

Testosterone, ticks and travels: a test of the immunocompetence–handicap hypothesis in free-ranging male sand lizards

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The immunocompetence–handicap hypothesis suggests that androgen-dependent male characters constitute honest signals of mate and/or rival quality because of the imposed costs through immune suppression associated with elevated testosterone levels. We demonstrate in a field experiment that male sand lizards (*Lacerta agilis*) exposed to elevated testosterone suffered from increased mass loss and tick load compared to control males. Although the first of these two results could be due to an elevated basal metabolic rate from increased plasma testosterone levels, the increased parasite load was statistically independent of the loss in body condition and is likely to be due to compromised immune function. Testosterone-treated males showed greater mobility than control males, and greater mobility resulted in higher mating success. Our experiment thus lends support to the immunocompetence–handicap hypothesis, suggesting that male testosterone levels have been moderated by balancing selection for reproductive success and sustained immune function.

Keywords: immunocompetence–handicap hypothesis; sand lizard; mass loss; tick load

1. INTRODUCTION

In a congress plenary talk in 1989, Tim Halliday predicted that in order to infer the evolutionary origin and consequences of mate choice and competition for mates, future studies on sexual selection would need to integrate fields such as physiology, genetics and ecology (Halliday 1989). Indeed he was proven right and perhaps one of the clearest examples is the immunocompetence–handicap (ICH) hypothesis, proposed by Folstad & Karter in 1992. This hypothesis targets the interplay between plasma levels of hormones (primarily steroids), and their positive effects on sexual characters such as ornamentation and status signals, and concomitant effects on reproductive success. However, increased levels of androgens may have costs associated with a depressed immune function resulting in an increased susceptibility to disease and parasites (suppression of the major histocompatibility complex class II system; e.g. Browning & McMichael 1996), and ultimately a reduction in organismal fitness (Folstad & Karter 1992). Thus, androgen-dependent sexual characters may form honest signals of mate or rival quality, and plasma levels of androgens such as testosterone may be subject to balancing selection over evolutionary time.

Recently, three different approaches have been taken in order to test predictions from the ICH hypothesis: first, correlative studies of activity (size) of immune organs (such as the spleen and the Bursa Fabricius in birds; Møller & Erritzoe 1996); second, experimental challenging of the immune system with novel antigens (Svensson *et al.* 1998; Hasselqvist *et al.* 1999); and third, experimental manipulation of steroid (primarily testosterone) plasma levels (Hasselqvist *et al.* 1999; Evans

et al. 2000). These studies have resulted in considerable debate because some carefully controlled experiments were unable to confirm immunosuppression by elevated levels of plasma testosterone (Hasselqvist *et al.* 1999) and other studies failed to find a relationship between testosterone levels, parasite load and lymphocyte counts (Weatherhead *et al.* 1993; Saino *et al.* 1995). Recently, Evans *et al.* (2000) demonstrated that experimental manipulations of testosterone levels in house sparrows also affected the levels of corticosterone, which in turn were related to the degree of immunosuppression. After controlling for the effect of corticosterone, Evans *et al.* (2000) found that testosterone enhanced the birds' ability to produce antibodies. Other studies, however, found support for steroid-induced immunosuppression resulting in increased parasite load (Saino & Møller 1994), lowered lymphocyte counts, decreased levels of immunoglobulin (Saino *et al.* 1995) and reduced resistance to infections (Barnard *et al.* 1994). Thus, to date, attempts at testing the assumption of the ICH hypothesis have given mixed results.

In the present study, we report on an experimental manipulation of plasma levels of testosterone using silastic implants in free-ranging male sand lizards (*Lacerta agilis*). The study was explicitly conducted to test the effect of increased plasma levels of testosterone on male reproductive success, and whether increased androgen levels would be associated with costs caused by depressed immune-system function.

