

Trade-offs among immunity, hormones and alternative reproductive
tactics of male Cape ground squirrels (*Xerus inauris*).

by

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Abstract

Immunity and reproduction are important and costly life-history traits and a large part of eco-immunology focuses on trade-offs between investing in reproduction versus immunity. Male Cape ground squirrels (*Xerus inauris*) are a good organism to test these trade-offs as they invest heavily in sperm competition, evidenced by their large testes, and exhibit alternative reproductive tactics. My research objective was to determine 1) if testosterone suppresses immunity, or if immunity suppresses testosterone, and 2) if alternative reproductive tactics differ in their investments in immunity and reproduction. I found evidence that testosterone was suppressed after an immune challenge, and that manipulatively increased testosterone had no effect on immunity, body condition or parasites. The dispersing tactic males were in worse condition, had higher parasite abundances, but showed no difference in their innate immune ability to non-dispersing males. Males incur increased costs with dispersal and may focus their energy into reproduction instead of immunity.

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Chapter 1- Thesis introduction

Ecological immunology (also known as eco-immunology) is a growing interdisciplinary field of research that approaches immunological questions by taking into consideration the host's physiology, disease ecology, and environmental factors that contribute to an organism's ability to mount an immune response (Demas and Nelson, 2012; Norris and Evans, 2000). An animal's immune response is composed of both their innate and acquired immune systems (Demas and Nelson, 2012; Demas et al., 2011). Innate immunity is a rapid, non-specific response that serves as the front line defense and includes lysozyme, complement, phagocytic cells, inflammatory mediators and natural killer cells (Demas and Nelson, 2012; Demas et al., 2011). The acquired immune response is a slower, pathogen specific response involving lymphocyte B and T cells to directly target specific pathogenic cells, and both the innate and adaptive immune systems are complementary responses that work together to eliminate harmful non-self cells (Demas et al., 2011).

Eco-immunology is based in the concept of life history theory, which describes how an animal allocates a finite amount of resources and time between three main competing biological processes: maintenance, growth and reproduction (Stearns, 1992). Immunity is costly, and an organism is forced to make trade-offs between immunity and other important life history traits like reproduction, growth and dispersal (Demas and Nelson, 2012; Lochmiller and Deerenberg, 2000; Norris and Evans, 2000). As both immunity and reproduction are important and energetically costly life-history traits, there has been considerable focus within eco-immunology on the trade-offs between investing in immediate reproductive benefits and investing in maintenance and immunity to increase potential for future reproductive benefits (Demas and Nelson, 2012; Lochmiller and Deerenberg, 2000; Norris and Evans, 2000; Stearns, 1992).

The immunocompetence handicap hypothesis (ICHH) proposes a trade-off between the positive effects of increased testosterone on a male's reproductive potential, via increased sperm production as well as secondary sexual characteristics such as sexual displays, and the negative effects on a suppressed immune system (Folstad and Karter, 1992). The ICHH incorporates Zahavi's (1975) handicap hypothesis that states that elaborate secondary sexual traits, like a peacocks tail plumage, which evolve through sexual selection confer a handicap that acts as a test of the male's quality. This test ensures that only high quality males are able to maintain the costly traits and survive, acting as honest signalling of quality to females (Zahavi, 1975). The ICHH is also derived from Hamilton-Zuk's (1982) parasite model that females choose males with "good genes" based on the quality of secondary sexual traits, such as brightness of plumage, that were dependent on health and parasite loads (Hamilton and Zuk, 1982; Smith, 1985). Folstad and Karter (1992) initially proposed the ICHH as the mechanism behind "honest" signalling of male quality to females. Only high quality males (i.e., males in good body condition and with good genes) can afford to suppress their immune system and remain attractive to females; this model excludes cheaters as poor quality males would not be able to bear the immunosuppression.

The second chapter of this thesis addresses the ICHH through manipulative experiments to look at the relationship between testosterone and immunity in male Cape ground squirrels (*Xerus inauris*). Cape ground squirrels are a good model organism to address this hypothesis as males invest heavily in sperm production, maintain reproductive readiness year round (Manjerovic and Waterman, 2012; Waterman, 1998), and invest in reproductive effort at the expense of their immunity as spleen size, an indirect estimate of immunity, decreases with increasing testes size (Manjerovic and Waterman, 2012). Additional evidence that testosterone may be playing a role in their immunocompetence is a sex bias observed in this species where

sexually mature males have three times the number of ectoparasites as females and significantly more than immature, non-scrotal males, suggesting testosterone may be related to parasite loads (Hillegass et al., 2008). I tested two alternative hypotheses in addition to the ICHH in chapter two. The immuno-suppressive hypothesis, based on Boonekamp et al. (2008) predicts a suppressive effect of immune activation on testosterone levels, suggesting that it is the immune response suppressing testosterone. The final hypothesis tested in chapter two is the stress-linked ICHH based on the indirect interaction between testosterone and cortisol, triggering the immuno-suppressive effects of cortisol (Roberts et al., 2007). The main objective of chapter two is to test if there is a trade-off between testosterone and immunity and to test if testosterone suppresses immunity, or vice versa.

A considerable amount of research within eco-immunology has focused on variation in behaviours to secure reproductive benefits and the trade-off with maintenance and immunity (Demas and Nelson, 2012; Lochmiller and Deerenberg, 2000; Norris and Evans, 2000; Stearns, 1992). Species that naturally show variation in reproductive behaviours and fitness provide interesting model systems in which to study trade-offs between behaviour and immunity in the wild (Demas and Nelson, 2012). Alternative reproductive tactics describe when conspecifics of the same sex use different behaviours to achieve reproductive success (Taborsky et al., 2008) and present a great opportunity to study trade-offs between immunity and reproduction, and growth and dispersal, as they relate to the variable tactics within a one species and sex. Male Cape ground squirrels present two alternative reproductive tactics and are unique because they do not differ in testosterone levels (Scantlebury et al., 2008) or reproductive fitness (Manjerovic and Waterman, 2015). This lack of difference in fitness and testosterone allow us to test how other differences between tactics, like home ranges, resting metabolic rate and social structure

(Scantlebury et al., 2008) are related to immunity within a species. The third chapter looks at the trade-off of between alternative reproductive tactics and immunity in male Cape ground squirrels.

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Chapter 2- Trade-offs between immunity and testosterone in male Cape ground squirrels (*Xerus inauris*)

Abstract

The immunocompetence handicap hypothesis (ICHH) proposes that testosterone has both beneficial effects on male reproductive potential and negative effects by suppressing the immune system. Support for the ICHH has been variable and it has also been proposed that testosterone may be acting indirectly via cortisol to suppress the immunity described as the stress-linked ICHH. An alternative hypothesis proposed is that increased investment in immunity results in the suppression of testosterone. We tested these hypotheses in male Cape ground squirrels through two separate manipulations; first by increasing circulating testosterone using silastic testosterone implants and second by triggering a strong immune response using a lipopolysaccharide injection. Responding to an immune challenge significantly reduced testosterone, supporting the immunity suppression hypothesis, while increasing circulating testosterone had no effect on immunocompetence, body condition, ectoparasite abundances or cortisol levels, failing to support either the ICHH or stress-linked ICHH. Our results add to the growing body of literature that challenges the widely accepted ICHH, and we conclude that the trade-off between testosterone and immunity is mediated through immune activation and not through testosterone in male Cape ground squirrels. Being able to test the ICHH, stress-linked ICHH and immune suppression hypotheses in a single free ranging mammal gives us a unique opportunity to examine the mechanisms mediating this trade-off.

Introduction

Life history theory describes how an animal allocates a finite amount of resources and time between three main competing biological processes: maintenance, growth and reproduction (Stearns, 1992). As both immunity and reproduction are important and energetically costly life-history traits, there has been considerable focus on the trade-off between immediate reproductive potential versus investing in maintenance and immunity to ensure future reproductive potential (Demas and Nelson, 2012; Lochmiller and Deerenberg, 2000; Norris and Evans, 2000; Stearns, 1992). A general pattern of sexual bias in immunity has been observed, with females generally being more immunocompetent, having longer life spans and lower parasite loads than males (Folstad and Karter, 1992; Nunn et al., 2009). Although the cause of this sex bias in immunity is unclear, one explanation is that the observed sex bias is established through testosterone acting as an immunosuppressant (Folstad and Karter, 1992).

The immunocompetence handicap hypothesis (ICHH) proposes a trade-off between the positive effects of increased testosterone on a male's reproductive potential, via increased sperm production as well as secondary sexual characteristics such as sexual displays, and the negative effects on a suppressed immune system (Folstad and Karter, 1992). Folstad and Karter (1992) initially proposed the ICHH as the mechanism behind "honest" signaling of male quality to females. Only high quality males (i.e., males in good body condition and with good genes) can afford to suppress their immune system and remain attractive to females. There has been a lot of correlational evidence that high levels of testosterone are associated with increased vulnerability to pathogens (Folstad and Karter, 1992; Roberts et al., 2004). However, a meta-analysis by Roberts et al. (2004) on experimental studies of the ICHH found only weak support across taxa

and found no support of this hypothesis in mammals, although this lack of support was possibly due to the few studies in which the ICHH had been experimentally tested in mammalian species.

An alternative hypothesis to the ICHH is that it is the immune response is suppressing testosterone, not vice versa. A meta-analysis by Boonekamp et al. (2008) found that overall there was strong support of the suppressive effect of experimental immune activation on testosterone levels. Although initially proposed by Folstad and Karter (1992) as a negative feedback to the ICHH it has received far less attention and offers an alternative and important mechanism on how the trade-off between reproduction and immunity is modulated. Luterman et al. (2012) found that highveld mole-rats (*Cryptomys hottentotus pretoriae*) that were naturally infected by cestodes had lower testosterone levels than those who were not infected. They also found that when faced with an additional immune challenge (lipopolysaccharide injection) there was a significant decrease in testosterone levels as well as an increase in cortisol levels. Similar decreases in testosterone levels have been seen after immune challenges in Indian peafowl (*Pavo cristatus*; Ros et al., 2008) and short-tailed fruit bats (*Carollia perspicillata*; Greiner et al., 2010). Owen-ashley et al. (2006) found when male white-crowned sparrows were faced with an immune challenge (LPS) they show a rapid increase in plasma corticosterone, and a decrease in territorial aggression and song behaviors.

A third alternative explanation is that increased testosterone may not be directly interacting with the immune system. Increased testosterone has been shown to positively affect corticosterone in several avian species (Casto et al., 2001; Duffy et al., 2000; Evans et al., 2000; Owen-Ashley et al., 2004). Acute stress as measured by circulating levels of cortisol or corticosterone may actually enhance immunocompetence, by redirecting energy and resources (Martin, 2009), but chronically high cortisol levels can be immunosuppressive (Demas and

Nelson, 2012; Martin, 2009). Evans et al. (2000) initially found that increased testosterone had immunosuppressive effects in house sparrows (*Passer domesticus*) but after they controlled for the effect of increased corticosterone they found that increased testosterone actually had an immunoenhancing effect and it was the increased corticosterone that was negatively affecting the immunity. This indirect interaction of testosterone, via cortisol or corticosterone, and the immune system has been called the stress-linked ICHH (Evans et al., 2000; Roberts et al., 2007). Testing the ICHH, the stress-linked ICHH, and the immunosuppressant hypotheses together will provide important insight into the mechanism that regulates the observed trade-offs between reproduction and immunity.

