Effect of testosterone on immunocompetence, parasite load, and metabolism in the common wall lizard (*Podarcis muralis*)

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Abstract: Testosterone can benefit individual fitness by increasing ornament colour, aggressiveness, and sperm quality, but it can also impose both metabolic and immunological costs. However, evidence that testosterone causes immunosuppression in freely living populations is scant. We studied the effects of testosterone on one component of the immune system (i.e., the cell-mediated response to phytohaemagglutinin), parasite load, and metabolic rate in the common wall lizard, *Podarcis muralis* (Laurenti, 1768). For analyses of immunocompetence and parasitism, male lizards were implanted at the end of the breeding season with either empty or testosterone implants and were returned to their site of capture for 5–6 weeks before recapture. For analyses of the effects of testosterone on metabolic rate, male lizards were captured and implanted before hibernation and were held in the laboratory for 1 week prior to calorimetry. Experimental treatment with testosterone decreased the cell-mediated response to the T-cell mitogen phytohemagglutinin and increased mean metabolic rate. No effects of testosterone on the number of ectoparasites, hemoparasites, and resting metabolic rate could be detected. These results are discussed in the framework of the immunocompetence handicap hypothesis and the immuno-redistribution process hypothesis.

Résumé : La testostérone peut avantager la fitness d'un individu en augmentant l'intensité des couleurs des caractères sexuels secondaires, l'agressivité et la qualité du sperme, mais elle peut aussi imposer des coûts métaboliques et immunologiques. Cependant, les preuves que la testostérone provoque une immunosupression sont rares. Nous avons étudié les effets de la testostérone sur la réaction cellulaire à la phytohémagglutinine (une composante du système immunitaire), sur la charge parasitaire et sur le taux de métabolisme du lézard des murailles, *Podarcis muralis* (Laurenti, 1768). Pour les analyses d'immunocompétence et de parasitisme, les lézards mâles ont été implantés, sur leur site, à la fin de la période de copulation soit avec un implant de testostérone, soit avec un implant vide; ils ont été recapturés 5 à 6 semaines plus tard. Pour les expériences sur les effets de la testostérone sur le taux de métabolisme, les lézards ont été capturés et implantés avant l'hibernation et maintenus en laboratoire durant une semaine avant les mesures calorimétriques. Le traitement expérimental avec la testostérone diminue la réponse immunologique cellulaire à la phytohémagglutinine, un mitogène pour les cellules T, et augmente le taux de métabolisme moyen. Aucun effet de la testostérone sur le nombre d'ectoparasites et de parasites sanguins ni sur le taux de métabolisme au repos n'a pu être mis en évidence. Ces résultats sont discutés dans le contexte de l'hypothèse du handicape de l'immunosupression et de l'hypothèse du processus d'immuno-redistribution.

Introduction

The endocrine and immune systems interact in ways that are crucial for reproductive success. Through testosterone and other androgenic steroids in males, the endocrine system controls the development and expression of ornamentation and behavioural displays in addition to its obligate role in sperm production (Zeller 1971; Stokkan 1979; Wingfield et al. 1990; Ketterson and Nolan 1992), and in seasonal breeders, reproductive effort is generally positively associated with circulating testosterone. High levels of testosterone, therefore, potentially bring fitness benefits in terms of mating success. For its part, the immune system defends against parasites and disease, and parasitic infection is known to adversely affect male ornamentation, behavioural displays, and sperm quality (Baudoin 1975; Kennedy et al. 1987; Zuk et al. 1990; Liljedal et al. 1999).

Despite its clear reproductive benefits, high levels of testosterone have been associated with several adverse effects in males. First, testosterone can increase metabolic rate, resulting in mass loss and a decrease in survival rate (Marler and Moore 1988; Abelenda et al. 1992; Marler et al. 1995; Buchanan et al. 2001). Second, it can suppress the immune system, reduce immunocompetence, and thus, increase susceptibility to parasites (Zuk 1990, 1996; Folstad and Karter

Received 8 April 2004. Accepted 25 October 2004. Published on the NRC Research Press Web site at http://cjz.nrc.ca on 27 January 2005.

