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Reproductive effort affects immune response and parasite infection in a lizard: a phenotypic manipulation using testosterone

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Life-history theory predicts that there is a trade-off between reproductive effort and several traits that determine fitness. Infectious disease has gained acceptance as a crucial factor linking both variables. In most instances phenotypic manipulation is necessary to convincingly demonstrate a causal relationship of reproductive effort on parasitism. However, experimental studies that manipulate reproductive effort or parasite load have been rarely conducted in reptiles. In this study, we manipulated reproductive effort of male lizards (*Psammodromus algirus*) through testosterone implants, and measured the associated response in some haematological variables and parasite load. Testosterone-supplemented males had lower scores than control males in factor 1 of a PCA for different blood parameters. This factor is correlated with the number of white blood cells, especially lymphocytes, and with plasma glucose levels. Experimental males also had higher scores in factor 3 that is mainly related to protein catabolism. Scores of males in component 1 tended to be correlated negatively with tick load, while scores in component 3 were correlated positively with the number of haemogregarines in the blood. These results suggest that higher investment in reproduction decreases the immune defences, and conduces to the use of structural resources, which may render individuals more susceptible to some haemoparasites. This is consistent with the idea that an increase in reproductive effort mediated by testosterone has a negative effect on the ability to counteract parasite infections.

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The existence of trade-offs between life-history traits is a central assumption in life-history theory (Williams 1966, Stearns 1992). Several studies have demonstrated that an increase in reproductive effort compromises offspring fitness, future reproduction or survival (reviewed by Lessells 1991, Stearns 1992). However, the specific ways in which physiological processes interact with ecological factors is still poorly understood. In recent years, infectious diseases have gained increased acceptance among evolutionary ecologists as crucial elements in establishing how variations in reproductive effort affect allocation of energy among different life-history traits (e.g., Hamilton and Zuk 1982, Folstad and Karter 1992, Gustafsson et al. 1994, Wedekind and Folstad 1994). 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

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sexual characters and can increase mobility, aggressiveness and sexual behaviour (reviewed by Ketterson and Nolan 1992). The energy costs associated with this increase of testosterone levels cannot, however, be compensated by additional food intake, as males implanted with testosterone decrease their food intake, and gain less weight than untreated controls (Gentry and Wade 1976, Marler and Moore 1988, Abelenda et al. 1992). It has been proposed recently that testosterone production is traded against immune defence, as this hormone directly deteriorates some white blood cell types or because it detracts resources that otherwise would be allocated to immune defence (Folstad and Karter 1992, Wedekind and Folstad 1994).

Because individuals differ in their abilities to allocate resources to reproduction, the use of phenotypic correlations between life-history traits usually renders ambiguous or contradictory results. In most instances phenotypic manipulation of one of the involved traits is necessary to convincingly demonstrate the existence of fitness costs associated with increased reproductive effort. The manipulation of clutch or brood size has been used successfully to demonstrate several costs of reproduction in birds (Gustafsson et al. 1994, Norris et al. 1994, Oppliger et al. 1996, Ots and Horak 1996). However, this procedure is not informative about life-history trade-offs in males that do not make a significant parental investment. Exogenous testosterone is an alternative method of manipulating reproductive effort that has been used in birds and reptiles (Marler and Moore 1988, Saino et al. 1995, Zuk et al. 1995). In lizards, this hormone stimulates aggressiveness (Moore and Marler 1987), activity (Marler and Moore 1988, 1991), which allows for the acquisition of larger and superior home ranges (Fox 1983), and leads to increased energy expenditure and more negative energy balance (Marler et al. 1995). Nevertheless, in most studies of parasitism and reproductive effort in reptiles, cause and effect are not clearly established (Telford 1970, Schall and Dearing 1987, Sorci et al. 1996), though experimental hormonal alterations and reproductive inhibition associated with malarial parasitism have been shown (Dunlap and Schall 1995).