Male Cape ground squirrels (*Xerus inauris*) are a particularly appropriate organism to test the immunosuppressive effects of testosterone and the testosterone suppressive effects of mounting an immune response. They invest heavily in sperm production (indicated by extremely large testes) and maintain reproductive readiness year round (Manjerovic and Waterman, 2012; Waterman, 1998). Testes size and testosterone levels have been shown to be positively correlated in numerous mammals (Soay sheep, Preston et al., 2012; European ground squirrels, Strauss et al., 2008; Red deer, Malo et al., 2009). Evidence suggests that there is a negative correlation between male investment in reproduction and their immune response as spleen size, an indirect estimate of immunity, decreases with increasing testes size (Manjerovic and Waterman, 2012). Parasite load appears to be sex biased; sexually mature males have three times the number of ectoparasites as females and significantly more than immature, non-scrotal males, suggesting testosterone may be related to parasite loads (Hillegass et al., 2008).

The objective of our research was to evaluate the direction of the interaction between testosterone and immune system function in free-ranging Cape ground squirrels through

manipulative studies. We predicted that if testosterone increases reproductive potential and suppresses immunity, as suggested by the ICHH, then higher observed testosterone levels will be related to larger testes, higher parasite abundance, and lower measure of immunity. Similarly, if the stress-linked ICHH is correct, then cortisol and testosterone should be positively correlated. In contrast, if investing energy in an immune response suppresses testosterone, the immune suppression hypothesis (ISH), then individuals given an immune challenge will demonstrate lower circulating testosterone levels, smaller testes and higher cortisol levels than individuals not given an immune challenge.

Methods

Male Cape ground squirrels were studied on the S.A. Lombard Nature Reserve (3660 ha, 18 km north west of Bloemhof, South Africa, 27°35'S, 25°23'E) from June to October 2013. Squirrels were trapped using live traps (Tomahawk Live Trap Inc., WI; 15 X 15 X 50 cm) and handled using a cone-shaped handling bag (Koprowski, 2002). Animals were permanently marked using PIT tags (AVID Inc. Folsom, LA; Hillegass et al., 2008), and freeze marking for identification (Quick Freeze; Miller-Stephenson Product, Morton Grove, IL; Rood and Nellis 1980), and temporarily marked dorsally using black hair dye (Rodol D; Lowenstein & Sons Inc., New York, NY).

Body mass was recorded (± 5.0 g) using a spring scale (Pesola AG, Baar, Switzerland) and spine length was measured from the occipital condyles to the base of the caudal vertebrae. Body condition was determined by taking the residuals of the regression between spine length and mass (Schulte-Hostedde et al. 2005; Manjerovic & Waterman 2012). We quantified ectoparasites by combing from head to tail along the three planes of the back with a metal flea comb (Hillegass et al., 2008) and by examining the groin and inner thigh of each male,

depositing any ectoparasites directly into 95% ethanol for immediate quantification. We examined the mean abundance of the total ectoparasites, fleas and lice as well as prevalence which represents the proportion of hosts with parasites.

To quantify immunocompetence and measure circulating testosterone, we collected approximately 1ml of blood from the femoral vein using a sterile 26 gauge needle and 1 ml syringe. Hematocrits of blood were used to estimate percent red blood cells (% RBC) as an additional measure of body condition (Beldomenico et al., 2010; Gilot-Fromont et al., 2012). As plasma is required for both hormone and some immunocompetence measurements, we separated the plasma from the whole blood by centrifugation at 6000 rpm for 5 minutes (Spectrafuge mini, Labnet International USA).

Immunity Measures

White blood cell differentials (WBC) of each individual were estimated by counting 100 cells on a single layer of whole blood smears stained with Diff-Quick and recording the number of leukocytes, neutrophils, basophils, eosinophils and monocytes (Bachman, 2003). Bacteria-killing assays were used to measure complement and lysozyme activity in the organism by evaluating the plasma's ability to kill a known dilution of a novel bacteria (Millet et al., 2007), giving us an estimate of the individual's ability to recognize and kill microorganisms, a measure of innate immune ability (Liebl and Martin, 2009; Matson et al., 2005; Merrill et al., 2014; Tieleman et al., 2005). We used the procedure of Liebl and Martin (2009), preparing a working solution of 10^5 bacteria/ml of *E. coli* (Lyophilised *E. coli* ATCC #8739, Microbiologics), and using 24.5 μ l of plasma to make a 1:4 dilution to test the plasma's ability to inhibit bacterial growth overnight. Each sample was tested in quadruplet and incubated in 96-well plates and

absorbance was measured at 595 nm using a spectrophotometer (Multiskan ascent, Thermo Labsystems). There were two blanks in quadruplet (growth medium only) and two controls in quadruplet (bacteria and growth medium only) on each plate and we calculated the percent of bacterial growth inhibited by comparing the absorbance of the samples and the absorbance of the controls.

Hormone assays

We used a commercially available coated tube assay kit (Coat-a-Count TKTT1, Diagnostic Products Corporation, Los Angeles, CA) to determine plasma testosterone concentration as previously described (Scantlebury et al., 2008). The assay-detectable range was 10 to 1600 nmol/L testosterone and cross reactivity of the Coat-a-Count testosterone antibody was 16% with 11-ketotestosterone, <5% with dihydrotestosterone and 19-hydroxyandrostenedione, and less than 1% with other steroids tested according to the manufacturer. Serial dilution of Cape ground squirrel plasma demonstrated good parallelism with the testosterone standard curve (data not shown). Assay sensitivity (90% binding) was 1.39 nmol/L and the intra-assay coefficient of variation was 8.4%. All samples were run in a single assay.

Plasma cortisol is difficult to measure in wild animals as cortisol levels spike quickly due to the stress of capture and handling (Romero and Reed, 2005) and the blood sample must be taken within the first 3 minutes of handling to get an accurate measure of baseline cortisol (Scantlebury et al., 2008). To avoid the confounding acute stress response of trapping and handling we measured cortisol metabolites from feces. Fecal cortisol was extracted from 0.2 g dried feces using 1.5ml of 95% ethanol, following the protocol outlined in Mateo and Cavigelli

(2005). Feces were collected from under the traps as the squirrels defecate readily when trapped. Concentrations of cortisol (ng/ml) were estimated using a radioimmunoassay technique described in Ryan et al. (2011). Cortisol antibody dilutions of 1:6400 were used and had a cross-reactivity of 5.7% for 11-deoxycortisol, 3.3% for corticosterone, 36% for prednisolone, and <0.7% for cortisone according to the manufacturer. Serial dilution of Cape ground squirrel fecal cortisol demonstrated good parallelism with the cortisol standard curve (data not shown). Inter-assay and intra-assay coefficients of variation were 15.4% and 5.1%, respectively. Fecal samples represent cortisol levels from approximately 10h – 24 h before collection (Dantzer et al., 2010; Harper and Austad, 2000; Palme, 2005; Touma et al., 2003). We were unable to validate the fecal cortisol measurements in this study, as Cape ground squirrels are difficult to keep in captivity (personal communication: Dr. Jane Waterman) and as there were multiple projects happening simultaneously on our population, we were unable to remove animals for validation during the field season without disrupting the other research. There have been a number of studies that have validated the use of fecal glucocorticoids in squirrels: red squirrels (*Tamiasciurus hudsonicus*; Dantzer et al., 2010), Richardson's ground squirrels (*Urocitellus richardsonii*; Hare et al., 2014), Columbian ground squirrels (*Urocitellus columbianus*; Bosson et al., 2009), Belding's ground squirrels (*Urocitellus beldingi*; Mateo and Cavigelli, 2005), and Eastern Chipmunk (*Tamias striatus*; Montiglio et al., 2012). Also, as we are only comparing fecal cortisol among males within one population, we control for possible problems of how sexes may differentially metabolise cortisol (Goymann, 2012).

Hormone Manipulation

During preliminary sampling, we found that the average circulating testosterone levels of male Cape ground squirrels was 1.88 ± 0.34 ng/ml (min:0.33 ng/ml , max: 8.7 ng/ml , n= 33).

Thus, we prepared silastic implants to raise the testosterone level by two standard deviations, to 5.7ng/ml, as suggested by Grear et al. (2009). This elevated testosterone level is within the range of naturally occurring testosterone levels we have found in this species (max. 8.7ng/ml). To raise the testosterone of males, we injected testosterone implants subcutaneously in the dorsal region using a sterile 12-gauge needle. Each male received two 2.5 cm long implants (1.47 mm inner diameter, 1.96 mm outer diameter) made of medical grade silastic tubing (HelixMark) and sealed with a medical grade non-toxic silicone adhesive (Bluestar Silicone, Inc.). Seven males received implants filled with crystalline testosterone (Sigma T-1500) and nine received empty implants to act as controls. Implants were constructed by sealing one end of the tubing with the silicone adhesive, filling the tube with testosterone and sealing the remaining end. We calculated tube length using previous studies of mammals (rabbits, mice, rats and degus) in which similar implants were used to raise testosterone, but we adjusted the length of tubing by the body mass of the male Cape ground squirrels to maintain delivery of the appropriate dose (Jechura et al., 2003; Limonta et al., 1986). We recaptured both testosterone-implanted and control squirrels approximately a month later to compare the effects of prolonged testosterone increase on treatment and control males.

Immune challenge

Lipopolysaccharide (LPS) is a cell wall component of gram-negative bacteria that can stimulate a strong immune response in an animal (Lutermann et al., 2012). This method is often used to test the acute immune response as it cannot replicate within the host and does not infect the animal (Adelman and Martin, 2009). To stimulate the immune response Cape ground squirrels were injected subcutaneously with a dose of 1mg/kg of LPS (from *Escherichia coli* serotype 026:B6, Sigma Chemical) dissolved in 0.9% saline (Lutermann et al., 2012). Similar

doses have been previously used in birds and small mammals (Lutermann et al., 2012; Owen-Ashley et al., 2006) and control animals received a subcutaneous injection of saline. Both LPS-treated and control males were recaptured approximately 1 week after injection to measure the effects of mounting an immune response on testosterone levels.

Statistics

Non-normal data were log transformed (testosterone levels, cortisol levels, total parasite abundances and relative scrotal size) or arcsine transformed (percent RBC, WBC and growth inhibition percentages) to satisfy normality. Transformation of the measured monocyte percentages did not satisfy normality so these data were analysed using a non-parametric Mann-Whitney U test. For log or arcsine transformed data, a two-sided Welch's t-test (Welch, 1947) was used to compare pre- and post-treatment levels. Results are reported as mean \pm standard error and an alpha value of 0.05 was used to determine significance. All statistical analyses were conducted in R (version 3.1.1). We compared prevalence between treatment and control with an exact unconditional test using Quantitative Parasitology version 3.0 (Rózsa et al., 2000).

Results

Effects of testosterone manipulation

Seven males were given testosterone implants, nine received control implants and all were handled on average 35.9 ± 2.73 days after treatment. Initial plasma testosterone concentration in the experimental animals (1.51 ± 0.27 ng/ml) was not significantly different from plasma testosterone concentration in control animals (1.03 ± 0.21 ng/ml; Two-sided Welch's t-test, $t_{13,56} = 1.43$, $p = 0.175$). However, testosterone implants significantly raised the

circulating plasma testosterone by 3.47 ± 0.51 ng/ml when compared to the control group that increased by 0.48 ± 0.25 ng/ml ($t_{13.88}: 5.89, p < 0.001$) when sampled a second time (Figure 2.1).

