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The effects of testosterone on a viral infection in greenfinches (*Carduelis chloris*): an experimental test of the immunocompetence-handicap hypothesis

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The immunocompetence-handicap hypothesis suggests that the honesty of quality signals could be guaranteed if testosterone (T) suppresses immune function while enhancing male ornaments. In addition, it has been proposed that the cost of enhancing ornaments should be highest for males with small ornaments. Recently, the assertion that T causes obligate immunosuppression has been questioned. In this study, we tested whether elevated T levels would increase susceptibility to a viral infection, and whether this hypothesized effect would be most pronounced in males with small ornaments. We surgically inserted T implants into 15 male greenfinches (*Carduelis chloris*) and control implants into a further 15 males. All birds were then infected with a naturally occurring virus (Sindbis virus, *Alphavirus* genus), and each bird's daily viraemia (blood virus concentration) was measured for seven days. The specific antibody response was measured for eight weeks. T-implanted males did not exhibit increased viraemia or decreased antibody response, and males with small and large ornaments did not respond differently to T implantation. We did, however, find that T implantation decreased viraemia early in the course of the infection and increased viraemia late in the infection. Thus, our results demonstrate that T may act both to increase and to decrease viraemia.

Keywords: viral infections; greenfinches; testosterone; immunocompetence; sexual selection

1. INTRODUCTION

Males with more elaborate ornaments are often preferred as partners by females, and the expression of male ornaments has, in many studies, been found to be negatively correlated with parasite load (i.e. males with large ornaments have low parasite load) (Andersson 1994; Hamilton & Poulin 1997; Møller *et al*. 1999). The mechanism behind this correlation is, however, unclear. It has been argued that ornaments used as quality indicators must be costly to maintain, otherwise all males would develop large ornaments (Zahavi 1975; Kirkpatrick & Ryan 1991). A mechanism to explain the cost of male ornaments was proposed by Folstad & Karter (1992) in the immunocompetence-handicap hypothesis. They suggested that male ornaments would be costly if they required the presence of a hormone, such as testosterone (T), that exerted immunosuppressive effects.

An essential assumption of the immunocompetence-handicap hypothesis is that T will suppress immune functions at naturally occurring concentrations, thereby allowing increased intensities of parasitic infections (Folstad & Karter 1992). This assumption has recently been questioned as it has received little empirical support, especially from studies on birds (Hillgarth & Wingfield 1997; Braude *et al*. 1999; Hasselquist *et al*. 1999). Although early reviews of hormone–immune-system interactions suggested that T exerts mainly immunosuppressive effects (Grossman 1985; Alexander & Stimson 1988), recent reviews have emphasized that T can both enhance and suppress immune functions (Marsh 1992; Olsen & Kovacs 1996).

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The relationship between the naturally occurring T plasma concentrations of males and parasite load, or immunity, has been investigated in at least three studies on birds. In two of these studies, T concentrations were not correlated with parasite load (Weatherhead *et al*. 1993; Saino & Møller 1994) and in one study, breeding males with high natural T levels were more likely to respond to an antigen challenge than males with basal T levels (Peters 2000). When T concentrations have been artificially increased by T implants, some studies have found evidence that T suppresses the production of immune components (Zuk *et al*. 1995; Saino *et al*. 1995; Peters 2000), while others have found no such effects (Ros *et al*. 1997; Hasselquist *et al*. 1999). Although it has been demonstrated that T can alter immunity, it is often unclear how this change will affect fitness (Sheldon & Verhulst 1996; Norris & Evans 1999).

According to the immunocompetence-handicap hypothesis, elevated concentrations of T should be immunologically costly (Folstad & Karter 1992). Also, the costs of increasing T concentrations should be unequally distributed, such that low-quality males incur higher costs than high-quality males (Grafen 1990). The study by Saino *et al*. (1995) demonstrated that T may induce this pattern. They found that low-quality barn swallows with short tail streamers experienced larger increases in parasite load, and that low-quality males had a greater reduction in survival, than high-quality males when both groups were given T implants.

In our experimental study, we manipulated natural T concentrations and monitored the viral clearance rate and the specific antibody response of greenfinches (*Carduelis chloris*) after a challenge with a naturally occurring avian virus, Sindbis virus (Togaviridae: *Alphavirus*).

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During the experiment, parasite exposure was equal for all males, so we were able to compare the abilities of individuals to resist a natural infection.