2. METHODS

The experiment was conducted at Asketunnan, a rocky peninsula 50 km south of Gothenburg on the Swedish west coast (see Olsson (1994) for a more detailed description of the study site). Male sand lizards were captured by hand or by noose

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within a week after emergence from hibernation (first week of May 1997) but prior to the onset of the mating season, and brought to the laboratory. The lizards were weighed to the nearest gram, measured to the nearest millimetre (snout–vent length, total length) and photographed against a neutral grey background using Ektochrome 200 ASA slide film. All ectoparasites (ticks) were counted. A 100 µl blood sample was obtained by puncturing *sinus angularis* in the corner of the upper jaw (Olsson 1994), collected in a capillary tube, centrifuged, and then the plasma stored at -20°C until the radioimmunoassay was conducted.

Forty male sand lizards were given silastic implants the day after capture. Prior to implantation the lizards were cold-anaesthetized in a refrigerator to a rectal temperature of $+4^{\circ}\text{C}$ (Sinervo *et al.* 1992). A 15×15 mm area on the right body side was washed with 70% alcohol, and a 3 mm incision made between two scale rows using the tip of a large syringe as a scalpel. A 6 mm silastic implant (Dow Corning, Midland, MI, USA; inner diameter 1.5 mm, outer diameter 2.0 mm, closed with Dow Corning 734 RTV Sealant) with a 4 mm column of crystalline testosterone (Sigma Products article no. T 1500; Sigma–Aldrich Pty Ltd, Castle Hill, NSW, Australia) was inserted subcutaneously, laterally, mid-body in half of the males ('T males'). Alternate males drawn at random from the sample were given an empty silastic implant ('control males'). In both groups the incision was closed immediately using a sterile tape. On top of the sterile tape, an oval cloth tape was attached carrying the lizard's individual identification number. All 20 males in each group were released at their site of capture within 24 h after implantation and were monitored every day during the three-week mating season when the weather permitted lizard activity.

Male sand lizards guard a mated female for between a few hours and several days (Olsson 1994; Olsson *et al.* 1996b). Since copulation lasts for only *ca.* 2 min, the mate guarding provides an opportunity to score mating success in cases where the brief copulation goes undetected in the field. We have demonstrated elsewhere, using DNA fingerprinting, that male mate guarding is a reliable predictor of paternity (Gullberg *et al.* 1997).

In the present study, male mating success was positively correlated with the number of observations (Spearman's rank-order correlation coefficient, $r_s = 0.53$, $p = 0.0002$, $n = 40$). Therefore, in order to ensure that variance in male mating success was not simply an effect of variance in the number of observations, we divided the number of mate guardings by the number of observations and used this ratio as an index of male mating success. For a subset of males ($n = 14$) we obtained detailed data on partner identity, and in these males could look for a trade-off between number of guarding days per female and male coloration, the best predictor of success in male mate acquisition (Olsson 1994).

In order to evaluate the success of the testosterone treatment and its associated effects on morphological traits, coloration, tick load, mobility and reproductive success, we attempted to recapture all males at the end of the mating season (end of May). Nine out of the 20 T males and 12 out of the 20 control males were recaptured before skin shedding, making it possible to count the number of ticks acquired since implantation. Four additional T males and one additional control male were recaptured immediately after shedding. All recaptured males were re-measured and re-photographed at the second capture as before. A male's 'badge size' was estimated by taking the ratio of the green versus the remaining part of the photographed lizard's body side. Two traits

did not conform to normality: 'movement' (accumulated distance between sightings) and 'tick load' (the number of additional ticks acquired). These traits were therefore square-root transformed (movement) and log transformed (tick load), respectively, which successfully normalized the data (Shapiro–Wilks' statistic, W , normal = 0.97 and 0.92, respectively, and $p = 0.60$ and 0.07, respectively; Proc Univariate (SAS 1990)).