There were no differences in the initial prevalence of ectoparasites between testosterone (Fleas: 85.7%, Lice: 85.7% and total: 100%) and control treatment (Fleas: 66.7%, Lice: 66.7%, Total: 88.9%; unconditional exact p-value = 1 for all three comparisons). Similarly, the testosterone treatment had no effect on the final prevalence of fleas (100%), lice (85.7%) and total ectoparasites (100%; unconditional exact p-value = 1 for all three comparisons) and there was no change in prevalence for the control group (Fleas: 100%, $p = 0.09$; Lice: 77.8%, $p = 1$; total: 100%, $p = 1$). There were no differences in any of the initial measurements of parasite abundance, bacteria growth inhibition, WBC differential, body condition, %RBC, scrotal size or fecal cortisol levels between the testosterone-manipulated and control males (Table 2.1). Additionally, increased testosterone had no significant effect on any of the above parameters when compared to control animals (Table 2.1). After combining the initial testosterone and control treatment levels there was no correlation between testosterone and cortisol levels (Pearson's correlation: $r = -0.008, N = 16, p = 0.98$).

Effects of Immune Challenge

Seven males received LPS injections and nine males received a saline injection as a control and all were re-handled on average 7.7 ± 0.38 days after receiving injections. There was no difference in the initial body condition between LPS and control treatments ($t_{13.9} = 1.64, p = 0.124$). However, the LPS injection had a significant negative effect on the body condition (body condition decreased by 47.56 ± 9.61) compared to controls (increased by $36.99 \pm 12.32; t_{13.9} = -5.41, p < 0.001$; Figure 2.2) at the second time-point. Similarly there was no difference in initial

% RBC between the LPS (47.14 ± 1.18 %) and the control treatments (47.33 ± 1.08 %; $t_{13.3} = -0.12$, $p = 0.905$) but the LPS treatment had a significant effect on the % RBC (decreased by 11.0 ± 2.81 %) when compared to the control treatment (decreased by 2.89 ± 1.6 %; $t_{9.86} = -2.54$, $p = 0.03$; Figure 2.3). There was no difference in the initial testosterone levels between the LPS treatment (0.95 ± 0.18 ng/ml) and control group (0.85 ± 0.12 ng/ml; $t_{11} = 0.36$, $p = 0.73$) although the LPS treatment had a significant negative effect on testosterone levels, decreased by 0.59 ± 0.13 ng/ml, when compared to the control group which increased by 0.15 ± 0.34 ng/ml ($t_{10.63} = -2.73$, $p = 0.02$, Figure 2.4). Initial relative scrotal size did not differ between LPS (17.02 ± 1.47 mm³) and control manipulation (17.54 ± 0.74 mm³/g; $t_{8.98} = -0.316$, $p = 0.76$) and there was no difference in the effect of LPS on relative scrotal size (-1.12 ± 2.68 mm³/g) when compared to the change seen in the control group (-1.54 ± 1.96 mm³/g; $t_{10.9} = -0.125$, $p = 0.9$). Initial cortisol levels did not differ between the LPS (0.79 ± 0.18 ng/g) and control treatment (0.67 ± 0.10 ng/g; $t_{8.88} = 0.52$, $p = 0.61$) and there was no effect of LPS on cortisol (increased by 0.286 ± 0.201 ng/ml) compared to the control group (decreased by 0.022 ± 0.114 ng/ml; $t_{10.21} = 1.33$, $p = 0.21$). After combining the initial LPS and control treatment levels, there was no correlation between testosterone and cortisol levels (Pearson's correlation: $r = 0.05$, $N = 16$, $p = 0.86$).

Discussion

Our results fail to support the ICHH as increased testosterone had no effect on parasite abundance, body condition, or any of the immunocompetent measures. This result is consistent with the meta-analysis conducted by Roberts et al. (2004), who found only weak support for the ICHH in birds and no support in mammals or reptiles. Other more recent studies have also failed to find a relationship between increased testosterone and immunocompetence measurements in mammals and birds (Nunn et al., 2008; Oers et al., 2011; Roberts et al., 2007; Ros et al., 2006),

and some studies have even found that elevated testosterone had immuno-enhancing effects, completely opposite to predictions from the ICHH (Bilbo and Nelson, 2001; Evans et al., 2000). Nonetheless, the ICHH remains widely accepted as one of the main mechanisms behind sex biased immunocompetence and parasitism and there are recent studies that have found support for testosterone playing an important role in parasite infections and fitness: in white-footed mice (*Peromyscus leucopus*) increased testosterone maintained social behaviours that increased the transmission potential of ectoparasites (Gear et al., 2009), and increasing testosterone in mice (*Mus musculus*) increased their endoparasite intensity (Zhang and He, 2014) and in red grouse (*Lagopus lagopus*) increased testosterone had a negative effect on body condition (Martínez-Padilla et al., 2014).

Previous research on Cape ground squirrels had supported the ICHH through demonstrating sex biased parasitism (Hillegass et al., 2008) and strong correlational evidence of a trade-off between investment in sperm competition, indicated by large testes and immunity (Manjerovic and Waterman, 2012). Male Cape ground squirrels do not demonstrate sex-biased parasite levels until they reach maturity, which suggests the natural increase in testosterone may be responsible for the increase in parasites as allogrooming received does not differ between juvenile, subadult or adult males within a social group (Hillegass et al., 2008). Our results indicate that testosterone does not suppress immunity, affect parasite abundance or affect body condition, which suggests that another mechanism other than testosterone is contributing to sex-biased parasitism and the observed relationship between immunity and testosterone in Cape ground squirrels.

Our experiments instead indicated that an immune challenge had a significant negative result on the circulating testosterone levels of male squirrels and supports the ISH. This pattern is

consistent with the ISH first proposed by Boonekamp et al. (2008) which showed a consistent suppressive effect of immunity on testosterone levels in domesticated mammals and birds. This alternative hypothesis has received a fair amount of support in recent studies. Lutermann et al. (2012) found that following an immune challenge (LPS), highveld mole rats demonstrated significantly decreased testosterone levels. Similarly, Müller et al. (2013) found that manipulatively infesting male canaries with ticks had a significant negative effect on their testosterone levels and sexual signals. Adult male humans have significantly lower testosterone levels during illness (Muehlenbein et al., 2010). Ros et al. (2008) found that peacocks displayed decreased testosterone after being immuno-challenged with sheep red blood cells and Greiner et al. (2010) also found that the activation of an immune response resulted in lowered plasma testosterone in a free-ranging fruit bat. It is important to note that this pattern is not universal as Zhang and He (2014) found that experimentally increasing parasite loads had no effect on testosterone levels in mice. Although we found a significant effect of the LPS challenge on circulating testosterone, we did not find an effect on average scrotal volume. This lack of change was possibly due to the acute and short term effects of the LPS challenge (Lutermann et al. 2012) which may not have been able to change scrotal volume significantly over the relatively short time period.

Results from the present study failed to support the stress-linked ICHH as increasing testosterone did not significantly affect fecal cortisol levels. Evans et al. (2000) initially found support that testosterone seemed to be immunosuppressive in house sparrows (*Passer domesticus*), supporting the ICHH, but when controlling for corticosterone, found that testosterone was actually enhancing immune status. As their increased testosterone manipulation caused an increase in corticosterone, and as corticosterone has been shown to be

immunosuppressive (Adamo, 2014; Martin, 2009), they concluded that testosterone was acting indirectly through corticosterone to suppress immunity. The stress-linked ICHH has been supported through numerous recent studies. Moore et al. (2015) found support for this hypothesis in perceived attractiveness of human male faces, and Owen-Ashley et al. (2004) found that testosterone implants significantly increased plasma corticosterone in song sparrows and suggested that the actions of testosterone were indirect through elevated corticosterone levels suppressing the immune system. Leary and Knapp (2014) found that while moderate increases of glucocorticoids were related to elaborate male traits and increased reproductive investment, high glucocorticoid levels were found to negatively affect reproduction and could have androgen-suppressive effects. When an organism is faced with an immune challenge they may experience increases in cortisol levels, which plays an important acute role in enhancing immunocompetence by redirecting energy and resources (Martin, 2009). Therefore, it is possible that cortisol may suppress testosterone following an immune challenge, however, our results show no effect of LPS treatment on fecal cortisol concentration, suggesting that cortisol is not playing an important role in either testosterone suppression nor immunosuppression, as predicted by the stress-linked ICHH, in the male Cape ground squirrels.

The ICHH, stress-linked ICHH and ISH are only a few of several, not mutually exclusive, hypotheses that address potential mechanisms behind male-biased parasitism and immunocompetence. Combes (2001) proposed an encounter filter suggesting that testosterone affects behaviours that lead to increases in parasite load through increased encounter rates. Increased testosterone is often linked with increased home range size, motility, and aggression, which are all behaviours that increase the potential to encounter parasites through increased exposure to the environment or through increased contact with conspecifics (Gear et al., 2009).

Increased exposure to parasites has an important fitness cost (Hudson et al., 2002) and a study by Moore and Wilson (2002) showed that sex-biased parasitism played an important role on sex-biased mortality in natural populations of mammals. Gear et al. (2009) found that male white-footed mice with increased testosterone levels had higher contact rates with conspecifics, altering their social networks, and increasing potential parasite transmission. Increased exposure to parasites associated with larger home ranges could possibly contribute to the sex-biased parasitism observed in the Cape ground squirrels, as adult males have a much larger home range than juvenile and female squirrels and, therefore, increase the probability of encountering parasites. However, subadult males, males that are an adult size but not yet fully sexually mature (Waterman, 1995) have higher parasite loads than subadult and adult females, despite having similar home ranges (Hillegass et al., 2008), which suggest that it is not simply home range that results in high parasitism in males.

Another possible mechanism to explain the observed trade-offs between testosterone and immunity is simply differential allocation of resources (Rolf, 2002). In species where males do not experience high levels of sexual selection, and where there is little to no competition for females, males do not have high levels of testosterone and, therefore, are able to allocate more resources towards immunity (Krasnov et al., 2012). Similarly, species that show seasonality to their energy investments in reproduction and testosterone levels are able to invest more in immune function during non-breeding seasons (Demas and Nelson, 2012). As Cape ground squirrels maintain reproductive readiness year-round and are under strong competition for females and sexual selection (Waterman, 1998), we expected to see a strong trade-off between testosterone and immunity. When forced to invest energy into an immune response, male Cape ground squirrels suffered a decrease in testosterone, which is likely a change in the allocation of

resources. Males may only be able to maintain high testosterone levels and reproductive investment when they are not immunologically challenged and, therefore, have additional resources at their disposal. The resource allocation hypothesis supports both the ICHH, ISH, and the general trend of a trade-off between testosterone and immunity.

The results of this manipulative study give us important insight into the mechanisms that may maintain the trade-offs between reproduction and immunity. The failure of our testosterone manipulation to impede any aspect of the immune response in Cape ground squirrels adds to the growing number of studies which challenge the long established ICHH. Instead, we find strong support for the ISH through the testosterone-suppressive effect of mounting an immune response. Although both the ICHH and ISH hypotheses predict a trade-off between reproduction mediated through testosterone and immunity, being able to test both hypotheses in a single free ranging mammal gives us a unique opportunity to examine the mechanisms mediating this trade-off.

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Figures and Tables

Table 2.2. Comparisons of ectoparasite abundance, body condition, cortisol and leukocytes between treated (increased testosterone) and control male Cape ground squirrels. We first compared difference between the initial measurements between the control and treatment animals, then we compared the change observed between the treated and control animals.

