of courting and haematological parameters in the third stage (Spearman's correlation coefficient) but again no significant correlations were found (RCC: r=-0.65, p=0.12; lymphocytes: r=0.36, p=0.21; WCC: r=0.089, p=0.76, monocytes: r=0.19, p=0.52, neutrophils: r=0.089, p=0.76; eosinophils: r=-0.021, p=0.94, basophils: r=0.12, p=0.69; platelet count: r=0.63, p=0.13). No comparisons could be made between haematological parameters of RF_{new} and other females during the third stage due to low sample size. Values are reported as mean per female ± standard deviation (SD). All statistical analyses were two-tailed and the level of statistical significance was taken as $p \leq 0.05$.

Results

Behaviour

The mean number of intervals of observed auto-grooming activities decreased following the removal of males from colonies, and again with the introduction of an unrelated male although the effect was not significant (Friedman ANOVA, χ^2=4.94, *P*=0.085) (Table 1). No significant difference was found between the mean number of intervals of observed allogrooming activities between the stages (Friedman ANOVA, χ^2=0.286, *P*=0.87) (Table 1). During the third stage however, there was a significantly higher mean number of intervals allocated to grooming males (2.80±2.95, *n*=5) than to grooming other females (0.53±1.00, *n*=17) (Mann-Whitney U test, U=18, Z=-2.17, *P*=0.03) as opposed to the first stage where there was no significant difference (Mann-Whitney U test, U=20.5, Z=-0.49, *P*=0.62) (Appendix B).

A description of the groomers and the grooming-recipients of each stage is given in Appendix B.

Table 1. The mean number of intervals a female was observed performing a behavioural act (autogrooming, allogrooming, aggression, submission, courting and mating) during a total of 300 minutes of observation. The values are presented separately for reproductive females (RF)(n=5) and non-reproductive females (NRF)(n=12).

	Stage 1		Stage 2		Stage 3	
Behaviour	RF	NRF	RF	NRF	RF	NRF
Autogrooming	13.0±10.8	10.6±6.5	7.2±4.2	5.7±4.6	3.4±3.2	5.0±4.9
Allogrooming	2.0±2.4	1.2±2.0	0.4±0.6	0.8±1.3	0.2±0.4	1.8±3.2
Aggression	1.6±2.5	1.5±2.1	0.4±0.6	0.9±1.4	3.4±1.8	3.8±7.3
Submission	0.6±0.9	1.2±2.1	0.4±0.6	0.7±1.4	0.8±1.3	0.4±0.7
Courting	0	0	N/A	N/A	1.2±1.8	2.0±2.9
Mating	0.2±0.4	0	N/A	N/A	0	1.1±1.8

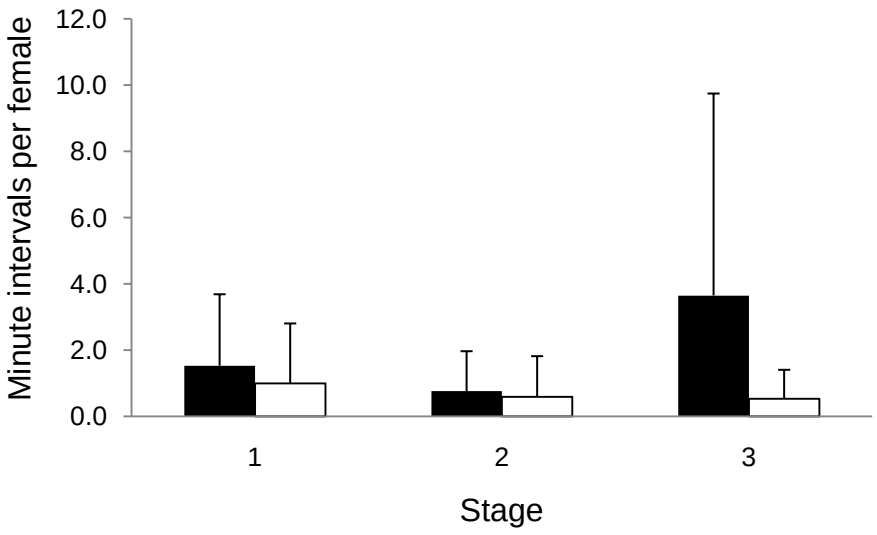

Figure 1. Frequency of aggressive (solid bar) and submissive (open bar) behavioural acts (mean ± SD) per female (all females, *n*=17) for the total five hours observation period by females in complete colonies (stage 1), all-female colonies (stage 2) and all-female colonies with an introduced male (stage 3)

There was a significant change in the mean number of intervals of observed aggressive behavioural acts by females across the three stages (Friedman ANOVA, χ^2=8.77, *P*=0.012) (Figure 1, Table 1). The mean number of intervals of aggression in stage 3 (3.7±6.1, *n*=17) was significantly higher than in stage 1 (1.5±2.2, *n*=17) (Wilcoxon test, Z=-2.30, *P*=0.022) and stage 2 (0.8±1.2, *n*=17) (Wilcoxon test, Z=-2.24, *P*=0.025) (Figure 1). There was, however, no significant difference in mean number intervals of aggressive behaviour between stage 1 and stage 2 (Wilcoxon test, Z=-1.26, *P*=0.21). The reproductive females were not always the most aggressive females in colonies, but were the most aggressive females after the introduction of an unrelated male in 3 out of 5 colonies (Appendix C). The mean number of intervals of submissive behavioural acts did not change in all-female colonies and after the introduction of unrelated males (Friedman ANOVA, χ^2=2.38, *P*=0.30) (Figure 1).

No courtship was observed during the initial first stage, while the number of courtship events observed during the third stage increased significantly to an average of 1.8±2.8 per female over the entire observation period for the third stage (Wilcoxon test, Z=-2.84, *P*=0.004) (Table 2). The total number of observed copulations for the entire observation period increased from a single copulation observed in colony A (A2 and breeding male) during the first stage to a total of 13 copulations (0.7±1.6 copulations per female) during the third phase, although the difference was not significant (Wilcoxon test, Z=-1.91, *P*=0.056) (Table 2). In four out of five colonies the introduced male mated with a female other than the original reproductive female (Table 2). No mating was observed during the third stage in the other colony (Colony C) (Table 2). In only one out of the five colonies (Colony B) was the introduced male observed to mate with more than one female (Table 2). There

were no significant differences found for any of the behavioural acts that were documented for this study between reproductive and non-reproductive females within the three stages or between RF_{new} and other females within the third stage (Table 3). E1 was the only female that produced a litter approximately three months after the experiment.

Table 2. The total number of times courtship and copulation was observed per female after the introduction of an unrelated male into all-female colonies (stage 3) over the entire observation period (300 minutes).

Colony	ID	Courtships	Copulation
A	A1	2	0
	A2*	2	0
	A3	0	2
	A4	1	0
	A5	0	0
B	B1	1	1
	B2	2	2
	B3*	0	0
C	C1	2	0
	C2	0	0
	C3*	0	0
D	D1	5	2
	D2	0	0
	D3*	0	0
E	E1	10	6
	E2	2	0
	E3*	4	0

*Reproductive female

Table 3. Results for comparisons (Mann-Whitney U tests) of the intervals of behavioural acts between reproductive (RF) and non-reproductive females for stages 1, 2 and 3 (RF's vs. NRF's) and between females who mated with the introduced male and other females in the colony (RF_{new} vs. F_{rest}) within the third stage.

	RF's vs. NRF's						RF_{new} vs. F_{rest}	
	Stage 1		Stage 2		Stage 3		Stage 3	
Behaviour	Z	*P*	Z	*P*	Z	*P*	Z	*P*
Grooming	-0.21	0.83	-0.85	0.34	-0.48	0.63	-0.05	0.96
Allogrooming	-0.83	0.41	-0.12	0.68	-1.20	0.23	-1.08	0.28
Aggression	-0.11	0.91	-0.42	0.68	-1.45	0.15	-0.75	0.45
Submission	-0.24	0.81	-0.12	0.90	-0.44	0.66	-0.93	0.35
Courting	N/A		N/A		-0.67	0.51	-1.39	0.17
Mating	-1.55	0.12	N/A		-1.64	0.10	N/A	

Hormone assays

There was no significant difference in the urinary testosterone concentrations when analysing all females (*n*=16) between stages (Friedman ANOVA, χ^2=5.00, *P*=0.78). However, urinary testosterone of reproductive females (*n*=5) differed significantly between the different stages (Friedman ANOVA, χ^2=7.60, *P*=0.02) (Figure 2). Urinary testosterone of stage 1 (264.43±201.50 ng/mg Cr) was significantly lower than that of stage 2 (417.74±465.25 ng/mg Cr) (Wilcoxon test, Z=-2.02, *P*=0.043) and stage 3 (253.74±182.27 ng/mg Cr) (Wilcoxon test, Z=-2.02, *P*=0.043) (Figure 2). There was no significant difference in urinary testosterone concentrations of reproductive females between stage 2 (911.36±446.89 ng/mg Cr) and stage 3 (866.38±829.12 ng/mg Cr) (Wilcoxon test, Z=-0.14, *P*=0.89). There was no significant difference in urinary testosterone concentrations of non-reproductive

females (*n*=11) between stages (Friedman ANOVA, χ^2=1.27, *P*=0.53) (Figure 2). Only in the second stage were urinary testosterone levels of reproductive females (724.03±646.27 ng/mg Cr) significantly higher than those of non-reproductive females (278.51±298.98 ng/mg Cr) (Mann-Whitney U-test, U=76.00, Z=-1.98, *P*=0.047) (Figure 2). In the third stage, although not significant, testosterone levels were higher for reproductive females (304.14±247.76 ng/mg Cr) than for non-reproductive females (194.90±142.44 ng/mg Cr) (Mann-Whitney U-test, U=11.00 Z=-1.87, *P*=0.062) (Figure 2).

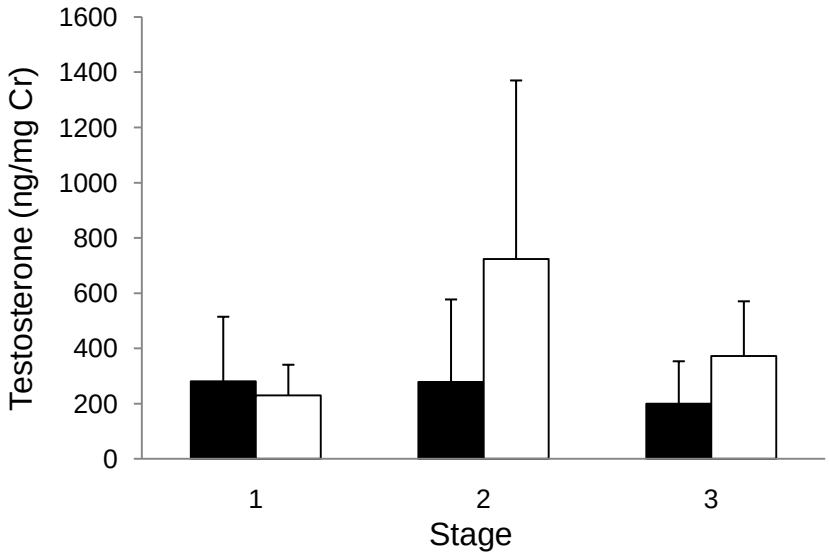

Figure 2. Mean urinary testosterone concentrations corrected for creatinine (±SD) of non-reproductive females (solid bars, *n*=11) and reproductive females (open bars, *n*=5) for stage 1, stage 2 and stage 3.

Urinary cortisol of all females (*n*=16) differed significantly between stages (Friedman ANOVA, χ^2=6.13, *P*=0.047). However, no significant differences were found in posthoc tests (Wilcoxon test, stage 1 vs. stage 2: Z=-1.14, *P*=0.26; stage 1 vs. stage 3: Z=-0.21 *P*=0.84; stage 2 vs. stage 3: Z=-1.55, *P*=0.12). Urinary cortisol concentrations did not change significantly between stages for reproductive females

(*n*=5) (Friedman ANOVA, χ^2=3.60, *P*=0.17) or for non-reproductive females (*n*=11) (Friedman ANOVA, χ^2=4.55, *P*=0.10) (Figure 3). No significant difference with respect to status was found for urinary cortisol within any of the stages (Figure 3).

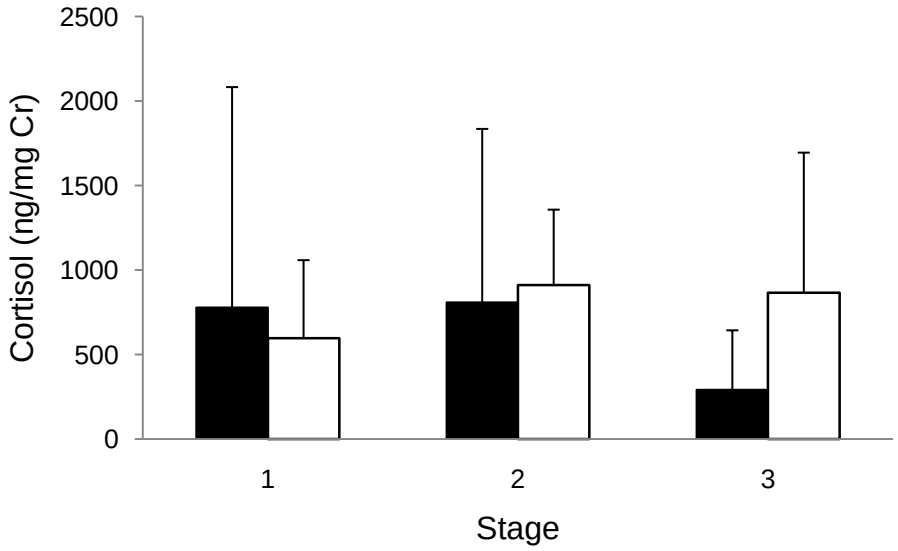

Figure 3. Mean urinary cortisol concentrations in urine (±SD) of non-reproductive females (white bars, *n*=11) and reproductive females (black bars, *n*=5) corrected for creatinine for stage 1, 2 and 3.

There was no significant difference in testosterone levels between females that were shown interest by the newly added males in stage 3 (RF_{new}) (183.69±268.86 ng/mg Cr, *n*=4) and females that did not mate with these males (265.14±151.78 ng/mg Cr, *n*=13) (Mann-Whitney U test, U=29.00, Z=-1.25, *P*=0.24). Similarly, there was no significant difference found between cortisol levels of RF_{new} (292.05± 232.80 ng/mg Cr, *n*=4) and other females (545.40±629.72 ng/mg Cr, *n*=13) (Mann-Whitney U test, U=32.00, Z=-1.01, *P*=0.34) during the third stage.

There was no correlation found between frequency of aggressive acts initiated and testosterone concentration (Spearman's correlation, *r*=-0.15, *P*=0.32) or cortisol concentration (Spearman's correlation, *r*=-0.036, *P*=0.81) (48 measurements from 16 individuals) or between frequency of courting and testosterone concentration

(Spearman's correlation, r=-0.064, P=0.81) (third stage only, n=16) or cortisol concentration (Spearman's correlation, r=0.16, P=0.56). There was no significant correlation found between overall urinary testosterone levels and overall urinary cortisol levels (Spearman's correlation, r=0.18, P=0.15).

Blood analysis

Overall the mean lymphocyte count of reproductive females (2.38±0.96 x10e9/l, 12 measurements from 5 individuals) was significantly lower than that for non-reproductive females (3.36±1.57 x10e9/l, 27 measurements from 11 individuals) (GEE, χ^2=4.52, df=1, P=0.033). The mean platelet count of reproductive females (713±318.96 x10e9/l, 12 measurements from 5 individuals) was significantly higher than that of non-reproductive females (491±121 x10e9/l, 27 measurements from 11 individuals) (χ^2=6.31, df=1, P=0.012). There was no significant difference between reproductive and non-reproductive females for any of the other blood measures (Table 4).

