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Effect of Testosterone on T Cell-Mediated Immunity in Two Species of Mediterranean Lacertid Lizards

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ABSTRACT One of the primary assumptions of the immunocompetence handicap hypothesis is that testosterone has an immunosuppressive effect, but conflicting results have been reported in a variety of bird species concerning the effect of testosterone on the humoral and the T cell-mediated components of the immune system. The T cell-mediated component of the immune system is particularly important during the breeding season, because the likelihood of injury during sexual competition is high and T cell-mediated immunity is essential for healing wounds and resisting infection. In this study we examined the effect of experimentally increased levels of testosterone during breeding season on T cell-mediated immunity in male lizards of two Mediterranean lacertid species, *Psammodromus algirus* and *Acanthodactylus erythrurus*. The hormonal treatment significantly increased testosterone of the experimental individuals. T cell-mediated responses to phytohemagglutinin stimulation were significantly suppressed in testosterone-treated males of both species. Furthermore, there was a significant negative relationship between individual variability in T cell-mediated responsiveness and plasma testosterone concentration. The present study is the first to demonstrate testosterone-induced suppression of T cell-mediated immunity in lizards. *J. Exp. Zool. 301A:411–418, 2004.*

INTRODUCTION

It has been hypothesized that during breeding season the reproductive effort could compromise an individual's immune defence, thereby decreasing its ability to offer an adequate immune response and increasing its susceptibility to infectious disease (Gustafsson et al., '94; Deerenberg et al., '97; Nordling et al., '98). In males of vertebrate species without parental care, reproductive effort is in general directly associated with circulating testosterone levels, because testosterone can induce the production of secondary sexual characters and can increase mobility, aggressiveness and sexual behaviors (review by Ketterson and Nolan, '92). T-lymphocyte cell-mediated immunity constitutes one of the main components of immunity in vertebrates (Roitt et al., '89; Wakelin, '96). The ability to mount a T-lymphocyte cell-mediated immune response to a mitogenic stimulus may have important fitness consequences (Gonzalez et al., '99), because it constitutes a generalized short-term response to grafts, allergens and wounds. Therefore, individuals with better T cell-mediated responses should be at an advantage. Such advantage may be particularly important during the breeding season, as the likelihood of injury during sexual competition is high and T cell-mediated immunity is essential for healing wounds and resisting infection (Zuk and Johnsen, '98).

In lizards, testosterone levels are generally highest at the time of year when males have enlarged testes and show marked territorial and sexual behaviors (McKinney and Marion, '85; Moore, '88; Moore and Lindzey, '92). When testosterone levels have been experimentally elevated, testosterone-treated lizard males are more successful in defending their territories (Moore and Marler, '87), having larger home-ranges sizes (DeNardo and Sinervo, '94) and acquiring higher-quality home ranges (Fox, '83) than control males.

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However, elevated testosterone levels can have fitness costs as well as benefits, because males with relatively high testosterone levels may have reduced survivorship (Marler and Moore, '88; Marler et al., '95; Salvador et al., '96). It has been proposed that testosterone production is traded against immune defence, as this hormone might have an immunosuppressive effect (Folstad and Karter, '92). The immune system could thus serve as a physiological mechanism mediating reproductive costs, and there is some empirical support for this hypothesis (e.g., Richner et al., '95; Deerenderg et al., '97; Nordling et al., '98).

Information on the seasonal changes induced in different aspects of immunity, humoral and cellular, reveals conflicting results in a variety of bird species. While in some species testosterone induced suppression of both cell-mediated and humoral immunity (Duffy et al., 2000), in other cases seasonal changes affected only humoral responses, and cell-mediated immunity was maintained (Zuk and Johnsen, '98). Finally, other studies have failed to find a link between testosterone and immunity (Hasselquist et al., '99).

Exogenous manipulations of testosterone to demonstrate a causal relationship of reproductive effort on immune defence have been scarce in reptiles compared to birds or mammals. Phenotypic manipulation in a Mediterranean lizard (*Psammodromus algirus*) showed variations in ectoparasite load and haematological parameters with testosterone (Salvador et al., '96; Puerta et al., '97; Salvador et al., '97; Veiga et al., '98). Experimentally elevated testosterone affected ectoparasite loads also in male sand lizards (*Lacerta agilis,* Olsson et al., 2000) and in free-ranging northern fence lizards (*Sceloporus undulatus hyacinthinus,* Klukowski and Nelson, 2001). The effect of testosterone on experimentally activated T cell-mediated immune response, however, remains poorly examined in lizards. In this study we investigated the ability to mount a T-lymphocyte cell-mediated immune response when testosterone was increased in males of two species of Mediterranean lacertid lizards, *Acanthodactylus erythrurus* and *Psammodromus algirus.*