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 doi: 10.1139/Z04-152

1992; Wedekind and Folstad 1994). The general significance of the immunosuppressive effects of testosterone in freely living populations of animals, however, is unclear because of the lack of strong evidence for an immunosuppressive effect of testosterone (Hillgarth et al. 1997; Hasselquist et al. 1999; Evans et al. 2000; Westneat et al. 2003) and because of the possible indirect effects of testosterone on behaviour that cause increased morbidity and mortality in males of many species (Zuk 1990). Moreover, there are some studies showing that immunity is positively correlated with testosterone (Dunlap and Schall 1995; Zuk et al. 1995; Klein and Nelson 1998), and contrary to expectation, McCurdy et al. (1998) found evidence of female-biased parasitism in an analysis of published avian data. Indeed, functional relationships and the directions of causality involving testosterone, immunocompetence, parasitism, metabolic rate, and several components of fitness are very intricate. The elucidation of clear causality requires experimental manipulation.

The aim of the present study is to investigate the effect of experimentally manipulated testosterone on one component of immunocompetence in the freely living male common wall lizard, *Podarcis muralis* (Laurenti, 1768), by measuring the cell-mediated immune response, hemo- and ecto-parasite loads, and oxygen consumption at rest.

Materials and methods

Species and study sites

The common wall lizard is a widely distributed, small (snout–vent length (SVL) of adults 48–67 mm) lacertid inhabiting dry, sunny, and stony habitats. For the immunocompetence experiment, 59 adult males were collected in early April 2001 at the end of the mating period from a population (P1) located in Rivaz (Switzerland) along a rocky bank of the Léman Lake. The area of capture was artificially delimited to a band 100 m × 5 m, and the population under study was therefore surrounded by other suitable habitat. As we have not measured the effect of testosterone on mating behaviour or reproductive success, we chose to manipulate testosterone in males after the mating period to avoid interfering with reproduction. It is possible that testosterone implantation after the mating period produces different results than during the reproductive season. For studies on oxygen consumption, 53 adult males were collected in early September from a population (P2) occurring along a 100-m dry-fit retaining wall separating two parts of a vineyard in Echandens (Switzerland). Lizards were caught with a slip noose on the end of a fishing rod. At the time of capture, body mass and SVL were measured for each lizard, and all mites attached under the ventral scales were counted. Lizards were toe-clipped for individual identification and a drop of blood was collected for the analyses of initial blood parasite load.

Hormonal treatment

At the time of capture, alternate males were assigned to either the testosterone or the control group for subsequent implantation with Silastic® tubules. Prior to implantation, lizards were cold-paralysed in a cold box containing ice. The left side of the body was disinfected with 70% alcohol and a 3-mm incision was made between two scales. Silastic® implants (total length 3 mm, inner diameter 1.39 mm, outer diameter 1.85 mm) were inserted into the peritoneal cavity through the incision and the surgical wound was then closed with surgical glue (Nexaband®; Veterinary Products Laboratories, Phoenix, Arizona). Implants were filled with either crystalline testosterone for the testosterone group (29 lizards in April and 10 in September) or left empty for the control group (30 lizards in April and 10 in September). Tubules were sealed with silicone gel (NuSil Technology, Carpinteria, California), leaving an enclosed chamber 1.0–1.5 mm long from which the testosterone diffused. In April, lizards were implanted and released the same day at their site of capture; in September, all lizards were kept in the laboratory for 1 week to measure oxygen consumption.