Total WCC's differed significantly between stages (GEE, χ^2=11.61, df=2, P=0.003) (Figure 4). Mean WCC of stage 2 (6.99±2.33 x10e9/l, n=12) was significantly lower than stage 1 (8.44±2.89 x10e9/l, n=14) (LSD, P=0.001) and although not significant, somewhat lower than stage 3 (8.09±3.40 x10e9/l, n=14) (LSD, P=0.096). There was no significant difference in total WCC's between stage 1 and 3 (LSD, P=0.18). MCV increased significantly across the three stages (χ^2= 11.05, df=2, P=0.004) (Figure 5) with MCV of stage 1 being significantly lower than that of stage 2 (LSD, P=0.036) and stage 3 (LSD, P=0.002). There was, however no significant difference between MCV of stage 2 and stage 3 (LSD, P=0.36). No

significant effect of stage was found for any of the other measures. Furthermore, no effects of either reproductive status or stage were found on the individual white blood cell types (monocytes, mature and immature neutrophils, eosinophils and basophils) (Table 4).

Table 4. GEE's showing the effect of reproductive status (df=1) and stage (df=2) on red blood cell count (RCC), lymphocytes, white blood cell count (WCC: neutrophils, monocytes, eosinophils and basophils), platelet count and mean corpuscular volume (MCV).

	Factors	
Blood parameter	Reproductive status	Stage
RCC (x10e12/l)	χ^2= 3.06, p = 0.08	χ^2= 0.72, p = 0.97
Lymphocytes (x10e9/l)	χ^2= 4.52, p = 0.03*	χ^2= 2.34, p = 0.31
WCC (x10e9/l)	χ^2= 0.056, p=0.81	χ^2=11.61, p=0.003**
Monocytes	χ^2= 0.15, p = 0.90	χ^2= 2.77, p = 0.87
Neutrophils (mature)	χ^2= 0.95, p = 0.33	χ^2=3.77 , p=0.15
Neutrophils (immature)	χ^2= 0.48, p = 0.49	χ^2=0.88 , p=0.64
Eosinophils	χ^2= 0.003, p=0.96	χ^2= 1.94, p = 0.38
Basophils	χ^2= 1.09, p = 0.30	χ^2= 1.22, p = 0.54
Platelet count	χ^2= 6.31, p=0.01*	χ^2= 3.04, p=0.21
MCV(fl)	χ^2= 3.14, p = 0.08	χ^2= 11.05, p = 0.004**

*Significant at p<0.05
**Significant at p<0.01

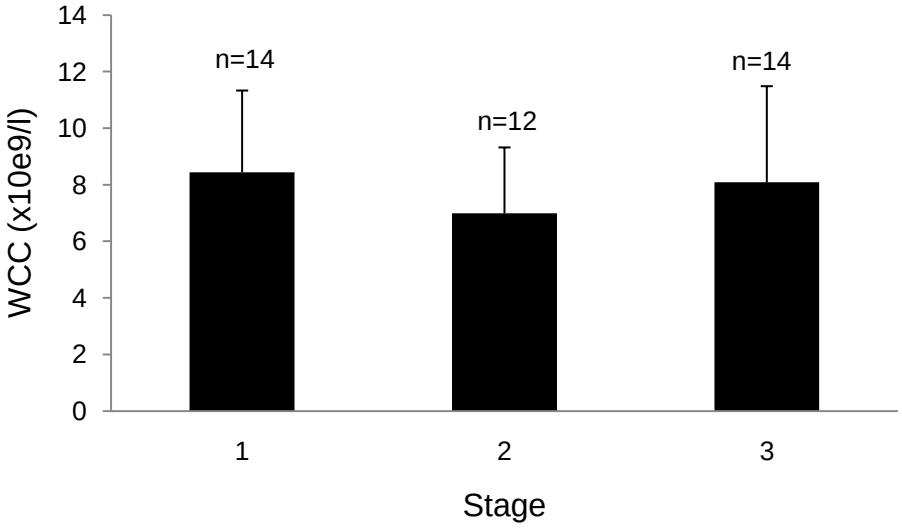

Figure 4. White blood cell counts (WCC) from females during stage 1, 2 and 3.

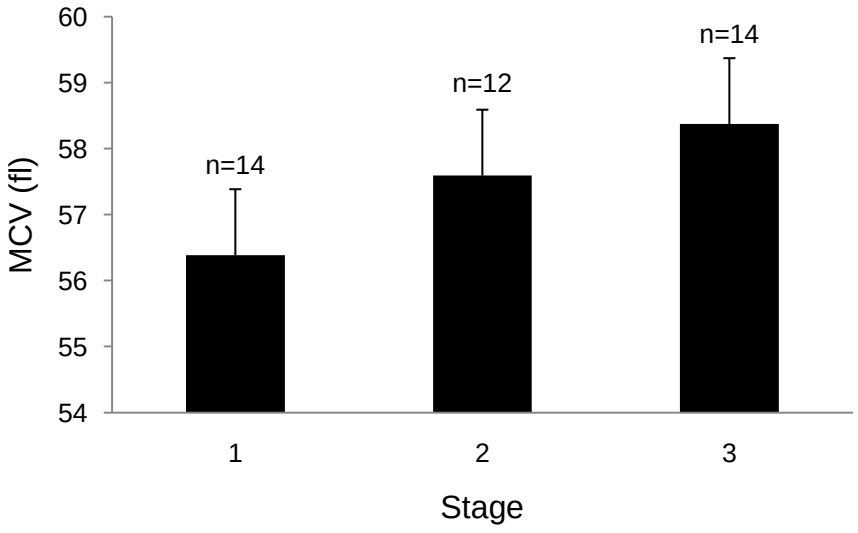

Figure 5. Mean corpuscular volume (MCV) of blood, measured in femtoliters (fl), taken during Stage 1, three weeks later (stage 2), and a further three weeks later (stage 3).

Discussion

The manipulation of the colony composition of the highveld mole-rat had a significant effect on the behavioural patterns of female mole-rats. After the introduction of an unrelated male into all-female colonies, female-female aggression increased significantly. In spite of reproductive females exhibiting aggressive behaviour most frequently during the third stage, all but one lost their “reproductive status” to another female. Aggression was therefore not related to how successful a female was in mating with the unfamiliar male, suggesting that non-reproductive females are not reproductively inhibited through subjugation by reproductive females.

Although the time spent on allogrooming was unaffected by the removal and additions of reproductive males, females groomed unfamiliar reproductive males more during the third stage than the familiar male during the first stage. Allogrooming plays an important role in determining and maintaining social structures in mammals (Kutsukake & Clutton-Brock 2006, Madden & Clutton-Brock 2009). It is therefore possible that female highveld mole-rats use allogrooming in an attempt to establish a sexual pair-bond with the introduced unfamiliar male. This appeared to be the case in colony E where the introduced male mated with the only female that was observed grooming him, although it was not necessarily apparent in the other colonies. It is of course possible that these interactions were missed since behavioural observations were only done in randomly chosen one hour sessions. The change in grooming behaviour, the dramatic increase in aggressive behaviour following the introduction of an unrelated male and the increase of bouts of courtships and copulations showed

that the experimental manipulations stimulated reproductive competition between females under a laboratory setting.

In captive colonies of naked mole-rats, urinary testosterone concentrations were found to be an important determinant of reproductive status (Clarke & Faulkes 1998). Lutermann *et al.* (under revision) found higher plasma testosterone levels for breeding females of the Damaraland mole-rat as well as the Natal mole-rat (*Cryptomys hottentotus natalensis*) when compared to non-reproductive colony members. In the highveld mole-rat there was no relationship between urinary testosterone concentrations and the reproductive status during the first stage, when mole-rats were kept with their original reproductive male. Similarly, Clarke *et al.* (2001) also found no such relationship for functionally complete colonies of the Damaraland mole-rat. Testosterone levels of reproductive females did, however, increase significantly after the removal of males from colonies and the introduction of unfamiliar males into all-females colonies compared to the baseline levels measured in stage 1. However, no correlation was found between urinary testosterone levels and aggression in female highveld mole-rats. The absence of a straight correlation does not exclude that such a relationship exists, but only suggests that they are not correlated in a linear fashion. Futhermore, the lack of a direct relationship could be due to the small sample size. Also, testosterone may be positively related to aggression within individuals rather than between them (Beehner *et al.* 2005). Although testosterone is often assumed to play the same role in promoting agonistic behaviour in females as in males, its role in aggression in females is not clearly defined and studies often produce conflicting results (Albert *et al*. 1989, Davis & Marler 2003, Gill *et al.* 2007, Goymann *et al.* 2008). Some studies intimate that progesterone may be more important than testosterone in modulating female

aggression (Albert *et al.* 1992, Davis & Marler 2003, Goymann *et al.* 2008, Vandenbergh 1971). For example, recently Goymann *et al.* (2008) showed that even though testosterone levels were not affected, progesterone levels in female African black coucals (*Centropus grillii*) were negatively correlated with agonistic behaviour. They proposed that progesterone and testosterone interact to modulate aggression. Oestradiol and oestrogen are also thought to play a role in female aggression, especially in combined action with testosterone (Albert *et al.* 1992, Albert *et al.* 1993, Davis & Marler 2003). Although reproductive status did not affect urinary testosterone levels overall, the levels of reproductive females were significantly higher than that of non-reproductive females in all-female colonies during the second and tended to be higher during the third stage. This indicates that reproductive females may have anticipated reproductive competition during the second stage following the removal of their original breeding partner.

Studies have shown that dominant breeding females of cooperative breeding species such as ring-tailed lemurs (*Lemur catta),* African wild dogs (*Lycacon pictus*), and common marmosets (*Callithrix jacchus*) have higher glucocorticoid levels than subordinate individuals (Cavigelli *et al.* 2003, Creel *et al.* 1997, Saltzman *et al.* 1998, Starling *et al*. 2010). Like the highveld mole-rat, African wild dogs and common marmosets exhibit a high reproductive skew where the dominant female is normally the only one that reproduces (Cavigelli *et al.* 2003). It has been suggested that reproductive hormones influence glucocorticoid levels (Saltzman *et al.* 1998), although other factors such as temperature, season, sex and age probably also have a considerable effect (Starling *et al*. 2010). Glucocorticoid levels of ring-tailed lemurs increased during the breeding season suggesting that reproductive competition may play a role in determining glucocorticoid levels of individuals (Starling *et al*. 2010).

Glucocorticoids are also known to be a useful measure of the level of stress that animals may experience (Sapolsky *et al.* 2000, Starling *et al*. 2010). Reproductive females were expected to have higher urinary cortisol concentrations than non-reproductive females and urinary cortisol concentrations were expected to rise as a result of reproductive competition. Surprisingly, breeding status did not affect urinary cortisol concentration nor did it change between the stages. Urine samples were only taken at the end of each stage to minimize disturbances. It is possible that raised urinary cortisol levels of some individuals may have been elevated earlier during the stage and returned to normal baseline levels after two weeks

Increased numbers of white blood cells are believed to be indicative of an increase in immunological investment (Bachman 2003, Norris & Evans 2000). Interpretation of haematological parameters requires caution since increased cell numbers could also indicate an infection (Beldomenico *et al.* 2008, Norris & Evans 2000). However, no signs of diseases were observed in the test subjects, and it was therefore assumed that increased cell numbers indicated higher investment in immune function rather than infection. Overall, reproductive females had lower lymphocyte counts than non-reproductive females indicating that being reproductively active may negatively influence the adaptive arm of the immune system. These results are similar to the study of Ardia *et al*. (2003) that showed a reduced ability of lymphocytes to produce antibodies with increasing breeding effort. Total WCC's decreased significantly in all-female colonies, but increased again after the introduction of unfamiliar males. Therefore this change cannot be attributed to increased reproductive competition or stress. It is however, possible that the stress caused by the change in colony composition caused total WCC's to drop (Dhabar *et al.* 1995). Total WCC's were also not affected by reproductive status. However,

platelet count of reproductive females was significantly higher than that of non-reproductive females. Blood platelets are a component of the inflammatory response especially and are important in responses against invading bacteria (Promislow 1991). Reproductive females, being the more dominant animals in a given colony (Malherbe & Bennett 2007, Moolman *et al*. 1998), probably run a higher risk of acquiring open wounds by aggressive encounters and of acquiring sexually transmitted diseases than non-reproductive females and can therefore be expected to invest more energy in some pro-inflammatory systems (Nunn *et al.* 2000, Nunn *et al.* 2003). Increased MCV after every stage indicates that the frequency of blood sampling (three weeks) may have affected the mole-rats to some degree as higher MCV values are associated with macrocytosis that in turn can be caused by acute blood loss (Barger 2003, Carmel 1979).

The behavioural and hormonal results of this study indicate that reproductive competition was successfully stimulated under laboratory conditions in females of the highveld mole-rat. The complexity of the vertebrate immune system makes it difficult to assess immunocompetence, and even more so, to interpret correlations found between a specific immune parameter and other life history components as a single immune measure only provides a single piece of a much larger vista. Total WCC's decreased after the removal of males from colonies during the second stage, indicating that stress from the removal of males from colonies had a negative effect on some aspects of immune function of females. Changes in colony composition and the increase of reproductive competition amongst females had no effect on any other blood cells. Because of the importance of the immune system to an organism's survival, it is highly unlikely that the entire system will be compromised (Braude *et al.* 1999, Martin *et al*. 2008). It makes sense to rather keep some aspects in good

working condition, and divert energy away only from components that are less important during reproduction and that will be easiest to make up for later (Braude *et al.* 1999, Martin *et al*. 2008). The results of this study found reproductive females to have lower numbers of lymphocytes than non-reproductive females, supporting the notion that a trade-off exists between the adaptive immune system and reproduction. No differences were found in the other blood cells (monocytes, neutrophils, basophils and neutophils) except for the number of platelets that were actually higher. These results support Braude *et al.* 1999's "immuno-redistribution theory". Since this study was also successful in stimulating reproductive competition amongst females of the highveld mole-rat, according to changes observed in the behaviour of females, trade-offs involving other immune parameters and even other physiological components in this social animal can now be investigated in this way.

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Appendix A

An ethogram for observed behaviours. The following behavioural acts were identified during the preliminary observation period as behaviours important in agonistic interactions between animals (grooming, aggression and submission) and reproductive activity (courting and copulation).

Behaviour	Description
Autogrooming	A mole-rat nibbling and licking its own pelage, limbs or claws. A mole-rat licking its front paws and then repeatedly wiping paws over the head. When mole-rats scratched themselves with hind feet it was also considered as grooming behaviour.
Allogrooming	One mole-rat nibbling and licking the pelage of another mole-rat usually on and around the head, neck, shoulders and snout. Allogrooming was recorded from the perspective of both the groomer and the receiver of allogrooming.
Aggressive behaviour	*Open mouth gapes* The most common aggressive behavioural act that was observed. Mole-rat lifts its head and opens its mouth widely showing both the upper and lower incisors and makes sudden jumps using all four legs simultaneously while making a bite action in the air. *Tail biting* Tail biting, when one mole-rat bites and pulls on the tail of another mole-rat *Stealing* One individual forcefully takes food from another individual. Aggression was not always directed towards a specific mole-rat, and was often completely ignored by other mole-rats. It was therefore only recorded who the aggressor was, who responded to the aggression as well as the response (aggression/submission). No physical fights between mole-rats or injuries were observed during this experiment.

Submissive behaviour	A mole rat lifts its tail dorsally and gives several high pitched squeaks usually in response to another mole-rats' aggressive behaviour. A mole-rat could also respond to an aggressive "open mouthed gape" by putting one or both of its front paws on the face of the attacker and gently pushing the aggressor away.
Courting	Female, facing away from the male and emitting high pitched squeaks, lifts her tail dorsally and then reverses repeatedly trying to get in under the male.
Copulating	After the females exhibits the courting behaviour the male mounts the female by biting the female on the skin on the back of the neck and grasping her flanks with his forepaws and thrusting of the male's pelvic region follow.

Appendix B

The groomers (G) and recipients (R) of allogrooming behaviour and the number of intervals (MI) that a groomer was observed grooming the recipient for stage 1, 2 and 3. M(A) to M(E) represents the introduced reproductive males originally from colonies A to E.