MATERIALS AND METHODS

The study was carried out in May-June 1998 on males from two Mediterranean lizard species, *Psammodromus algirus* and *Acanthodactylus erythrurus* (medium-sized lizards of adult snout-vent up to 82 mm). Individuals were captured at representative areas of their typical environment. Although sharing part of their distributional ranges in the Iberian Peninsula, *Psammodromus algirus* is more dependent on dense vegetation cover (Mellado, '80) than *Acanthodactylus erythrurus.* Males of *P. algirus* (snout-vent length 64.31 $\pm$ 8.86 mm; n = 29) were captured in a deciduous oak forest in Madrid, central Spain. Males of *A. erythrurus* (snout-vent length 66.59 $\pm$ 8.12 mm; n = 34) were captured in a dune field with sparsely distributed vegetation in Alicante, eastern Spain. Captures took place at the end of May. Individuals were kept in the laboratory in individual terrarium. To facilitate thermoregulation, they were maintained under incandescent illumination 6 hours a day. They were offered water and *Pyralis* larvae *ad libitum*. After two weeks of acclimatization, the experiment started.

Males were assigned to experimental or control treatment randomly. A total of 17 males of *Acanthodactylus erythrurus* and 14 males of *Psammodromus algirus* were experimentally testosterone-increased, although sample sizes change in the analysis due to unsuccessful testosterone level or immune response measurements. We checked in both species that there were not differences in body size of each category of individuals ($p > 0.05$ in both cases).

Hormonal treatment

Circulating levels of testosterone were manipulated using a non-invasive method for sustained elevation of steroid hormone levels in reptiles modified from the procedure described by Knapp and Moore ('97). Through this method, steroid hormones are transdermally delivered to the lizards. The high concentration of lipids in the lizard skin (Mason, '92) allow lipophilic molecules to pass through the scales of the integument into the blood stream. Testosterone was transdermally delivered to the lizards in a mixture of the steroid hormone and sesame oil. We did not use dermal adhesive bandages as done by Knapp and Moore ('97) to decrease the risk of stress associated with the application, presence, and removal of a patch. This technique allows for a less invasive means of elevating testosterone levels than implants.

We diluted testosterone (Sigma T1500) in commercial sesame oil (4.5 u g testosterone / 1 u l sesame oil). Experimental males of both species received 4.5 u l of the hormone dilution every two days during three weeks, while control males were delivered the same amount of sesame oil as

a placebo. The dilution and control oil were placed on the dorsal side of each treatment male with a pipet for accurate delivery. The oil was immediately absorbed into the integument so that no excess oil remained on the surface of the scales before we placed the males back into their terraria. At the end of treatment, blood samples were obtained from experimental males so that hormone levels could be measured by radioimmunoassay.

Immunocompetence test

We evaluated T cell-mediated immunity using a delayed cutaneous hypersensitivity response (Roitt et al., '89; Benjamini and Leskowitz, '91; Saino et al., '97; Sorci et al., '97; Gonzalez et al., '99). This response is a measure of T-cell proliferation, and is assessed by subcutaneously injecting a mitogen and measuring the swelling that occurs within 24h (Benjamini and Leskowitz, '91). Larger localized swelling indicates a more robust immune response.

T-cell response was assessed using the following assay: thickness of the left and right foot were measured (in a central point marked with a pen in the ventral face) using a spessimeter (cod. SM112, Alpa S.p.A., Milan, Italy), with an accuracy of 0.01 mm. The right foot was then injected at the marked point with 0.03 ml of a solution containing 50 mg of phytohaemagglutinin (PHA; Sigma, L-8754) in 10 ml phosphate buffered saline (PBS); the left foot was injected with 0.03 ml of PBS. Twenty-four hours later we again measured the thickness of both feet at the marked inoculation sites. Reaction to PHA was expressed as the difference between the change in thickness of the right PHA-inoculated foot (thickness 24 h after inoculation minus thickness just before inoculation) and the change in thickness of the left foot (again, thickness 24 h after inoculation minus thickness just before inoculation).