Blood samples were not taken for measurements of plasma hormones in intact or experimental lizards. However, in *Podarcis sicula sicula* (Rafinesque, 1810), plasma testosterone varies from about 85 ng/mL prior to reproduction to 30 ng/mL during the mating period to 15 ng/mL during the post-mating period (Manzo et al. 1994). During in vitro incubations, testosterone diffuses from the type of Silastic® implant used here at an initial rate of about 30 ng/h, and this rate declines exponentially over a period of many months until the implants are depleted (H.B. John-Alder, unpublished data). (In a field experiment on *Sceloporus undulates* (Bosc and Daudin in Sonnini and Latreille, 1801), testosterone continued to diffuse at a rate of about 1 ng/h after 420 days of implantation (H.B. John-Alder, unpublished data).) In experiments on *S. undulatus* and *Sceloporus virgatus* Smith, 1938, which are similar in size to common wall lizards, we have consistently induced experimental levels of plasma testosterone between 25 and 40 ng/mL using Silastic® implants identical to those used here (H.B. John-Alder, unpublished data). Thus, induced levels of plasma testosterone in the present experiments were in all likelihood within the normal physiological range of natural variation found in common wall lizards.

Immunocompetence (T-cell response)

Five to 6 weeks after implantation (in May), 12 males in the testosterone group and 22 males in the control group were recaptured. We again measured body mass, counted the number of ectoparasites, and collected a drop of blood to determine hemoparasite load for each male. Subsequently, 12 males in the testosterone group and 19 males in the control group were taken into the laboratory to measure their response to phythohemagglutinin (PHA). Three males in the control group were released at the capture site because we did not have sufficient terrarium space in the laboratory to keep all captured lizards. Immunocompetence was measured as the in vivo T-lymphocyte-mediated response to a 20-μL injection of a suspension of PHA (PHA-P, Sigma Chemical Co., St. Louis, Missouri; 30 mg of PHA in 5 mL of 1× phosphate-buffered saline) in the foot pad. PHA has a mitogenic effect on T-lymphocytes, and the injection stimulates macrophage infiltration and dense perivascular accumulation of lymphocytes (Stadecker et al. 1977; Goto et al. 1978). The response to PHA has been shown to be a reliable indicator of one component of immunocompetence (see Lochmiller et al. 1993). Control injections were not made in the contralateral foot pad because this kind of control test

has been shown to be uninformative (Smits et al. 1999). The thickness of the foot pad at the inoculation point was measured with a digital calliper to the nearest 0.01 mm both before and 24-h after the injection. The change in thickness of the foot pad was used as the measure of immunocompetence (Christe et al. 1998). Measurements were made in triplicate, and the mean was used in the analyses.

Hemoparasite count

The smear collected during toe-clipping was subsequently fixed in methanol and stained with May-Grünwald Giemsa (Colorap de Bioréac, Lausanne, Switzerland). Stained slides were examined using oil immersion microscopy (630× magnification) and blood parasites were counted. Hemogregarine parasites are naturally widespread in common wall lizards. This protozoan (phylum Sporozoa) has a complex life cycle that involves a microscopic blood-feeding mite vector (Manwell 1977). Parasites are usually intra-erythrocytic (Manwell 1977), but free forms can be observed in severe infections (A. Oppliger, personal observation). The genus of hemogregarine was determined by the morphology of gametocytes found in red blood cells (Manwell 1977). Parasitaemia was estimated by counting the number of parasites observed per 10 000 red blood cells. A slide was considered negative when no parasites were observed after 5 min of searching (approximately 300 fields of 400 cells/field).

Energetics

Experimental conditions

Twenty males were assigned randomly to one of the two hormonal treatments (10 males in the testosterone group and 10 males in the control group; see above for details). They were kept in individual terraria (20 cm × 15 cm × 20 cm) with a substrate of sandy soil and shelters for refuge. Water and small crickets, *Acheta domesticus* (L., 1758), were provided ad libitum. A heat lamp provided warmth 6 h/day, allowing the lizards to thermoregulate. One week later, after having controlled for any body mass difference owing to hormonal treatment ($F_{[1,16]} = 1.35$, $p = 0.26$), oxygen consumption was measured.

