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RESEARCH ARTICLE

Trade-offs between immunity and testosterone in male African ground squirrels

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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. However, support for the ICHH has been variable and an alternative hypothesis suggests that testosterone may be acting indirectly via cortisol to suppress immunity (the stress-linked ICHH). A third hypothesis is that increased energetic investment in immunity results in the suppression of testosterone. We tested these hypotheses in male Cape ground squirrels (*Xerus inauris*) through two separate manipulations: first, by triggering a strong immune response using a lipopolysaccharide (LPS) injection and, secondly, by increasing circulating testosterone using silastic testosterone implants. Responding to an immune challenge significantly reduced testosterone, supporting the immune suppression hypothesis, while increasing circulating testosterone had no effect on immunocompetence, body mass, ectoparasite abundances or cortisol levels, failing to support either the ICHH or stress-linked ICHH. Our results add to the increasing body of literature that challenges the 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 free-ranging mammal gives us a unique opportunity to examine the mechanisms mediating this trade-off.

KEY WORDS: Immunocompetence handicap hypothesis, *Xerus inauris*, Cortisol, Stress-linked ICHH, LPS, Lipopolysaccharide

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 sex bias in immunity has been observed, with females generally being more immunocompetent, having longer lifespans 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' 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. 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). A recent meta-analysis by Foo et al. (2017) concluded that, in experimental studies, testosterone had a medium immunosuppressive effect across species. This conclusion differs from an earlier meta-analysis by Roberts et al. (2004) on experimental studies of the ICHH that found only weak support across taxa and no support for this hypothesis in mammals. The Foo et al. (2017) meta-analysis also found that correlational studies failed to show any effect of testosterone on immunity, which highlights the need for more manipulative studies.

An alternative hypothesis to the ICHH is that it is the immune response that 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, this hypothesis has received far less attention and offers an alternative and important mechanism on how the trade-off between reproduction and immunity is modulated. Lutermann 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 immune challenge through the subcutaneous injection of a lipopolysaccharide (LPS) to stimulate the innate immune system, 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), short-tailed fruit bats (*Carollia perspicillata*; Greiner et al., 2010) and house sparrows (*Passer domesticus*; Needham et al., 2017). The direction of this effect, of increased immunity causing a decline in testosterone, could be the result of specific chemicals suppressing testosterone or the energetic costs of mounting an immune response may leave less energy to maintain high levels of testosterone (resource allocation constraints; Burness et al., 2010; Demas and Nelson, 2012).

Another alternative explanation for the trade-off is that increased testosterone may not be directly interacting with the immune

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system. Increased testosterone increases 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 (*P. domesticus*) but, after they controlled for the effect of increased corticosterone, they found that increased testosterone actually had an immune-enhancing 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 immune suppression hypothesis (ISH) together will provide important insight into the mechanism that regulates the observed trade-offs between reproduction and immunity.

Male Cape ground squirrels [*Xerus inauris* (Zimmerman 1780)] 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 are 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 Cape ground squirrel male investment in reproduction and their immune response because spleen size, an indirect estimate of immunity, decreases with increasing testes size (Manjerovic and Waterman, 2012). Ectoparasite 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 that testosterone and investment in reproductive behaviours 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 artificially raised testosterone levels will be related to larger testes, higher parasite abundance and lower measures 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 (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.

MATERIALS AND METHODS

We studied male Cape ground squirrels on the S.A. Lombard Nature Reserve (3660 ha, 18 km northwest 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, USA; 15×15×50 cm) and handled using a cone-shaped handling bag (Koprowski, 2002). We identified animals with passive integrated transponder (PIT) tags (AVID Inc., Folsom, LA, USA; Hillegass et al., 2008) and freeze marks for permanent identification (Quick Freeze, Miller-Stephenson Product, Morton Grove, IL, USA; Rood and Nellis, 1980). We also temporarily marked squirrels dorsally using black hair dye for identification at a distance (Melchior and Iwen, 1965; Rodol D, Lowenstein & Sons Inc., New York, NY, USA).

We recorded body mass (±5.0 g) using a spring scale (Pesola AG, Baar, Switzerland). 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 ticks are rarely found) as well as prevalence, which represents the proportion of hosts with parasites.

To quantify immunocompetence and measure circulating testosterone, we collected approximately 1 ml of blood from the femoral vein using a sterile 26-gauge needle and 1 ml syringe. We used blood hematocrit as an estimate of the percent red blood cells in circulation (% 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 min (Spectrafuge Mini, Labnet International, Edison, NJ, USA).