	Stage 1			Stage 2			Stage 3		
Colony	G	R	MI	G	R	MI	G	R	MI
A	A2	A1	1	A1	A5	2	A1	A3	3
	A2	A4	3	A4	A3	1	A1	M(E)	5
	A4	A2	2				A3	M	1
B	B2	B1	1	B1	B2	1	B1	B2	1
	B2	M(B)	1	B1	B3	1			
C	C3	C4	1	No allogrooming			C1	M(B)	1
	M(C)	C2	1						
D	No allogrooming			D3	D2	1	D2	D1	1
				D1	D3	1	D3	M(C)	1
							M(C)	D2	1
E	E1	E2	1	E1	E2	3	E1	E2	3
	E1	E3	1	E1	E3	1	E1	M(D)	6
	E1	M(E)	3	E3	E1	1			
	E2	E1	1						
	E2	E3	2						
	E2	M(E)	1						
	E3	E2	4						
	E3	M(E)	1						
	M(E)	E3	3						
Total			27			12			23

Appendix C

The number of intervals per stage that individual female mole-rats were observed exhibiting aggressive or submissive behaviour.

Colony	ID	Aggression			Submission		
		Stage 1	Stage 2	Stage 3	Stage 1	Stage 2	Stage 3
A	A1	4	3	10	7	0	0
	A2*	6	1	5	1	0	1
	A3	2	0	3	3	0	1
	A4	7	1	25	1	0	0
	A5	1	4	0	0	1	2
B	B1	1	0	1	0	0	0
	B2	0	1	1	0	0	1
	B3*	0	0	5	0	1	0
C	C1	2	0	3	0	0	0
	C2	0	0	0	0	0	0
	C3*	0	0	1	2	0	0
D	D1	0	0	0	1	1	0
	D2	0	2	0	0	0	0
	D3*	1	1	2	0	0	0
E	E1	0	0	2	2	5	1
	E2	1	0	0	0	1	0
	E3*	1	0	4	0	1	3

*****Reproductive female

Chapter 2

The response of body temperature to a lipopolysaccharide challenge in the highveld mole-rat: the effects of season and reproductive status

Abstract

Fever is believed to play an important role in both the activation and proliferation of various immune cells. Fever can be experimentally induced in animals by subcutaneous lipopolysaccharide (LPS) administration. LPS activates the innate immune response in animals bringing about a febrile response and is commonly used as an indicator of the strength of the innate immune system. It is believed that the immune system varies on a seasonal basis in response to varying seasonal stressors. On that note, it has been postulated that animals may reduce immunocompetence to cope with the increasing energetic demands of reproduction. Immunomodulation has been well studied in laboratory animals, but has rarely been measured in wild animals. After a baseline period, thirty-seven wild-caught highveld mole-rats were subjected to a saline and LPS challenge with body temperature changes being monitored. The effects of sex, breeding status and season to the administration of LPS were investigated. Increases in body temperatures were observed during baseline measurements and following saline administration indicating that the stress of handling alone caused a hyperthermic response. Further significant increases in rectal body temperature were found after injection with a dose of 1 mg kg^{-1} LPS indicating that an immune response had been triggered by the treatment. Absolute rectal temperatures after both saline and LPS were higher in

summer, but no seasonal effect was found for the LPS-induced fever itself, indicating that seasonal energy availability might play an important role in mediating seasonal changes in body temperature but not immune responses. No evidence was found for sex-differences or a trade-off between reproductive status and the ability to launch a fever response. LPS only challenges a single aspect of the immune system, and more in-depth studies are required to fully understand the effects of season and reproductive status on other components of the immune system.

Keywords: Bathyergidae, subterranean rodent, innate immunity, life-history trade-offs, pyrogen, fever, stress-induced hyperthermia

Table 1. Abbreviations

LPS	Lipopolysaccharide
T_b	Rectal temperature measurement
RF	Reproductive female
RM	Reproductive male
NRF	Non-reproductive female
NRM	Non-reproductive male
M_{PT}	Mean value for baseline T_b measurements
ΔT_{mean}	Differences between post-treatment T_b values and mean baseline T_b values
ΔT_{-10}	Differences between post-treatment T_b values and last T_b measurement prior to injection
T_{-60}	Initial rectal temperature measurement done 60 minutes prior to injection with either LPS or saline
T_{-10}	Last rectal temperature measurement before injection with either LPS or saline, done 10 minutes prior to injection.
T20 to T360	Represents the rectal temperature measurement made 20 to 360 minutes after treatment.

Introduction

Although the specific biological purpose of fever is unclear, it is believed to be beneficial to hosts that are infected with pathogens or parasites (Kluger *et al.* 1996). It has been postulated that the energetically expensive act of maintaining elevated body temperatures for a prolonged period in response to infections would not have evolved or persisted unless there was some net evolutionary benefit to the host (Kluger *et al*. 1996). The adaptive value of fever is demonstrated by studies which have shown a positive relationship between core body temperature and survival along with shortened disease duration when animals (both endotherms and ectotherms) are infected with a pathogen (Hasday *et al.* 2000, Jiang *et al.* 2000, Kluger *et al.* 1975, Swenson *et al*. 2007). Furthermore, studies have found an increase in mortality rate when fever in infected animals is inhibited by anti-pyretics (Crocker & Digout 1998, Kurosawa *et al*. 1987, Kluger & Vaughn 1978).

Cytokines are a group of proteins/glycoproteins that mediate interactions between cells during immune responses (Male *et al.* 2006). Many of these cytokines (IL-1, IL-6, TNF-α, TNF-β, etc.) are also pyrogenic cytokines that act directly on the thermoregulatory centre of the anterior hypothalamus causing an increase in core body temperature (Male *et al*. 2006). The obvious association of a febrile response with infections, along with the range of immunological functions of pyrogenic cytokines links fever to immune function and inflammation (Dinarello 2004, Hanson 1993, Male *et al.* 2006). The febrile response of an infected host is likely to play an important role in temperature-dependant immunological responses. For example, elevated body temperatures in a host function in heating certain immune cells to a critical level to control lymphokine (signalling molecules other than antibodies

produced by lymphocytes) expression in immune defences in particular regions of the infected host (Hanson 1993). Furthermore, an increase in body temperature to normal febrile levels leads to quicker lymphocyte division (Male *et al*. 2006), enhances several macrophage functions (Hasday *et al.* 2000) and regulates cytokine secretion and function of dendritic cells (Tournier *et al.* 2003). On the other hand, macrophage and lymphocyte action and proliferation decreases when exposed to temperatures above 41°C (Hasday *et al*. 2000) and dendritic cells reduce cytokine production at temperatures above 40°C (Tournier *et al.* 2003). Therefore febrile responses should be maintained within narrow margins by the physiological systems to maximise effectiveness and prevent damage to cells (Hasday *et al*. 2000).

Lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, is an exogenous pyrogen that is often used to mimic bacterial infection and cause fever in subjects (Dinarello 2004, Martin *et al.* 2008a). When injected into an animal or human, LPS triggers the innate immune system by activating the production of pro-inflammatory cytokines thereby causing an inflammation and a febrile response (Male *et al*. 2006). Recognition of LPS by the immune system is a complex process that involves LPS binding to LPS-binding protein that then passes it on to membrane associated receptors on leukocytes and endothelial cells which in turn activates a pro-inflammatory cascade (Male *et al.* 2006). Binding of LPS to toll-like receptor 4 (TLR4) is believed to be the decisive event in the immune activation as macrophages and B-cells from TLR4 knocked-out mice do not react to LPS (Hoshino *et al.* 1999). Mechanisms of the immune system are very well studied in laboratory models such as mice, rats and rabbits (Hoshino *et al.* 1999, Kurosawa *et al.* 1987, Książek *et al.* 2003) but are rarely researched in wild animals.

The ability of individuals to mount an immune response often varies on a seasonal basis (Greenman *et al.* 2004, Martin *et al.* 2008b, Nelson 2004, Nelson and Demas 1996). It has been suggested that survival of individuals can be optimized if seasonal immune stressors are anticipated and opposed by an increase in immune function (Nelson 2004). In the laboratory, when all other factors are held constant, immune function appears to be enhanced during short day conditions in the majority of small mammals (Nelson & Demas 1996, Nelson *et al.* 2002). In the wild, however, the pattern seems to be reversed with rodents and birds showing a reduction in immunocompetence on short day conditions (Nelson 2004). The harsher climatic conditions along with poorer nutrition and lower energy intakes most likely outweigh the endogenous increase in immune function during winter (Nelson *et al.* 2002). The strong seasonal patterns of many infectious diseases and parasite infections possibly influence fluctuations in immune function to some extent (Altizer *et al.* 2006, Dowell 2001, Martin *et al.* 2008b). Energetic dynamics probably play an important role in mediating seasonal patterns in immunocompetence (Nelson *et al.* 2002).

The energetic costs of maintaining normal immune processes in the absence of infections are still debated (Derting & Compton 2003) but actively mounting an immune response is without a doubt energetically expensive (Derting & Compton 2003, Lochmiller & Deerenberg 2000, Nelson *et al*. 2002). This is experimentally illustrated by studies that show an increase in energetic demand when animals are subjected to immunological challenges (Książek *et al.* 2003, Martin *et al.* 2003). A significant increase in resting metabolic rate was found by Demas *et al.* (1997) after an antibody response in mice (*Mus musculus*) following keyhole limpet haemocyanin challenge. House sparrows (*Passer domesticus*) challenged with phytohaemagglutin (a mitogen that activates cell-mediated immune responses) showed a significantly

elevated resting metabolic rate (Martin *et al*. 2003). Fever alone causes an increase in resting metabolic rate of up to 20% (Buchanan et al 2003, Kluger *et al.* 1996) and it can therefore be deduced that at the very least, a febrile reaction is an energetically expensive activity.

Assuming that energy is one of the most important factors mediating seasonal patterns of immune function, then trade-offs with other expensive life-history components that vary on a seasonal basis will also have a marked effect on immunocompetence (Martin *et al.* 2008b). One trade-off that has recently received increasing attention is the trade-off between immunocompetence and components of reproduction and reproductive effort (Martin *et al*. 2008b). Cai *et al.* (2009) demonstrated that there is an energetic trade-off between humoral immune responses and reproduction in adult male Brandt's voles (*Lasiopodomys brandtii*). Voles challenged with human immunoglobulin G had lower testes and epididymis mass than controls (Cai *et al*. 2009). Pregnant and lactating rats (*Rattus rattus)* exhibited lower LPS-induced fevers compared to virgin rats (Martin *et al*. 1995). Lactation and pregnancy in Siberian hamsters (*Phodopus sungorus*) kept under long day photoperiodic conditions suppressed antibody production responses to keyhole limpet hemocyanin (KLH) (Drazen *et al.* 2003). Williams *et al.* (1999) however, did not find a negative relationship between antibody production and reproductive effort in female European starlings (*Sturnus vulgaris*).

Males and females optimise their reproductive success in very different ways and one can therefore expect that gender differences in the investment strategies between immunity and reproduction will arise (Mckean & Nunney 2005, Martin 2000). Females gain fitness through longevity (Rolff 2002), and therefore females tend to have greater humoral and cell-mediated immunity than males (Martin 2000).

Males, on the other hand, gain fitness by increasing their mating success and will therefore rather invest energy in reproductive mechanisms such as secondary sexual characteristics than into the immune function (Rolff 2002, Folstad & Karter 1992). Males are also normally more susceptible to parasitism than females, possibly as a result of the immunosuppressive effect of androgens such as testosterone (Folstad & Karter 1992, Møller *et al*. 1998).

The highveld mole-rat (*Cryptomys hottentotus pretoriae*) is a social, subterranean rodent that occurs in the summer rainfall regions of the highveld in South Africa (Janse van Rensburg *et al.* 2002). Colonies are made up of a single reproductive female, up to three putative reproductive males and a number of non-reproductive males and females. The reproductive division of labour within this species allows comparisons to be made between reproductive and non-reproductive individuals to investigate differences in their physiological traits. The highveld mole-rat is a seasonal breeder with the majority of litters being born between July through to November (Janse van Rensburg *et al.* 2002). These two traits make the highveld mole-rat an ideal model to independently study the effects of reproduction and season on physiological components such as the immune system. The aim of this study was to determine if fever could be induced in the highveld mole-rat at a dose of 1 mg kg^{-1} LPS and to express the nature of the LPS-induced fever. Furthermore I examined the effects of sex, reproductive status and season on LPS-induced fever response in the highveld mole-rat. Because males tend to favour mating effort over immune investment I predicted LPS-responses to be more pronounced in females. Reproductive mole-rats were expected to divert resources from immune function into reproductive activities. Accordingly I predicted that reproductive mole-rats would exhibit weaker LPS-induced febrile responses than non-reproductive mole-rats. I

also expected LPS-induced fever responses to be higher in the warmer, wetter summer months when food is more abundant than during the dry winter months.

Materials and methods

Capture and housing

Mole-rats were captured from the Tygerpoort area in Tshwane (S25°46'35.45" E28°21'37.34"), South Africa from October 2008 to August 2009 using Hickman live-traps (Hickman 1979) baited with sweet potato. Mole-rats were captured bi-monthly to ensure that samples could be obtained from different seasons throughout the year. Six complete colonies were trapped over the entire trapping period. Traps were checked three times a day, and the bait was replaced on a daily basis. During the capture process, if no further activity arose from the system after three days had passed the colony was considered complete and the traps removed. Captured animals were housed together within their natal colonies in plastic crates (49.5 x 28 cm) and were provided with wood shavings and paper towelling for nesting material. Animals were fed an *ad libitum* diet of fresh potato and apple on a daily basis and all old food was removed. Room temperature was maintained under constant conditions at 25 ± 1°C to exclude body temperature fluctuation as a result of ambient temperature. The light cycle was maintained at 12:12h LD. Animals were maintained in the laboratory for a minimum of one and a maximum of two weeks prior to the start of an experiment. Mole-rats with a body mass under 50g were considered juvenile and excluded from the study. Mole-rats were classified into four groups according to their breeding status namely: reproductive female (RF), reproductive male (RM), non-reproductive female (NRF) and non-reproductive male (NRM). Reproductive

females were discerned by the presence of elongated teats and a perforate vagina, while reproductive males were characterised by being the largest males in the colony (Moolman *et al*. 1998, Rosenthal *et al*. 1992).

Experimental design

On the day of the experiment, animals were removed from their colonies, weighed and placed in separate crates. Rectal temperatures (T_b) of animals were measured using a chromel-alumel thermocouple connected to APPA 51 thermometer (APPA Technology Corporation, Taiwan). The end of the thermocouple was lubricated with petroleum jelly and carefully inserted one centimetre into the rectum whilst the animals were hand-restrained. To determine baseline rectal temperature for each animal, measurements were made at intervals of ten minutes for a period of one hour (six measurements, -60 minutes to -10 minutes). Ten minutes after the last baseline measurement, animals received an intra-peritoneal administration of either a dose of 1 mg kg^{-1} LPS (from *Escherichia coli* serotype 026:B6, Sigma Chemical) dissolved in sterile 0.9% saline or pure sterile 0.9% saline (0.01ml per gram body mass) as control. Twenty minutes after the injection the T_b was measured again and this body temperature measurement was repeated at intervals of 20 minutes for a period of 360 minutes (T20-T360). Intervals were changed from 10 minute intervals for baseline measurements to 20 minute intervals for post-treatment measurement to reduce the stress impact on the mole-rats. After the last measurement (T360) the mole-rats were returned to their respective crates with the rest of the colony members. The experiment was repeated two days later with individuals that had previously been injected with LPS now receiving saline and *vice versa* such that all animals were subjected to both treatments over the

experimental period. To avoid possible order effects, half the subjects were first injected with saline (n=18) and the remainder with LPS (n=19). Room temperature was kept constant throughout experiments at around 25 ± 1°C. Animals received *ad libitum* food during the experimental period. Experiments occurred during the photophase of the light cycle and started at around 10:00am to reduce the possible effects of circadian variations in body temperature. Even though it is stressful to captive animals not used to handling, I still favoured the rectal temperature method over implanting transmitters as it has no long-term effect on the physiology of the animal (Adams *et al*. 2001).