In this experiment, we predicted that elevated levels of T would have an immunosuppressive effect. Thus, we expected T-implanted males to have increased viraemia and reduced or delayed production of antibodies. We also predicted that the immunosuppressive effect of T would be strongest in males with small ornaments.

2. METHODS

(a) *Study organisms*

Greenfinch males have brighter plumage than females and previous studies on greenfinches have shown that the brightness of male plumage can affect mating success (Eley 1991). Males that were involved in extra-pair copulation and polygyny had brighter plumages than other males (Eley 1991). Male plumage brightness has also been found to be negatively related to blood parasite load (Merilä *et al.* 1999) and positively related to testis size (Merilä & Sheldon 1999). For the Sindbis virus, we have found that males with large bright tail patches are more efficient at virus clearance (Lindström & Lundström 2000). Thus, in terms of Sindbis-virus clearance, males with large tail patches are of high quality.

For this study, 32 male greenfinches were caught in mist-nets in the Uppsala area (59°50′ N, 17°50′ E) in December 1997. We ringed all birds and measured tarsus lengths with a digital calliper to the nearest 0.1 mm. Ages were determined for all birds using plumage characteristics (Svensson 1992). As we have found that tail patch length is the plumage character that correlates most closely with Sindbis-virus clearance rate (Lindström & Lundström 2000), we used a ruler to measure the length of the right tail patch to the nearest mm. This patch is yellow and extends from the tail base. Throughout the experiment, birds were kept in individual indoor cages (50 cm × 50 cm × 50 cm) on a natural day-length cycle. Food (seed mixture) and fresh water were always available. The infections were performed in March 1998, a time corresponding to the start of the breeding season for greenfinches in this area.

Sindbis virus is a mosquito-borne virus that occurs in Europe, Africa, Asia and Australia (Olson & Trent 1985). It infects several bird species in Sweden, mainly species of thrushes (*Turdus*) and finches (Fringillidae) (Lundström 1999). The infection prevalence for greenfinches in the study area is around 10% (J. O. Lundström and K. M. Lindström, unpublished data). During infection, greenfinches escape from predator attacks with reduced take-off speed and their locomotion activity tends to be decreased (K. M. Lindström, J. O. Lundström, I. T. van deer Veen and B.-A. Legault, unpublished data). Thus, the viral infection may affect the fitness of avian hosts through increased predation. For the infections, we used the Edsbyn 82/5 strain of the Sindbis virus. Genetically, this is a typical northern European Sindbis-virus strain (Norder *et al.* 1996).

(b) *Experimental procedure*

Birds were matched into 16 pairs based on resemblances with respect to age, tail patch length and tarsus length. Within each pair, one bird was randomly chosen to receive the T treatment, while the other bird acted as a control. This was done to obtain two groups with equal compositions of birds. All birds were implanted with 10 mm silicone tubes (Dow Corning, Horgen, Switzerland; 1.47 mm internal diameter, 1.96 mm external diameter) that were either empty (control group) or filled with T (Sigma Chemical T1500, Sigma, St Louis, MO, USA) (T group) and sealed with silicone. Implants were inserted subcutaneously into the breast. Two weeks after implantation, we took blood samples from all birds to detect Sindbis-specific antibodies from prior exposures. Two out of the 32 birds tested positive for antibodies and had no detectable viraemia after infection. This showed that these two birds had already been exposed to the virus in nature, so they were excluded from further analyses. After the screening blood sample was taken, we infected each bird with a 0.1 ml subcutaneous injection of infectious-virus solution diluted in saline (0.09% NaCl). Each injection contained approximately 104 plaque-forming units of virus. Blood samples were then taken from all birds once every 24 h for seven days to quantify the levels of infectious virus. Samples were also taken at one, two, three, four and eight weeks after infection to quantify the levels of Sindbis-specific neutralizing antibodies. During each sampling, 0.1 ml of blood was taken from the jugular vein with a 27G needle on a 0.5 ml syringe. Whole blood was diluted 1:10 in Hank's balanced salt solution supplemented with 5% Hepes buffer and 10% foetal calf serum (Life Technologies^{TM}, Gaithersburg, MD, USA). Samples were kept on ice for less than 3 h before storage at −70 °C. Handling and blood sampling took less than 5 min per bird on all occasions.

Blood samples were assayed for virus concentrations in duplicates at tenfold dilution using a plaque assay (Lundström *et al.* 1990). Sindbis-virus specific antibody titres were expressed as the reciprocal dilution, giving 80% reduction in plaque numbers as compared to the average count of controls in a plaque-reduction neutralization test (Earley *et al.* 1967; Francy *et al.* 1989). In a previous experiment, viraemia and antibody production were uncorrelated (Lindström & Lundström 2000).