Immunity measures

We estimated white blood cell (WBC) differentials of each individual by counting 100 WBCs on a single layer of whole blood smears stained with Diff-Quick and recording the number of lymphocytes, neutrophils, basophils, eosinophils and monocytes (Bachman, 2003). The clinical pathology lab at Onderstepoort, University of Pretoria, South Africa performed all WBC differentials. As basophils and eosinophils occurred at low levels, we focused on lymphocyte, neutrophil and monocyte values. We used bacteria-killing assays to measure complement and lysozyme activity in the organism by evaluating the plasma's ability to kill a known dilution of novel bacteria (Millet et al., 2007). This latter test gives us an estimate of an 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 (*Escherichia coli*) ml^{-1} (Lyophilised *E. coli* ATCC #8739, Microbiologics, St Cloud, MN, USA) and using 24.5 µl of plasma to make a 1:4 dilution to test the plasma's ability to inhibit bacterial growth overnight. We incubated each sample in quadruplet in 96-well plates and measured absorbance at 595 nm using a spectrophotometer (Multiskan Ascent, Thermo Labsystems, Philadelphia, PA, USA). We used two blanks in quadruplet to act as negative controls (growth medium only) and two positive 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 positive controls.

Hormone assays

We used a commercially available coated tube assay kit (Coat-a-Count TKTT1, Diagnostic Products Corporation, Los Angeles, CA, USA) to determine plasma testosterone concentration as previously described (Scantlebury et al., 2008). The assay-detectable range was 10–1600 nmol l^{-1} 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^{-1} and the intra-assay coefficient of variation was 8.4%. We ran all samples in a single assay and units were converted to ng ml^{-1}.

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 min 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 faecal glucocorticoid metabolites (FGMs). We extracted FGMs from 0.2 g dried faeces using 1.5 ml of 95% ethanol, following the protocol outlined in Mateo and Cavigelli (2005). We collected faeces from under the traps, as the squirrels defecate readily when trapped. We estimated concentrations of FGMs (ng ml^{-1}) using a radioimmunoassay technique previously validated and used in Richardson's ground squirrels (*Urocitellus richardsonii*; Clary et al., 2014; 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 FGMs 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. FGMs represent cortisol levels from approximately 10–24 h before collection (Dantzer et al., 2010; Harper and Austad, 2000; Palme, 2005; Touma et al., 2003). It is important to note that, as we compared FGMs within a single population among males only, the potential confounding factor of sex and differential metabolism of cortisol did not arise in our study (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^{-1} (min: 0.33 ng ml^{-1}, max: 8.7 ng ml^{-1}, N=33). We prepared silastic implants to raise the testosterone level by two standard deviations, to approximately 5.7 ng ml^{-1}, 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. 8.7 ng ml^{-1}). To raise the testosterone levels of males, we injected testosterone implants subcutaneously in the dorsolateral 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, Carpinteria, CA, USA) and sealed with a medical grade non-toxic silicone adhesive (Bluestar Silicone Inc., Lyon, France). Testosterone implants were constructed by sealing one end of the tubing with the silicone adhesive, filling the tube with crystalline testosterone (Sigma T1500, Sigma-Aldrich, St Louis, MO, USA) and sealing the remaining end; control animals received empty implants. We calculated tube length using previous studies of mammals [rabbits (*Oryctolagus cuniculus*; Limonta et al., 1986), rats (*Rattus norvegicus*; Wang et al., 1994) and degus (*Octodon degus*; Jechura et al., 2003)] in which similar implants were used to raise circulating testosterone. However, we adjusted tube length for the squirrels in our study to approximate our target dose of 5.7 ng ml^{-1} in the plasma for the duration of our study period (minimum of 30 days). We alternated treating males with the testosterone and control implants to ensure that both treatment groups were paired temporally to control for any effect of date. We recaptured both testosterone-implanted and control-implanted squirrels approximately 36 days after injection to confirm that we had approximated our target dose and to compare the effects of prolonged testosterone increase on treatment and control males.