To ensure that there were no differences between T males and control males in traits relating to mating success and parasite load at the start of the experiment, we performed a series of *t*-tests on the relevant traits. At the first sampling, males in the two groups did not differ in mean plasma testosterone levels, number of days separating implantation and re-sampling, badge size, snout–vent length, mass, or tick load ($0.09 < t < 1.44$, $0.16 < p < 0.93$).

(a) Radioimmunoassay

Samples were assayed by radioimmunoassay using a single antibody (testosterone antibody from Wien Laboratories Inc., Wien, Austria) as briefly described below. Standard curves were sensitive within the range 3–500 pg. Plasma levels of testosterone were measured after extraction and partial purification of steroid fractions on diatomaceous earth–glycol microcolumns. Plasma samples were first diluted (1:10) with phosphate-buffered saline because of the relatively high testosterone titres. Then, a sample from the diluted plasma was equilibrated overnight with *ca.* 2000 cpm of titrated testosterone (New England Nuclear) to assess the percentage recovery after chromatography, and distilled water was added to a total volume of 400 µl. Plasma samples were then extracted in dichloromethane. The organic phase was dried under a stream of nitrogen at 40°C , and thereafter re-dissolved in ethyl acetate and isooctane. This solution was transferred to the microcolumns, and steroids were eluted in order of increasing polarity. In this study, only the testosterone fractions were included. The purified testosterone extracts were again dried under a stream of nitrogen and reconstituted in phosphate-buffered saline. To estimate interassay variation, four samples from a plasma pool from mallards were included in each assay. The interassay variation was found to be 13.0% and the intra-assay variation in both cases was less than 10%. Three samples of hormone-free plasma were taken through each assay. In all cases blanks measured less than 3 pg of testosterone.

3. RESULTS

(a) Effects of testosterone implants on plasma testosterone levels

Recapture of the lizards took place on average 26 and 24 days after release for control males and T males, respectively (s.e.m. = 2.49 and 1.31, respectively, $t = 0.83$, $p = 0.41$; final capture date was 12 June for both T males and control males). At recapture, plasma testosterone levels were significantly higher in T males than in control males (figure 1; 7.4 ng ml^{-1} versus 13.5 ng ml^{-1} , respectively, $t = 3.7$, $p = 0.001$; for sample sizes see § 2). Thus, the radioimmunoassay confirmed that our manipulation resulted in an experimental increase in plasma testosterone levels. We also noted that the variation around the means was substantial in both groups (s.d. = 3914 and 4075, respectively) but not significantly different from each other ($F = 1.1$, $p = 0.91$) and, as a result, there were substantial overlaps between the groups in their distributions of testosterone levels.

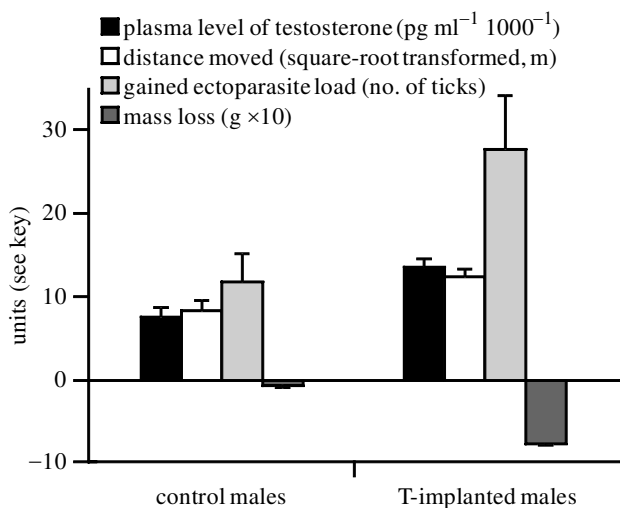


Figure 1. Differences between control males and testosterone-implanted males in plasma levels of testosterone at recapture, accumulated distance moved between the first and last observations, number of ectoparasites gained between release and recapture, and the loss of body mass in the same interval. All differences between the two categories were statistically significant (see § 3(a)–(d) for test statistics).