Rates of oxygen consumption could be measured in two lizards simultaneously. They were placed in separate respirometers (see below), which prevented all contact (visual, olfactory, auditory) between lizards. Measurements were made in the near total absence of light and sound. Each measurement of oxygen consumption lasted 3.0 ± 0.1 h (mean ± SD), during which lizards had no access to food or water. Data recorded during the first hour of each experiment was excluded from the analysis, as it often corresponded to a period of stress resulting from the entry into the metabolic chamber. Body mass of each lizard was measured both before and after measurements of oxygen consumption. Because there was no significant difference between these measurements (Wilcoxon's signed rank test, $V = 45$, $N = 18$, $p = 0.99$), body mass prior to measurement was used in the analyses.

Measurement of oxygen consumption

Oxygen consumption was measured using an open-flow respirometer (0.4 L air volume) containing a small plastic shelter (reassuring effect). A lizard was placed inside and the chamber was then immersed in a thermoregulated water bath at 18 ± 0.1 °C. This temperature was chosen because it represented the mean daily temperature in summer. Outside air was dried over silica gel and pushed through the two metabolic chambers at a flow rate of approximately 115 mL/min. The flow rate was controlled and measured continuously by a calibrated mass flow controller (model 5850E; Brooks Instruments, Veenendaal, the Netherlands) connected to a controller (model 5878, Brooks Instruments). The effluent air was sequentially passed through a KOH column (to remove the expired CO_2) and a silica gel column. Finally, oxygen concentration was measured through an oxygen analyser (Gas Purity Analyser Xentra 4100; Servomex, Esslingen, Switzerland). The oxygen analyser was calibrated monthly using pure nitrogen gas (≈95%) and pure oxygen gas (≈95%).

The oxygen concentration was recorded on BioBench® version 1.0 (National Instruments Corporation 1997) via a Rack-Mount BNC 2090 accessory (National Instruments Corporation, Austin, Texas) and a PCI-MIO-16XE-50 data acquisition card (National Instruments Corporation). It allowed us to obtain precisely the minimum (equivalent to resting metabolic rate, RMR) and mean oxygen concentrations in the effluent air. Rates of oxygen consumption (mL O_2/(g·h)) were calculated following Depocas and Hart (1957).

Results

Effect of testosterone on recapture rate and body mass

In the immunosuppression experiment involving population P1, body mass and SVL did not differ between treatment groups at the beginning of the experiment, and the difference between initial and final body mass did not differ between groups (Table 1). The number of days between implantation and recapture did not differ between treatment groups (testosterone group: 44.9 ± 4.9 days; control group: 46.0 ± 3.7 days; $t_{[32]} = 0.742$, $p = 0.464$). However, significantly fewer males from the testosterone group than from the control group were recaptured ($p = 0.01$; Table 1).

Effect of testosterone on immune system and parasite loads

Testosterone implantation had a significant adverse effect on response to PHA but had no demonstrable effect on hemoparasites or ectoparasites. On average, PHA injection caused nearly twice as much thickening of the foot pad in males from the control group than from the testosterone group (ANCOVA hormonal treatment factor: $F_{[1,28]} = 9.2$, $p = 0.005$; mass covariate factor: $F_{[1,28]} = 0.001$, $p = 0.98$; Table 2). Changes in parasite loads between the beginning and end of the experiment did not differ significantly between treatment groups (hemogregarine: $t_{[32]} = -0.351$, $p = 0.728$; mites: $t_{[32]} = 0.427$, $p = 0.672$; Table 2). However, the number of hemoparasites for the two groups was significantly greater in May than in April ($Z_{[33]} = 2.30$, $p = 0.02$; Fig. 1), although the number of ectoparasites was not significantly different between April and May (0.41 ±

Table 1. Body-size parameters and recapture rate of control and testosterone-implanted common wall lizards (*Podarcis muralis*) used in the immunocompetence experiment.