Blood collection

Blood samples were obtained four days after the hormone treatment, two days after the immunocompetence test. Blood was collected from the ocular arcs and was drawn into heparinized microhematocrit tubes that were sealed at one end with putty and centrifugated. The resulting plasma was collected into microcentrifuge tubes and measured to the nearest microliter. Plasma samples were stored at -20°C until they were shipped on dry ice to Rutgers University, where testosterone levels were measured.

Radioimmunoassays

Plasma levels of testosterone were measured using published radioimmunoassay (RIA) procedures (Wingfield and Farner, '75; Moore, '86; Ball and Wingfield, '87; Smith and John-Alder, '99). Extracted samples were dried under stream of unfiltered air, and steroids were separated via Celite (Sigma) chromatography after samples had been reconstituted in 10% ethyl acetate: 90% isooctane (v/v). Recovery of testosterone was measured for each sample. After chromatographic separation, samples were dried, reconstituted in assay buffer, and allowed to equilibrate overnight before RIAs.

We assayed testosterone using tritiated-labeled hormones (Dupont, New England Nuclear, Wilmington, Del.) and antisera from Wien Laboratories (Succasunna, N.J.). Intra-assay variation was 11% and inter-assay variation was 13%.

Data analysis

We used ANOVA to investigate the effect of hormonal treatment on the immune response. All variables supported normality and ANOVA assumptions with the exception of testosterone level, that was log-transformed. Analysis were done using STATISTICA statistical package.

RESULTS

Hormone analysis revealed that treatment significantly elevated plasma testosterone concentrations in the experimental individuals of *Acanthodactylus erythrurus* ($F_{1,29}=7{,}74$, $p<0.0001$; Fig. 1a) and *Psammodromus algirus* ($F_{1,22}=96.67$, $p<0.0001$; Figure 1b). The level of testosterone was similar in both species ($F_{1,25}=0.20$, $p=0.66$) and was not correlated with body weight or size of the individuals ($p>0.05$ in both cases).

Swelling reaction was positively correlated with body size ($r=0.29$, $p=0.025$, $n=58$) and therefore body size was considered as a covariate in the analyses.

T cell-mediated responses to phytohemagglutinin stimulation were significantly reduced in testosterone-treated males compared to controls in *Acanthodactylus erythrurus* (ANCOVA with body size as a covariante: $F_{1,30}=10.14$, $p=0.0034$; Fig. 2a) and *Psammodromus algirus* (ANCOVA