(b) Reproductive success: effects of badge size and male mobility

An initial comparison of the reproductive-success score between T males and control males revealed no significant effect of elevated testosterone levels on male reproductive success (Wilcoxon two-sample test, d.f. = 1, $Z = 0.03$, $p > 0.05$). However, T males moved significantly longer distances than did control males ($t = 2.6$, $p = 0.01$): on average 144 m compared to 64 m for control males (figure 1). There was thus the possibility that male testosterone levels can influence male reproductive success via male movement patterns. To increase our power to detect such effects, we standardized male movement by group (T versus control) setting mean to zero and standard deviation to one, after which we pooled the data. It then became apparent that males with greater mobility obtained matings with more individual females, in accordance with previous work (Spearman's rank-order correlation coefficient, $r_s = 0.55$, $p = 0.03$, one-tailed test) (Olsson & Madsen 1998).

T males did not develop a larger badge than control males ($t = 0.30$, $p = 0.76$; average badge sizes (\pm s.d.) $58\% \pm 0.11$, versus $60\% \pm 0.10$; badge size (a ratio) was left untransformed because the original data conformed to normality; Shapiro–Wilks' statistics, W , normal = 0.96, $p = 0.42$). The lack of a testosterone effect on badge size agrees with our previous work (Olsson & Silverin 1997). However, because our previous studies have revealed a strong effect of both badge size and mobility on reproductive success in sand lizards and other reptiles (reviewed in Olsson & Madsen 1998), we looked for independent effects of these traits on the reproductive-success score. A standardized multiple regression analysis with badge size and male movement as predictor variables was highly statistically significant ($p < 0.0097$, table 1) with both these traits remaining significant when a non-parametric, partial correlation analysis was applied (table 1). Male

Table 1. Analysis of standardized sexual selection gradients

(The tests of independent association between relative movement and badge size, respectively, and reproductive success were performed using Spearman's partial rank-order correlation analysis. Model, d.f. = 2, error = 25, sum of squares = 0.38, mean squares = 0.19, $F = 5.61$, $p = 0.0097$, $r^2 = 0.31$.)

traits	β	s.e.m.	p
movement	0.069	0.032	0.04
badge size	0.090	0.032	0.02

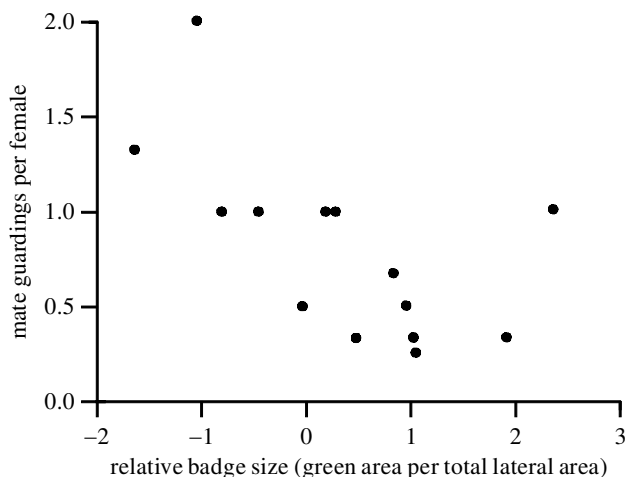


Figure 2. Relationship between a male's relative badge size and the number of days he invested in mate guarding per female ($r_s = -0.69$, $p = 0.006$).

body mass was deliberately left out of the analysis because it was uncorrelated with reproductive success ($r_s = 0.24$, $p = 0.21$) but correlated with badge size, which may have introduced collinearity.