Immune challenge

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 phase response (APR) of an organism's immune response as, unlike bacteria, it cannot replicate within the host and does not cause an infection in the animal (Adelman and Martin, 2009). To stimulate the APR, we injected Cape ground squirrels subcutaneously with a dose of 1 mg kg^{-1} LPS (from *E. coli* serotype 026:B6, Sigma-Aldrich) 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). Our control animals received a subcutaneous injection of saline. We alternated treating males with the LPS and control injections to ensure both groups were paired temporally to control for any effect of date. We attempted to recapture all individuals approximately 48 h post-injection to measure the peak response to LPS injection. Both LPS-treated and control males were difficult to recapture and were successfully recaptured approximately 1 week post-injection, which still allowed us to measure the effects of mounting an immune response on testosterone levels.

We conducted the LPS challenge first (late June to early July), and conducted the testosterone manipulation mid-July to September. Owing to the difficulty in repeatedly trapping males, nine individuals were used in both the testosterone and LPS manipulations and seven individuals were unique to each manipulation. If individuals from the LPS challenge were included in the testosterone manipulation, we waited a minimum of 3 weeks post-injection, and did not include them if they demonstrated any lasting effects (i.e. low body mass) from the first manipulation. Of the nine individuals used in both manipulations, only three had the LPS injection (the others received the saline control) and all three had no lasting effects from the LPS. All protocols were approved by the University of Manitoba's Animal Care and Use Committee (Protocol #F10-030 to J.M.W.) and permission for the project was provided by Northwest Province Parks Board.

Statistics

Non-normal data were log transformed (testosterone levels, FGM levels, total parasite abundances and relative scrotal size) or arcsine transformed (% 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 or Wilcoxon signed rank test. For log or arcsine transformed data, a two-sided Welch's t-test (Welch, 1947) was used to compare pre-treatment levels between control and treatment. We used paired t-tests to compare pre- and post-treatment within individuals of both treatment and control groups. Results are reported as means±s.e.m. and an alpha value of 0.05 was used to determine significance. All statistical analyses were conducted in R (version 3.1.1; http://www.R-project.org/) or JMP v13.0 (SAS Institute Inc., Cary, NC, USA). We compared prevalence between treatment and control with an exact unconditional test using Quantitative Parasitology version 3.0 (Rózsa et al., 2000).

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Table 1. Comparison of initial values between our testosterone manipulation group and our control group

	Initial values				
	Testosterone	Control	d.f.	*t*-value	*P*-value
Total ectoparasite	11.86±3.53	10.67±2.24	13.9	0.26	0.8
Flea abundance	5.57±1.28	5.89±2.36	13.83	0.62	0.55
Lice abundance	6.14±2.56	4.78±1.75	12.81	0.32	0.76
% Bacteria growth inhibition	47.7±0.08	44.7±0.9	13.99	0.11	0.91
% RBC	44.29±1.58	42.11±1.43	13.05	1.02	0.32
Mass (g)	675.0±23.6	682.8±13.4	9.74	–0.28	0.78
Relative scrotal size ($mm^3\ g^{-1}$)	16.19±1.02	15.02±1.49	13.37	–0.64	0.53
Faecal cortisol ($ng\ g^{-1}$)	0.721±0.12	0.621±0.063	9.51	0.66	0.53
Lymphocytes (%)	48.57±5.64	42.67±2.89	8.99	1	0.34
Neutrophils (%)	44.28±5.38	43.56±4.41	12.18	0.12	0.9
			N1–N2	*U*-value	
Monocytes (%)	4.57±0.72	10.89±2.65	7–9	20	0.24

Welch's t-test was used for ectoparasite abundance, immunity, RBC (red blood cell), body mass, cortisol and leukocytes in Cape ground squirrels; Mann–Whitney *U*-test was used for monocytes.

RESULTS

Effects of testosterone manipulation

We implanted testosterone implants in seven males and control implants in nine males during mid-July to mid-August, and all individuals were handled 35.9±2.73 days after treatment. Initial plasma testosterone concentration in the experimental animals (1.51±0.27 ng ml^{-1}) did not differ significantly from initial plasma testosterone concentration in control animals (1.03±0.21 ng ml^{-1}; two-sided Welch's *t*-test, $t_{13.56}$=1.43, *P*=0.18). Testosterone levels did not change in control animals in before and after samples (paired *t*-test, t_8=−1.94, *P*=0.09), whereas it increased significantly in testosterone-implanted individuals (paired *t*-test, t_6=6.85, *P*<0.0005; Fig. 1).