	Initial in treatment	Initial in control	Df	t-value	p-value	Change in treatment	Change in control	df	t-value	p-value
Total ectoparasite	11.86 ± 3.53	10.67 ± 2.24	13.90	0.26	0.80	- 0.857 ± 5.26	3.33 ± 3.21	11.17	-0.32	0.76
Fleas	5.57 ± 1.28	5.89 ± 2.36	13.83	0.62	0.55	0.714 ± 2.33	1.22 ± 2.22	13.99	-0.86	0.40
Lice	6.14 ± 2.56	4.78 ± 1.75	12.81	0.32	0.76	- 1.43 ± 3.84	2.11 ± 2.53	12.49	-0.44	0.67
% Bacteria growth inhibition	47.7 ± 0.08	44.7 ± 0.9	13.99	0.11	0.91	3.01 ± 11.8	- 3.33 ± 11.7	13.93	0.44	0.66
Body condition	-21.08 ± 21.31	2.77 ± 11.1	9.19	0.99	0.35	13.62 ± 17.41	-13.43 ± 11.28	10.68	1.30	0.22
% RBC	44.29 ± 1.58	42.11 ± 1.43	13.05	1.02	0.32	1.43 ± 2.08	4.5 ± 0.76	12.99	0.40	0.70
Relative scrotal size (mm³/g)	16.19 ± 1.02	15.02 ± 1.49	13.37	-0.64	0.53	- 3.91 ± 1.5	-3.43 ± 1.37	12.04	0.27	0.79
Fecal cortisol (ng/g)	0.721 ± 0.12	0.621 ± 0.063	9.51	0.66	0.53	- 0.011 ± 0.19	0.007 ± 0.16	12.56	0.05	0.96
Lymphocytes (%)	48.57 ± 5.64	42.67 ± 2.89	8.99	1.00	0.34	3.43 ± 8.59	6.89 ± 4.09	8.83	-0.38	0.72
Neutrophils (%)	44.28 ± 5.38	43.56 ± 4.41	12.18	0.12	0.90	- 10.29 ± 7.54	- 8.89 ± 4.32	9.52	-0.19	0.86
Eosinophils (%)	1.43 ± 0.95	1.11 ± 0.59	10.36	0.29	0.78	1.71 ± 1.27	2.25 ± 0.88	11.00	-0.35	0.74
Basophils (%)	1.14 ± 0.60	1.78 ± 0.78	13.80	-0.65	0.53	0.57 ± 0.84	1.11 ± 1.3	13.10	-0.35	0.73
	Initial in treatment	Initial in control	N1-N2	U-value	p-value	Change in treatment	Change in control	N1-N2	U-value	p-value
Monocytes (%)	4.57 ± 0.72	10.89 ± 2.65	7 - 9	20.00	0.24	4.57 ± 2.17	0.44 ± 3.01	7 - 9	39.50	0.42

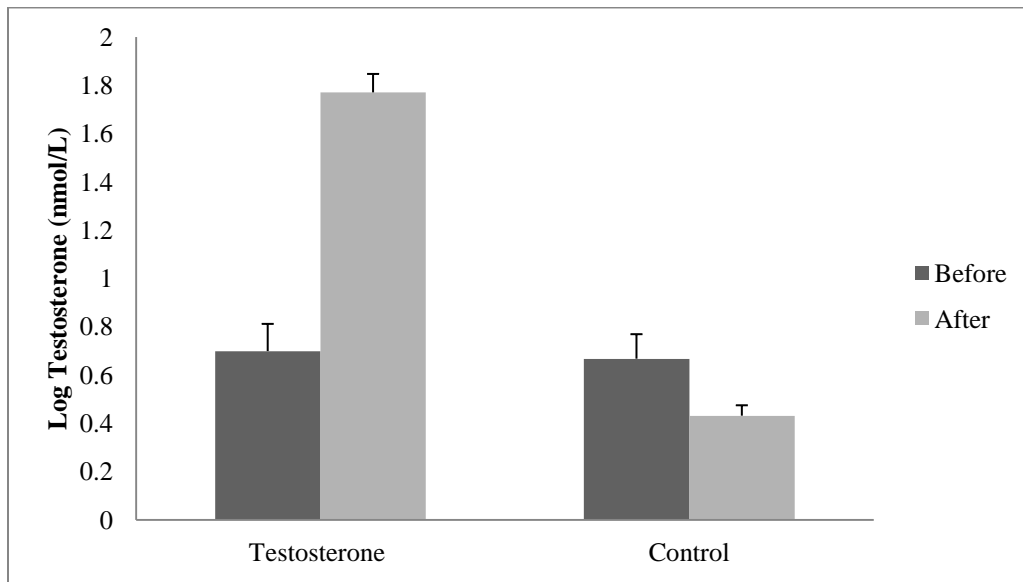


Figure 2.5. Testosterone levels between treated (testosterone implants; n= 7) and control (n= 9) male Cape ground squirrels.

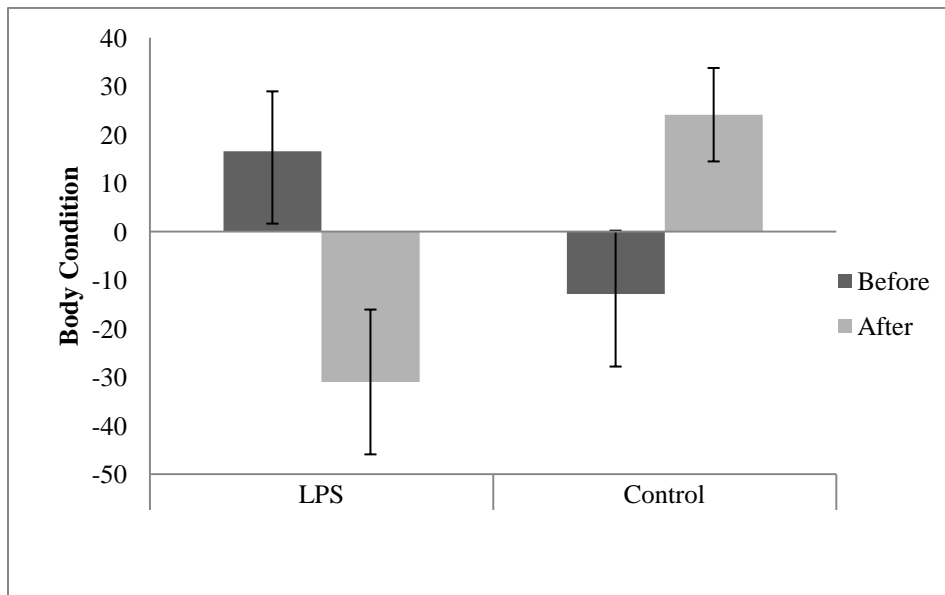


Figure 6.2. Initial and final body condition (residuals of spine length on body mass) of treated (LPS injections; n= 7) and control (n= 9) male Cape ground squirrels.

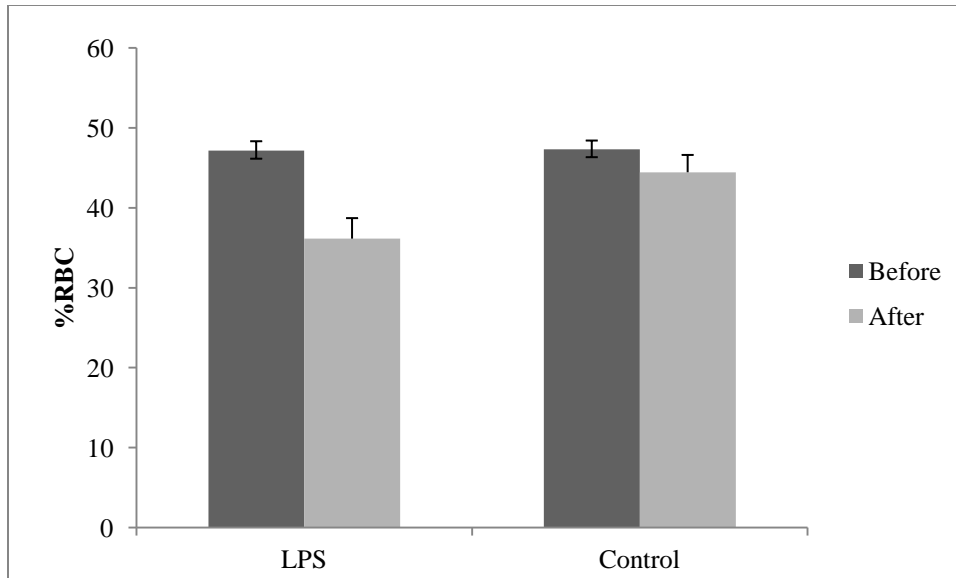


Figure 2.7. Hematocrits of treated (LPS injection; n= 7) and control (n= 9) male Cape ground squirrels.

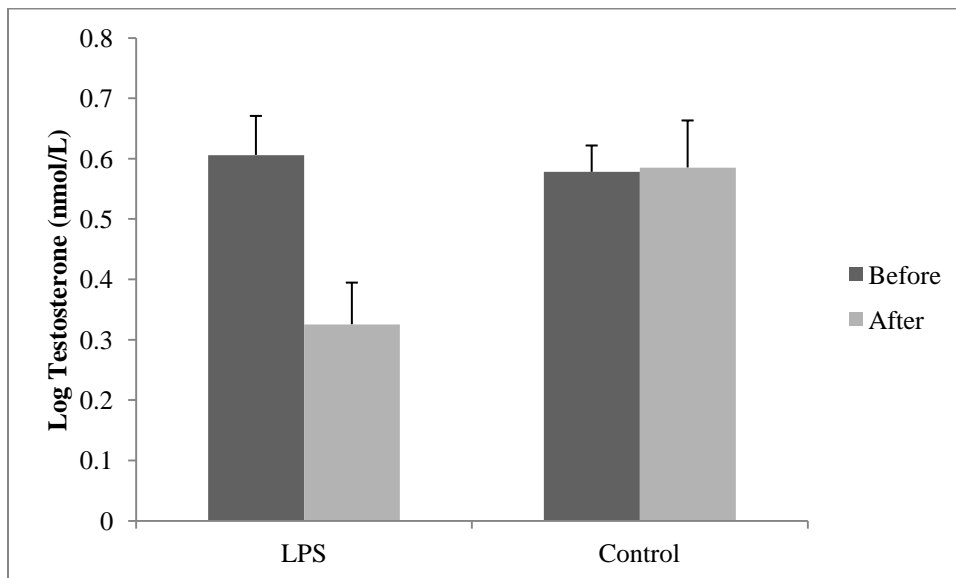


Figure 2.8. Testosterone levels between treated (LPS injection; n= 7) and control (n= 9) male Cape ground squirrels before and after treatment.

Chapter 3- Trade-offs between immunity and alternative reproductive tactics in male Cape ground squirrels: dispersed males suffer poorer body condition and higher ectoparasites than non-dispersers.

Abstract

Alternative reproductive tactics, the use of different reproductive behaviours between conspecifics of the same sex, present an opportunity to study the relationship between immunity, reproduction, growth and dispersal as they relate to the variable tactics within one species and sex. Male Cape ground squirrels (*Xerus inauris*) display two alternative tactics with some sexually mature males dispersing from their natal groups and joining bands of non-related males, while others do not disperse and remain in their natal groups past sexual maturation. Dispersed males have a higher resting metabolic rate, are more mobile, spend less time feeding, and have larger home ranges than non-dispersed males. Interestingly, there is no difference in reproductive investment or fitness between tactics, which allows us to test the trade-offs between alternative tactics and immunity when fitness is equal. We assessed differences in immunity, ectoparasites, condition, cortisol and testosterone to compare the trade-offs between tactics and immunity. We found that males who dispersed had higher total ectoparasite abundance, poorer condition and white blood cell differentials implying they are less healthy than non-dispersed males. There was no difference in cortisol or testosterone between tactics. Bacteria growth inhibition did not differ between tactics suggesting that both dispersed and non-dispersed males are equally resistant to parasites, but the lower condition indicates that dispersed males are less tolerant to parasites. Non-dispersed males also appear to benefit from increased allogrooming from females in their group. Dispersal causes increased costs to condition and parasite infection, but we suggest that the reproductive benefit of overlapping with more females may contribute to maintaining equal fitness between tactics.