Statistical analysis

All statistical analyses were performed with the aid of SPSS (Version 17.0, Chicago, Ill). The distributions of time-point sub-sets were tested for normality using the Shapiro-Wilk test (Shapiro & Wilk 1965). All sub-sets were normally distributed, except the subset recorded for 260 minutes after LPS treatment (T260). A box plot of the sub-sets revealed no extreme scores or outliers indicating that data for the sub-set was not markedly skewed, and I proceeded to analyse data using parametric tests (Kinear & Gray 2008). To compare baseline T_b values with response T_b values, the six measurements were averaged and compared separately to every time-point value using pairwise *t*-test comparisons. A general linear model (GLM) with repeated measures was used to analyse data with the within-subject factors of time (six levels for baseline and 18 levels for response) and treatment (two levels) and with between-subject factors of season (two levels), breeding status (four levels: BM, BF, NBM & NBF) (Kinnear & Gray 2008). To determine the effect of season, data were divided into measurements collected in summer (October-February, n=19) and that

collected in winter (April-August, n=18). Where significant differences were found for between subject effects of breeding status, Fisher's least significant difference (LSD) tests were used to carry out post-hoc comparisons. A large variation in initial baseline Tb's measurements was found (31.3-35.2°C) meaning that to express the fever response of an animal, response T_b's would have to be compared to a set-point. The mean baseline T_b's was calculated (M_{PT}) but found to be significantly different between pre-saline and pre-LPS treatments (Table 2). The least difference between pre-saline and pre-LPS measurements was found for the measurements made 10 minutes before treatments (T_{-10}) (Table 2). Post-treatment T_b responses to LPS and saline were therefore analysed and compared using a) absolute post-treatment T_b values, b) differences between post-treatment T_b values and M_{PT} (ΔT_{mean}) and c) differences between post-treatment T_b values and last T_b measurement prior to injection (ΔT_{-10}). Mauchly's criterion test of sphericity, a measure of the homogeneity of variances, was obtained for within subject effects and the corrected significant levels (Greenhouse-Geisser) were reported in case of the rejection of the symmetry assumption (Kinear & Gray 2008). Body mass was initially included as a covariate in the analyses. No significant effect of mass was found (GLM, T_b: F=2.34, df=1 P=0.14, ΔT_{mean}: F=0.09, df=1 P=0.93, ΔT_{-10}: F=1.23, df=1 P=0.28) and it was subsequently omitted from the analyses. Full-factorial interactions were analysed but only significant interactions were reported. Post hoc pairways *t*-tests were used to analyse the effects of significant interactions. Test results were considered significant if $P \leq 0.05$.

Results

Rectal temperatures prior to treatment

Although not significant, the mean body temperature (T_{-60}) of the mole-rats measured 60 min prior to the treatment with LPS (33.23±0.89°C, *n*=37) was slightly lower than the corresponding measurements prior to the saline administration (33.52±1.01°C, *n*=37) (*F*=3.90, *P*=0.058). There were, however, significant differences found for the T_{-60} values between the different reproductive groups (*F*=4.18, *P*=0.013), and a post hoc analysis revealed that the mean T_{-60} of NRM's (32.69±0.82ºC, *n*=10) was significantly lower than that for NRF's (33.60±0.78, *n*=14) (LSD, *P*=0.007) (Figure 1). There was no significant difference in T_{-60} measurements between any other reproductive groupings (RM vs. RF, RF vs. NRF and RM vs. NRM) and no seasonal effect was found (Table 2). No significant effect of treatment, season or reproductive status was found for T_{-10} (Table 2).

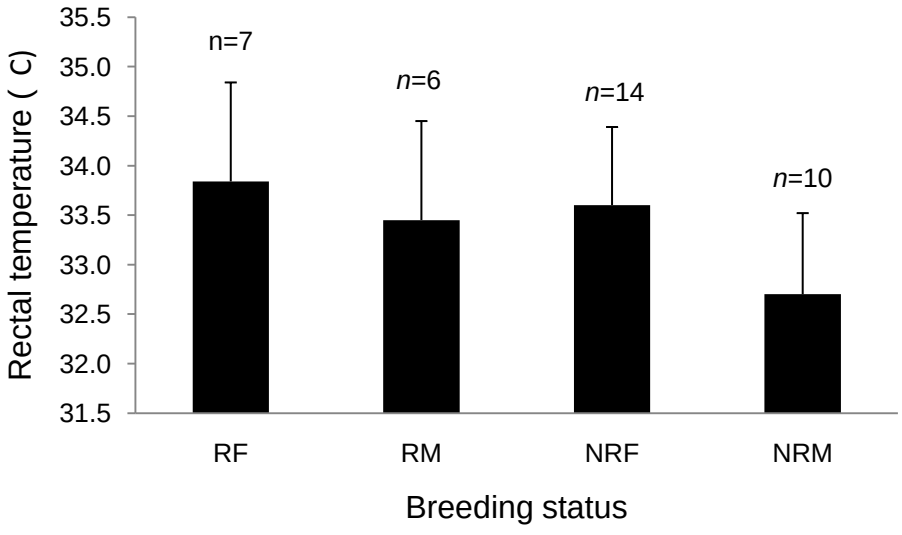

Figure 1. Initial rectal temperature measurements (T_{-60}) from reproductive females (RF), reproductive males (RM), non-reproductive females (NRF) and non-reproductive males (NRM).

Table 2. Analysis of variance with repeated measures of baseline T_b for the first baseline measurement (-60 minutes), the baseline measurement just prior to injection (-10 minutes) and for baseline T_b measured in six intervals of 10 minutes (overall) before LPS and saline treatment (n=37).

	F	df	*P*
Within subjects effects			
$Treatment_{(-60\ minutes)}$	3.90	1	0.058
$Treatment_{(-10\ minutes)}$	1.06	1	0.31
$Treatment_{(overall)}$	4.17	1	0.048*
$Time_{(overall)}$	36.23	5	<0.001**
Between subjects effects			
$Season_{(-60\ minutes)}$	0.78	1	0.38
$Season_{(-10\ minutes)}$	0.23	1	0.64
$Season_{(overall)}$	0.19	1	0.67
$Breeding\ status_{(-60\ minutes)}$	4.18	3	0.013*
$Breeding\ status_{(-10\ minutes)}$	2.28	3	0.14
$Breeding\ status_{(overall)}$	1.47	3	0.24

*Significant at p<0.05
** Significant at p<0.01

Baseline T_b's (T_{-60}-T_{-10}) were significantly affected by treatment (GLM, *F*=4.17, *P*=0.048) with T_b measurements being generally higher during pre-saline (34.03±0.84°C) treatment than during the pre-LPS treatment (33.79±0.74°C) (Table 2, Figure 2). In addition, time had a significant effect on baseline T_b's (GLM, *F*=36.23, *P*<0.001) (Table 2). There were no significant effects of season or breeding status found for the baseline T_b's (Table 2). However, there was a significant "time

by breeding status" interaction found (GLM, df=51, *F*=3.17, *P*<0.001). Post hoc *t*-tests showed no significant differences between consecutive baseline Tb's measured for RF's and only one significant difference between consecutive baseline Tb's for RM's (Appendix A). For non-reproductive animals, however, two (NRF) and three (NRM) out of five measurements were significantly different from the preceding baseline Tb's (Appendix A). No significant differences were found for corresponding saline and LPS baseline T_b's.

Baseline T_b measurements vs. response T_b measurements

The M_{PT} before saline treatment (34.03±0.84°C) was significantly lower than T20, T40, T60, T160 and T200-T300 after saline treatment (Figure 2, Appendix B). T_b for all post-LPS-treatment time-points increased significantly compared to M_{PT} before LPS treatment (33.79±0.87°C) (p<0.001 for all comparisons, Figure 2, Appendix B).

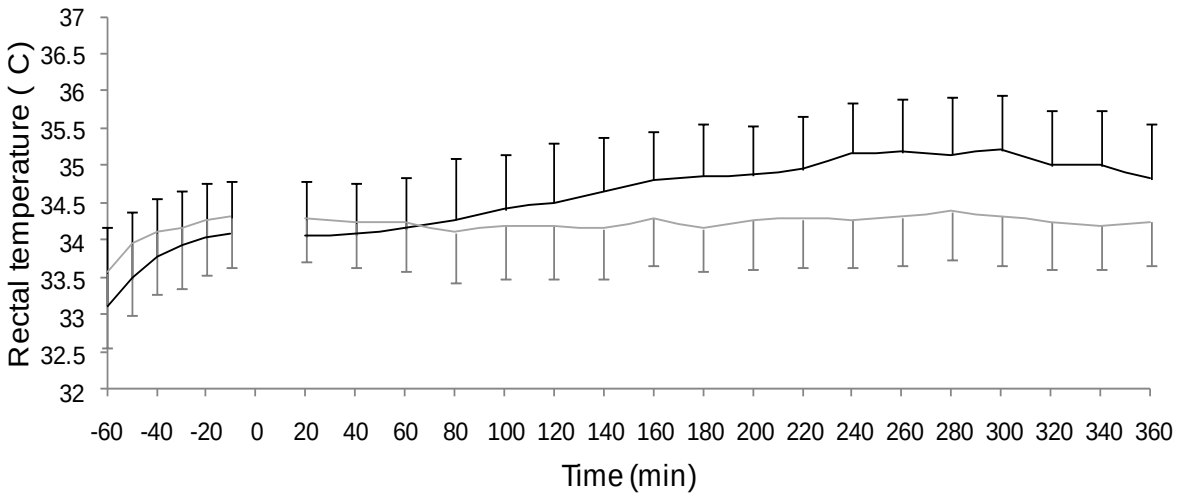

Figure 2. Baseline rectal temperature values (mean ± SD) in ten minute intervals (-60 to -10 minutes) and post-treatment rectal temperature measurements in twenty minute intervals (20 to 360 minutes) after intraperitoneal injection for saline (grey line) and LPS (black line) treatment (n=37).

Response (LPS vs. saline)

There was a highly significant effect of treatment on absolute post-treatment T_b values (Figure 2), ΔT_{mean} and ΔT_{-10} with LPS measurements being higher than saline measures for all three variables (Table 3). A significant effect for time was also found for all three variables (Table 3, Appendix C) with significant consecutive measurements for T100-120 (T_b) (t=-2.45, df=36, *P*=0.020), T140-T160 (ΔT_{mean} and ΔT_{-10}) (t=-3.37, df=36, *P*=0.002), T300-T320 (ΔT_{mean} and ΔT_{-10}) (t=2.58, df=36, 0.014) and T320-T340 (T_b) (t=2.57, df=36, *P*=0.015). A significant effect of season was found for absolute post-treatment T_b's but not ΔT_{mean} and ΔT_{-10}, with an upward shift in temperatures (for both post-saline and post-LPS treatment T_b's combined) during summer (Table 3). Neither of the measures was significantly affected by breeding status (Table 3). No significant seasonal difference was found for post-saline T_b's (GLM, *F*=2.05, df=1, *P*=0.16) or post-LPS Tb's (GLM, F=3.72, df=1, P=0.064).

Significant time*treatment effects were found for absolute T_b's, ΔT_{mean} and ΔT_{-10} (Table 4). Post-hoc paired *t*-tests for consecutive time-points of post-treatment T_b measurements showed that when animals were treated with saline, a significant increase in T_b value only occurred between T140 and T160 (t=-2.25, *P*=0.031) but none of the other values (*P*≥0.08 for all comparisons, same results for ΔT_{mean} and ΔT_{-10}) (Appendix C). In contrast, significant increases in post-treatment T_b occurred when animals were treated with LPS between T80 – T100, T120 – T140, T220 – T240, T300 – T320 and finally T340 – T360 (*P*≤0.024, Appendix D). Post-hoc paired *t*-tests of the corresponding saline-treatment T_b's and LPS-treatment T_b's showed significant differences from 120 minutes after treatment (T120) (t=-2.32, *P*=0.026) to 360 minutes after treatment (T360) (t=-5.17, *P*<0.001) (Appendix D). Significant

differences between corresponding pre-saline and pre-LPS measurements were observed from T80 to T360 for ΔT_{mean} values and from T100 to T360 for ΔT_{-10} values with values being higher after LPS than saline injection (Appendix E).

Table 3. Analysis of variance of a) absolute post-treatment T_b values, b) differences between post-treatment T_b values and mean baseline T_b values (ΔT_{mean}) and c) differences between post-treatment T_b values and last T_b measurement prior to injection (ΔT_{-10}) testing for the effects of treatment, time, season and breeding status.

	F	df	*P*
Within subjects effects			
Treatment$_a$	27.27	1	<0.001**
Treatment$_b$	72.40	1	<0.001**
Treatment$_c$	25.80	1	<0.001**
Time$_a$	3.77	17	<0.001**
Time$_b$	15.07	17	<0.001**
Time$_c$	15.07	17	<0.001**
Between subjects effects			
Season$_a$	4.46	1	0.043*
Season$_b$	0.74	1	0.40
Season$_c$	0.91	1	0.35
Breeding status$_a$	0.25	3	0.86
Breeding status$_b$	1.03	3	0.39
Breeding status$_c$	0.86	3	0.47

*Significant p<0.05
**Very significant p<0.01

Table 4. Significant interactions between treatment, time and season for a) absolute post-treatment T_b values, b) differences between post-treatment T_b values and mean baseline T_b values (ΔT_{mean}) and c) differences between post-treatment T_b values and last T_b measurement prior to injection (ΔT_{-10})

	F	df	*P*
Treatment*time$_a$	16.12	17	<0.001
Treatment*time$_b$	18.54	17	<0.001
Treatment*time$_c$	20.11	17	<0.001
Time*season$_a$	2.84	17	0.026
Time*season$_b$	3.02	17	0.021
Treatment*season$_b$	4.68	1	0.039

Significant time*season interactions were found for absolute post-treatment T_b values and ΔT_{mean} values and ΔT_{mean} values (Table 4). Post hoc *t*-tests for consecutive time-points of post-treatment T_b measurements showed that significant increases occurred during summer between points T80 – T100, T140 – T160, T200 – T220 and T300 – T320 ($P \leq 0.023$, Appendix F). No significant increases between consecutive time-points were found for winter T_b measurements ($p \geq 0.05$ for all comparisons, Appendix F). Significant differences in corresponding summer and winter T_b's were found from T220 to T300 and again at T340 ($P \leq 0.029$) for absolute T_b's (Appendix G). ΔT_{mean} in summer at T220 (0.98±0.62°C) was significantly higher than ΔT_{mean} in winter (0.63±0.59°C) (T-test, t=2.06, P=0.047) while no significant seasonal differences were found for corresponding summer and winter ΔT_{-10} values (Appendix G).

A significant treatment*season interaction was found for ΔT_{mean} values (F=4.68, df=1, P=0.039). Treatment*season interactions was not significant for the other two measures (*P*>0.05 for both). Mean post-saline ΔT_{mean} values was significantly higher in summer (0.37±0.72°C) than in winter (0.045±0.61°C) (T-test, t=6.33, *P*<0.001), while no seasonal difference was found for post-LPS treatment ΔT_{mean} values. Mean post-LPS ΔT_{mean} values were significantly higher than post-saline ΔT_{mean} values in summer (0.93±0.87°C vs. 0.37±0.72°C) (*t*-test, t=-14.20, *P*<0.001) and in winter (0.96±0.77°C vs. 0.045±0.61°C) (T-test, t=-17.88, *P*<0.001).

Discussion

A large inter-individual and day-to-day variation of the Tb's taken from subjects before treatments were found in the highveld mole-rat. Body temperature variations are not uncommon in rodents and may also indicate that the highveld mole-rat exhibits a daily body temperature rhythm (Refinetti 1997). Mole-rats were maintained under constant room temperatures and initial T_b's were taken at approximately the same time of the day. Therefore, if the variation is due to a daily body temperature rhythm, it is most likely independent of ambient temperature or time of day. These findings complicate the interpretation of LPS-response absolute T_b's, making it necessary to compare post-response T_b's to a reference point to rather express the magnitude of stress induced hyperthermia or LPS-induced fever. In this case, the best reference point was T_{-10} since there were no apparent differences found between treatments for this measurement. T_{-60} of non-reproductive females was higher than that of non-reproductive males. Similarly, body temperatures of female mole-rats from Zambia (*Cryptomys* sp.) were lower than

those of their male counterparts at 10ºC, although not at temperatures of 35 ºC (Marhold & Nagel 1995). Early studies have shown that females often exhibit higher body temperatures than males (Feuerbacher & Prinzinger 1981, Hänssler & Prinzinger 1979, Simpson & Galbraith 1905). Castrated male Japanese quail (*Coturnix coturnix japonica*) had lower body temperatures than uncastrated males (Feuerbach & Prinzinger 1981) while testosterone treatment reduced body temperature in castrated male Japanese quail (Hänssler & Prinzinger 1979). These studies indicate that testosterone possibly plays a role in determining sex-specific body temperatures.