In this study we experimentally manipulate the reproductive investment of *Psammodromus algirus* males by implanting them with subcutaneous testosterone and examine the relationships between hormone treatment, parasites and blood variables that give information on immune and metabolic responses. We have previously shown that, in *P. algirus*, testosterone stimulates the production of nuptial coloration and induces a more aggressive behaviour during the reproductive season (Salvador et al. 1996). A descriptive study on haematological variables of the same lizard population has been reported elsewhere (Puerta et al. 1996).

Methods

A field experiment with *Psammodromus algirus* lizards was conducted in a deciduous oak-forest (*Quercus pyrenaica*) near Navacerrada (40° 44′ N, 4° 00′ W), central Spain, during the 1994 mating season (April–May). From 14 to 20 March a 1.5-ha plot with markers on a grid every 10 m was established. Within this plot, 13 small adult males (70–75 mm snout-vent-length, SVL) and 29 large adult males (80–85 mm SVL) were captured by noosing between 21 and 29 of March, shortly after lizards emerged from hibernation. Individuals were weighed, SVL measured, and marked by toe-clipping. The number of ticks (*Ixodes ricinus*) present on each individual was also recorded (ticks were left in situ). Every second individual was assigned to a control (C-males, $n = 21$) and an experimental group (T-males, $n = 21$). Experimental and control males did not differ in SVL or weight when they were captured the first time. Both C- and T-males received a subcutaneous implant of Silastic tube (Dow Corning; 1.95 mm outer diameter; 1.47 mm inner diameter). Both ends of the tube were plugged with a wooden cap and sealed with Silastic adhesive. This ensures a fixed surface for testosterone diffusion, which allows a constant release of the hormone (Smith et al. 1977). Males were cold-anaesthetised and implanted through a small dorsal incision which was closed with a suture. T-males received an implant containing 5 mm (large males, 80–85 mm SVL) or 3 mm (small males, 70–75 mm SVL) of packed crystalline testosterone-propionate (Sigma Chemicals) while C-males received an empty implant of the corresponding size. The relation of testosterone amount to body weight was similar to that used in several studies in which no pharmacological effects were detected (Marler and Moore 1988). Between 1 and 4 h after capture, males were released within a 5-m radius of the capture site. C-males (large males, $n = 13$; small males, $n = 3$) and T-males (large males, $n = 9$; small males, $n = 3$) were recaptured in May to count ticks and take blood samples.

Blood was collected by cardiac puncture under ether anaesthesia using heparinised syringes (25 U/ml) 24 h after focal individuals were recaptured. Samples were analysed within 3 h after collection. Aliquots of blood were diluted 200 and 50 times for red and white cells, respectively, with Natt and Herrick's (1952) solution. Red (RBC) and white (WBC) blood cells were counted manually in a cell counting Thoma chamber using 96 small squares for red cells and all the large squares for white cells. Haematocrit was determined by centrifugation at 10 000 rpm for 12 min. Haemoglobin was assayed according to the colorimetric method of Drabkin (1945) using a haemoglobin standard from Sigma. Blood smears were fixed by immersion in methanol for 3 min and stained for 5 min with May–Grünwald (Sigma Chemicals, USA) stain and 45 min with Giemsa

Table 1. Factor loadings of the PCA for blood variables. Loadings larger than 0.5 are in bold.

	factor 1	factor 2	factor 3	factor 4
Proteins	−0.350	−0.103	**0.650**	−0.171
Urea	0.319	0.403	**0.508**	0.326
Uric acid	0.172	0.133	**0.871**	0.010
Glucose	**0.677**	0.015	−0.132	0.115
Azurophils	**0.830**	−0.205	0.043	−0.287
Eosinophils	**0.601**	0.080	−0.134	**0.532**
Lymphocytes	**0.843**	−0.066	0.163	0.011
Monocytes	0.450	0.311	**0.524**	−0.118
Heterophils	0.240	−0.248	**−0.563**	−0.366
Basophils	−0.073	−0.293	−0.293	**0.820**
Red blood cells	−0.125	**0.825**	0.240	0.008
Haemoglobin	0.136	**0.918**	0.092	−0.002
Haematocrit	−0.217	**0.808**	−0.001	−0.321
Eigenvalues	3.26	2.87	1.70	1.27
% total variance	25.1	22.1	13.0	9.8
Cumulative % of total variance	25.1	47.2	60.2	70.0