(c) *Hormonal assay*

Blood samples (*ca.* 0.3 ml) were collected from all birds at noon on the 19th day after infection using the same blood-sampling method as described above (§ 2(b)). For ten birds, a second sample was taken 25 days after infection. We obtained each sample within 5 min of capture. Blood samples were centrifuged after 20 min and the supernatant was frozen at −20 °C until assayed. After extraction in dichloromethane, plasma levels of T were measured by a single-antibody assay after chromatographic separation and purification on micro-columns of celite:glycol, where steroids were eluted in order of polarity. In this study, we included only T fractions. Samples were assayed in duplicates within a single assay. To estimate the intra-assay variation we included a standard plasma pool from mallards in triplicates. The intra-assay variation was found to be 10.8%. Three samples of hormone-free plasma were also analysed and, in all cases, the hormone-free samples measured less than 3 pg of T. The sensitivity of the standard curve was within the range 3–500 pg. To estimate the losses of hormones during the assay, tritiated T was added to the plasma immediately after the initial extraction.

T concentrations were higher in the T-treated group than in the control group (Mann–Whitney U-test, $U = 0.00$, $p < 0.001$, mean ± s.e.m. was 5.5 ± 0.1 ng ml^{-1} in the T group and 0.6 ± 0.4 ng ml^{-1} in the control group, $n = 15$ for both groups). For birds that were sampled twice, T concentrations within individuals were highly correlated between samplings (Spearman's rank, $r = 0.76$, $p = 0.01$, $n = 10$). A T concentration of 5.5 ng ml^{-1} is within the natural range for a medium-sized passerine in the field at the beginning of the breeding season (Deviche *et al.* 2000).

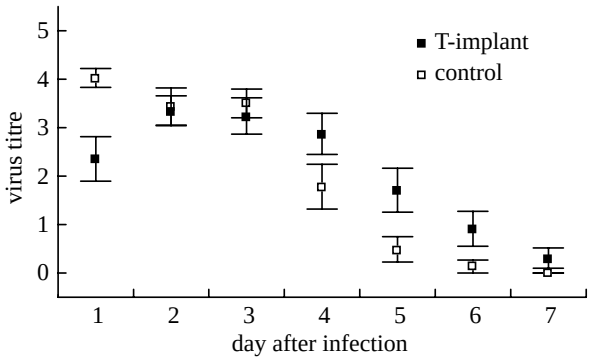

Figure 1. Mean (±s.e.m.) of $\log_{10}$-transformed Sindbis-virus titres (plaque-forming units per millilitre of blood) for testosterone-implanted and control male greenfinches, one to seven days after infection. There were statistically significant differences between groups for both early (sum of titres on days 1–3) and late (sum of titres days 4–7) viraemia (see §2(d)). The sample size was 15 males in each experimental group for all days.

(d) *Data analyses*

Fifteen males in each treatment group became viraemic after the infection. During the first three days after infection, the mean concentration of the virus increased or remained stable. Thereafter, mean virus concentrations decreased (figure 1). We divided viraemia into two phases: a proliferation phase, referred to as early viraemia (sum of virus titres on days 1–3), and a clearance phase, referred to as late viraemia (sum of virus titres on days 4–7). From the antibody titres, we calculated the antibody-production rate, defined as the highest antibody titre recorded for each male divided by the time (number of weeks after infection) at which this peak occurred. T concentrations within groups were not normally distributed, so we used non-parametric statistics to compare this variable between groups. We used $\log_{10}$-transformed values of daily virus titres for all analyses. The effect of treatment on viraemia was analysed by including early and late viraemia in a repeated-measures ANOVA. Antibody-production rate was analysed in a separate ANOVA model. We included tail patch length as a covariate in the ANOVA models and we used *t*-tests as post-hoc tests. Data were analysed according to Sokal & Rohlf (1995) using Statistica for Windows. To analyse the power of our tests we used G*power (Erdfelder *et al.* 1996).