Stress-induced hyperthermia is a common phenomenon in animals (Carere & Van Oers 2004, Long *et al*. 1990, Urison *et al*. 1993). Significant increases in T_b's before treatments with either saline or LPS were found. Therefore the stress of repetitive handling alone was enough to cause significant increases in body temperature. One way to possibly reduce the stress-effect on T_b's is to implant a microchip transponder instead of using the rectal probe method to acquire pre- and post-treatment T_b's (Chen & White 2006). Because of the relatively short duration of this experiment, rectal probe measurements were preferred above microchip transponder implantations since it is considered a less invasive method (Adams *et al*. 2001, Chen & White 2006).

Although handling had an augmenting effect on body temperature, LPS administration raised body temperature even further indicating that a febrile response was induced in the highveld mole-rat at a dose of 1 mg kg^{-1} LPS. Treatment affected the time it took for significant increases in rectal temperatures to occur with significant increases in T_b's occurring earlier and more often after LPS treatment compared to saline treatment. This is a strong indication that LPS

produced higher T_b increases than purely the stressed-induced hyperthermia. The nature of LPS induced fever may vary dramatically between species (Martin *et al.* 2008a). In fact hypothermic responses to LPS administration are not uncommon (Card *et al*. 2006, Martin *et al*. 2008a). Five closely related species of deer mice (*Peromyscus* spp.) showed dramatic variation in the direction, intensity and duration of LPS-induced changes in T_b's (Martin *et al.* 2008a). *P. leucopus* exhibited an LPS-induced febrile response that persisted for almost seven and a half hours after treatment (Martin *et al*. 2008a). Fever induced by a gram-positive bacterial toxin in the naked mole-rat persisted for about three hours (Urison *et al.* 1993). In the highveld mole-rat it appeared that post-LPS T_b's began to stabilize after five hours.

A significant effect of season was found for overall post-treatment T_b values (saline and LPS), with an upward shift in responses to both saline and LPS combined in summer. The same pattern was however not observed for overall ΔT_{mean} or ΔT_{-10} values. Similarly, T_b's after LPS treatment were higher during summer months while no seasonal change was observed in the magnitude of change in body temperatures (ΔT_{mean} and ΔT_{-10}) after LPS treatment. T_b's increased more sharply in response to either treatment during the summer months while no significant increases were found for consecutive measurements during winter months. This indicates that highveld mole-rats are able to adjust their body temperature seasonally but not necessarily their febrile response. There is however, a weak indication that this might be different for the stress response as indicated by the significant interaction between treatment and season for ΔT_{mean}. In summer ΔT_{mean} values after saline treatment were higher than in winter, while there was no seasonal difference in ΔT_{mean} values after LPS treatment. No significant treatment by season interactions was found for the other two measurements (T_b and ΔT_{-10}).

Siberian hamsters exhibit a reduced duration of LPS-induced fever when maintained under short day lengths (Bilbo *et al.* 2002). It was also argued that during winter, when energy is not always readily available, it could be more detrimental than beneficial for animals to prolong and maintain energy-expensive symptoms such as a febrile response. Food restriction depresses body temperature in hamsters, and the combination of restricted food and exercise lowers acute phase responses to intra-peritoneal administration of LPS (Conn *et al*. 1995). Reduced energy availability should also account for seasonal variation in an animal's ability to initiate increases in body temperature, be it disease or stress related.

Although I did not find seasonal differences in the amplitude of febrile responses, it is possible that the highveld mole-rat rather exhibits a seasonal pattern in the duration of LPS-induced fever. Mole-rats generally have low body temperatures compared to other mammals due to adaptations to their subterranean habitat where heat conductance is restricted (Van Aardt *et al.* 2007, Zelová *et al*. 2007). Mole-rats may therefore avoid raising their body temperatures above a certain point during summer months to avoid overheating in burrow systems. Other symptoms that may develop in response to LPS include anorexia, reduced water intake and decreased activity (Aubert *et al*. 1997, Bilbo *et al.* 2002, Martin *et al.* 2008b). It is therefore possible that seasonal patterns in other LPS-induced symptoms may occur rather than a febrile response.

In contrast to many other studies (Cai *et al*. 2009, Drazen *et al.* 2003, Martin *et al*. 1995) no evidence for a trade-off between reproduction and the innate immune system, specifically the ability to mount a fever response, was found for the highveld mole-rat. Physiological suppression of reproduction does not occur in males of the highveld mole-rat (Janse van Rensburg *et al*. 2003) possibly explaining why there

was no difference in febrile responses between BM's and NBM's. One might, however expect to find differences in febrile responses between RF's and NRF's since NRF's are suppressed physiologically (Van der Walt *et al.* 2001). However, no differences in febrile responses between RF's and NRF's were found. One reason could be that differences between reproductive and non-reproductive animals may only become noticeable once RF's are pregnant or lactating, while there was no such animals in this study. Another reason could be that differences between reproductive status lies in other LPS-induced symptoms and not necessarily a febrile response. Many other studies also did not find negative relationships between immunocompetence and reproduction. For example, Lutermann & Bennett (2008) found no difference in spleen mass between reproductive and non-reproductive Natal mole-rats (*Cryptomys hottentotus natalensis*). In fact, spleen size of breeding females tended to be larger than that of non-breeding females (Lutermann & Bennett 2008). They suggested non-breeding individuals might invest more energy in other activities such as burrowing and finding food thereby reducing the workload of breeding individuals and allowing them to invest more energy in immune function.

Despite gender differences in T_{-60} values, stress response as well as the response to LPS was apparent in both sexes and thus appeared unaffected by the initial body temperature. Male laboratory mice developed more severe hypothermic and airway inflammatory responses to oral LPS administration than females (Card *et al*. 2006). LPS-induced hyper-responsiveness was however, reduced in gonadectomised males and increased in females who received exogenous testosterone, suggesting that testosterone mediated sex-specific differences in murine responses to LPS (Card *et al*. 2006). In the current study there was no gender differences found in saline or LPS responses even after correcting for initial

body temperature differences (ΔT_{mean} and ΔT_{-10}). Again, it is possible that gender differences may be found only when one investigates some of the other LPS-induced symptoms or that gender differences might occur in the duration of the febrile response rather than the amplitude.

In conclusion, no evidence was found that the ability to launch an LPS-induced fever response varied between seasons. Although some research supports the notion that animals generally have greater immune system capabilities in winter, it is more plausible that different arms of the immune system will exhibit different annual patterns (Braude *et al.* 1999, Martin *et al*. 2006, Smith 2003). Similarly I found no difference between reproductive and non-reproductive or sex-specific differences in the highveld mole-rats ability to produce a febrile response. This supports the concept that androgens, specifically testosterone, probably has a smaller effect on immune-modulation than what was previously thought (Braude *et al.* 1999, Martin *et al.* 2008a). It is important to bear in mind that single immune assays do not measure an animal's overall ability to launch an immune response and therefore a lack of change in an immune measure should not necessarily be interpreted as unchanged immunocompetence (Adamo 2004, Martin *et al*. 2006). Trade-off's between life-history components may also be very small and only become measurable over a lifetime (Zuk & Stoehr 2002). To date not many studies have investigated the nature of LPS-induced fever in wild animals, and therefore these findings contribute significantly to our current knowledge of immunocompentence and life-history trade-offs especially in bathyergids.

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Appendix A

Pairwise *t*-tests comparing six consecutive baseline rectal temperatures measured at time-points 60 minutes prior to treatment (T_{-60}) to 10 minutes prior to treatment (T_{-10}) for reproductive females (*n*=7), reproductive males (*n*=6), non-reproductive females (*n*=14) and non-reproductive males (*n*= 10).

Pair	t	*P*
Reproductive females		
$T_{-60} - T_{-50}$	-1.82	0.12
$T_{-50} - T_{-40}$	-2.09	0.081
$T_{-40} - T_{-30}$	1.36	0.22
$T_{-30} - T_{-20}$	-0.93	0.39
$T_{-20} - T_{-10}$	-0.74	0.48
Reproductive males		
$T_{-60} - T_{-50}$	-2.68	0.044
$T_{-50} - T_{-40}$	-1.26	0.27
$T_{-40} - T_{-30}$	-3.14	0.026*
$T_{-30} - T_{-20}$	1.19	0.29
$T_{-20} - T_{-10}$	-0.51	0.63
Non-reproductive females		
$T_{-60} - T_{-50}$	-3.57	0.003**
$T_{-50} - T_{-40}$	-1.79	0.097
$T_{-40} - T_{-30}$	-2.37	0.034*
$T_{-30} - T_{-20}$	0.15	0.88
$T_{-20} - T_{-10}$	-1.04	0.32
Non-reproductive males		
$T_{-60} - T_{-50}$	-5.03	0.001**
$T_{-50} - T_{-40}$	-4.57	0.001**
$T_{-40} - T_{-30}$	-1.29	0.23
$T_{-30} - T_{-20}$	-3.99	0.003**
$T_{-20} - T_{-10}$	1.26	0.24

*Significant at $p<0.05$
** Significant at $p<0.01$

Appendix B

Pairwise *t*-tests comparing the mean value for baseline T_b measurements (M_{PT}) with post-treatment T_b values for time-points from 20 minutes after treatment (T20) to 360 minutes after treatment (T360) (n=37).

Pair	Saline t	Saline *p*	LPS t	LPS *p*
M_{PT} – T20	-3.32	0.002**	-3.09	0.004**
M_{PT} – T40	-2.28	0.029*	-3.14	0.003**
M_{PT} – T60	-2.16	0.037*	-3.78	0.001**
M_{PT} – T80	-1.08	0.29	-4.88	<0.001**
M_{PT} – T100	-1.61	0.18	-5.92	<0.001**
M_{PT} – T120	1.44	0.16	-6.94	<0.001**
M_{PT} – 140	1.04	0.31	-7.07	<0.001**
M_{PT} – T160	-2.04	0.049*	-9.16	<0.001**
M_{PT} – T180	-1.13	0.27	-8.92	<0.001**
M_{PT} – T200	-2.48	0.018*	-8.41	<0.001**
M_{PT} – T220	-2.17	0.037*	-9.02	<0.001**
M_{PT} – T240	-2.03	0.049*	-11.46	<0.001**
M_{PT} – T260	-2.28	0.028*	-10.39	<0.001**
M_{PT} – T280	-2.72	0.010*	-10.14	<0.001**
M_{PT} – T300	-2.59	0.014*	-9.98	<0.001**
M_{PT} – T320	-1.54	0.13	-8.69	<0.001**
M_{PT} – T340	-1.20	0.24	-8.51	<0.001**
M_{PT} – T360	-1.63	0.11	-7.02	<0.001**

*Significant at p<0.05
** Significant at p<0.01

Appendix C

Pairwise *t*-tests comparing consecutive time-point values (T20-T360) for absolute rectal temperature measurements (T_b's), differences between post-treatment T_b values and mean baseline T_b values (ΔT_{mean}) and differences between post-treatment T_b values and last T_b measurement prior to injection (ΔT_{-10}) (df=36, n=37).

Pair	T_b's t	*P*	ΔT_{mean} & ΔT_{-10} T	*P*
T20 – T40	0.29	0.78	0.53	0.60
T40 – T60	0.53	0.60	-1.05	0.30
T60 – T80	-1.05	0.30	-0.16	0.88
T80 – T100	-0.17	0.87	-2.47	0.018*
T100 – T120	-2.45	0.020*	-0.99	0.33
T120 – T140	-0.99	0.33	1.20	0.24
T140 – T160	-1.20	0.24	-3.37	0.002**
T160 – T180	-3.37	0.002	0.80	0.43
T180 – T200	0.80	0.43	-1.54	0.13
T200 – T220	-1.53	0.14	-1.03	0.31
T220 – T240	-1.03	0.31	-1.72	0.093
T240 – T260	-1.72	0.094	-0.53	0.60
T260 – T280	-0.53	0.60	-0.49	0.63
T280 – T300	-0.49	0.63	-0.11	0.91
T300 – T320	-0.11	0.98	2.58	0.014*
T320 – T340	2.57	0.015*	0.53	0.60
T340 – T360	0.53	0.60	1.34	0.19

*Significant at p<0.05
**Significant at p<0.01

Appendix D

Pairwise *t*-tests comparing consecutive time-point values (T20-T360) for post-treatment T_b measurements for saline and LPS (df=36, n=37).

Pair	Saline t	*P*	LPS t	*P*
T20 – T40	1.16	0.25	-0.29	0.77
T40 – T60	-0.053	0.96	-1.33	0.19
T60 – T80	1.80	0.08	-1.45	0.16
T80 – T100	-0.99	0.32	-2.37	0.024*
T100 – T120	0.045	0.97	-1.49	0.14
T120 – T140	-0.46	0.65	-2.50	0.017*
T140 – T160	-2.25	0.031*	-2.50	0.017*
T160 – T180	1.90	0.65	-0.68	0.50
T180 – T200	-1.74	0.09	-0.42	0.68
T200 – T220	-0.22	0.83	-1.33	0.19
T220 – T240	0.26	0.80	-3.17	0.003**
T240 – T260	-0.66	0.52	-0.18	0.86
T260 – T280	-1.48	0.15	0.54	0.59
T280 – T300	0.84	0.41	-0.98	0.33
T300 – T320	0.94	0.36	3.11	0.004**
T320 – T340	0.82	0.42	-0.04	0.97
T340 – T360	-0.67	0.51	3.23	0.003**

*Significant at p<0.05
**Significant at p<0.01

Appendix E

Pairwise *t*-tests comparing corresponding time-point values for saline ($T20_{SALINE}$ - $T360_{SALINE}$) and LPS ($T20_{LPS}$ - $T360_{LPS}$) for absolute post-treatment T_b values, differences between post-treatment T_b values and mean baseline T_b values (ΔT_{mean}) and differences between post-treatment T_b values and last T_b measurement prior to injection (ΔT_{-10}) (*n*=37).

Pair	Absolute T_b's t	*P*	ΔT_{mean} t	*P*	ΔT_{-10} t	*P*
$T20_{LPS}$ - $T20_{SALINE}$	1.92	0.063	-0.03	0.98	0.84	0.41
$T40_{LPS}$ – $T40_{SALINE}$	1.20	0.24	-0.81	0.42	0.12	0.90
$T60_{LPS}$ – $T60_{SALINE}$	0.61	0.54	-1.60	0.12	-0.50	0.62
$T80_{LPS}$ – $T80_{SALINE}$	-0.95	0.35	-2.92	0.006*	-1.72	0.09
$T100_{LPS}$ – $T100_{SALINE}$	-1.73	0.09	-3.59	<0.001**	-2.26	0.030*
$T120_{LPS}$ – $T120_{SALINE}$	-2.32	0.026*	-4.35	<0.001**	-3.01	0.005**
$T140_{LPS}$ – $T140_{SALINE}$	-3.73	0.001**	-5.59	<0.001**	-4.06	<0.001**
$T160_{LPS}$ – $T160_{SALINE}$	-3.95	<0.001**	-7.50	<0.001**	-4.76	<0.001**
$T180_{LPS}$ – $T180_{SALINE}$	-5.89	<0.001**	-7.84	<0.001**	-5.13	<0.001**
$T200_{LPS}$ - $T200_{SALINE}$	-4.34	<0.001**	-7.70	<0.001**	-5.06	<0.001**
$T220_{LPS}$ - $T220_{SALINE}$	-4.72	<0.001**	-7.52	<0.001**	-5.26	<0.001**
$T240_{LPS}$ - $T240_{SALINE}$	-6.83	<0.001**	-9.49	<0.001**	-6.51	<0.001**
$T260_{LPS}$ - $T260_{SALINE}$	-6.21	<0.001**	-9.04	<0.001**	-6.55	<0.001**
$T280_{LPS}$ - $T280_{SALINE}$	-5.31	<0.001**	-7.73	<0.001**	-5.58	<0.001**
$T300_{LPS}$ – $T300_{SALINE}$	-6.49	<0.001**	-8.09	<0.001**	-6.41	<0.001**
$T320_{LPS}$ – $T320_{SALINE}$	-6.73	<0.001**	-7.56	<0.001**	-5.84	<0.001**
$T340_{LPS}$ – $T340_{SALINE}$	-6.98	<0.001**	-7.66	<0.001**	-6.27	<0.001**
$T360_{LPS}$ – $T360_{SALINE}$	-5.17	<0.001**	-6.76	<0.001**	-5.10	<0.001**

*Significant at p<0.05
**Significant at p<0.01

Appendix F

Pairwise *t*-tests comparing consecutive time-point values (T20-T360) for post-treatment T_b measurements for summer (*n*=19) and winter (*n*=18).