Introduction

Eco-immunology approaches immunological questions by taking into consideration the host's physiology, behaviour, disease ecology, and environmental factors that contribute to an organism's ability to mount an immune response (Demas and Nelson, 2012; Norris and Evans, 2000). The driving force behind eco-immunology is the idea that immunity is costly, and an organism is forced to make trade-offs between immunity and other important life history traits like reproduction, growth and dispersal (Demas and Nelson, 2012; Lochmiller and Deerenberg, 2000; Norris and Evans, 2000). As both immunity and reproduction are important and energetically costly life-history traits, there has been considerable focus on the conflict between investing in behaviours to secure immediate reproductive benefits and investing in maintenance and immunity to increase potential for future reproductive benefits (Demas and Nelson, 2012; Lochmiller and Deerenberg, 2000; Norris and Evans, 2000; Stearns, 1992). Species that naturally show variation in reproductive behaviours and fitness provide interesting model systems in which to study trade-offs between behaviour and immunity in the wild (Demas and Nelson, 2012).

Alternative reproductive tactics describe when conspecifics of the same sex use different behaviours to achieve reproductive success (Taborsky et al., 2008). Studies addressing alternative tactics focus on variation in fitness as an ultimate cause leading to the evolution of alternative reproductive tactics (Gross, 1996), as well as the role of physiology and behaviour as the proximate causes (Scantlebury et al., 2008a; Taborsky and Brockmann, 2010; Taborsky et al., 2008). Alternative reproductive tactics have been characterized into three separate strategies: 1) Alternative strategies where tactics are genetically distinct from each other and have equal fitness, 2) Mixed strategies where tactics are genetically monomorphic and have equal average

fitness, and 3) Conditional strategies where tactics are genetically monomorphic and one tactic has increased fitness when compared to the other (Gross, 1996). Species displaying alternative reproductive tactics present an opportunity to study the relationship between immunity and reproduction, and growth and dispersal, as they relate to the variable tactics within one species and sex.

Species that display conditional or mixed strategies of alternative tactics provide the best models to study immunological trade-offs as individuals are able to display either or both tactics throughout their lifetime (Gross, 1996) allowing for immunological comparisons between genetically similar individuals. Previous studies examining differences in immunocompetence between alternative reproductive tactics have often focused on conditional strategies featuring dominant versus subordinate tactics or territorial versus satellite tactics (reviewed in Demas and Nelson, 2012 and Taborsky and Brockman, 2010). It has been suggested that dominant tactics offset the increased energetic and immunological costs associated with their tactic either proximately through access to high quality resources or ultimately through the increased fitness benefits associated with dominant tactics (Demas and Nelson, 2012). Differences in cortisol, testosterone and behaviour are proximate causes attributed to the variation in immunocompetence between tactics (Demas and Nelson, 2012; Ros et al., 2006). Mixed strategies are thought to be rare in wild populations (Gross, 1996), yet offer a unique opportunity to address how immunological differences between tactics contribute to the proximate causes that maintain alternative tactics when the tactic is not genetically determined and when fitness is equal.

Male Cape ground squirrels (*Xerus inauris*) are an ideal study organism to address eco-immunological questions about the trade-offs between immunity and alternative reproductive

tactics as sexually mature males either disperse from their natal social groups and join roving bands of non-related males, (hereafter: dispersed males) or they remain in their natal social groups past sexual maturation (hereafter: non-dispersed males) (Scantlebury et al., 2008a; Waterman, 1995, 1997). Non-dispersed and dispersed males do not differ in their reproductive investment, as there are no differences in testosterone levels or testes size (Scantlebury et al., 2008a). Male Cape ground squirrels, regardless of tactic, do not display dominance hierarchies (Manjerovic, 2010), are not territorial, and do not engage in male-male fighting for access to females (Waterman, 1997). In addition, there is no difference in their average fitness, suggesting that male Cape ground squirrels may follow a mixed strategy (Manjerovic and Waterman, 2015). Male Cape ground squirrels also differ from conditional strategies as there is no clear dominance hierarchy among males (Manjerovic, 2010) and they are not territorial (Waterman, 1997). Previous research suggests there are no differences in parasite abundances (endoparasites or ectoparasites) between tactics (Hillegass et al., 2008), but dispersed males have a higher resting metabolic rate, are more mobile, spend less time feeding (Scantlebury et al., 2008a), and have larger home ranges (Manjerovic and Waterman, 2015) than non-dispersed males. Scantlebury et al. (2008a) concluded that dispersed males may be incurring increased energetic costs associated with life, which may be indicative of having less energy available to allocate to maintenance and immunity as predicted by life history theory (Stearns, 1992).

The objective of this study is to examine the relationship between the immunity and alternative male tactics in Cape ground squirrels. If dispersal increases energetic costs and decreases the energy available to allocate to immunity, then males that display the dispersing tactic will present poorer body condition, higher parasite levels, and lower measures of immunocompetence than males that choose the non-dispersing tactic. As hormones such as

testosterone and cortisol often differ between tactics and affect the immunity of an organism (Demas and Nelson, 2012), we included estimates of these variables in our research design to attempt to understand the relationship between alternative tactics and immunity.

Methods

The Cape ground squirrel is a highly social species that occurs throughout arid regions of southern Africa (Waterman, 1995). Females live in small social groups composed of related individuals, approximately 1-6 adult females and up to 9 subadults of either sex (Hillegass et al., 2008; Waterman, 1995). Males reach sexual maturity at 8-10 months of age and either disperse to join non-kin "roving" bands or remain non-dispersed in their natal social group for up to five years (Manjerovic and Waterman, 2015; Waterman, 1995).

We trapped and handled male Cape ground squirrels on the S.A. Lombard Nature Reserve, a population that has been studied since 2002 (3660 ha, 18 km north west of Bloemhof, South Africa, 27°35'S, 25°23'E), between May-August 2012 and May-October 2013 (during the Austral winter) using Tomahawk traps (Tomahawk Live Trap Inc., WI; 15 X 15 X 50 cm) and cone-shaped handling bags (Koprowski, 2002). We permanently marked animals using PIT tags (AVID Inc. Folsom, LA; Hillegass et al., 2008). As well, animals were freeze marked for identification (Quick Freeze; Miller-Stephenson Product, Morton Grove, IL; Rood and Nellis 1980), and temporarily marked dorsally for observations using black hair dye (Rodol D; Lowenstein & Sons Inc., New York, NY). We determined the reproductive tactic of males based on observations made using 10X50 binoculars and 15-45X60 spotting scopes from hides on top of vehicles or observation towers as described by Scantlebury et al. (2008a). We also used radio telemetry to determine where and with whom they were sleeping. Males were classified as non-dispersed if they continued to sleep and associate with their natal group, and as dispersed males

if they were known to be sleeping alone, or only with other adult males. If we were unable to determine male status with confidence, they were excluded from the analysis.

We determined body condition by taking the residuals of the ordinary least squares regression between spine length, measured from the base of the skull to the base of the caudal vertebrae, and mass \pm 5.0 g using a spring scale (Pesola AG, Baar, Switzerland) (Manjerovic and Waterman, 2012; Schulte-hostedde et al., 2005). Ectoparasites were quantified by combing along the three planes of the back (left, center and right) from head to tail with a metal flea comb (as described in Hillegass et al. 2008) and by careful examination of the inner thighs and groin region. Ectoparasites were deposited directly into 70% ethanol for quantification and storage. We focused on ectoparasites (fleas and lice) as male Cape ground squirrels are predominately affected by ectoparasites and have low endoparasite loads (Hillegass et al., 2008). We examined the mean abundance of parasites across the entire sample, mean intensity (which represents the mean number of ectoparasites of infected individuals only) and prevalence which represents the proportion of hosts with parasites.

A blood sample (0.5-1ml) was collected from the femoral vein using a sterile 26 gauge needle and a 1ml syringe to quantify immunocompetence and circulating testosterone. Plasma, used in both hormone and immunocompetence assays, was separated from whole blood by centrifugation at 6000 rpm for 5 minutes (Spectrafuge mini, Labnet International USA) and frozen at -20°C until processed at the University of Pretoria.

White blood cell differentials (WBC) of each individual in both 2012 and 2013 were completed by technicians in the clinical pathology laboratory of the University of Pretoria by counting 100 cells on a single layer of whole blood smear stained with Diff-Quick (and

recording the number of lymphocytes, neutrophils, basophils, eosinophils and monocytes (Bachman, 2003)). Eosinophils and basophils occurred in very small proportions (on average $1.64 \pm 0.22\%$ and $0.88 \pm 0.16\%$, respectively) and were therefore excluded from the analysis. Neutrophils are part of the innate immune system which responds quickly to pathogens and increases in response to infection and inflammation (Beardsell and Howell, 1984). Lymphocytes are composed of T and B cells, which are important components of the adaptive immune system and increase in response to ectoparasites (Christe et al., 2002) and monocytes primarily respond to inflammation and infection (Davis et al., 2008; Falcone et al., 2001).

We used bacteria killing assays in 2013 to measure innate immune ability (Liebl and Martin, 2009; Matson et al., 2005; Merrill et al., 2014; Tieleman et al., 2005) as they measure complement and lysozyme activity by evaluating the plasma's ability to kill a known dilution of a novel bacteria (Millet et al., 2007). We followed the procedure outlined by Liebl and Martin (2009), preparing a working solution of 10^5 bacteria/ml of *E. coli* (Lyophilised E coli ATCC #8739, Microbiologics), and using 24.5 μ l of plasma to make a 1:4 dilution to test the plasma's ability to inhibit bacterial growth overnight. Each sample was tested in quadruplet, incubated in 96-well plates and absorbance was measured at 595 nm using a spectrophotometer (Multiskan ascent, Thermo LabSystems). There were two blanks in quadruplet (growth medium only) and two controls in quadruplet (bacteria and growth medium only) on each plate. We calculated the percent of bacterial growth inhibited by comparing the absorbance of the samples and the absorbance of the controls.

To measure circulating testosterone we used a commercially available coated tube assay kit (Coat-a-Count TKTT1, Diagnostic Products Corporation, Los Angeles, CA) as previously described (Scantlebury et al., 2008 and Chapter 2). The assay detectable range was 10 to 1600

nmol/L testosterone and cross reactivity of the Coat-a-Count testosterone antibody was 16% with 11-ketotestosterone, < 5% with dihydrotestosterone and 19-hydroxyandrostenedione, and less than 1% with other steroids tested according to the manufacturer. Serial dilution of Cape ground squirrel plasma demonstrated good parallelism with the testosterone standard curve (data not shown). The intra-assay coefficient of variation was 8.4% and the inter-assay coefficient was 12%.

Plasma cortisol is difficult to measure in wild animals as cortisol levels spike quickly due to the stress of capture and handling (Romero and Reed, 2005) and the blood sample must be taken within the first three minutes of handling to get an accurate measure of baseline cortisol (Scantlebury et al., 2008a). To avoid the confounding acute stress response of trapping and handling we measured cortisol metabolites from feces. Fecal samples represent cortisol levels from approximately 10 – 24 h before collection (Dantzer et al., 2010; Harper and Austad, 2000; Palme, 2005; Touma et al., 2003) and provide a reliable short term estimate of stress (Dantzer et al., 2010; Keay et al., 2006; Sheriff et al., 2010). We extracted fecal cortisol following the protocol outlined in Mateo and Cavigelli (2005); 0.2 g dried feces with 1.5ml of 95% ethanol. Feces were collected from under the traps (9h30-15h30) as the squirrels frequently defecated while in traps (Pettitt et al., 2007). We estimated concentrations of cortisol (ng/ml) using a radioimmunoassay technique described in Ryan et al. (2011). Cortisol antibody dilutions of 1:6400 were used and had a cross-reactivity of 5.7% for 11-deoxycortisol, 3.3% for corticosterone, 36% for prednisolone, and < 0.7% for cortisone according to the manufacturer. Serial dilution of Cape ground squirrel fecal cortisol demonstrated good parallelism with the cortisol standard curve. Inter-assay and intra-assay coefficients of variation were 15.4% and 5.1%, respectively.