There were no differences in the initial prevalence of ectoparasites between testosterone (fleas: 85.7%, lice: 85.7% and total: 100%) and control (fleas: 66.7%, lice: 66.7%, total: 88.9%; unconditional exact *P*=1.0 for all three comparisons) treatment. Similarly, the testosterone treatment had no effect on the final prevalence of fleas (100%), lice (85.7%) and total ectoparasites (100%; unconditional exact *P*=1.0 for all three comparisons), and there was no change in prevalence for the control group (fleas: 100%, *P*=0.09; lice: 77.8%, *P*=1.0; total: 100%, *P*=1.0). We also found no differences in any of the initial measurements of parasite abundance, bacteria growth inhibition, WBC differential, body mass, % RBC, scrotal size or FGM levels between the testosterone-manipulated and control males (Table 1). Additionally, we found no effect of increased testosterone on any of the above parameters when comparing initial or final levels in testosterone-treated animals, except for a decline in scrotal size (Table 2). However, the final scrotal size of the treated group did not differ from control animals ($t_{12.9}$=0.55, *P*=0.59) as the scrotal size declined in both groups. After combining the initial levels in the testosterone and control treatment groups, there was no correlation between testosterone and FGM levels (Pearson's correlation: *r*=−0.008, *N*=16, *P*=0.98).

Effects of immune challenge

We injected seven males with LPS and nine males with a saline injection as a control during late June and early July, and we re-handled all males on average 7.7±0.38 days after they received their injection. There was no difference in the initial body mass between LPS and control treatments ($t_{13.9}$=1.64, *P*=0.12). However, the LPS injection had a significant negative effect on the body mass of treated animals (pre- and post-comparison, paired *t*-test, t_6=−8.82, *P*<0.0001), but we did not see a change in controls (pre- and post-comparison, paired *t*-test, t_8=−0.18, *P*=0.86; Fig. 2). Similarly, we found no difference in initial % RBC between the LPS (47.14±1.18%) and the control (47.33±1.08%; $t_{13.3}$=−0.12, *P*=0.91) treatments; however, after the LPS treatment there was a decrease in % RBC compared with pre-treatment values (paired *t*-test, t_6=−3.91, *P*=0.0079; Fig. 3). We did not find a change between

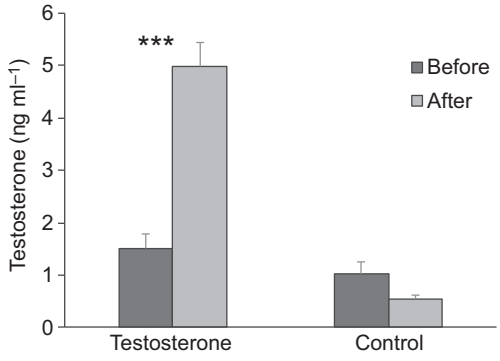

Fig. 1. Testosterone levels between testosterone-implanted and control male Cape ground squirrels. Asterisks indicate significant differences (***P<0.001). Data are presented as means±s.e.m. (N_T=7; N_C=9). For raw data, see Table S1.

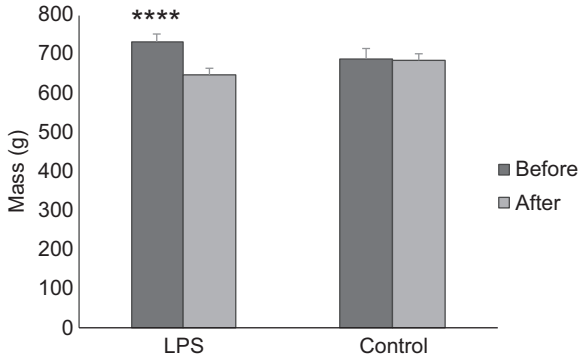

Fig. 2. Mass changes (g) of lipopolysaccharide (LPS)-injected and control (saline-injected) male Cape ground squirrels from pre- to post-treatment. Asterisks indicate significant differences (****P<0.0001). Data are presented as means±s.e.m. (N_{LPS}=7; N_C=9). For raw data, see Table S2.