3. RESULTS

For total viraemia, there was no difference between the treatment group and the control group ($F_{1,27}=0.17$, $p=0.68$, figure 2*a*). In the post-hoc power analyses of this result, we found that we would have been able to detect increases in total viraemia above 15% with 80% power at the $p=0.05$ level (one-tailed test). There was a significant interaction between treatment (T versus C) and measurement time (early versus late viraemia) ($F_{1,27}=81.5$, $p=0.0002$), thus the effect of treatment was different for early and late viraemia. In the post-hoc *t*-tests, we found that T-implanted birds had lower early viraemia ($t=8.94$, $p=0.019$, figure 2*b*) but higher late viraemia ($t=-2.42$, $p=0.022$, figure 2*c*). For antibody-production rate, we found no significant effect of treatment ($F_{1,27}=0.07$, $p=0.80$, figure 2*d*). Within the control group, antibody-production rate tended to be negatively correlated with early viraemia ($r=-0.49$, $p=0.06$, $n=15$). However, there were no such tendencies ($p<0.22$) for correlation between antibody-production rate and total or late viraemia. Thus, again, we found that antibody production was poorly correlated with viraemia.

The covariances between tail patch length and both total ($F_{1,27}=5.97$, $p=0.02$) and late ($F_{1,27}=6.22$, $p=0.02$) viraemia were significant and negative (late viraemia, $\beta=-0.43$, $r^2=0.19$, $p=0.02$; total viraemia, $\beta=-0.42$, $r^2=0.19$, $p=0.02$). This supports the assumption that males with small tail patches were of low quality in terms of Sindbis-virus clearance. However, the covariance between tail patch length and early viraemia was not significant ($F_{1,27}=1.91$, $p=0.18$), and there was no significant covariance between tail patch length and antibody-production rate ($F_{1,27}=0.76$, $p=0.39$).

We predicted that the immunosuppressive effect of T would be most pronounced in males with small ornaments. Since an immunosuppressive effect of T could only be detected for late viraemia, we examined the relationship between tail patch size and late viraemia in more detail. In the T-treated group, there was a significant and negative correlation between tail patch size and late viraemia ($r=-0.56$, $p=0.03$, $n=15$), while birds in the control group showed no such correlation ($r=-0.16$, $p=0.56$, $n=15$). Thus, the immunosuppressive effect of T appeared to be most pronounced for males with small tail patches, as predicted. However, the interaction between treatment group and tail patch length was not significant for early viraemia ($F_{1,26}=0.32$, $p=0.57$), total viraemia ($F_{1,26}=0.15$, $p=0.69$) or late viraemia ($F_{1,26}=1.18$, $p=0.29$). Thus, overall, we found no clear evidence that males responded differently to treatment depending on their tail patch length.

4. DISCUSSION

Males that had received T implants had lower virus titres early in the infection, compared to controls. This result shows that increases in T acted to reduce Sindbis-virus replication immediately after infection. It has previously been demonstrated that immune components can be temporarily redistributed to peripheral tissues in response to T (Braude *et al.* 1999), thus, viraemia may initially have been controlled by immune cells allocated to peripheral tissue. Although the T implants initially prevented virus replication, T-implanted males had higher virus titres late in the infection. The differences that we found could be caused either by direct interactions between T and immunity or by a coupled release of stress hormones interacting with immunity in T-implanted birds (Poiani *et al.* 2000). There was no difference in total viraemia between the treatment and control groups, hence we cannot conclude that T was costly in terms of immunosuppression. In the power analyses, we found that we would have been unable to detect increases in total viraemia below 15%. Although we found no significant difference in total viraemia, the mean of the T group was 6% higher than that of the control group. However, a potential effect of T as small as 6% would account for only a minor part (1%) of the natural variation in viraemia (>600% in this study).