	Initial snout–vent length (mm)			Initial mass (g)			Change in mass (g)			Recapture rate		
	Mean ± SE	*n*	*p**	Mean ± SE	*n*	*p**	Mean ± SE	*n*	*p**	Ratio	%	*p*†
Control	63.77±3.70	22	0.252	6.18±1.09	22	0.194	0.04±1.66	22	0.264	22/30	73	0.013
Testosterone	64.54±3.53	12		6.75±1.41	12		–0.52±0.55	12		12/29	41	

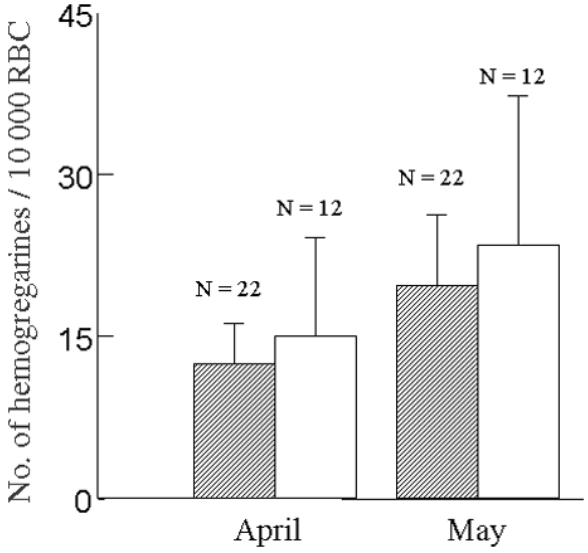
*p values from Student's t test.
†$\chi^2_{[1]} = 6.166$.

Table 2. Immune response to injection of phythohemagglutinin and changes in parasite loads in control and testosterone-implanted common wall lizards.

	Thickening of foot pad (mm)			Change in hemoparasite load (no. of hemogregarines / 10 000 RBC)			Change in ectoparasite load (no. of mites/lizard)		
	Mean ± SE	*n*	*p*	Mean ± SE	*n*	*p*	Mean ± SE	*n*	*p*
Control	0.047±0.005	19	0.005	8.54±3.89	22	0.728	0.16±0.24	22	0.672
Testosterone	0.023±0.006	12		6.85±2.85	12		0.36±0.31	12	

Note: p values < 0.05 are significant.

Fig. 1. Mean (+SE) number of hemogregarines per 10 000 red blood cells (RBC) observed in April (before hormonal treatment) and May (6 weeks after hormonal treatment) in the common wall lizard (*Podarcis muralis*) experimentally implanted with testosterone (open bars) or with empty implants (hatched bars). Numbers above bars indicate sample sizes.

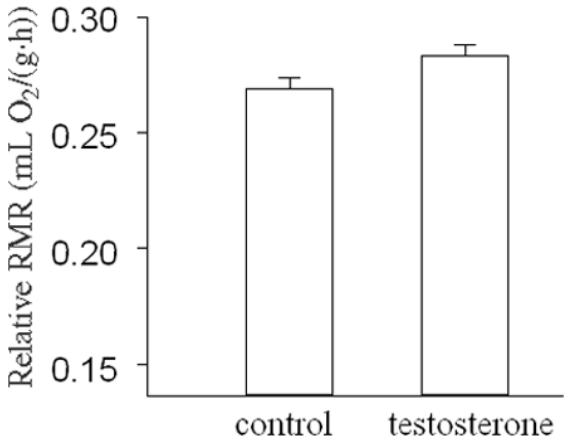


0.57 mites/lizard in April versus 0.7 ± 0.26 mites/lizard in May; $Z_{[33]} = 0.47$, $p = 0.63$).

Effect of testosterone on oxygen consumption

The minimum rate of oxygen consumption (RMR) did not differ between treatment groups ($F_{[1,16]} = 0.15$, $p = 0.71$; Fig. 2). However, the rate of O_2 consumption, on average, was about 50% higher in males from the testosterone group than from the control group ($F_{[1,16]} = 6.99$, $p = 0.01$; Fig. 3). The rate of O_2 consumption was 51.4 ± 14.2% greater than the RMR in the control group and 100.3 ± 15.2% greater than the RMR in the testosterone group. Thus, metabolic rates did not differ; however, mobility rates did, which resulted in the higher mean metabolic rate in the testosterone group.