(Merck, Germany) diluted 1:4.5 with phosphate buffer, pH 6.8. WBC differential counts were counted with an oil immersion lens (1000×). At least 400 WBC were counted from each sample. On mounted slides, half a smear, chosen at random, was scanned entirely at 200× along the longitudinal of the slide, looking for extraerythrocytic protozoa (Merino and Potti 1995). Numbers of intraerythrocytic parasites were estimated under oil at 1000× by counting the number of parasites per 8000 erythrocytes (Godfrey et al. 1987). The only haemoparasites found were haemogregarines. Plasma was obtained by centrifugation at 13 000 rpm during 10 min and stored at −20°C until analysed. Plasma proteins were assayed as described by Lowry et al. (1951). Glucose, urea and uric acid were assayed with commercial kits (Cromatest, Knickerbocker Lab., Spain).

We examined normality of variables by means of normality plots and the Lilliefors test (SPSS statistical package). Variables that were not distributed normally were transformed by square root or logarithm. Variables became normally distributed after transformation. To reduce the number of haematological variables to a few variation axes, we computed a principal component analysis (PCA) that selected factors with eigenvalues above 1. The resulting matrix was rotated using the varimax procedure. Scores of the 22 large males and 6 small males along the four resulting factors were log-transformed after which they were distributed normally. Relationships between factor scores, counts of blood parasites and ticks were tested using ANCOVAs to control for treatment effects. We excluded from these correlation tests males whose SVL was below 80 mm because these individuals did not develop coloration and practically do not reproduce even after testosterone addition (Salvador et al. 1997, pers. obs.). Also, in contrast with large males, these small males did not develop the head coloration nor increased infestation rates by ticks in response to exogenous testosterone (Salvador et al. 1996, 1997).

Results

The PCA extracted four components that accounted for 70% of the variance. The first factor had high loadings for plasma glucose concentration, and number of lymphocytes, azurophils and eosinophils, while the remaining WBC types (with the exception of basophils) also had positive values. Lymphocytes were the most abundant WBC type, accounting for 80% of total WBC counts (see Puerta et al. 1996). The second factor had high loadings for RBC, haemoglobin and haematocrit levels; all the remaining variables had low loadings. The third factor had high positive loadings for uric acid and protein plasma concentration. The number of monocytes with high positive values tended to oppose the number of heterophils. The fourth factor had high loadings for number of basophils, and to a lesser degree for eosinophils (Table 1).

A MANOVA with the four components of the PCA as the dependent variables revealed a significant effect of the testosterone treatment (Wilkinson Lambda, $F_{4,23} = 6.39$, $P = 0.001$). Testosterone-supplemented individuals had lower scores on the factor 1 axis than control individuals ($F_{1,26} = 13.3$, $P = 0.001$; Fig. 1). The control and experimental individuals showed little overlap along the axis, suggesting that it represents a testosterone dose dependent axis. The low levels of glucose concentration of individuals on the negative side of the factor are consistent with both the reported decrease in food intake (Gentry and Wade 1976, Abelenda et al. 1992) and the increased physical activity after testosterone treatment (Marler and Moore 1988). The experimental and control individuals did not differ in their scores on component 2. The experimental individuals had higher scores for factor 3 than controls, though in this case the difference is less clear-cut than for factor 1 ($F_{1,26} = 7.7$, $P = 0.01$; Fig. 1). The high loadings for products of protein metabolism, especially uric acid, on this axis associated with individuals with higher average

testosterone levels, suggest that this factor represents an intense protein turnover for muscle accretion. The opposing values of the monocyte and heterophil loadings are probably related to the connection of this factor with haemoparasites (see below).