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Table 2. Initial and final values from our testosterone manipulation group and controls

	Testosterone			Control		
	Initial	Final	*P* (d.f., *t*-ratio)	Initial	Final	*P* (d.f., *t*-ratio)
Total ectoparasite	11.9±3.3	11.0±2.6	0.88 (6, −0.16)	10.7±2.2	14.0±4.3	0.33 (8, 1.04)
Flea abundance	5.6±1.3	6.3±2.2	0.77 (6, 0.31)	5.89±2.36	7.1±2.1	0.59 (8, 0.55)
Lice abundance	6.1±2.6	4.7±1.7	0.72 (6, −0.37)	4.78±1.75	6.9±2.7	0.84 (8, 0.84)
% Bacteria growth inhibition	47.7±8.1	50.7±9.5	0.80 (6, 0.26)	44.7±8.5	41.4±7.2	0.78 (8, −0.28)
% RBC	44.3±1.6	45.7±0.9	0.52 (6, 0.69)	42.1±1.4	46.3±1.5	**0.0006 (7, 5.95)**
Body mass (g)	675.0±23.6	710.0±20.6	0.09 (6, 2.0)	682.8±13.4	692.2±9.1	0.43 (8, 0.83)
Relative scrotal size ($mm^3\ g^{-1}$)	16.2±1.0	12.3±0.9	**0.04 (6, −2.54)**	15.0±1.5	11.6±0.8	**0.04 (8, −2.50)**
Faecal cortisol ($ng\ g^{-1}$)	0.72±0.1	0.71±0.12	0.95 (6, −0.06)	0.62±0.06	0.62±0.14	0.96 (7, 0.05)
Lymphocytes (%)	48.6±5.6	52.0±5.4	0.70 (6, 0.40)	42.7±2.9	49.6±4.1	0.13 (8, 1.68)
Neutrophils (%)	44.3±5.4	34.0±4.0	0.22 (6, −1.36)	43.6±4.4	34.7±4.4	0.07 (8, −2.06)
			P (*N*, *S* statistic)			*P* (*N*, *S* statistic)
Monocytes (%)	4.6±0.7	9.1±1.7	0.16 (6, 8.5)	10.9±2.6	11.3±2.1	0.52 (8, 6.0)

Paired *t*-tests were used to determine whether the treatment had an effect on the ectoparasite abundance, % bacteria growth inhibition, % RBC (red blood cells) and leukocytes in Cape ground squirrels; Wilcoxon signed rank test was used for monocytes. Bold numbers indicated significant values ($P<0.05$).

before and after treatment values in the % RBC of control individuals (paired *t*-test, $t_8=-1.80$, $P=0.11$). We also did not find a difference in the initial testosterone levels between the LPS treatment group (0.95 ± 0.18 ng ml^{-1}) and control group (0.85 ± 0.12 ng ml^{-1}; $t_{11}=0.36$, $P=0.73$). The testosterone of individuals in the control treatment did not change (paired *t*-test, $t_8=0.44$, $P=0.67$), whereas LPS-treated animals had a significant decrease when comparing before and after levels (paired *t*-test, $t_6=-4.66$, $P=0.0035$; Fig. 4). Initial relative scrotal size did not differ between LPS (17.02 ± 1.47 $mm^3\ g^{-1}$) and control (17.54 ± 0.74 $mm^3\ g^{-1}$; $t_{8.98}=-0.32$, $P=0.76$) groups, and there was no effect of LPS on relative scrotal size (treatment: -1.12 ± 2.68 $mm^3\ g^{-1}$; control: -1.54 ± 1.96 $mm^3\ g^{-1}$; $t_{10.9}=-0.13$, $P=0.9$). Initial FGM levels did not differ between the LPS (0.79 ± 0.18 ng g^{-1}) and control (0.67 ± 0.10 ng g^{-1}; $t_{8.88}=0.52$, $P=0.61$) treatment. We found no change in FGM levels pre- and post-treatment in either the LPS treatments (final FGM: 1.08 ± 0.26 ng g^{-1}; paired *t*-test, $t_5=1.42$, $P=0.22$) or controls (final FGM: 0.65 ± 0.08; paired *t*-test, $t_7=-0.19$, $P=0.86$). After combining the initial levels in the LPS and control treatment groups, we found no correlation between testosterone and FGM levels (Pearson's correlation: $r=0.05$, $N=16$, $P=0.86$).