We have demonstrated elsewhere that an experimental increase in badge size increases a male's probability of winning contests (Olsson 1994). Thus, it may be expected that the size of a male's badge is related to his mate-search versus mate-guarding tactics. We therefore calculated an index of male 'time investment per female' by dividing the total number of days a male was observed with females by the number of days he mate-guarded each female. Interestingly, males with larger badges invested fewer guarding days per individual female (figure 2; $r_s = -0.69$, $p = 0.006$, $n = 14$). When body mass was controlled for in a partial correlation analysis, the correlation between badge size and mate guarding became even more strongly negative ($r_s = -0.76$, $p = 0.001$). Thus, males more capable of defending and/or acquiring partners seem to allocate more time to mate acquisition rather than investing additional time in mate guarding. This result was not an effect of larger-badged males being more conspicuous to us, because there was no correlation between badge size and number of sightings ($r_s = 0.19$, $p = 0.32$, $n = 29$).

(c) Male costs of high testosterone levels

(i) Parasite load

Nymphs of the tick *Ixodes ricinus* (Bauwens *et al.* 1983; Olsson 1992) were prevalent in the sand-lizard population

and sometimes reached levels that were pathological. This was supported by an observation of a male not included in the experiment. He was lethargic and had 85 ticks when captured. Blood sampling revealed a pink rather than the normal deep red colour of the blood, suggesting that he was anaemic, and he was found dead the following day. However, our observations cannot demonstrate to what extent the ticks were the cause of mortality.

Males in both groups accumulated ticks during the experiment (there were no differences between T males and control males in the number of ticks they carried at the start of the experiment). However, T males gained 27.9 ticks on average (± 6.4 , s.e.m.), whereas control males gained only 11.8 ticks on average (± 3.4 , s.e.m.) during the course of the experiment ($t = 2.4$, $p = 0.018$; figure 1). This could have been caused by T males moving greater distances, resulting in a higher encounter rate of ticks. However, this is contradicted by a non-significant correlation between tick load and distance moved ($r_s = -0.01$, $p = 0.96$).

(ii) *Mass loss*

Males in both groups lost body mass during the mating season (figure 1), however, T males lost significantly more (on average 0.8 ± 0.53 g) than did control males (0.08 ± 0.98 g; $t = 2.32$, $p = 0.029$). The average body mass of males in both groups at the start of the experiment was 10.6 g; the mass loss thus represents *ca.* 8% of the total body mass in T males and < 1% in control males. This result could be explained by testosterone effects on male movement patterns; greater mobility would be expected to incur a higher energy expenditure. However, there was no relationship between movement and mass loss ($r_s = 0.15$, $p = 0.43$) and hence there was no support for this hypothesis.

The increased ectoparasite load in T males could be due to a direct effect on immune function (as predicted by the ICH hypothesis) or it could be an indirect effect on overall loss of condition, resulting in males becoming poorer at fighting pathogens. In order to test for effects of mass loss on ectoparasite load, we first performed a heterogeneity-of-slopes test with tick load as response variable and the interaction between mass loss and treatment (T versus control) as predictor. When this test proved non-significant ($F = 1.8$, $p = 0.19$), we performed an analysis of covariance, with treatment and mass loss as predictors of tick load. In this model, treatment was statistically significant ($F = 4.9$, $p = 0.04$) whereas mass loss was not ($F \approx 0.0$, $p = 0.98$). Thus, the difference in tick load between T males and control males seems more likely to be a direct effect of testosterone on immune function, rather than as secondary effect of T males losing more in bodily condition.

4. DISCUSSION

At the onset of our study, we predicted from the ICH hypothesis that males with elevated testosterone levels should acquire more mates via increased mobility, and suffer increased costs from suppressed immune function. Both these predictions seem to be upheld.