Statistics

To assess the effect of male reproductive tactic and sampling year on ectoparasite abundance and intensity we used a generalized linear mixed model (GLMM) with a Poisson distribution. To assess body condition, immunocompetence measurements, testosterone and cortisol we used linear mixed models (LMM) using Gaussian distributions. To determine if status had an effect on each response variable we performed a type 3 ANOVA (R-*Package lme4* version 1.1-7) and reported the Wald's Chi-square value. We included individual tag number as a random factor in all analyses to account for repeated measures across the two sampling years, and we also controlled for year in the models and found there were no significant interactions between year and male status. Testosterone and cortisol levels were log-transformed to satisfy normality, while %RBC and all WBC proportions were arcsine transformed to satisfy normality. Because bacteria growth inhibition was only measured in 2013, a Wilcoxon rank sum test was used to compare the differences between male reproductive tactics as the data were non-normal. Statistical analysis was done using R (version 3.1.1). We compared prevalence of parasites between tactics with an exact unconditional test using Quantitative Parasitology version 3.0 (Rózsa et al., 2000).

Results

We measured 34 adult males (15 non-dispersed and 19 dispersed) in 2012 and 36 adult males (16 non-dispersed and 20 dispersed) in 2013. As Cape ground squirrels are a long-lived species, some of the individuals were sampled in both 2012 and 2013. Over the two years of the study we handled 56 unique individuals, with only two individuals changing tactics, becoming dispersed between 2012 and 2013.

There was no difference in body mass between non-dispersed (713.47 ± 7.94 g) and dispersed males (693.08 ± 7.62 g; $X^2 = 1.85$, $df = 1, 65$, $p = 0.17$), or average spine length between non-dispersed (18.43 ± 0.12 cm) and dispersed males (18.42 ± 0.11 cm; $X^2 = 0.003$, $df = 1, 64$, $p = 0.98$). Dispersed males were in worse body condition (-9.16 ± 6.97) than non-dispersed males (11.23 ± 7.29 ; $X^2 = 3.85$, $df = 1, 64$, $p = 0.049$; Figure 3.1). Dispersed males had a higher prevalence of fleas (97.4%) than non-dispersed males (74.2%; unconditional test, $p = 0.004$). Similarly dispersed males had higher prevalence of lice (79.5%) than non-dispersed males (45.2%; $p = 0.003$). The mean abundance of ectoparasites (total ectoparasites: fleas and lice) was higher in dispersed males (Table 3.1; Figure 3.2). The mean intensity of lice was higher in dispersed males and there was no difference in flea intensity between tactics (Table 3.1).

There was no difference in the plasma testosterone concentration (nmol/L) between alternative male tactics in Cape ground squirrels (non-dispersed; 4.97 ± 1.07 nmol/L, dispersed; 4.59 ± 0.779 nmol/L; $X^2 = 0.993$, $df = 1, 65$, $p = 0.813$). Similarly, dispersed (0.98 ± 0.079 ng/g) and non-dispersed (1.08 ± 0.088 ng/g) males did not differ in fecal cortisol levels ($X^2 = 0.99$, $df = 1, 62$, $p = 0.319$).

We found significant differences in the WBC differential between male tactics. Dispersed males had significantly lower proportions of lymphocytes (dispersed; $38.3 \pm 0.94\%$, non-dispersed; $54.6 \pm 3.07\%$; $X^2 = 16.7$, $df = 1, 65$, $p < 0.0001$), higher proportion of neutrophils (dispersed; $51.1 \pm 2.95\%$, non-dispersed; $32.87 \pm 3.22\%$; $X^2 = 16.5$, $df = 1, 65$, $p < 0.0001$) and lower proportion of monocytes (dispersed; $7.74 \pm 0.94\%$, non-dispersed; 10.39 ± 1.11 ; $X^2 = 4.67$, $df = 1, 65$, $p = 0.03$) than non-dispersed males (Figure 3.3). There was no difference between male tactics for bacteria killing ability (dispersed; $38.5 \pm 6.6\%$, non-dispersed; $41.9 \pm 3.3\%$; $W_{28,48} = 212$, $p = 0.10$).

Discussion

The poor body condition, higher ectoparasite abundance and differences in WBC differential were all indicative of dispersed males displaying a worse body condition and immune status than non-dispersed males, which supports the hypothesis that dispersal affects the immune status of male Cape ground squirrels. The poorer body condition of dispersed males was consistent with findings that they have higher resting metabolic rate than non-dispersed males (Scantlebury et al., 2008a), which is indicative that they are metabolising energy at a higher rate, and do not have additional energy to store (Demas and Nelson, 2012). Dispersed males may also have less energy available as they spend less time feeding than non-dispersed males and they engage in more energetically expensive behaviours such as having larger home ranges and spending more time moving (Scantlebury et al., 2008a). Interestingly, Manjerovic and Waterman (2015) found that dispersed males were in better body condition (smaller data set), which may be a reflection that body condition, along with other factors that influence alternative reproductive tactics, may not be fixed, but instead change from year to year (Schradin and Lindholm, 2011). In species displaying alternative reproductive tactics, better body condition is most commonly attributed to the tactic with the best fitness payoff, while individuals with poorer condition are forced to make the "best of a bad job" and are less reproductively successful (Taborsky and Brockmann, 2010). In anurans (*Bufo woodhousii* and *Bufo cognatus*) the males that followed the "calling" tactic resulted in the highest fitness and were in better body condition than the satellite males who were in poorer body condition and not able to meet the energetic costs of calling (Leary et al., 2005). Similarly, in African striped mice (*Rhabdomys pumilio*), males in the best condition become territorial males and receive highest fitness, while lower quality males become either roamers or remain philopatric (Schradin and Lindholm, 2011). While dispersed and non-dispersed male Cape ground squirrels do not differ in their fitness (Manjerovic and Waterman,

2015), body condition may have important implications for their survivorship and immune abilities. Hares (*Lepus granatensis*) in poor body condition are less likely to escape a predator than individuals in better condition (Alzaga et al., 2007), and individuals in poorer body condition likely have fewer resources available to mount costly immune defenses to protect against parasites and infections (Demas and Nelson, 2012; Sheldon and Verhulst, 1996). A study on Sundevall's jird (*Meriones crassus*) showed that a host may only suffer negative effects from parasitism when in poor condition and not able to access additional energy to offset the costs of the parasites (Hawlena et al., 2008).

Dispersed male Cape ground squirrel males had higher ectoparasite abundances (lice and fleas) than non-dispersed males; possibly the higher parasite abundance was the result of their poorer body condition leaving them unable to mount a strong immune response to resist ectoparasites (Sheldon and Verhulst, 1996). Alternatively, the higher parasite abundances may be contributing to the poorer body condition, similar to the findings of female Columbian ground squirrels (*Urocitellus columbianus*; Neuhaus, 2003). Although interesting to note that a recent study by Raveh et al. (2015) found that ectoparasites had no effect on body condition or reproductive success in female Colombian ground squirrels. Dispersed males may also have higher ectoparasite abundances as a consequence of their larger home ranges overlapping with more conspecifics and increasing the exposure to ectoparasites compared to non-dispersed males, with their smaller home ranges (Manjerovic and Waterman, 2015; Moore and Wilson, 2002). Non-dispersed males may also have lower parasite abundances due to their better body condition and more energy available to fight off infestation (Hawlena et al., 2008; Sheldon and Verhulst, 1996). Non-dispersed males are also very likely benefiting from staying in their natal group. Hillegass et al. (2008) found that adult females spend significantly more time allogrooming than

any other sex or age class, particularly more than adult males in their all-male bands. As non-dispersed and dispersed males do not differ in the amount of time spent autogrooming (Hillegass et al., 2008), the lower ectoparasite abundances on non-dispersed males may reflect the increased allogrooming received from the females within its natal group. Grooming has been suggested to be more important than the immune response for controlling ectoparasites as Hawlena et al. (2007) found that Sundevall's jird does not mount an immune response to flea infestation and rely solely on grooming to resist fleas.

Increased parasite loads can significantly affect survival, growth and fitness of the host (Agnew et al., 2000; Hawlena et al., 2006; Watson, 2013). Parasites, by definition, impose some cost on their host by consuming the host's resources (Combes, 2001) and parasites have significant negative effects on the reproductive success of female Cape ground squirrels (Hillegass et al., 2008). The increased parasites of dispersed males could possibly be affecting their reproductive success as it has been shown that female mice preferentially mate with non-parasitized males (Ehman and Scott, 2002). A meta-analysis by Moore and Wilson (2002) showed a positive correlation between parasites on male mammals and male mortality, suggesting that the increased parasite abundances of dispersed males could have negative impacts on their survivorship and that there is an increased cost of dispersing early when compared to males that delay dispersal. Parasites do not always have the expected negative effects on host survival and fitness and an additional stressor, such as a food shortage or contaminants, can be required for parasites to affect the hosts negatively (Marcogliese and Pietrock, 2011). Sundevall's jird only show negative effects from parasitism when paired with worse body condition and the negative effects were lost if they had a surplus of energy or access to resources (Hawlena et al., 2008). Similarly, the poorer body condition of dispersed Cape

ground squirrel males could be acting as an additional stressor that may amplify the negative effects of parasites.

Dispersed and non-dispersed male Cape ground squirrels displayed distinctly different WBC profiles. Interpretation of WBC differentials has varied widely in the past and it is important to understand that they are not measures of an organisms immune ability but instead a snapshot of its current immune status, either healthy or fighting an infection (Davis et al., 2008). Dispersed males presented a high proportion of neutrophils and a lower proportion of lymphocytes relative to non-dispersed males, which is indicative of an infection (Davis et al., 2008). In response to an infection, neutrophils increase while lymphocytes are directed out of the blood stream to target tissues (Davis et al., 2008), resulting in a high neutrophil to low lymphocyte pattern. Such a pattern is associated with infection in house finches (*Carpodacus mexicanus*; Davis et al., 2004), and chickens (*Gallus gallus domesticus*; Al-Murrani et al., 2002). Similarly, the higher proportion of monocytes displayed by dispersed Cape ground squirrel males is indicative of a response to inflammation and infection (Davis et al., 2008; Falcone et al., 2001). The WBC differential of dispersed males is an indication that they are responding to an infection and is consistent with their higher parasite abundances.

Despite the differences in body condition, parasite abundance, and WBC differential between the tactics, the results from our bacteria growth inhibition assays indicated that the innate immune systems of both non-dispersed and dispersed males were equally capable of fighting off novel pathogens. Host defense against parasites/pathogens has been conceptualized into two different strategies; resistance and tolerance (Råberg et al., 2009). Resistance occurs when the host's immune system directly targets parasites and pathogens to limit their population growth and/or to eliminate them all together (Bordes et al., 2012; Råberg et al., 2009). However,

a tolerance strategy does not reduce parasite and pathogen loads, but instead attempts to limit the damage incurred by the host (Bordes et al., 2012; Råberg et al., 2009). As immunity measures, such as bacteria growth inhibition, are often used as measurements of resistance, our results indicate that both tactics are equally resistant to parasites (Bordes et al., 2012). Tolerance is commonly measured as body condition, where better body condition is associated with better tolerance (Bordes et al., 2012) and our results suggest that dispersed males may be less tolerant to ectoparasites than non-dispersed males. Due to the increased energetic demands of dispersal, males that follow that tactic may be less able to, or less willing to tolerate ectoparasites, leaving them more vulnerable to environmental stressors than non-dispersed males (Marcogliese and Pietrock, 2011).