Thirty-five of 36 males had ticks when they were captured in March. The number of ticks per male was 2.9 ± 0.3 (mean $\pm$ SE) at this time. In May, when males were recaptured, all males had ticks and the number of ticks per male averaged 20.1 ± 1.4. The number of ticks per male when they were recaptured and the increase in the number of ticks since the first capture did not correlate with factor 1 scores (ANCOVA, $B = 0.02$, $P = 0.93$ and $B = -0.09$, $P = 0.73$, respectively; $n = 22$). The correlations with the other factors were neither significant (all $Ps > 0.18$). Twelve of 28 recaptured males (42.9%) were infected with haemogregarines. Infestation rates (mean $\pm$ SE, C-males: 0.31 ± 0.14; T-males: 0.33 ± 0.16) were correlated positively with factor 3 scores ($B = 0.59$, $P = 0.018$, $n = 22$) and tended also to correlate with factor 4 ($B = 0.36$, $P = 0.10$) but not with factors 1 and 2 (all $Ps > 0.37$). Haemogregarine infection rates correlated positively with number of ticks counted in the recapture ($B = 0.55$, $P = 0.019$), but not with the initial number of ticks ($B = 0.01$, $P = 0.95$), suggesting that ticks are involved as transmission vectors of the haemoparasite or that individuals are differentially susceptible to both parasite species in a similar manner.

Discussion

Relationships between blood variables and parasites

The results of the PCA analysis show that experimental and control individuals are significantly segregated by components 1 and 3. The segregation along component 1, with the experimental individuals having smaller values, suggests that the negative direction of this factor is indicating higher levels of plasma testosterone. Also, the decrease of plasma glucose levels in the negative direction of this factor is consistent with the fact that testosterone stimulates physical activity (Marler and Moore 1988) and reduces food intake (Gentry and Wade 1976, Abelenda et al. 1992). As males after their recapture were maintained unfed 24 h until blood samples were taken, it is possible that testosterone-treated males maintained a higher activity than control individuals. This might explain that testosterone males exhausted the glucose reserves they had when they were recaptured. However, this result is not sufficient evidence to argue that T-males were undernourished, because they might have a higher ingestion rate in the field that enabled them to maintain similar glucose plasma levels as control individuals (but see Marler and Moore 1988, Marler et al. 1995).

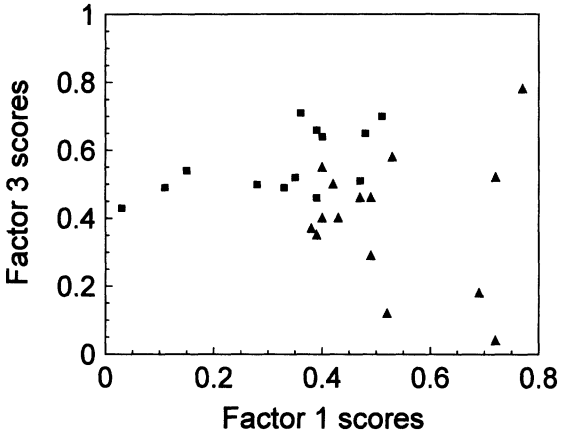

Fig. 1. Plot of the testosterone-implanted males (squares) and the control males (triangles) in the plane defined by factors 1 and 3 of the PCA for blood parameters.

The negative direction of the component 1 is associated with a general decrease in WBC counts, which is more pronounced for lymphocytes, the most abundant white cell type in this species (Puerta et al. 1996). This result is consistent with a negative effect of testosterone on the immune response, but it does not enable us to discriminate between direct or indirect effects of the hormone (see below).

Experimental males also showed higher scores in the third component, mainly characterised by high plasma levels of protein catabolites. It is plausible that the anabolic effect of testosterone (Mooradian et al. 1987, Puerta et al. 1996), together with testosterone enhancement of physical activity, causes an increased protein turnover rate that, in turn, results in greater plasma levels of protein catabolic products. The high scores on this component of some WBC types, namely heterophils and monocytes, as opposed to the low scores of other types, seem to indicate that this component is also related to an unspecific phagocytic response, as this is the role of these WBC (Sypek and Borysenko 1988).