DISCUSSION

Our results fail to support the ICHH because increased testosterone had no effect on parasite abundance, body mass or any of our immunocompetence measures. This result differs from the conclusion of Foo et al. (2017), who found that testosterone has a medium immnosuppressive effect across species. There are other 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 (Grear et al., 2009), and increasing testosterone in house mice (*Mus musculus*) increased their endoparasite intensity (Zhang and He, 2014). In red grouse (*Lagopus lagopus*), increased testosterone had a negative effect on body condition (Martínez-Padilla et al., 2014) and higher levels of testosterone were positively correlated to increasing ectoparasite intensity in female meerkats (*Suricata suricatta*) (Smyth et al., 2016). However, other studies have also failed to find a relationship between increased testosterone and immunocompetence measurements in mammals and birds (Nunn et al., 2009; van Oers et al., 2011; Roberts et al., 2007; Ros et al., 2006), and some studies have even found that elevated testosterone had immune-enhancing effects, completely opposite to predictions from the ICHH (Bilbo and Nelson, 2001; Evans et al., 2000).

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 enhancing 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 that the natural increase in

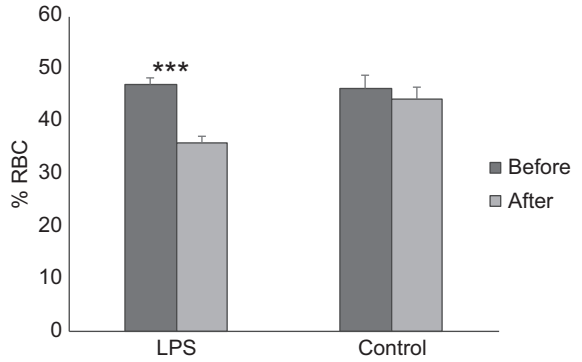

Fig. 3. Hematocrit [percent red blood cells in circulation (% RBC)] of LPS-injected and control (saline-injected) male Cape ground squirrels. Asterisks indicate significant differences (***$P<0.001$). Data are presented as means±s.e.m. ($N_{LPS}=7$; $N_C=9$). For raw data, see Table S3.

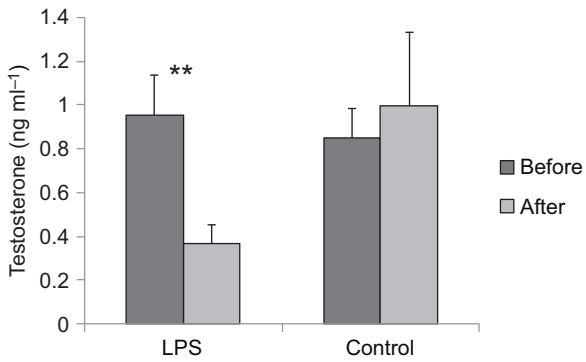

Fig. 4. Testosterone levels between LPS-injected and control (saline-injected) male Cape ground squirrels. Asterisks indicate significant differences (**$P<0.005$). Data are presented as means±s.e.m. ($N_{LPS}=7$; $N_C=9$). For raw data, see Table S4.

testosterone may be responsible for the increase in parasites (Hillegass et al., 2008). Our results indicate that testosterone does not suppress immunity, affect parasite abundance or affect body mass, 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. However, if mounting an immune response to parasites were to negatively affect testosterone or an animal's energy budget, males may choose to tolerate parasites rather than resist them (Best et al., 2010), particularly to maintain year-round reproductive readiness in a species where sperm competition success is critical to reproductive success, such as in the Cape ground squirrel (Manjerovic and Waterman, 2012).

Our experiments indicated that an immune challenge had a significant negative effect on the circulating testosterone levels of male squirrels. 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 (*C. hottentotus pretoriae*) demonstrated significantly decreased testosterone levels. Similarly, Müller et al. (2013) found that manipulatively infesting male canaries (*Serinus canaria*) 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 (*Pavo cristatus*) displayed decreased testosterone after being immuno-challenged with sheep RBCs, 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 (*Carollia perspicillata*). 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 (*Mus musculus*). Although we found a significant effect of the LPS challenge on circulating testosterone, we did not find an effect on average scrotal volume. Interestingly, Needham et al. (2017) found that testosterone levels of house sparrows (*P. domesticus*) decreased after a first and second LPS challenge, but there was no effect on sperm quality, suggesting that there may be mechanisms in place to avoid consequences of pathogen exposure on reproductive output. The lack of change observed in scrotal size of treated animals in our study may also be 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 affect FGM levels. Evans et al. (2000) initially found support that testosterone was immunosuppressive in house sparrows (*P. domesticus*), supporting the ICHH, but, after controlling for corticosterone, they found that testosterone was actually enhancing immune status. As their increased testosterone manipulation caused an increase in corticosterone, and as corticosterone can 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 (*Melospiza melodia*) 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 negatively affected 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. Our results show no effect of LPS treatment on faecal FGMs, suggesting that cortisol is not playing an important role in either testosterone suppression or immunosuppression, as predicted by the ISH and stress-linked ICHH, in 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 (Grear et al., 2009). Zohdy et al. (2017) found that, in brown mice lemurs (*Microcebus rufus*), aggression was positively correlated with testosterone and cortisol, and aggressive individuals had higher lice infestations and contributed significantly more lice to conspecifics. Increased exposure to parasites has an important fitness cost (Hudson et al., 2002) and sex-biased parasitism appears to play an important role on sex-biased mortality in natural populations of mammals (Moore and Wilson, 2002). Grear 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 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). This difference between subadult males and females suggests that it is not simply home range that results in high parasitism in males. It is also important to note that male Cape ground squirrels are not aggressive and associate with other males year-round (Waterman, 1995).