Testosterone is considered a proximate mechanism affecting immunity through a trade-off between the positive effects of increased testosterone on a male's reproductive potential, via increased sperm production as well as secondary sexual characteristics such as sexual displays, and the negative effects of a suppressed immune system (Folstad and Karter, 1992). Folstad and Karter (1992) initially proposed this trade off as the mechanism behind "honest" signaling of male quality to females. Alternative reproductive tactics often differ in their testosterone levels as a result of differences in aggression and territoriality between dominant and subordinate tactics (Taborsky et al., 2008). The alternative male tactics of Cape ground squirrels showed no difference in their circulating testosterone (this study), consistent with Scantlebury et al. (2008a) and neither male tactic displays aggression. The immune suppression hypothesis suggests that individuals that are having their immune system challenged by an infection or parasites should be displaying lower levels of testosterone (Chapter 2). Despite facing higher infections, dispersed males do not differ in their testosterone levels (this study) or relative scrotal size (Scantlebury et

al., 2008a) compared to non-dispersed males. Similar to the dispersed males in this study, Zhang and He (2014) found that experimentally increasing parasite loads had no effect on testosterone levels in mice. It is possible that ectoparasites may not trigger a strong immune response, as long-lived parasites have been shown to down-regulate a host's immune response to protect themselves (arthropods; Wikel, 1999; helminths; Maizels and Yazdanbakhsh, 2003). This down-regulation of the host's immune system by macroparasites would not only facilitate survival of the parasite but also prevent the immunological suppression of testosterone.

The trade-off between immunity and testosterone as described by the immune suppression hypothesis (Chapter 2) may be mediated through differential allocation of resources (Rolf, 2002). A review by Agnew et al. (2000) found that across several species there was evidence that when faced with a chronic infection, like parasites, hosts preferentially allocate resources toward reproduction. There is evidence in rodents of investment in larger testes being correlated with greater species richness of parasites (Bordes et al., 2011, 2012). Male Cape ground squirrels may preferentially allocate resources to reproduction despite the costs to their body condition and parasite abundances. Traditionally pre-copulatory male-male competition for females such as dominance hierarchies, territoriality, and male-male aggression (Wong and Candolin, 2005) give advantages to males in good body condition (Schradin and Lindholm, 2011). As an example, Richardson's ground squirrels (*Urocitellus richardsonii*) invest heavily in pre-copulatory behaviours to access females as evidenced by their high aggression and intense fighting during breeding season (Michener, 1983). Male Cape ground squirrels present an interesting variation of this norm as they do not display dominance hierarchies in this population (Manjerovic, 2010), are not territorial, and do not engage in male-male fighting for access to females (Waterman, 1997). However, there is evidence that they invest heavily in post-

copulatory competition via sperm competition, as indicated by their large testes (Manjerovic and Waterman, 2015; Manjerovic et al., 2008). It may be that dispersed males continue to allocate resources toward reproduction and sperm production as well as immunity, at a cost to their body condition, to minimize the ultimate fitness costs of reduced reproductive investment.

There were no differences in fecal cortisol between tactics. Cortisol has often been considered a proximate mechanism affecting immunity (Demas and Nelson, 2012), as acute cortisol can enhance immunocompetence, through redirection of energy and resources, while chronically high cortisol levels have been shown to be immunosuppressive (Demas and Nelson, 2012; Martin, 2009). Our cortisol results are inconsistent with Scantlebury et al. (2008a and 2008b) who found that non-dispersed males had significantly higher plasma cortisol than dispersed males. Scantlebury et al. (2008b) attributed the increased cortisol in non-dispersed males to higher levels of aggressive behaviours from females. Variation in group size and composition year to year could explain the difference seen in cortisol levels between this study and Scantlebury et al. (2008a and 2008b). Our cortisol levels were measured from feces, which estimates the average cortisol over 10-24 hours (Dantzer et al., 2010; Harper and Austad, 2000; Palme, 2005; Touma et al., 2003), while plasma cortisol used in Scantlebury et al. (2008a and 2008b) is a snapshot of the immediate cortisol levels. It is possible that using a longer term estimate of cortisol averages out acute cortisol spikes from aggressive encounters. Our testosterone and cortisol results indicate there are other proximate mechanisms, such as allogrooming and energy allocation discussed above, affecting the differences in immune status observed in the alternative male tactics of the Cape ground squirrels.

As male Cape ground squirrels tactics do not differ in their reproductive investment as estimated by testes size and testosterone (Scantlebury et al., 2008a; this study) or reproductive

fitness (Manjerovic and Waterman, 2015), they provide us with a unique opportunity to study the relationship between immunity and alternative reproductive tactics in a species with a mixed reproductive strategy. In conditional strategies, it is often thought that the benefits of increased fitness gained by the dominant tactic offset the energetic and immunological costs associated with the dominant tactics (Demas and Nelson, 2012; Scantlebury et al., 2008a). Our results suggest that even when reproductive investment and fitness are equal, alternative male tactics result in differences in immune status and body condition that may reflect differences in the energetic demands of dispersal and social benefits of remaining in their natal group. We also demonstrated that dispersed males incur a lot of costs associated with dispersal which throws into question why males would disperse at all. Dispersed males are able to encounter a larger number of potential mates as they have larger home ranges than non-dispersed males (Scantlebury et al., 2008a), which may offset the cost of dispersal and maintain equal fitness between tactics. As non-dispersed males received increased aggressive behaviours from females (Scantlebury et al., 2008b) and the presence of non-dispersed males contributes to the reproductive suppression of subadult females (Pettitt and Waterman, 2011) it is possible that males are eventually forced out of social groups and do not disperse. Future studies looking at the social group composition when males disperse could help clarify when and why males become dispersed.

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Figures and Tables

Table 3.1. Mean abundance and mean intensity of ectoparasites (fleas and lice) of dispersed and non-dispersed male Cape ground squirrel. GLMM with a Poisson distribution. Reported as means \pm SE.

		Non-dispersed	Dispersed	χ^2	Df	p-value
Mean Abundance	Fleas	2.94 \pm 0.868	6.28 \pm 0.514	7.07	1, 66	0.007
	Lice	1.35 \pm 0.519	3.59 \pm 0.395	4.26	1, 66	0.039
Mean Intensity	Fleas	3.95 \pm 0.55	6.44 \pm 0.875	2.23	1, 57	0.135
	Lice	3.00 \pm 0.646	4.52 \pm 0.539	6476.1	1, 41	<0.001

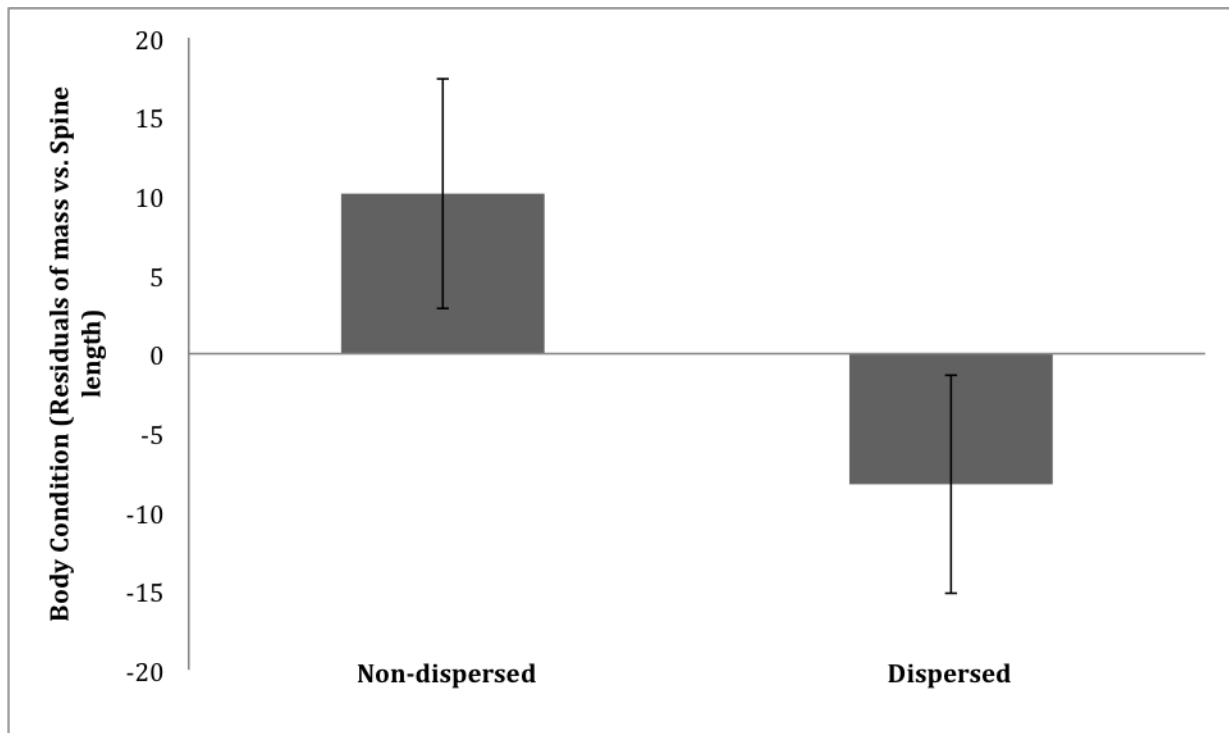


Figure 3.1. Body condition estimates (\pm SE) of dispersed (n= 39) and non-dispersed male (n= 31) Cape ground squirrels, calculated as the residuals of body mass (g) versus spine length (cm). Positive values indicate better body condition.