Pair	Summer		Winter	
	t	*P*	t	*P*
T20 – T40	0.90	0.38	-0.30	0.77
T40 – T60	-0.15	0.88	-1.25	0.23
T60 – T80	-1.69	0.11	1.23	0.24
T80 – T100	-3.22	0.005**	-0.57	0.58
T100 – T120	-0.23	0.82	-1.12	0.28
T120 – T140	-0.73	0.48	-0.96	0.35
T140 – T160	-3.34	0.004**	-1.57	0.14
T160 – T180	0.07	0.95	1.05	0.31
T180 – T200	-1.45	0.16	-0.67	0.52
T200 – T220	-3.14	0.006**	0.77	0.45
T220 – T240	-1.09	0.29	-1.35	0.20
T240 – T260	0.08	0.93	-0.82	0.43
T260 – T280	-0.67	0.51	-0.15	0.88
T280 – T300	-0.08	0.94	-0.07	0.95
T300 – T320	2.49	0.023*	1.24	0.23
T320 – T340	-0.36	0.72	0.98	0.34
T340 – T360	1.56	0.14	0.25	0.81

*Significant at p<0.05
**Significant at p<0.01

Appendix G

Pairwise *t*-tests comparing corresponding time-point values for summer ($T20_{SUM}$ – $T360_{SUM}$) (*n*=19) and winter ($T20_{WIN}$ – $T360_{WIN}$) (n=18) (T20-T360) for absolute post-treatment T_b values, differences between post-treatment T_b values and mean baseline T_b values (ΔT_{mean}) and differences between post-treatment T_b values and last T_b measurement prior to injection (ΔT_{-10}).

Pair	Absolute T_b's		ΔT_{mean}		ΔT_{-10}	
	t	*P*	t	*P*	t	*P*
$T20_{SUM}$ - $T20_{WIN}$	0.10	0.92	-0.54	0.59	-0.58	0.56
$T40_{SUM}$ – $T40_{WIN}$	-0.35	0.73	-1.08	0.29	-1.28	0.21
$T60_{SUM}$ – $T60_{WIN}$	-0.70	0.49	-1.41	0.17	-1.72	0.094
$T80_{SUM}$ – $T80_{WIN}$	0.28	0.78	-0.24	0.81	-0.28	0.78
$T100_{SUM}$ – $T100_{WIN}$	0.91	0.37	0.55	0.59	0.66	0.52
$T120_{SUM}$ – $T120_{WIN}$	0.56	0.58	0.15	0.88	0.16	0.87
$T140_{SUM}$ – $T140_{WIN}$	0.52	0.61	0.04	0.97	0.06	0.96
$T160_{SUM}$ – $T160_{WIN}$	1.03	0.31	0.38	0.71	0.43	0.67
$T180_{SUM}$ – $T180_{WIN}$	1.50	0.14	0.87	0.39	0.84	0.41
$T200_{SUM}$ - $T200_{WIN}$	1.95	0.059	1.09	0.28	1.10	0.28
$T220_{SUM}$ - $T220_{WIN}$	3.31	0.002**	2.06	0.047*	2.11	0.042
$T240_{SUM}$ - $T240_{WIN}$	2.86	0.007**	1.75	0.089	1.94	0.060
$T260_{SUM}$ - $T260_{WIN}$	2.33	0.026*	1.30	0.20	1.41	0.17
$T280_{SUM}$ - $T280_{WIN}$	2.28	0.029*	1.35	0.18	1.41	0.17
$T300_{SUM}$ – $T300_{WIN}$	2.28	0.029*	1.43	0.16	1.55	0.13
$T320_{SUM}$ – $T320_{WIN}$	1.71	0.095	0.98	0.34	1.05	0.30
$T340_{SUM}$ – $T340_{WIN}$	2.58	0.014*	1.49	0.14	1.52	0.14
$T360_{SUM}$ – $T360_{WIN}$	1.90	0.066	1.01	0.32	1.03	0.31

*Significant at p<0.05
**Significant at p<0.01

Chapter 3

Interactions between parasitism and host sociality, sex and season

Abstract

Increased parasitism is widely believed to be a cost of sociality, since increased interactions provide more opportunities for parasites to spread. Some studies however, show that the diversity and intensity of parasites decrease with increasing group size, especially in social animals. Other factors that may affect the level of parasitism are gender of host, reproductive status of host and the season. Males are believed to be more susceptible to parasitic infestation than females and reproductive animals are believed to have lower defences against parasitism due to energetic trade-offs with reproductive activities. Many parasites are known to have seasonal cycles of infestation that coincide with the breeding season of their host. The highveld mole-rat is a social, subterranean rodent that lives in groups of up to fourteen individuals. Mole-rats were captured throughout the year and screened for ectoparasites and gastrointestinal parasite prevalence and intensity. The focus of this study was to elucidate the influence that group size, reproductive status, season and colony membership had on the prevalence and intensity of parasites associated with the highveld mole-rat. While no effect of group size was found on cestode infestation, larger mole-rat colonies had lower mite intensities than smaller colonies. Colony membership affected both mite and cestode intensities, demonstrating the importance of inter-colonial contact between host groups for parasite transmission. Within groups, reproductively active animals were predicted to have higher parasite loads compared to non-breeding individual. Reproductive status did not affect either

parasite prevalence or intensity. Mite infestation was higher in summer, and cestode infestations were higher in winter, but no seasonal effect was found for the prevalence of either parasite group. Because of the increased risk of parasitism due to social aggregation, it is likely that social animals like the highveld mole-rat evolved strategies to minimise parasite transmission within and between groups.

Keywords: Bathyergidae, higveld mole-rat, mites, cestodes, transmission, group size

Introduction

Parasites are an essential component of a healthy ecosystem and today it is widely recognised that they play a major role in shaping community and population structure (Altizer *et al.* 2003, Hudson *et al*. 2006, Poulin 1999). Parasites may either spend the majority of their life associated with one or more host species, or be free-living for the majority of their life and only associate with hosts for short periods of time (Anderson & May 1978). Parasites depend on their hosts for their nutritional needs and the relationship is essentially damaging to the host (Anderson & May 1978). In response to the damaging effects of parasites, hosts may develop several behavioural and physiological counter strategies (Hart 1992, Male *et al*. 2006). Common behavioural patterns that animals employ in attempt to avoid, control or eliminate parasites include grooming, anorexia, and mate choice on the grounds of evidence for resistance to parasite infection, to name but a few (Hart 1992). The main physiological adaptation of the hosts is immune responses to parasites. A variety of immune defence mechanisms are activated by parasites, with inflammation playing a very prominent role in controlling parasitic infestations in the host (Male *et*

al. 2006). In response, parasites have also developed strategies to bypass the protection of the host (Thomas *et al*. 2002, Smith Trail 1980). Parasites and their hosts therefore co-evolve together and these interactions continually shape the life-histories of both parasite and host. One of the greatest challenges that parasites face is that of dispersal to new hosts (Smith Trail 1980). Transmission modes are largely grouped into direct transmission where parasites are spread from host to host via direct contact and indirect transmission where the host encounters the parasite in its environment or becomes infected through a vector (Altizer *et al*. 2003). Transmission is relatively easy for free-living parasites such as mites, fleas, ticks and lice, but parasites without free-living stages must use other methods for dispersal (Smith Trail 1980).

An increased risk of parasitism is generally considered to be a cost of sociality since individuals that occur in groups are believed to be more likely to be infested with parasites since there is an increased risk of transmission between animals living in close proximity to one another (Altizer *et al*. 2003, Christe *et al.* 2007, Côté & Poulin 1995). Higher frequencies of social interactions provide greater opportunities for parasites to spread and transmission is expected to be more common in larger groups with more social contacts (Altizer *et al.* 2003, Whiteman & Parker 2004). Adult females of five European bat species that aggregate in nursery colonies had higher prevalence (five out of five species) and intensities (three out of five species) of parasitic mites (*Spinturnix* spp.) than solitary males (Christe *et al.* 2007). Some studies, however, have revealed that the intensity of infestation by mobile parasites such as ticks and mites can in fact decrease as host group size increases. For example, a decrease in flea intensity was found with an increase in host density for the striped field mouse (*Apodemus agrarius*) and the yellow-necked mouse (*A.*

flavicolis) (Stanko *et al*. 2002). Another recent study showed a negative correlation between group size and parasite prevalence in red colobus monkeys (*Procolobus rufomitratu*) (Snaith *et al*. 2008). Clearly parasite transmission in social animals is not always simply a factor of group size or density.

A host's social behaviour may reduce the risk of becoming infested as well as control or eliminate current infestations. Ectoparasites are often very effectively removed by auto-grooming (Hart & Hart 1988, Stanko *et al*. 2002). In social groups where animals groom one another, there is the added benefit of being able to reach and groom areas that an individual itself would not be able to reach (Hart & Hart 1988). The social behaviour and the degree of sociality is, however, not the only determinant of parasite transmission as the mating system and social organization (size and composition) of groups will also have major effects on the distribution of parasites within and between populations (Altizer *et al.* 2003, Ezenwa 2004*a*). For example, promiscuous species run a much higher risk of being infected by micro- or macro-parasites when compared to monogamous species due to the higher contact rates between individuals (Altizer *et al*. 2003). Finally, contact rate between social groups can be a very important factor in determining risk of parasitism. Social group-living animals have the added benefit that, with the exception of dispersal events, an animal's interactions with conspecifics is limited to group members. Therefore, if a contagious parasite is absent from the group, the group has a good chance to remain "clean" if social interactions with other possible carriers are avoided. If between-group contact rate is sufficiently low, it may lead to a social species having a lower risk of parasitism than a non-social species (Altizer *et al*. 2003, Ezenwa 2004*a*, Wilson *et al*. 2003).

Since parasites can have a dramatic effect on host fitness, one would expect a trade-off between the ability to fight parasites and other life-history components. The potential trade-off between immunological defence mechanisms against parasites and reproductive effort in particular has received a great deal of attention (Nordling *et al*. 1998, Sheldon & Verhulst 1996). Some of the classic examples of this trade-off are lactating bighorn ewes (*Ovis canadensis*) having higher faecal counts of lungworm larvae than their non-lactating counterparts (Festa-Bianchet 1989) and increased parasite prevalence in male great tits (*Parus major*) with enlarged brood sizes (Norris *et al.* 1994). A more recent study showed that pregnant greater mouse-eared bats (*Myotis myotis*) had higher ectoparasite intensities than non-reproductive females of the same group (Christe *et al.* 2000). Prevalence of naturally acquired porcine cysticerosis, *Taenia solium*, nearly doubled in rural pigs during pregnancy (Morales *et al*. 2002). Castration of male pigs had the same effect, indicating that sex steroid hormones are to some degree responsible for mediating changes in parasite defence (Morales *et al*. 2002). These studies imply that increased reproductive effort decreases the ability of an animal to defend against parasites through reduced availability of resources, since energetic and nutritional resources are expected to be subdivided amongst these two life history components.

Male mammals are generally more susceptible to parasitism than females (Klein 2000, Møller *et al.* 1998, Poulin 1996, Zuk & McKean 1996) with exceptions (Christe *et al*. 2007, Morales-Montor *et al.* 2004). Sex-specific parasitism may arise from differences in allocation of resources to immune function, immunosuppressive effects of sex-hormones, direct effects of sex hormones on parasite proliferation and due to differences in exposure of parasites (Christe *et al.* 2007, Møller *et al.* 1998, Morales-Montor *et al.* 2004). Prevalence and intensity of nematode infestations are

often higher in male mammals (Poulin 1996). Møller *et al.* (1998) assessed spleen sizes in 226 species of birds and found that adult males had significantly smaller relative spleen sizes than their female counterparts. Morales-Montor *et al.* (2004) stressed however, that there are far too many exceptions to this "rule" and that aspects like extrinsic environmental factors and social aggregations of hosts probably play a far more important role in patterns of intra-specific abundance of parasites.

Many parasites are known to exhibit seasonal patterns of abundance (Altizer *et al*. 2006, Davidson *et al.* 1980). Making broad assumptions on these seasonal patterns of prevalence and intensity may be somewhat unreliable as it appears that different parasites have different seasonal patterns (Altizer *et al*. 2006). Some studies have shown that parasite population booms coincide with the seasonal reproduction patterns of their hosts (Altizer *et al.* 2006, Møller *et al*. 2003). It is hypothesised that these patterns of parasitism are a consequence of the lowered immunity of hosts that occur as a result of increased reproductive effort, which in turn increases the hosts' susceptibility to infestation (Altizer *et al.* 2006, Christe *et al.* 2000). Other parasites may be more prevalent during months when temperatures are lower and food is scarcer to the host animal, since malnourishment can also compromise the host's immune system (Murray *et al.* 1998, Coop & Kyriazakis 2001, Ezenwa 2004*b*). Furthermore, parasites respond to external environmental cues such as temperature and humidity to proliferate, thereby affecting their season patterns of abundance (Thomas *et al*. 2002). It is very clear that major differences occur in the survival strategies of parasites and that numerous factors influence their seasonal patterns. The seasonal patterns of parasites can therefore give us insight into the nature of parasite-host co-evolution.

The aims of this study were to assess how the social structure of the highveld mole-rat (*Cryptomys hottentotus pretoriae*; Family: Bathyergidae) affects parasite intensity and prevalence. In this chapter *intensity* of parasites is defined as the number of parasites per infested host while *prevalence* refers to percentage of mole-rats infested with the parasite (Margolis *et al*. 1982). Highveld mole-rats occur alone or in groups of up to fourteen individuals in the wild (Moolman *et al*. 1998), thereby allowing one to assess the effect of group size on parasite intensity and prevalence. Considering the fact that allogrooming is prevalent amongst members of colonies (H. Viljoen, personal observation), I expected the intensity and prevalence of ectoparasites to decrease with increasing colony size. The effect of group-size on endoparasites was, however, not tested for in the current study due to the lack of variation found in colony sizes of mole-rat groups used for endoparasite assessment. Colonies of the highveld mole-rat typically have a single reproductive female, up to three putative reproductive males as well as non-reproductive males and females (Moolman *et al*. 1998). By comparing reproductive individuals to non-reproductive individuals I aimed to determine if reproductive status influenced parasite intensity and prevalence. If reproductive animals have fewer resources available to combat parasites after allocation of some resources to reproduction, one can expect reproductive animals to be more likely to be infested with parasites and also harbour larger numbers of parasites than their non-reproductive counterparts. If testosterone (directly or indirectly) adversely affects an animal's ability to fight parasites (Saino *et al*. 1995), gender differences in infestation can also be predicted. Males were predicted to be more frequently infested with parasites and furthermore carry heavier infestations. Finally, the seasonal patterns of intensity and prevalence of parasites associated with the highveld mole-rat were investigated. In this chapter parasites

refer to macro-parasites (specifically ectoparasites and gastrointestinal parasites) and not micro-parasites such as bacteria and viruses as with the broader definition.

Materials and methods

Capturing and housing

Mole-rats were captured from the National Botanical Garden (NBG) (25°44'13.49"S 28°16'42.25"E) (June 2008 to May 2009), Tygerpoort (S25°46'35.45", E28°21'37.34") (October 2008 to June 2009) and Centurion (S25°50'42", E28°11'2") (August 2009) in Tshwane, South Africa. The mole-rats were captured using Hickman live-traps (Hickman 1979) baited with sweet potato. Traps were checked three times daily, with the bait being replaced every day. To ensure that all colony members were captured, traps were only removed after three consecutive days had passed without further signs of activity in or near traps. It was then assumed that the entire colony was captured. Colonies were housed in plastic crates (49.5 x 28 cm) with wood shavings and paper towelling for nesting material. Animals were fed an *ad libitum* diet of fresh potato and apple on a daily basis. Wood shavings and paper towelling were replaced weekly and old food was removed daily. Room temperature (25 ± 1°C) and light cycles (12:12h LD) was maintained under constant conditions.