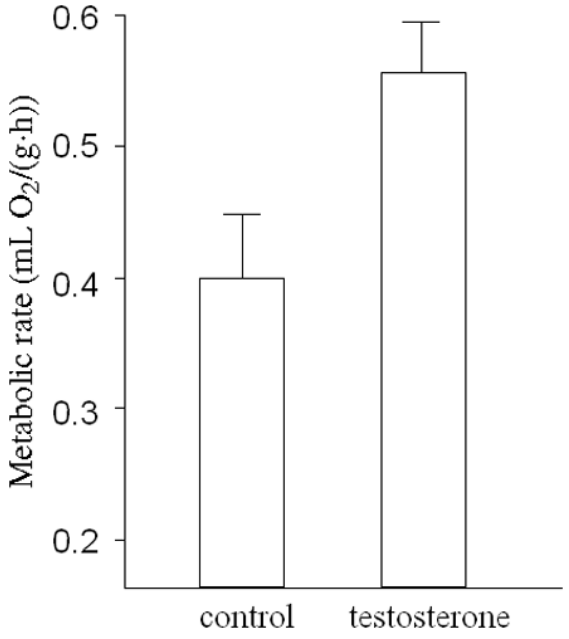
Fig. 2. Mean (+SE) relative resting metabolic rate (RMR) for common wall lizards implanted with testosterone or with empty implants. Sample size for each group is 9.



Discussion

The immunocompetence handicap hypothesis (ICH) (Folstad and Karter 1992) postulates that testosterone has the dual and conflicting effects of stimulating the expression of sexually selected traits while reducing immunocompetence. A direct prediction of ICH is that testosterone levels in freely ranging animals should be correlated with parasite loads. Tests of ICH as it applies to lizards have focused mainly on ectoparasitism (e.g., Salvador et al. 1996; 1997; Klukowski and Nelson 2001; Olsson et al. 2000) and rarely on immune function and immunocompetence per se (Veiga et al. 1998). Thus, no study has presented sufficient evidence to fully evaluate ICH and its alternatives (e.g., Braude et al. 1999), and apparent discrepancies among studies cannot presently be reconciled. Results of the present study indicate that testosterone reduces one component of immunocompetence without affecting parasite load in freely

Fig. 3. Mean (+SE) metabolic rate for common wall lizards implanted with testosterone or with empty implants. Sample size for each group is 9.



ranging common wall lizards. These results neither fully support nor fully refute ICH but indicate that alternative hypotheses must be taken into consideration.

The cell-mediated immune response to PHA injection showed, for the first time in a lizard species, the negative effect of testosterone. This technique is considered a useful method of evaluating thymus-dependent immune function (Goto et al. 1978). It is easily applied in the field and does not provoke potential confounding effects associated with physiological stress (Merino et al. 1999). However, this simplistic method does not measure the numerous components of the immune system (Norris and Evans 2000) and thus cannot fairly differentiate between changes in immunocompetence versus immuno-redistribution (Braude et al. 1999), which suggests that testosterone causes white blood cells to be temporarily shunted to different compartments where they are likely to be more useful for countering antigens. The importance of this distinction becomes evident in a review of the literature. Several laboratory studies in different animal species have reported that testosterone can decrease immunocompetence (Grossman 1985; Alexander and Stimson 1988; Saad and Shoukrey 1988; Slater and Schreck 1993; Arnold and Holt 1995; Klukowski and Nelson 2001). However, a field study (Hasselquist et al. 1999) reported no relationship between plasma testosterone and humoral immunocompetence in freely ranging males of red-winged blackbirds, *Agelaius phoeniceus* (L., 1766). Furthermore, McCurdy et al. (1998) unexpectedly found evidence of female-biased blood parasitism during the breeding season in an analysis of published avian data, which they suggested could have resulted from sex differences in exposure to parasite vectors or from estrogen-based effects on immunity. In the house sparrow (*Passer domesticus* (L., 1758)), manipulation of testosterone did not produce a cell-mediated response, but testosterone had significant effects upon the humoral response (Buchanan et al. 2003). Other field studies have shown that implantation of testosterone in the small lizard *Psammodromus algirus* (L., 1758) affects various blood parameters, especially the quantity of lymphocytes, which are the most important cells involved in immune defense (Puerta et al. 1996; Salvador et al. 1996; Veiga et al. 1998). In this case, the decrease in circulating lymphocytes in the blood could result equally well from an immuno-redistribution process (Braude et al. 1999), a reallocation of resources towards other functions with more direct influences on fitness (Wedekind and Folstad 1994), or as a direct immunosuppressive effect of testosterone as stipulated by the ICH (Folstad and Karter 1992).