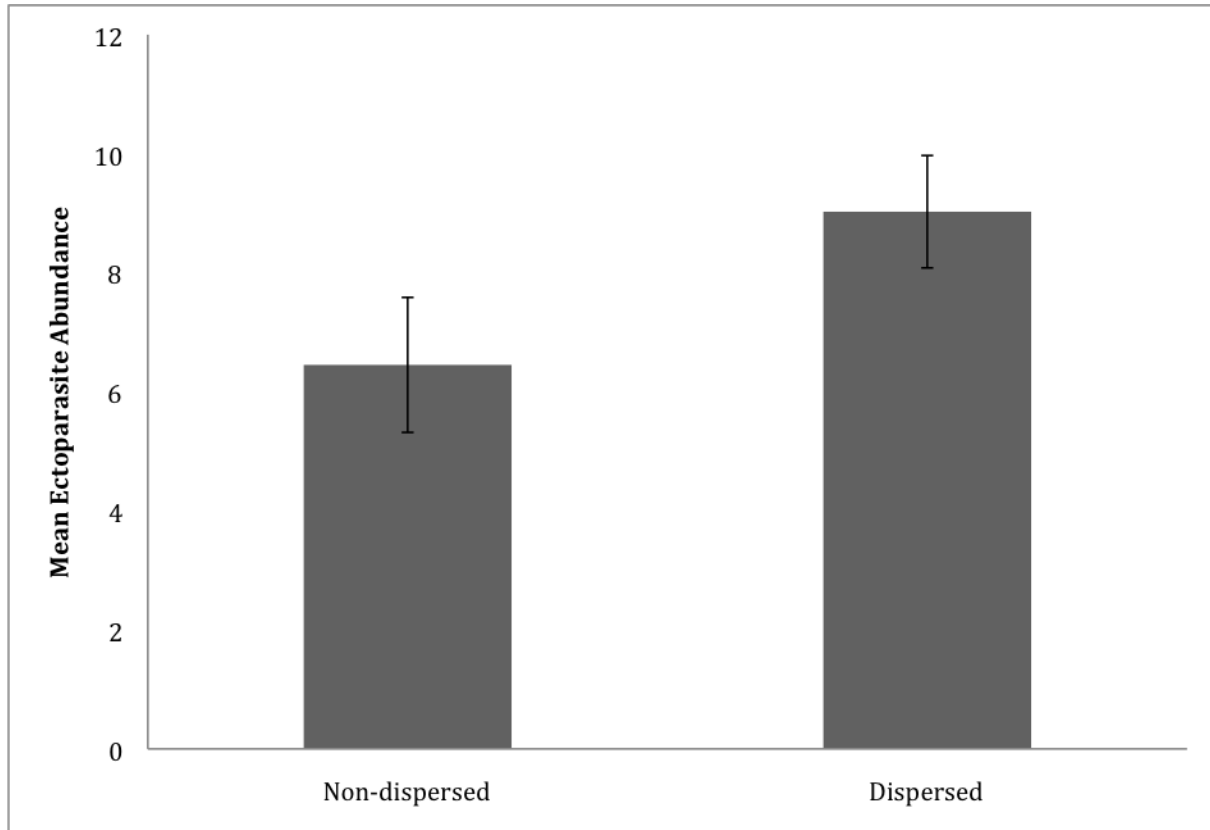


Figure 3.2. Mean total ectoparasite abundance of dispersed (n= 38) and non-dispersed male Cape ground squirrels (n= 30). $\chi^2= 17.6$, $df= 1,66$, $p < 0.0001$. Displayed are means \pm SE.

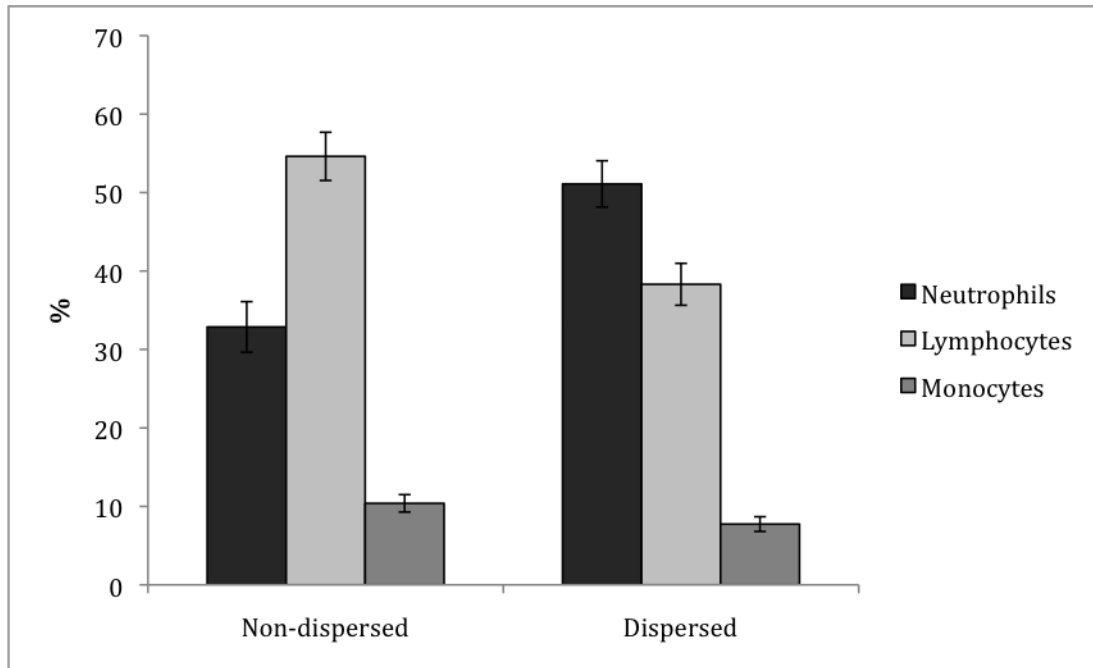


Figure 3.3. WBC differentials of dispersed (n= 39) and non-dispersed (n= 31) male Cape ground squirrels. Displayed are means \pm SE.

Chapter 4- Thesis Conclusions

We found strong support for the immune suppression hypothesis (ISH) in male Cape ground squirrels as testosterone levels were negatively affected by an immune challenge. In contrast to our predictions, we found no support for the immunocompetence handicap hypothesis (ICHH) as elevated testosterone levels had no effect on body condition, ectoparasite or immunity estimates. Similarly, the increased testosterone levels had no effect on cortisol levels, failing to support the stress-linked ICHH. In recent years, the ICHH has received only weak support across taxa (Roberts et al., 2004), and our results add to the growing body of literature that challenges the widely accepted ICHH. This is particularly important as the ICHH is often maintained as a common explanation for sex-biased parasitism and may not truly be representing the mechanisms that contribute to the observed relationship between testosterone and immunity. Our results instead agree with the increasing number of studies that support the ISH (Boonekamp et al., 2008; Lutermann et al., 2012). To my knowledge, my thesis is the first to test both the ICHH and ISH in the same species and adds to our understanding of one of the mechanisms that mediates the trade-offs between reproduction and immunity.

My thesis also addressed how differences in reproductive behaviour can affect the immunity of an individual. Interestingly, the alternative reproductive tactics of the male Cape ground squirrels do not differ in their fitness or testosterone levels (Manjerovic and Waterman, 2015; Scantlebury et al., 2008a; this study) which allowed us to examine how alternative reproductive tactics affect immunity when reproductive success is equal. Dispersed males had significantly poorer body condition, higher ectoparasite abundances and lower immune status which all supported our hypothesis that dispersal affects the immune status of male Cape ground squirrels. As dispersed males have higher resting metabolic rates, and engage in more

energetically costly behaviours such as larger home ranges and more time spent moving (Scantlebury et al., 2008a), we concluded that there is a trade-off between the increased energetic demands of dispersal and immunity in males. Dispersed and non-dispersed males did not differ in their estimates of innate immune ability and non-dispersed males likely benefit from receiving increased allogrooming from females in their natal group (Hillegass et al., 2008). In contrast to the increased energetic and immunity costs, dispersed males encounter a larger number of potential mates as they have larger home ranges than non-dispersed males (Manjerovic and Waterman, 2015), which may be able to offset the cost of dispersal and maintain equal fitness between tactics. Non-dispersed males may also receive inclusive fitness benefits by giving alloparental care in their natal groups (Pettitt and Waterman, 2011). Our results from Chapter 3 highlight the costs associated with dispersal and pose the question, why would males ever disperse? It is possible that non-dispersed males do not choose to become dispersed but that they are eventually forced out of social groups as they receive increased aggression from females (Scantlebury et al., 2008b), possibly because their presence contributes to the reproductive suppression of subadult females (Pettitt and Waterman, 2011). Future studies looking at how social group composition, kinship and female aggression affect the timing of male dispersal could help answer the question as to when and why males become dispersed.

Although we found strong support for the ISH in Chapter 2 that a strong immune challenge decreased testosterone levels, in Chapter 3, dispersed males had higher parasite abundances but no difference in their testosterone levels when compared to non-dispersed males. This result is in conflict with the prediction of the ISH, but it is interesting to consider that parasites may not trigger a strong immune reaction as parasites have been shown to down-regulate the host immune response to maximize their own fitness (Maizels and Yazdanbakhsh,

2003; Wikel, 1999). This down-regulation of the host immune system by parasites could prevent the immune suppression of testosterone and experimentally raising ectoparasites in mice has failed to have any effect on the testosterone levels (Zhang and He, 2014). It would be interesting if future studies looked at the level of parasitism required to trigger a strong immune response that could in turn suppress testosterone. Future research should also look at the differences of endoparasites and ectoparasites on testosterone suppression. Our manipulation outlined in chapter two triggered an acute and strong immune response. It would be interesting to compare and contrast the effects of an acute immune challenge versus a chronic immune challenge on testosterone levels. It is possible that a chronic infection will also suppress the testosterone levels as observed with an acute immune challenge but it is also possible that when faced with a chronic infection, males would make life history trade-offs to prioritize reproduction (Agnew et al., 2000) and we may see an increase in testosterone and reproductive behaviours instead of suppression.

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Appendix I

Calculating tube length of testosterone silastic implants

From our preliminary testosterone samples we had found that the average testosterone level of male Cape ground squirrels (CGS) was 1.8ng/ml. We aimed to raise the testosterone levels by two standard deviations, to 5.7ng/ml, as suggested by Grear et al. (2009). The testosterone implants were implanted subcutaneously in the dorsal region using PIT-tag injectors (AVID USA), a technique already used to implant PIT-tags in all the squirrels. Silastic tubing with a 1.47mm inner diameter, 1.96mm outer diameter was selected to fit neatly inside the PIT-tag injectors. This size of tubing is commonly used for silastic implants in mammals (Grear et al., 2009; Jechura et al., 2003; Limonta et al., 1986). Implants were constructed by sealing one end of the tubing with the silicone adhesive, autoclaving the half sealed tubing, filling the tube with testosterone (Sigma T-1500) and sealing the remaining end. Implants were loaded immediately in sterilized PIT-tag injectors and kept frozen until use.

We extrapolated implant length from previous studies of mammals (rabbits, rats and degus) that used the same diameter implants to artificially increase testosterone for approximately 30 days (Jechura et al., 2003; Limonta et al., 1986; Moger, 1976). We calculated the length of our implants (X) using the equation below. Bold terms are values used from previous studies.

$$X = \text{Length of implant} * \frac{\text{Mass of CGS}}{\text{Mass of study mammal}} * \frac{\text{Desired increase}}{\text{Observed increase}}$$

Examples:

Moger 1976

2 cm implants in Sprague-Drawly rats (*Rattus norvegicus*) with a mass of 82g

Observed testosterone increase was 0.43 to 3.96ng/ml = 9.21x increase

Cape ground squirrels = 700g

Desired testosterone increase was 1.8 to 5.2 ng/ml= 3.17x increase

$$X = (2\text{cm})(700\text{g}/82\text{g})(3.17/9.21)$$

$$X = 5.88 \text{ cm}$$

Limonta et al. 1986

8cm implants in New Zealand white rabbits (*Oryctolagus cuniculus*) with a mass of 2800g

Observed testosterone increase was 5 to 8 ng/ml = 1.6x increase

Cape ground squirrels = 700g

Desired testosterone increase was 3.17x

$$X = (8\text{cm})(700\text{g}/2800\text{g})(3.17/1.6)$$

$$X = 3.96$$

Jechura et al. 2003

10mm implants in *Octodon degus* with a mass of 230g

Observed testosterone increase was 2x

Cape ground squirrels = 700g

Desired testosterone increase was 3.17x

$$X = (10\text{mm})(700\text{g}/230\text{g})(3.17/2)$$

$$X = 48.2 \text{ mm} = 4.82\text{cm}$$

From these calculations we concluded that to increase testosterone levels of male Cape ground squirrels from 1.8ng/ml to 5.2ng/ml over the course of 30 days we needed implants that were approximately 4 to 6 cm in length. We implanted two 2.5cm implants and observed an average testosterone increase of 1.51ng/ml to 4.98ng/ml.

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