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Is testosterone immunosuppressive in a condition-dependent manner? An experimental test in blue tits

Mark Roberts* and Anne Peters
Max Planck Institute for Ornithology, Vogelwarte Radolfzell, 78315 Radolfzell, Germany
*Author for correspondence (e-mail: m.l.roberts@imperial.ac.uk)

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SUMMARY

In this experiment we manipulated testosterone (T) and condition in juvenile male blue tits (*Cyanistes caeruleus*) during the moult, to test whether T's supposed immunosuppressive qualities are condition-dependent. To achieve this, we used T and control implants in combination with a dietary manipulation. We measured responses to both phytohaemagglutinin (PHA) and humoral immune challenges during the period of the treatments (moult) and also in the following breeding season (spring). During moult, males fed the enhanced diet were in better condition but there was no difference in humoral response between the dietary groups. T males produced a greater humoral antibody response than control (C) males. In the spring, males that had been previously treated with high T again exhibited higher antibody responses than C males. High T levels during moult were associated with a low PHA response but only in males with low body mass: heavier males that had high T exhibited the highest PHA responses. In the spring, the pattern of PHA responses was reversed; responses were highest in males that had low body mass but also had high T levels, and the lowest responses were by males that had both high T and were relatively heavy. Our results suggest that the effects of T on immunity can be either immunoenhancing or immunosuppressive, depending upon the condition of the individual, its life history stage, as well as on the immune challenge employed.

Key words: testosterone, blue tit, condition, immunity, PHA, SRBC, carotenoids, protein.

INTRODUCTION

A key assumption of the immunocompetence handicap hypothesis (ICHH) (Folstad and Karter, 1992) that the male sex hormone testosterone (T) is immunosuppressive, is supported by surprisingly scant evidence in birds (Roberts et al., 2004). The ICHH is a handicap model that suggests that the expression of male secondary sexual traits used by females to choose potential mates is T-dependent, and T simultaneously has negative effects on immune function (Folstad and Karter, 1992). Therefore, only relatively superior males are capable of both fully expressing their sexual signals and resisting or tolerating a weakened immune system, or are not negatively affected by high T (Folstad and Karter, 1992). Although some evidence has been found that supports this assumption from studies that have manipulated T levels and immunochallenged the experimental subjects (Duffy et al., 2000; Peters, 2000; Casto et al., 2001; Buchanan et al., 2003; Owen-Ashley et al., 2004; Deviche and Cortez, 2005), other studies have found no such immunosuppression of high T-treated males (Hasselquist et al., 1999; Buchanan et al., 2003; Greenman et al., 2005; Roberts et al., 2007a). Indeed a recent meta-analysis confirmed that evidence supporting this assumption of the ICHH is, at best, weak (Roberts et al., 2004).

Several variants of the original ICHH have been proposed in an attempt to explain the lack of consistent results obtained when testing the ICHH (e.g. Braude et al., 1999; Kurtz, 2000; Poiani et al., 2000; Alonso-Alvarez et al., 2006; Alonso-Alvarez et al., 2007). In particular, recent studies have suggested that T-mediated immunocompetence may be condition-dependent (Duckworth et al., 2001; Alonso-Alvarez et al., 2007). The implication is that only males in good condition (and therefore of higher phenotypic and possibly genetic quality) have sufficient resources to maintain high levels of T and, consequently, have high quality sexual signals and remain fully immunocompetent. T may be immunosuppressive but only in males in poor condition that cannot afford to invest in reproduction as well as immune function.

In the present study, we aimed to disentangle the effects of T and condition on immune response. By manipulating both body condition and T levels in moulting juvenile male blue tits (*Cyanistes caeruleus* Linnaeus 1758), we tested different components of immunity in relation to T, condition and a combination of both factors: if T alone is always immunosuppressive, then a high T treatment group should have the lowest immune response regardless of the individuals' condition; if T is only immunosuppressive in individuals in poor condition, then we would expect a significant interaction between T treatment and dietary treatment, such that T males on a poor diet would have the lowest immune response; and finally, if condition alone and not T mediates immunocompetence, then dietary treatment should have the only significant effect on immunity. In addition to this, we tested whether enhancement of condition also elevates T levels (see Pérez-Rodríguez et al., 2006) and conversely whether artificial elevation of T levels affected body condition. Several studies have found a deleterious effect of T manipulation on condition (Mougeot et al., 2004; Owen-Ashley et al., 2004; Roberts et al., 2007a) whereas correlational studies have found that males with high T levels are also in better condition than those with relatively low T levels (e.g. Duckworth et al., 2001). Moult is a costly period during a bird's life-history, with dramatic physiological costs, such as a daily energy expenditure of 2–3 times basal metabolic rate during peak moult (Lindstrom et al., 1993), a 3–4-fold increase in protein turnover (Murphy and Taruscio, 1995), decreased thermoregulatory ability caused by impaired feather insulation (Klaassen, 1995) and reduced ability to produce a

physiological stress response (Romero, 2002). The moult period is therefore an ideal opportunity to examine the effects of condition on immunity, because presumably the costs of an immune response during this time will be particularly costly and therefore highly condition-dependent.