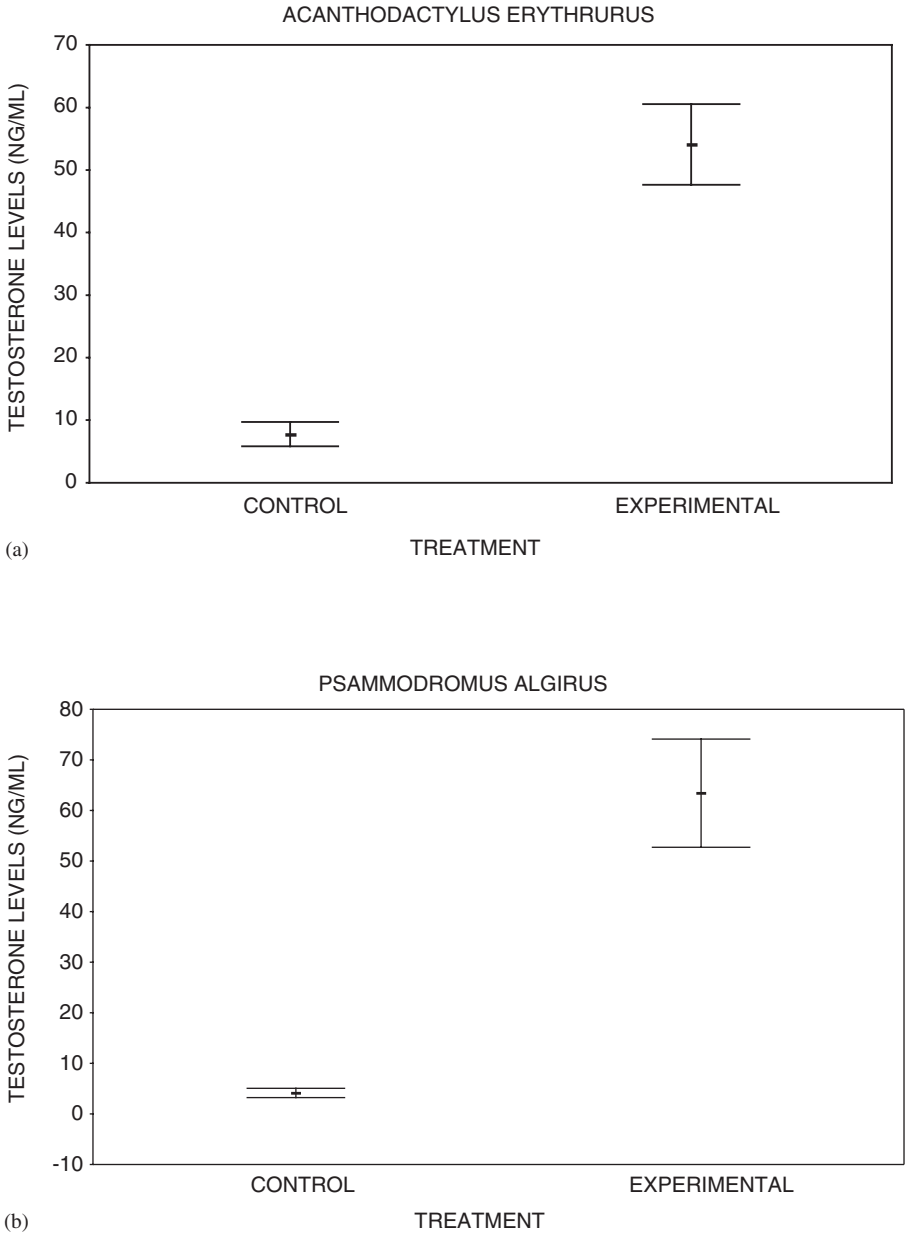

Fig. 1. Mean and Standard Error Blood Testosterone Levels (ng/ml) for males of (a) *Acanthodactylus erythrurus:* Control (N=16) and Experimental (N=15), and (b) *Psammodromus algirus:* Control (N=12) and Experimental (N=12).

with body mass as a covariante: $F_{1,22}=9.90$, $p=0.0047$; Fig. 2b).

Closer examination of the individual concentrations of testosterone indicated variability within each species. Therefore, a regression was performed for each species to investigate whether the individual variability in testosterone concentrations could predict individual variability in T cell-mediated responses to phytohemagglutinin stimulation. Body size was included in the model. A significant negative correlation was observed between plasma testosterone concentrations and T cell-mediated response in *Acanthodactylus erythrurus* ($F_{2,27}=5.35$, $p=0.011$, $R=0.28$; Fig. 3a), where the effect of testosterone levels was marginally significant ($p=0.055$) and body size positively and significantly entered in the model ($p=0.006$), and *Psammodromus algirus* ($F_{1,19}=7.94$, $p=0.011$, $R=0.26$; Fig. 3b), where testosterone level significantly entered in the model ($p=0.011$).