A male's green lateral coloration and mobility, independently of each other, contributed significantly to a

male's success in acquiring matings in the present study, even when the reproductive-success score was corrected for total number of sightings. Thus, although we could not demonstrate a significant effect of testosterone on male reproductive success *per se*, the strong link between testosterone levels and male mobility, and between mobility and reproductive success, suggest that such a relationship cannot be rejected. There was a direct relationship between badge size and mating success, but no relationship between testosterone levels and the size of the badge. This latter result is in agreement with our previous studies (Olsson & Silverin 1997), but differs from those of two studies in which testosterone effects on male nuptial coloration were confirmed (Cooper *et al.* 1987; Salvador *et al.* 1996). In our 1997 study, however, we controlled for growth rate in partial correlation analyses of the colour–testosterone-level relationship. At least in sand lizards, allocation to reproduction or coloration is strongly size and age dependent and, hence, allocation to growth needs to be explicitly considered in the analysis. When this was done, there was no longer an effect of testosterone on coloration.

In accordance with predictions from the ICH hypothesis, increased levels of testosterone resulted in costs in terms of increased parasitization and mass loss. We did not, however, monitor internal parasites that are known to cause loss of body mass in reptiles (Reichenbach-Klinke & Elkan 1965) and we acknowledge the possibility that this may explain the higher mass loss in T males. However, although ectoparasites may compromise sand lizard health, for example by acting as vectors for blood parasites (Reichenbach-Klinke & Elkan 1965), the number of ticks to which sand lizards are exposed are nowhere near those often reported in birds, for example (several thousand per nest; Møller 1990). Only rarely do sand lizard ectoparasites cause overt signs of phenotypic deterioration; only one male appeared anaemic and we cannot disentangle cause and effect of its health and parasite load. Previous work has also reported mass loss in testosterone-treated lizards (Marler & Moore 1988, 1989, 1991), with an associated increase in mortality. However, these effects were alleviated by experimental food supplementation (Marler & Moore 1988, 1989, 1991), suggesting that the mortality effect was due to depletion of energy rather than increased susceptibility to pathogens. Thus, mass loss seems to be more parsimoniously explained by an increased metabolic rate due to the steroid administration (Chandola *et al.* 1974; Thapliyal *et al.* 1974). In the lacertid lizard *Psammotromus algirus*, Salvador *et al.* (1996) allowed males to keep their testosterone implants for longer than in the present study, which resulted in increased lizard mortality. Salvador *et al.* also demonstrated significant positive effects of testosterone on male nuptial coloration, suggesting that any function that coloration may have in mate acquisition (possibly via status signalling; Salvador *et al.* 1996; Díaz 1993) ought to be reinforced by elevated testosterone levels. Although mating success was not monitored in the *Psammotromus* study, our results corroborate their conclusion that increased levels of testosterone in lizards may indeed result in elevated mating success, although the causal link mediated by the steroid may differ between these two species (colour versus mobility).

In a recent survey of the literature (Olsson & Madsen 1998), we demonstrated that mate guarding is a widespread phenomenon in reptiles and plays a significant part in the mating tactics of many primarily non-territorial species. However, previous studies have focused primarily on the presence or absence of this phenomenon in a species or on its association with male body size. In sand lizards, a previous study (Olsson *et al.* 1996a) failed to find significant relationships between male guarding time and operational sex ratio, time to ovulation, or female size and concomitant clutch-size characteristics. In the present study, however, there was a very strong inverse relationship between the main determinant of male reproductive success, his lateral coloration ('badge'), and the duration of his guarding activity. This may suggest that incorporation of more female partners is more important to a male in terms of marginal fitness benefits than the guarding activity *per se*, and that males with the best chances of acquiring partners also invest the least in staying put with a female.

In summary, we demonstrate that plasma testosterone levels in sand lizards are likely to be under balancing selection. This is a result of increased costs from a complex interaction between higher activity levels, possibly a higher basal metabolic rate and a compromised immune function that together result in pronounced mass loss and an increase in parasite load in males with higher testosterone levels. However, these costs seem to be balanced on the 'Darwinian ledger sheet' by a higher reproductive success from incorporation of more partners.

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