Another possible mechanism to explain the direction of our observed trade-offs between testosterone and immunity (immunity causing a decrease in testosterone) is simply differential allocation of resources (Rolff, 2002). This explanation predicts the same direction of effects as the ISH. Although the suppressive mechanisms of an immune response, such as the release of cytokines during an immune response, have been documented to suppress steroid production in vertebrates (Demas and Nelson, 2012), exposure to LPS infection has also been documented to affect energy budgets (Burness et al., 2010). In brown-headed cowbirds, where females invest more heavily in reproduction than males, females have lower immune function than males, suggesting that they reduce their allocation to self-maintenance in order to maintain high reproductive output (Merrill et al., 2013). Female Cape ground

squirrels have increased resting metabolic rates and quadruple their reproductive output when parasite-free, suggesting that they are energetically constrained by parasites (Hillegass et al., 2010; Scantlebury et al., 2007). Likewise, in other species where males do not experience high levels of sexual selection, and where there is little or 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; Manjerovic and Waterman, 2012), 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.

It is possible that Cape ground squirrels avoid a strong trade-off between testosterone and immunity by maintaining low levels of testosterone compared with species that are seasonal breeders. In seasonal breeding ground squirrels, the mating period when males are scrotal and must maintain high levels of testosterone is only a few weeks. Testosterone levels in these seasonal breeders range from almost double to quadruple that of Cape ground squirrel [e.g. *Spermophilus citellus* (3 ng ml^{-1}; Strauss et al., 2008), *Spermophilus fulvus* (3.3 ng ml^{-1}; Vasilieva et al., 2014), *Citellus dauricus* (3.9 ng ml^{-1}; Zhang et al., 2017), *Callospermophilus lateralis* (4.5 ng ml^{-1}; Licht et al., 1982), *Otospermophilus beecheyi* (5.9 ng ml^{-1}; Holekamp and Talamantes, 1992), *Urocitellus parryi* (8.6 ng ml^{-1}; Boonstra et al., 2001) and *Callospermophilus saturatus* (10–20 ng ml^{-1}; Barnes et al., 1988)]. We found that Cape ground squirrels averaged much lower levels of testosterone (1.88 ng ml^{-1}), similar to those found in the year-round breeding tropical tree squirrel *Funambulus pennanti* (1.51 ng ml^{-1}; Seema and Chandana, 2013). The lower levels of testosterone in the latter two species could be a means to cope with continually having to be scrotal and the high costs of testosterone production, and avoidance of trade-offs between testosterone and immune competence. However, our manipulation raised the testosterone level of Cape ground squirrels to that of seasonal breeders and we still did not see any impact on the immune response at these elevated levels of testosterone.

Conclusion

The results of this manipulative study give us important insights 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 that an increased immune response negatively affected testosterone, either directly through suppression or through the diversion of resources to immunity at a cost to testosterone levels. Although both the ICHH and ISH 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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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: K.A.O., J.M.W., W.G.A., N.C.B.; Methodology: K.A.O., J.M.W., W.G.A., N.C.B.; Validation: W.G.A., N.C.B.; Formal analysis: K.A.O., J.M.W., W.G.A.; Investigation: K.A.O., J.M.W.; Resources: J.M.W., W.G.A., N.C.B.; Writing - original draft: K.A.O.; Writing - review & editing: J.M.W., W.G.A., N.C.B.; Supervision: J.M.W.; Project administration: J.M.W.; Funding acquisition: J.M.W.

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Supplementary information

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