Animals captured from the NBG were part of a long-term mark-recapture study and thus were released after experimental procedures. I attempted to recapture as many as possible throughout the following year. On the first capture of an individual, a toe clip was taken by clipping the outer toe of one of the hind feet. The tissue was immediately stored at -30°C for future population genetic studies. Individuals were also marked by implanting a TX1400L microchip transponder (Identipet, Johannesburg, South Africa) under the skin of the neck. Each chip has a

unique code that can be read by a hand-held reading device allowing for fast and effective identification of recaptured animals. Prior to release, animals were weighed, sexed and parasite loads were assessed (see below). Animals were subsequently released into their burrows at the point of capture within two days after marking and/or collection of ectoparasites and at least within three weeks from capture.

Ectoparasite assessment

Ecto-parasite sampling commenced within a week of capture. Mole-rats were placed into a jar with halothane-soaked cotton wool for between 10 to 20 seconds until animals were unconscious but had not expired. The animals were then removed from the jar and dipped exactly 20 times (to standardise the procedure and avoid any bias) into a bath of tepid soapy water while holding their heads up to avoid water getting into their respiratory tracts. After the mole-rats recovered consciousness, tissue paper was used to remove excess water from their pelage and the animals were subsequently placed into a temperature controlled incubation chamber (35°C) for a few minutes until their pelages were dry. The soapy water was filtered through a No. 25 U.S. Standard Sieve (710 micron screen) for ectoparasites and these were then collected and placed in 70% ethanol for preservation. Mite specimens were cleared in lactic acid and then mounted on slides with a small amount of Hoyer's medium and placed in an oven at 37°C for approximately three days to dry. Clearing and mounting techniques are described in more detail by Zhang (2003). Mites were counted and identified under a light microscope (magnification 100X).

Gastrointestinal parasites

Mole-rats were sacrificed by placing them in a killing jar with an overdose of halothane. The alimentary tract (from the stomach to the rectum) was removed from the abdomen. Contents of the stomach, small intestine, caecum and large intestines were removed and examined under a dissection microscope (40X magnification) for the presence of gastrointestinal parasites. Examining intestinal contents for the presence of endoparasites in this manner is a standard parasitological procedure (Klimpel *et al*. 2007). All collected gastro-intestinal parasites were counted and sent to The Royal Veterinary College (Herts), London, UK, for identification.

Prior to sacrificing mole-rats, faecal samples were collected and it was determined whether or not the mole-rats were infested with cestodes by identifying proglottids in the faecal pellets. This measure of cestode prevalence was then compared with the actual infestation to determine the accuracy of this method for determining whether an animal is infested with cestodes or not. 96% of the predictions were accurate (44 out of 46). The inaccurate predictions were for two mole-rats scored negative for cestode infestations but in fact had very low cestode intensities (≤3 cestodes per mole-rat). I concluded that the method was accurate enough to determine cestode prevalence in mole-rats captured from the NBG that could not be sacrificed for a complete endoparasites assessment. Faecal samples from these mole-rats were collected, and animals were scored either positive or negative for cestode infestation.

Statistical analysis

Reproductive status of mole-rat was classified as being reproductive males (RM), reproductive females (RF), non-reproductive males (NRM) and non-

reproductive females (NRF). RM were characterised by their size and bulging inguinal testes and RF by the presence of elongated teats and a perforate vagina (Moolman *et al*. 1998). Two seasons were used in the analyses: summer (September to March) and winter (April to August). The majority of ectoparasites found were mites. A small number of lice were found and their numbers are described, but their small number precluded a meaningful statistical analysis. Similarly the majority of gastrointestinal parasites found were cestodes, and only descriptive statistics are provided for the small number of nematodes found.

All statistical analyses were performed using SPSS (Version 17.0, Chicago, Ill). General estimating equations (GEE's) were used to analyse the data where individuals were repeatedly used for sampling and where natal colony of subjects were specified as repeated measures (Zeger & Liang 1986). The use of GEE's has several advantages, with most importantly for our study being 1) the ability to analyse dependent variables with more than one predictor variable over time and 2) the ability to use all available data for analyses in spite of missing data points for some subjects (Liu *et al*. 2009, Zeger & Liang 1986). Each of the variable's distribution was chosen according to Pan's quasi likelihood under the independence model information criterion (QIC) with the lowest QIC score considered to be the best (Ballinger 2004). GEE's are also robust against the wrong choice of correlation structure, and results remain very similar regardless of the distribution chosen for the model (Liu *et al*. 2009, Zeger & Liang 1986).

A GEE following an inverse Gaussian probability distribution (QIC=49.35) and an identity-link with mite intensities as the dependent variable (after adding one to all data points to get rid of zero counts) was employed to determine the effects of reproductive status (coding sex and status into one variable) and season. The effect

of the natal colony on mite intensity (as the only predictable variable) was tested in a separate GEE to avoid parameter overload on the model (Lumley 1996). A GEE with a binomial distribution (QIC=264.57) and a logit-link was used to determine the effects of reproductive status and season on the prevalence of mites (absent/present) on animals. Individual and colony was specified as repeated measures while colony size and body mass of animals were added as covariates in both models.

Cestode prevalence (QIC=148.49) and intensity (QIC=11.71) was analysed in the same manner as described for mite intensity and prevalence. Colony size was not added as a covariate in the GEE for cestode intensity due to the limited variation in colony sizes for this part of the analysis (five out of seven colonies had a colony size of six) and only colony was specified as a repeated measure for this model. The limited sample size precluded the analyses of the effect of colony membership on parasite prevalence. Only two-way interactions were tested for. No significant interactions between factors were found and are therefore not reported.

Results

Ectoparasite assessment

Eighty-five animals were assessed for ectoparasites between one and five times during this study (Appendix 2). Lice were found on four animals from the same colony captured in June'09 (two lice on each of three of animals, and one louse on the fourth) (Appendix 2). Lice were identified as being from the genus *Linognathus* (Scholtz & Holm 1995). One louse was found on an animal captured in April 2009 and another one on an animal in August 2009. No lice were found on any of the animals captured from the NBG. The majority of ectoparasites found on mole-rats

were mites (99.2%). Three species of parasitic mites found on the mole-rats were identified as *Androlaelaps scapularis* (Berlese), *A. capensis* (Berlese) and *A. marshalli* (Berlese) with *A. scapularis* being by far the most prevalent species (Table 1). Since all mites belonged to the same genus, data for mites were lumped together for analyses.

Table 1. Three species of mites found on highveld mole-rats, and a breakdown of the number of adult males, adult females, protonymphs (PN) and deutonymphs (DN) of the respective species.

Species	Total	Males	Females	PN	DN
Androlaelaps scapularis (Berlese)	1013	23	839	96	55
A. capensis (Berlese)	16	0	12	3	1
A. marshalli (Berlese)	25	4	18	2	1

Mites were found on 66.9% of all animals captured. The prevalence of mites was not affected significantly by season, reproductive status, colony size or body mass (Table 2). Mite intensity was significantly higher in summer (15.82±25.80, n=47) than in winter (5.32±12.47, n=77) (GEE, X^2=7.22, df=1, P=0.007) (Figure 1) and a significant effect of colony size on mite intensity was also found (GEE, X^2=7.94, df=1, P=0.005) with mole-rats from smaller colonies being heavier burdened with mites (Figure 2). Reproductive status, sex and body mass had no significant effect on the mite intensity (Table 2). Colony membership had a significant effect of on the mite intensity (GEE, X^2=713671.23, df=22, P<0.001) suggesting that mite loads were more similar between colony members compared to between groups.

Table 2. GEE's with individual and colony as repeated measures showing the effect of season reproductive status, colony size and mass on mite intensity as well as on prevalence of mites (absent/present).

Effect	Mite prevalence χ^2	df	*P*	Mite intensity χ^2	df	*P*
Season	2.53	1	0.11	7.22	1	0.007**
Reproductive status	0.73	3	0.87	0.46	3	0.93
Colony size	1.47	1	0.23	7.94	1	0.005**
Body mass	0.016	1	0.90	1.53	1	0.22

*Significant at p<0.05
** Significant at p<0.01

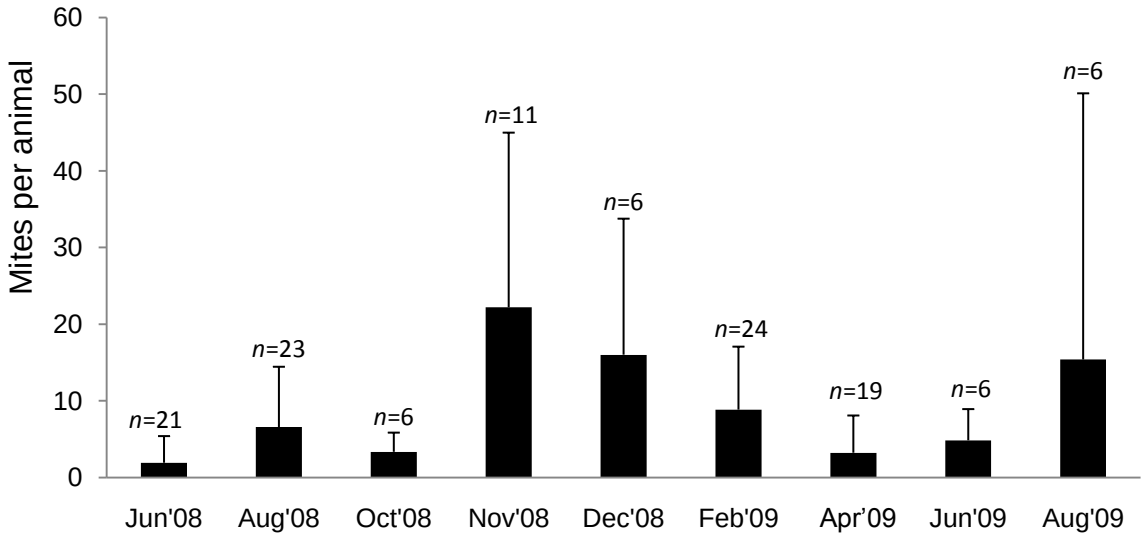

Figure 1. Graph showing the number of mites (mean ± SD) in summer (October'08, November'08 December'08 and February '09) and winter (June'08, August'08, April'09, June'09 and August'09).

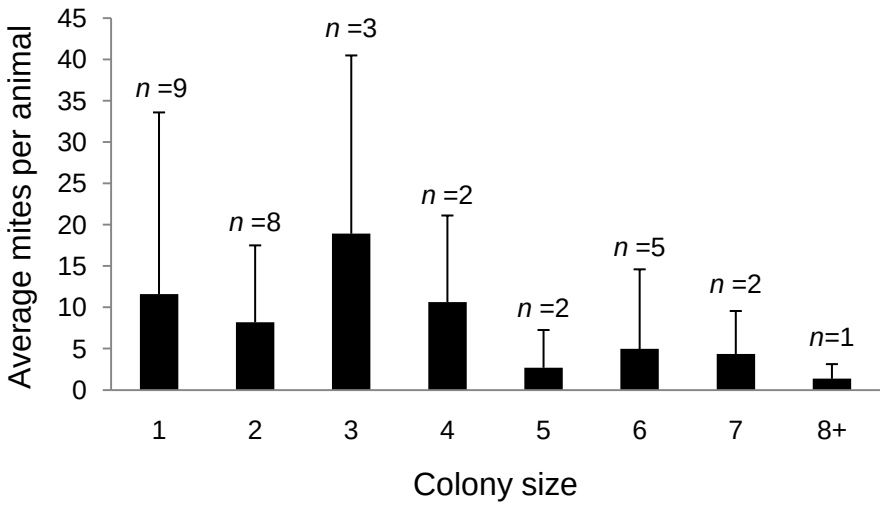

Figure 2. Graph showing mites per animal (mean ± SD) on animals from various colony sizes. Note that some of the colonies were captured more than once over the course of this study.

Gastrointestinal parasites

Out of the 46 mole-rats that were assessed for diversity and intensity of gastrointestinal parasites, cestodes and/or nematodes were found in the gastrointestinal tracts of 34 animals (Appendix 2). Three single nematodes identified as *Protospirura* sp. were found in the stomachs of three animals in October and February, while two *Heligmonina* sp. nematodes were found in the stomach of an animal caught in October (Appendix 2). Cestodes were identified as *Mathevotaenia* sp. (Cestoda: Anoplocephalata).

One hundred and thirty-one animals were assessed for prevalence of cestodes between one and 5 times during this study. The prevalence of cestodes was significantly higher in winter (cestodes present in 80.2% of animals sampled, n=86) than in summer (cestodes present in 46.7% of animals sampled, n=45) (GEE, X^2=14.00, df=1, P<0.001) (Table 3). There was no significant effect of reproductive status or colony size on cestode prevalence (Table 3). Body mass did, however,

affect cestode prevalence significantly (GEE, X^2=13.46, df=1, P<0.001) (Table 3), with infected mole-rats (96.43±22.06g, n=90) being heavier than uninfected mole-rats (84.59±22.37g, n=41) (Figure 3). Cestode intensity was significantly higher in winter (12.15±4.89, n=27) than in summer (1.61±2.75, n=19) (X^2=17.49, df=1, P<0.001) (Figure 4, Table 3). No significant effect on cestode intensity was found for reproductive status or body mass (Table 3). Colony membership was a significant predictor of cestode intensity (GEE, X^2=324.81, df=22, P<0.001) suggesting that the number of cestodes is more similar between colony members than between individuals originating from different colonies.

Table 3. GEE testing the effects of season (winter and summer), reproductive status and body mass on the cestode intensity (n=46) and the cestode prevalence (n=131)

Effect	Cestode intensity			Cestode prevalence		
	X^2	df	P	X^2	df	P
Season	17.49	1	<0.001*	14.00	1	<0.001*
Reproductive status	2.35	3	0.50	4.32	3	0.23
Body mass	0.074	1	0.79	13.46	1	<0.001*
Colony size				0.024	1	0.88

* Significant at p<0.01

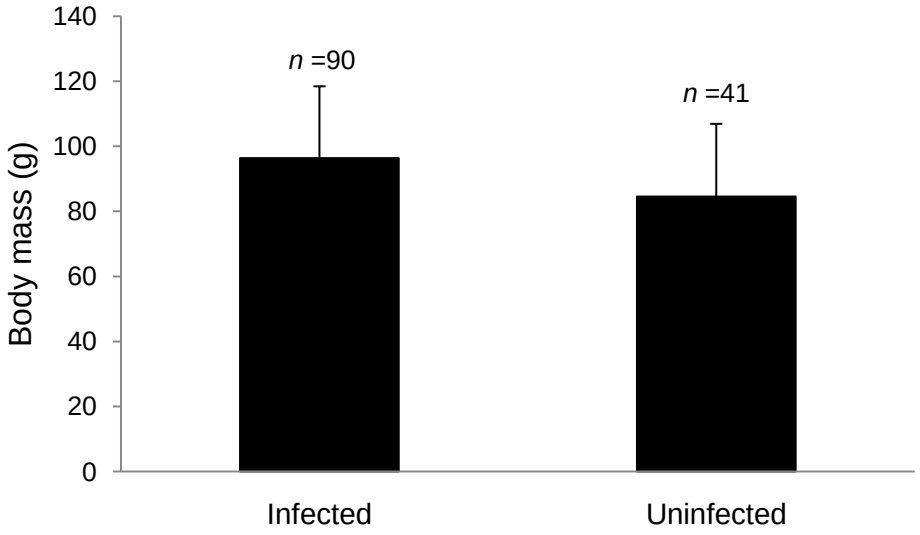

Figure 3. The body mass (mean ± SD) of infected mole-rats compared to that of uninfected mole-rats.