The second component has high loadings for RBC, haemoglobin concentration and haematocrit. This axis represents the variation in the amount of RBC and associated parameters. In mammals and birds, the testosterone addition increases the number of RBC (Mooradian et al. 1987, Puerta et al. 1996). However, this trend was not observed in this study. This may have been due to the negative effects that the higher number of blood sucking ticks present on experimental males have on the number of RBC (Salvador et al. 1996). The fourth component is related mainly to the number of basophils and eosynophils, and it possibly

reflects a specific kind of immune response against parasites, in agreement with the role of these WBC in parasitic infections (Sypek and Borisenko 1988, Gounni et al. 1994).

Reproductive effort, immunological consequences and parasites

Correlational studies in vertebrates have reported a negative association between coloration development or testosterone levels and number of lymphocytes (e.g., Zuk et al. 1995, Skarstein and Folstad 1996). However, it remains unresolved whether the low number of lymphocytes is the consequence of higher hormone levels in individuals with more developed coloration, or whether it reflects that more ornamented individuals have lower intensities of infection, resulting in low antigen stimulation of lymphocyte proliferation. Previous results indicated that testosterone-implanted individuals developed a more extended nuptial coloration (Salvador et al. 1996). Results presented here show that testosterone supplementation is also associated with a general decrease in the number of WBC types, especially lymphocytes. Although the relation between increased testosterone levels and lower exposure to parasites cannot be excluded completely, this result is consistent with the proposal that testosterone has a negative effect on some lymphocyte types (reviewed by Folstad and Karter 1992). However, it is also possible that the reduced levels of WBC in T-males is the consequence of a general physiological stress induced by the hormone or the result of a reallocation of resources towards other functions with higher incidence on fitness (Wedekind and Folstad 1994). The association between low plasma glucose levels and small numbers of WBC in component 1 supports this last argument, though it does not invalidate the former. Nevertheless, the evolutionary consequences of either phenomenon are probably the same, namely, building up an immunological handicap in the individuals with the highest testosterone levels.

Scores for component 3 were also higher in testosterone-treated individuals. This association may indicate that an enhanced reproductive effort brings about an increased protein turnover rate. This is consistent with the fact that testosterone stimulates activity and a rapid depletion of glucose and lipid reserves (Marler and Moore 1988, this study). Also, the correlation between component 3 scores and haemogregarine counts suggests that this enhancement in metabolism renders the individual more susceptible to these parasites. However, as experimental and control individuals did not significantly differ in haemogregarine loads, this conclusion is only tentative. The high values of monocytes for the third principal component associated with high levels of haemogregarines may indicate a specific response of these macrophages against the parasite, as has been found in other parasitic and non-parasitic infections in reptiles (Greiner et al. 1980, Glazebrook et al. 1981, Wolke et al. 1982, Evans 1983). Because monocytes have higher phagocytic capacity than heterophils (Sypek and Borysenko 1988), the negative value of heterophils could reflect that monocytes had the larger phagocytic task at the time of blood analysis. Changes in eosinophil numbers in relation to parasites are not clearly understood in reptiles (Cooper et al. 1985). However, the trend towards a significant correlation between component 4, mainly defined by basophils and eosinophils, and haemogregarine load is also consistent with both the specialised role of these WBC (Gounni et al. 1994), and with previous results in other vertebrates (Turner 1988, Saino et al. 1995). The effects of haemogregarines on their hosts are poorly known. The presence of gametocytes in the blood indicates the existence of schizonts in internal organs (Svahn 1974), where they may cause additional damage.

In conclusion, our results indicate that an increase of the reproductive effort induced by sexual hormones determines a reduction in the total number of WBC, mainly lymphocytes, which may evidence immunosuppression (Barnes 1986). The higher mortality detected among the testosterone-implanted individuals (40% vs 7.1% in controls; Salvador et al. 1996) could be a result of this handicap. A higher reproductive effort was also reflected in a greater metabolism of proteins and an associated increase of haemogregarine load, whose effects are, however, not known. Reproductive effort mediated by testosterone seems to compromise health, so giving support to the immunological handicap hypothesis of the evolution of secondary sexual traits. However, the conclusion that a reduced immune response causes reduced survival should be taken with caution since both variables might be linked by the effects of testosterone on overall condition, and the independent effects of condition on survival and health.

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