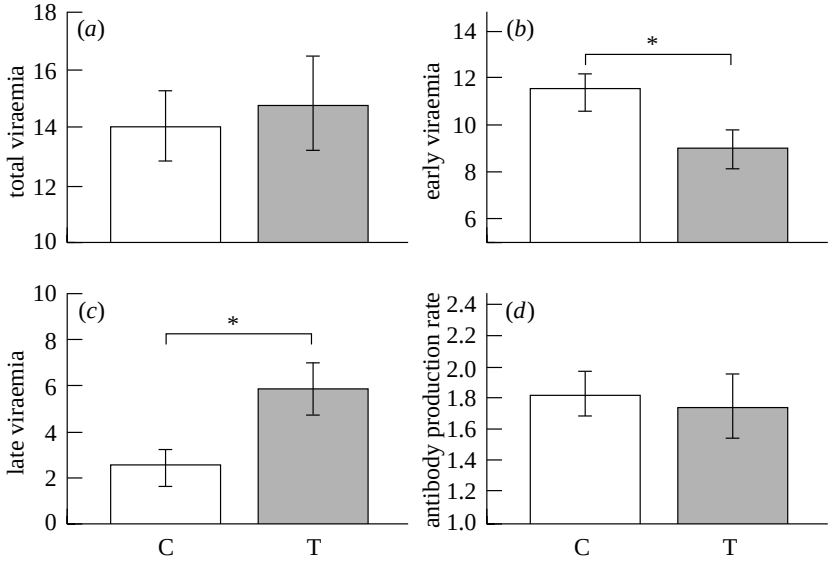

Figure 2. Mean (±s.e.m.) of (*a*) total viraemia: sum of $\log_{10}$-transformed Sindbis-virus titres (plaque-forming units per millilitre of blood) on days 1–7; (*b*) early viraemia: sum of $\log_{10}$ virus titres on days 1–3; (*c*) late viraemia: sum of $\log_{10}$ virus titres on days 4–7; and (*d*) antibody-production rate: peak antibody titre per week after infection for this peak, for greenfinches that were given empty implants (open bars) or testosterone-filled implants (closed bars) and subsequently infected with the Sindbis virus. The sample size was 15 males in each group, $^{*}p < 0.05$. T, testosterone; C, control.

In contrast to Saino *et al.* (1995), we found no clear evidence that T was more costly for males with smaller ornaments. For late viraemia, the immunosuppressive effect of the T treatment seemed to be most pronounced for males with small ornaments but there was no significant difference in the response to treatment that could be related to tail patch size. However, since our study was based on a small number of birds, and the immunosuppressive effect of treatment that we found was limited, this evidence is not conclusive.

Previous studies on birds in which T-implanted males have been given immune challenges have produced equivocal results. Hasselquist *et al.* (1999) found that the magnitude of the secondary antibody response to a non-pathogenic protein antigen was unaltered by T implants in male red-winged blackbirds (*Agelaius phoeniceus*). Peters (2000) found that the magnitude of the primary immune response after a challenge with sheep red blood cells was unaltered in T-implanted male superb fairy-wrens (*Malurus cyaneus*) but the likelihood of producing a detectable immune response was decreased in T-implanted males. In our study, T-implanted birds had delayed viraemia but antibody-production rate did not differ between treatment and control groups. Furthermore, although both the speed and the magnitude of the specific antibody response were measured, antibody-production rate was only poorly correlated with viraemia. Thus, the relevance of the variation in antibody-production rate between individuals cannot be explained.

In a field study on barn swallows (*Hirundo rustica*) by Saino *et al.* (1995), it was found that the T-implanted males had an increased parasite load and a decreased survival. Thus, increased T was costly for male birds. However, since the degree of exposure to a parasite can depend on a bird's behaviour (Hart 1997), it may be difficult to disentangle the behavioural and hormonal causes of immunosuppression in T-implant studies conducted in the field. T may influence male behaviour, for example by increasing territorial aggression (Wingfield *et al.* 1990). If, for example, a male's activity increases in response to higher T levels, such a male may be exposed to more parasites and infection intensities may be higher in males with high T levels, regardless of changes in their immunocompetence. Also, a bird's social status may be altered by increasing T levels (Poiani *et al.* 2000) and this may, in turn, influence its immunocompetence (Poiani *et al.* 2000; Evans *et al.* 2000; Zuk & Johnsen 2000). In a recent study where house sparrows (*Passer domesticus*) with manipulated T levels were held together in groups, ectoparasite load increased and antibody production decreased in relation to T level (Poiani *et al.* 2000; Evans *et al.* 2000). These apparent immunosuppressive effects of T were, however, best explained by increases in the levels of stress hormones, corticosteroids, and increases in social dominance in birds with high T levels. Thus, when measuring immunocompetence in T-implanted birds, social behaviour should be taken into account.

For greenfinches in this experiment, increased T levels delayed both the onset and the clearance of a viral infection. For the Sindbis-virus infection, and many other diseases, infected individuals may suffer increased predation (Møller & Erritzøe 2000). If the risk of predation is increased during an infection, a fast infection clearance may be important for survival. Also, if each parasite causes some degree of damage to the host, it would be important to reduce the intensity of the infection. In this study, we compared the total viraemia of infected individuals, to account for both intensity and duration.

In conclusion, this and other studies suggest that T can interact with immune functions in birds. This study does not support the assumption that T has obligate immunosuppressive effects. Instead, we have shown that T can have both positive and negative effects on an individual's

ability to resist infections and that the effects of T may differ depending on the time at which they are measured.

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