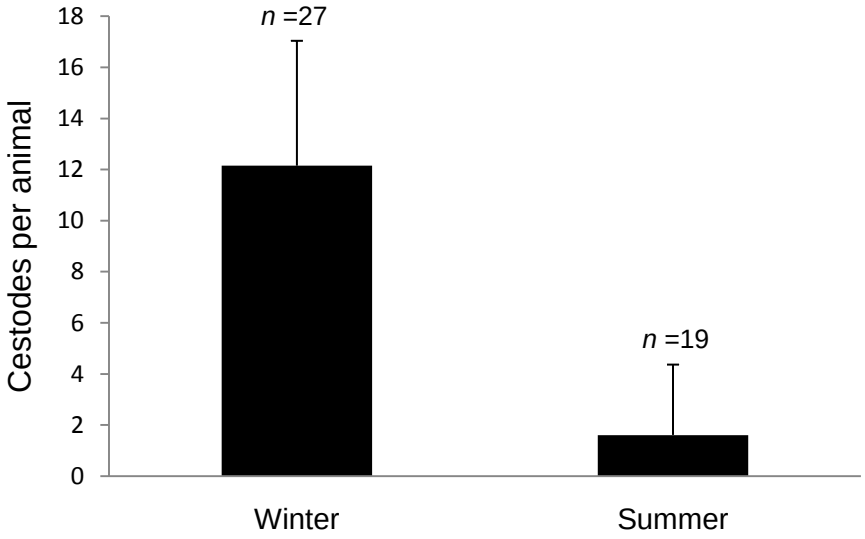

Figure 4. Number of cestodes (mean ± SD) per animal in summer and winter.

Discussion

I did not find a particularly rich diversity of ecto- or gastrointestinal- parasites associated with the highveld mole-rat possibly because their subterranean lifestyle limits parasite spread amongst and between groups (Scharff *et al*. 1997). However, one should bear in mind that I only covered a small segment of the distributional range of this social rodent and that further similar studies will likely add to the diversity of parasites that are associated with the highveld mole-rat. There is general paucity of information pertaining to parasites of the bathyergids. Similar studies also found low parasite species richness for example two nematodes (*Protospirura* and *Capillaria*) associated with the giant mole-rat (*Fukomys mechowii*) (Scharff *et al.* 2001) and a louse (*Eulinognatbus hilli*) that have been collected from *C. hottentotus* mole-rat (subspecies were not mentioned) in South Africa (Durden 1991). I found three species of mites, one genus of louse, one genus of cestode and two genera of nematodes. The vast majority of parasites found associated with the highveld mole-rat were mites and cestodes.

Cestodes collected for this study could only be identified to generic level, and it is more than likely that cestodes collected are of an undescribed species. An interesting question arises as to how these cestodes are transmitted from one mole-rat to another. Although the majority of cestodes have a life-cycle that involves one or more intermediate hosts, at least three cases have been documented where cestodes have been found to be capable of direct infection (Mackiewicz 1988, Craig & Akira 2007). The diet of mole-rats is thought to consist largely of geophytes and tubers (Bennett & Faulkes 2000) intimating that ingestion of an intermediate host

may be accidental. Mites are possible candidates for intermediate hosts of this life-cycle as mole-rats are likely to ingest a number of parasitic mites through grooming and allogrooming activities and soil mites while digging. Although the relationship between colony size and cestode prevalence and intensity could not be tested, colony membership was a highly significant predictor of cestode prevalence suggesting that infestations and re-infestations are likely to happen within the confines of the colony. Although it is possible that parasitic mites are intermediate carriers, it may also suggest that these cestodes are possibly capable of completing a one-host life-cycle as is the case of the cestode, *Vampirolepis nana* (Mackiewicz 1988). Furthermore, direct life-cycles are more likely to be selected for in coprophagous animals such as mole-rats (Mackiewicz 1988). Studies attempting direct infestation as well as infestation of a range of potential intermediate hosts are required to uncover the full details of this particular cestode's life-cycle.

Parasite loads for both cestodes and mites were more similar between colony members than between members of different groups indicating that transmission is linked to the habitat patches that group members exploit and the burrow systems that they share. This also suggests that transmission of these parasites is less dependent on group size than would be expected. Freeland (1979) suggested that social groups are comparable to biological islands and therefore parasite intensity and diversity will be largely affected not only by the size of groups, but also by transmission of parasites between different groups. This emphasises that inter-colonial contact rates between colonies is probably a far more important factor determining the transmission success of parasites than group size.

As predicted, I found that ectoparasite intensity decreased with increasing colony size in the highveld mole-rat. Similarly, Côté and Poulin (1995) consistently

found a negative relationship between group size and intensity of free-living parasites in a variety of host taxa. Bordes *et al*. (2007) found that ectoparasites species richness (but not endoparasites species richness) decreased with the level of sociality in 46 different rodent species, and speculated that social species have evolved the ability to reduce and impair ectoparasite transmission within groups. Some aspects of social living such allogrooming, parasite avoidance through dilution effects and limiting contact with unfamiliar individuals that may or may not be infested with parasites clearly can have a very positive effect on parasite-defence in the case of ectoparasites (Bordes *et al*. 2007, Hart 1992, Stanko *et al.* 2002). If the recipient of allogrooming is at least sometimes chosen randomly by the groomer, then the likelihood of being groomed by a conspecific increases with increasing colony size. In contrast to the review by Côté and Poulin (1995) there was no relationship found between ectoparasite prevalence and group size in the current study. Larger groups may therefore be able to reduce the number of ectoparasites using behavioural activities such as allogrooming, but mites still persisted in small numbers within the group regardless of group size. The effect of group size on parasitism also depends on the life-cycle of the parasite. One would expect host group-size to have less of an effect on prevalence and intensity of parasites with one or more intermediate hosts than on parasites with one-host life-cycles (Moore *et al.* 1988). Therefore one would expect intensity and prevalence of gastrointestinal parasites not to be affected as much by group size, depending on the type of parasite. Alternatively, there are no means such as grooming that may help to reduce endoparasite loads.

There are interesting seasonal patterns for the macro-parasites of the highveld mole-rat. Mite intensities, but not prevalence, were higher during warm and wet

summer months. Ectoparasite loads of Daubenton's bat (*Myotis daubentonii*) also peaked during summer months, coinciding with this mammal's lactation period and supporting the notion that increased energy demands of reproduction has negative effects on the host's ability to fend off ectoparasites (Lučan 2006). A very similar pattern was found for the abundance of the parasitic mite *Spinturnix psi* of the colonial bat *Miniopterus schreibersii* (Lourenço & Palmeirim 2007). In contrast, cestode intensity was higher during the harsher winter months. Murray *et al*. (1998) showed that free-ranging snowshoe hares (*Lepus americanus*) that received supplementary feeding were infested with lower mean numbers of the nematode, *Trichuris leporis*. Other nematodes were however, unaffected by supplementation. It does nonetheless suggest that at least some species of endoparasites benefitted when the host is malnourished providing one explanation of why cestode intensities were higher during winter months. Some studies indicate that reduced protein intake is especially associated with a reduced ability of hosts to resist parasitic infestations (Ezenwa 2004*b*, Coop & Kyriazakis 2001).

No relationship was found between body mass and mite intensity or prevalence, but mole-rats with higher body masses were found to have greater infestations with cestodes. Host-quality may be related to body mass and a heavier animal may provide a better nutrient resource than a smaller animal (Krasnov *et al*. 2003). Another explanation for this pattern is that age, rather than body mass, is the more important determinant in the intensity of cestodes in highveld mole-rats. Younger, and therefore smaller, mole-rats have a shorter period during which they could have been infested and subsequently re-infested with cestodes and are therefore more likely to not harbour cestodes when compared to older, and heavier, mole-rats (H.Viljoen *pers. observation.).* Alternatively, there may be differences in

foraging behaviour that make juveniles less susceptible to infestation. Although I did not use juveniles for the purpose of this study (>50g), out of six juveniles caught not a single one had signs of proglottids in their faeces. Body mass in mole-rats is not necessarily correlated with age (Bennett & Navarro 1997) but one would still expect to see a strong positive correlation between body mass and age in younger animals that are still growing.

Increased testosterone levels of breeding individuals has been suggested as a factor that may be detrimental to the ability to fight macro-parasites in males of some species (Saino *et al.* 1995), although the immunosuppressive effect of testosterone is still debated (Greenman *et al*. 2005, Morales *et al.* 2002). In this study, no gender difference was found for parasite prevalence or intensity. It is likely that small or no gender differences in parasite intensity and prevalence are simply not reported, explaining why there are little examples of this in the literature (Poulin 1996). One of the proposed reasons used to explain gender differences in parasitism is potential differences in life-histories between the sexes such as diet, behaviour and habitat exploitation (Zuk & McKean 1996). Similarly, reproductive status did not affect the intensity or the prevalence of ecto- or gastrointestinal parasites in the highveld mole-rat. The lack of gender (and reproductive state) differences in parasitism in the highveld mole-rat could be due to these groups sharing the same burrows, nests and probably similar diets. Furthermore, the role of subordinate males and females in a colony are similar, since they do not compete for breeding opportunities (Bennett & Faulkes 2000). Therefore one could expect even less differences in parasitism between non-reproductive individuals. Finally, reproductive status alone may not be sufficient to cause a notable trade-off with immune function

and differences in parasitism may rather be picked up when comparing pregnant and lactating females to non-breeding females (Lučan 2006).

Social animals exhibit an array of behavioural (and perhaps physiological) defences against parasites (Møller *et al*. 2001, Sheldon & Verhulst 1996) and clearly group size or density are not the only factors playing a role in parasite infestation and transmission within and between groups. In fact, larger groups clearly may have a positive effect on parasite prevalence and intensity in many social animals (Hart & Hart 1988, Mooring & Hart 1992, Snaith *et al*. 2008, Stanko *et al*. 2002, this study) and contact rate between social groups is likely to be more important in determining risk of parasitism than local density within a group. The low diversity of parasites found on the highveld mole-rat indicates that this species may have been especially successful in evolving ways to evade parasites. The lack of gender and reproductive differences in parasitism as found in this study suggests that there is little life-history and behavioural differences between males and females of the highveld mole-rat. Although no effect of reproductive status was found on parasite prevalence and intensity, future studies may benefit by focusing on comparing pregnant and lactating females to non-breeding females where differences are more likely to be picked up. Finally, the contrasting seasonal patterns of the mites and cestodes indicate that these groups of parasites may have very different survival strategies and that the host uses different strategies to combat specific parasites. This study adds to our understanding of the relationship between parasitism and sociality and how social animals have evolved virulence and defense systems against parasites.

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Appendix A

Colony ID's, site of capture and colony size of captured animals. An indication of whether an endoparasite and/or ectoparasite assessment was done for the animals from the respective colonies.

Colony	Site	Colony size	Endoparasite assessment	Ectoparasite assessment
A1*	NBG	2	Prevalence	Both
A2*	NBG	1-2	Prevalence	Both
A3*	NBG	3-9	Prevalence**	Both
A4*	NBG	1	Prevalence	Both
A5*	NBG	2-3	Prevalence	Both
A6*	NBG	1-2	Prevalence	Both
A7*	NBG	1	Prevalence	Both
A8*	NBG	1	Prevalence	Both
A9*	NBG	2-5	Prevalence	Both
A10*	NBG	1	Prevalence	Both
A11*	NBG	1	Prevalence	Both
A12	NBG	2	Prevalence	Both
A13	NBG	4	Prevalence	Both
A14	NBG	4	Prevalence	Both
A15	NBG	5	Prevalence	Both
A16	NBG	3	Prevalence	Both
B1	Tygerpoort	6	Both	Both
B2	Tygerpoort	6	Both	Both
B3	Tygerpoort	7	Both	Both
B4	Tygerpoort	6	Both	Both
B5	Tygerpoort	6	Both	Both
B6	Tygerpoort	6	Both	Both
C	Centurion	12	Both	None

*****Recaptured colonies
**One animal that died while in the lab where assessed for intensity of endoparasites

Appendix B

The total number of parasites found (n_p) on infected host mole-rats (n_a) for the period from May 2008 to August 2009.

Month	Cestodes: *Mathevotaenia* spp.		Nematodes: *Protospirura* & *Heligmonina* spp.		Mites: *Androlaelaps spp.*		Lice: *Linognathus spp.*	
	n_p	n_a	n_p	n_a	n_p	n_a	n_p	n_a
May'08	23	3	-	-	-	-	-	-
June'08	-	1	-	-	40	9	-	-
August'08	29	-	-	-	151	15	-	-
October'08	-	2	3	2	20	5	-	-
November'08	-	-	-	-	244	8	-	-
December'08	-	-	-	-	96	3	-	-
February'09	69	-	2	2	199	21	-	-
April'09	27	6	-	-	61	10	1	1
June'09	214	5	-	-	29	5	7	4
August'09	10	12	-	-	123	3	1	1

Conclusion

A comparison of the haematological parameters of the reproductive females with non-reproductive females revealed that the former have lower lymphocytes counts and higher blood platelet numbers, and therefore potentially invest less energy in their adaptive immune system and more energy into inflammatory cell production. One might expect to observe even greater differences when comparing pregnant or lactating females with non-reproductive females. Increased reproductive competition amongst female highveld mole-rats did not appear to affect the immune function negatively. In accordance with many other studies referred to in the first chapter, the results of this chapter suggest that the role which testosterone plays in the modulation of the immune system appears to be less than has been previously reported in the literature. Although there was no direct correlation between testosterone and aggression in females, testosterone concentrations of reproductive females increased significantly with the removal of the males from the colony and subsequent introduction of unfamiliar male. It is not clear exactly why this pattern was observed, but this emphasises the need for further research into this field to unravel the functions and effects of testosterone in female mammals. Behavioural observations of female mole-rats intimate that reproductive competition amongst this sex was instigated following the removal of the reproductive and related males and subsequent introduction of unrelated males into the colonies. This opens the field for future studies investigating life-history trade-offs between reproduction competition and other components of the immune function.

A fever was successfully raised in the highveld mole-rat using the exogenous pyrogen, lipopolysaccharide. No significant differences were found in the body

temperatures raised by fever in reproductive and non-reproductive individuals and therefore no evidence was found to suggest a trade-off between the ability to launch a fever response and reproductive activity. The lack of gender differences in the highveld mole-rats' ability to raise a fever response also implies that testosterone probably does not affect this component of the immune system significantly. Furthermore, no evidence was found for seasonal variation of the innate immune system, although the possibility of variation in other immune components cannot be excluded. This study revealed that highveld mole-rats exhibit a large inter-individual variation of body temperatures and therefore larger sample sizes may be required to investigate if and how fever responses are affected by reproductive status, gender and season in future studies. In addition, it may be necessary to include fever response duration as a factor into studies, as differences may arise in how long animals can afford to maintain elevated body temperatures.

The co-evolution between parasites and their hosts is likely to have a huge impact on how the host immune system functions and implements adjustments. Seasonal patterns of parasite infestation are therefore likely to have affects on the seasonal cycles of the immune function of hosts. The seasonal patterns of intensity of ectoparasites differed from that of internal gastrointestinal parasites. While mite infestations were more intense during summer, cestodes infested mole-rats at higher intensities during the winter, emphasizing how different parasites have divergent survival strategies and that seasonal fluctuations in host immune function will probably accommodate this. This study also showed that larger group size is not necessarily positively correlated with larger parasite infestation as some earlier studies have intimated. In the case of the highveld mole-rat, members from larger colonies were infested to a lesser degree with ectoparasites. Therefore ectoparasite

control may be only one of the driving forces of the social behavior exhibited by this animal. From the perspective of the ectoparasite, inter-colonial contact, rather than concentration of hosts is probably the major factor affecting transmission success. Other factors, such as the availability of intermediate hosts and behaviour of the host animal, are expected to play a more important role in transmission success of endoparasites. Reproductive status did not affect parasite load of individuals and therefore no evidence was found for a trade-off between reproduction and the ability to fight parasites in this species.

In conclusion, evidence of life history trade-offs between immunocompetence and reproduction was found only for the adaptive arm of the immune system in the highveld mole-rat. For none of the other aspects of the immune function investigated in this study were such trade-offs observed. In fact, reproductively active individuals tended to invest more resources into platelet production, an important aspect of the inflammatory system. This strongly supports the notion that increased reproductive effort results in a re-distribution of immune cells, rather than an overall trade-off with immune function. The apparent lack of seasonal cycles in the fever response may be attributed to the more stable underground environment of this species, although future studies may benefit from investigating possible seasonal patterns in aspects of the immune system not covered in this study. Similar to the effects of reproductive effort on the immune system, one can also expect seasonal differences on how energy is distributed between immune function components, with specific aspects being better maintained during certain times of the year as to accommodate the different seasonal cycles of parasites and diseases.