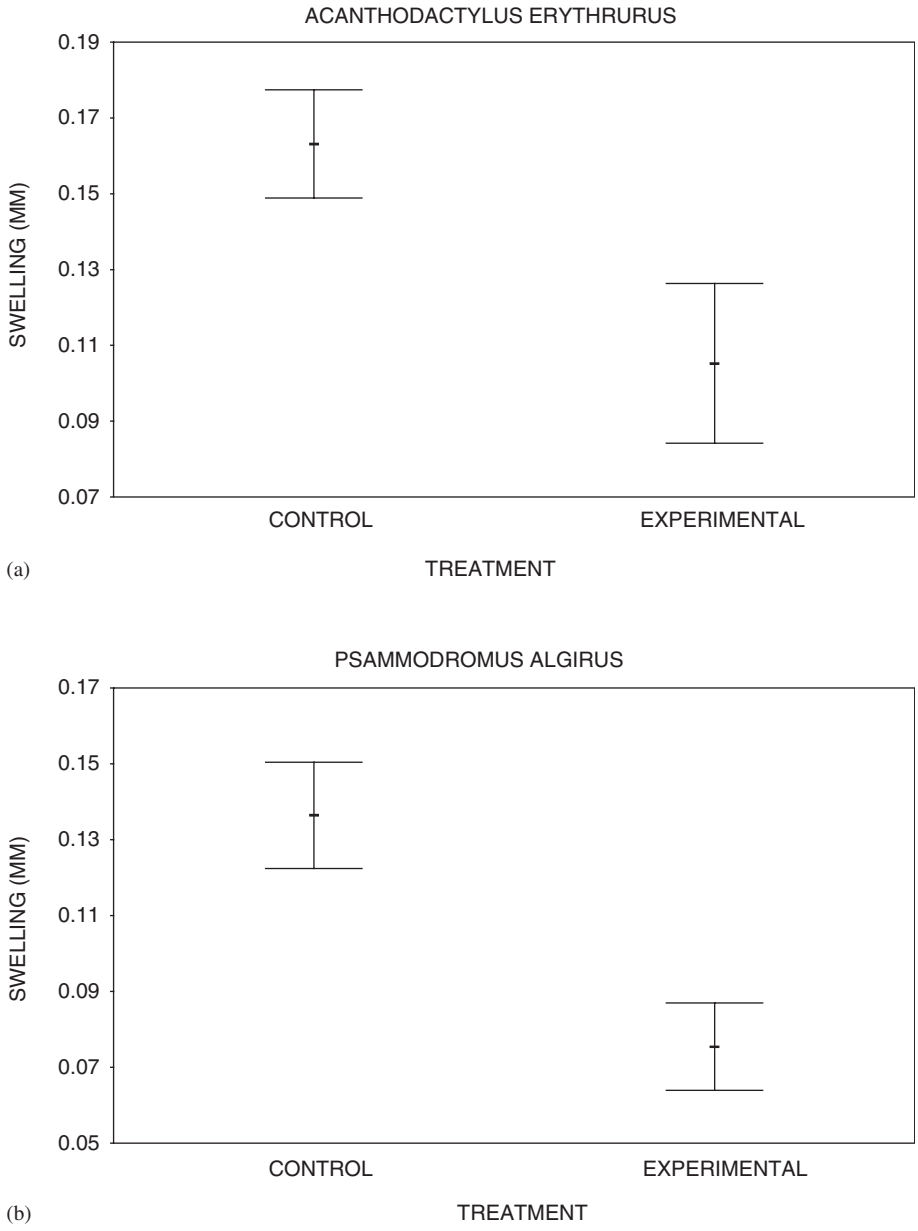

Fig. 2. Mean and Standard Error Swelling (mm) in males of (a) *Acanthodactylus erythrurus:* Control (N=16) and Experimental (N=17), and (b) *Psammodromus algirus* Control (N=14) and Experimental (N=11).

DISCUSSION

The limits of the normal physiological range of testosterone levels for adult breeding males of *Acanthodactylus erythrurus* and *Psammodromus algirus* have not been measured. The T levels raised in the study are high but are in the physiological range known to occur in nature in lizards (Smith and John-Alder, unpublished). Measurements in nature for *Anolis sagrei* revealed a peak around 25 ng/ml testosterone during the breeding season (Tokarz et al., '98). Considering that they are proximately half the body mass of the species studied here, we assume that hormone treatment raised testosterone concentrations to within the probable physiological range for the species.

T cell-mediated immune response decreased when testosterone levels were increased in males of both lizard species. Therefore, these species of lizards may not be able to maintain an optimal T-lymphocyte immune system during the breeding season. This result is consistent with the immunocompetence handicap hypothesis (Folstad and