Our results show that despite the purported negative effect of testosterone on response to PHA, we have not observed a significant effect on either endo- or ecto-parasite loads. However, our results must be interpreted with caution because our statistical analysis has low power to detect a difference between groups owing to the low numbers of mites observed. In a recent review (Roberts et al. 2004), a meta-analysis shows no effect of testosterone on direct measures of immunity, but it did increase ectoparasite abundance in several studies, particularly in reptiles. Recently, Buchanan et al. (2003) have also suggested the possibility of a trade-off between the different arms of the immune system. Thus, the reduced immune response in the heel of wall lizards without a general increase in parasite load could be due to the fact that immunocompetence level has not changed but that immune resources have been reallocated.

A study on *P. algirus* (Veiga et al. 1998) reported no significant effects of testosterone on parasite load, which is consistent with our findings. Other field studies have shown that testosterone increases parasite loads in lizard species, but it has not been possible to determine if increased parasitism was due to an immunosuppressive effect of testosterone or to an increased level of activity (Salvador et al. 1996; Olsson et al. 2000; Klukowski and Nelson 2001). Indeed, Marler and Moore (1989) and Denardo and Sinervo (1994) have demonstrated that testosterone increases the mean daily activity of Yarrow's spiny lizard (*Sceloporus jarrovii* Cope in Yarro, 1875), thereby increasing the probability of meeting parasites. Our results also indicate that testosterone increases the common wall lizard's activity, since the mean metabolic rate of common wall lizards with implants was increased relative to controls owing to an increase in activity. This result could explain the difference in recapture rate between males in the testosterone and control groups. Indeed the increased activity of males in the testosterone group can influence their mobility (Olsson et al. 2000), their mortality (Salvador et al. 1996), or can increase the dispersion of small males without territory (Hews 1993). In our case, we believe that testosterone has influenced the mobility of males, because in the spring after hibernation the recapture rate was not different for males in the testosterone group than for males in the control group (personal observation).

Our results show that the increased activity of lizards with testosterone has no significant effect on body mass. Two recent studies on the lizard *S. undulatus* (Klukowski and Nelson 2001) and on *Lacerta agilis* L., 1758 (Olsson et al. 2000) have shown that testosterone has a negative impact on body mass. However, in these two studies, males with testos-

terone suffered an increase in parasite load in association with mass loss. Thus, it is impossible to differentiate among increased parasitism, increased activity, and possibly decreased feeding rate as potential causes for the loss in body mass.

In conclusion, we have shown that in the common wall lizard testosterone has a localized immunosuppressive effect on the cell-mediated immune response. However, our results cannot clearly distinguish between evidence for the immunocompetence hypothesis versus a trade-off between the different components of the immune system. Future investigations must incorporate comprehensive analyses of various components of the immune system (acquired, humoral, and innate immunities) together with an assessment of the effects of testosterone on parasite load.

Acknowledgements

We thank Philippe Christe and Jérôme Goudet for constructive comments on an earlier draft of the manuscript. This research was supported by the Swiss National Foundation (grant MHV:32-63171.00 to A.O.), the Société académique Vaudoise, the Foundation Dr. Joachim de Giacomi de l'ASSN to A.O., and by the National Science Foundation (IBN 0135167) to H.B.J.-A.

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