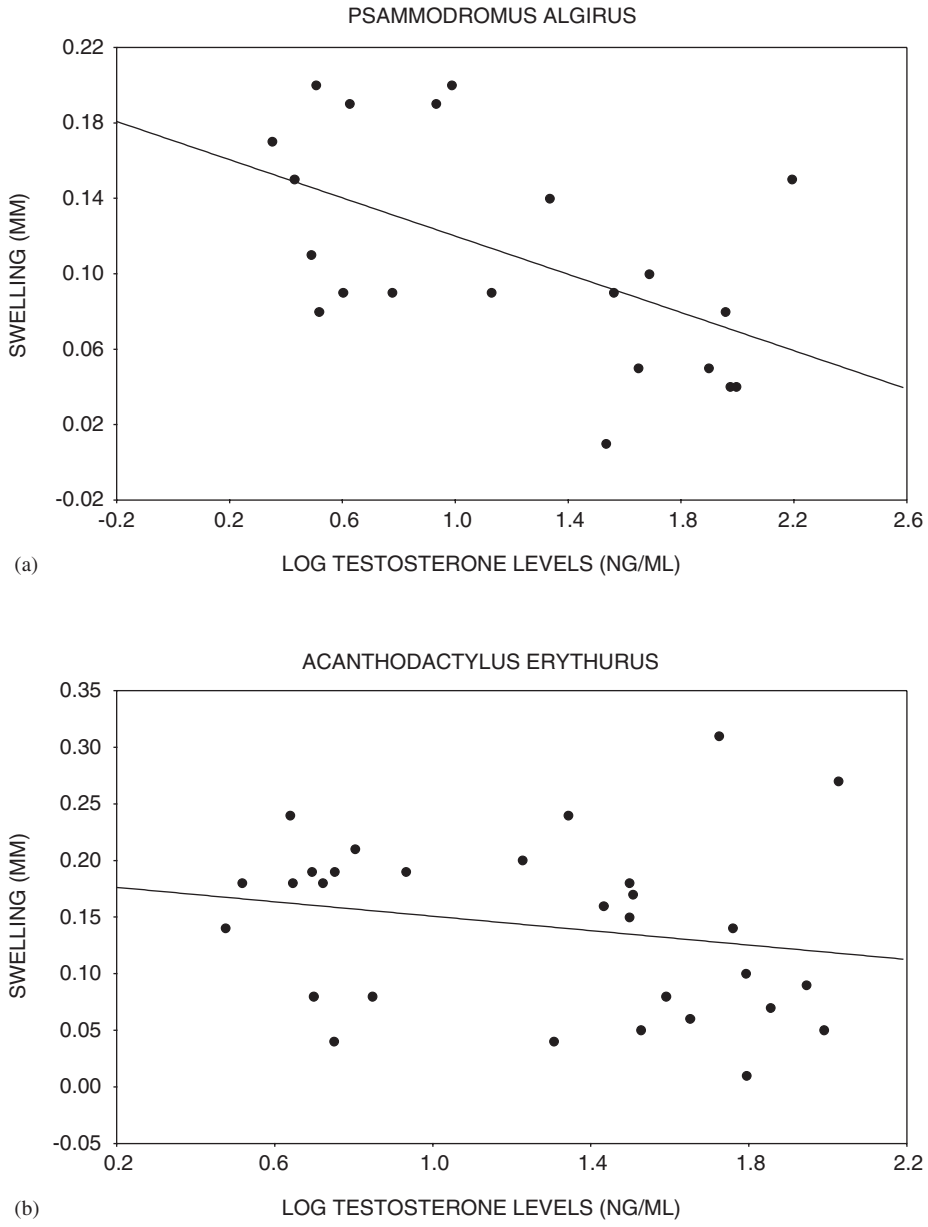

Fig. 3. Swelling values (mm) in relation to plasma testosterone levels (ng/ml) in (a) *Acanthodactylus erythrurus* (N=30) and (b) *Psammodromus algirus* (N=21). The linear regression line for *Psammodromus algirus* has the equation $y=0.17-0.05x$; r2=0.26, p=0.011.

Karter, '92), and with previous results about testosterone-induced suppression of white blood cells in lizards (Veiga et al., '98).

Phenotypic manipulations using testosterone to evaluate the trade-offs between reproductive investment and the immune response have been rarely performed in reptiles compared to other groups like birds. In previous studies with *Psammodromus algirus,* males were implanted with subcutaneous testosterone to physiologically increase reproductive investment (Salvador et al., '96; Veiga et al., '98). In *P. algirus*, testosterone stimulates the production of nuptial coloration and induces more aggressive behavior during the reproductive season suggesting that increased testosterone levels do increase the likelihood of greater reproductive investment (Salvador et al., '96). In addition, this increase in reproductive effort induced by testosterone determined an increase in ectoparasite load (Salvador et al., '96) and a reduction in the total number of white blood cells, mainly limphocytes (Veiga et al., '98), which

may evidence immunosuppression (Barnes, '86). These studies, however, lack of a final measure of testosterone plasma levels to verify the effectiveness of their treatment. In this study, we verify the effectiveness of our testosterone supplementation and although in captivity we show a distinct cause and effect relationship between elevated testosterone levels and immunosuppresion. Less is known about the effect of testosterone in *Acanthodactylus erythrurs* where, to our knowledge, testosterone manipulations have not been made.

The development of a meaningful assessment of immunity in the context of animals in the wild is still in its infancy, although of increasing interest to evolutionary biologists (Zuk, '94; Sheldon and Verhulst, '96; Schmid-Hempel, 2003). It has been stressed that it can be misleading to use a single aspect of immune response in evaluating immunocompetence (Zuk and Johnsen, '98), as different parts of the immune system may be important under different circumstances and may offer different responses to a single factor (Roitt et al., '89; Westerman and Pabst, '90; Siva-Jothy, '95). However, the results of our study complement the scarce existing information about the suppressive effect of testosterone on the reptile immune system (Veiga et al., '98).

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