To investigate whether treatment effects continued on into the breeding season (the treatments continued only until the end of the moult) and whether immune function was differentially affected between moult (thought to be an energetically expensive period) and the subsequent breeding season, we challenged the cell-mediated and humoral arms of the immune system both during the prebasic moult and in the following breeding season, when any relationships between T, condition and immunity may be more crucial in terms of sexual signalling.

MATERIALS AND METHODS

Study animals and housing

Full details of the methodology employed to obtain and raise the birds are given in Kurvers et al. (Kurvers et al., 2008). Briefly, wild-caught parents and chicks were kept in large, outdoor aviaries (300×300×190 cm) (Radolfzell, Baden-Wurttemberg, Germany) during spring 2006, where the parents continued to raise their chicks independently. After the fledglings could forage independently, four males from each of the 12 broods were selected randomly and placed separately in similar outdoor aviaries for the remainder of the experiment (i.e. one individual per cage). All remaining fledglings were released with their parents in the local area. Therefore, at the outset of the project there were 48 experimental males. In a fully factorial design, males from each family were equally allocated to one of two dietary treatments and one of two testosterone treatments in a balanced manner. Consequently there were 12 males in each treatment combination.

During the course of the experiment, eight birds died for unknown reasons [six T males and two control (C) males]. In addition, one C male was excluded from all analyses due to an injury early in the project. Final sample sizes are given in the figure legends. Removal of the birds from the wild and all animal experimental procedures were approved by the Regierungspräsidium Freiburg (Aktenzeichen 55-8852.15/05 and Registriernr. G-06/05, Aktenzeichen 35-9185.82/3/339, respectively).

General design

We implanted the birds with T or placebo pellets and placed them on an enhanced (E) or standard (S) diet before the beginning of the moult (July 2006). The treatments continued until the end of the moult (November 2006). We challenged the birds with sheep red blood cell (SRBC) and phytohaemagglutinin (PHA) injections mid-moult (August and September 2006) and again in the following spring (April and May 2007). Blood samples for T assay were taken once mid-moult (August 2006) and twice in the following spring (March and May 2007).

Testosterone treatment

On the 17 July 2006, T-treated males were given subcutaneous T implants, while C males received placebo implants that consisted of inert binding material only (both implant types: diameter, 3 mm; height, 1 mm). The implants (Innovative Research of America, Sarasota, FL, USA) were inserted through a small incision in the skin on the back between the wings. T implants contained 1 mg T; implant size was based on implants previously used in blue tits (Foerster and Kempenaers, 2004), and implants were designed to dissolve gradually over a period of 90 days. Between the 26 and 30 October 2006 careful visual inspection of the implantation site showed no definite evidence of any pellet remainders and they appeared to have completely dissolved.

Dietary treatment

The different dietary treatments commenced on the 15 July 2006. The S diet consisted of 20% protein, 0.4% vitamin, 42% carbohydrate and 20% fat (Pierce and McWilliams, 2005). The E diet contained 41% protein, 0.25% lutein, 2% vitamin, 15% carbohydrate and 15% fat. This diet was a novel dietary formulation that was designed to simulate an insect diet in protein content, contained higher vitamin and antioxidant (carotenoid) concentrations and was more easily digestible (lower fibre content and more water) than the standard diet. For a full description of the content of the diets, see Kurvers et al. (Kurvers et al., 2008). After moult was completed (November 2006), all males received a similar standard diet consisting of live mealworms, fat balls and sunflower seeds.

To determine condition, the males were weighed to the nearest 0.1 g using a Pesola spring balance (Baar, Zug, Switzerland) and their right tarsus lengths were measured to the nearest 0.1 mm with digital callipers (Mahr GmbH, Göttingen, Lower Saxony, Germany). The birds were weighed before SRBC injection on the 23 August 2006 and 18 April 2007 and before PHA injection on the 11 September 2006 and 11 May 2007. Their tarsus lengths were recorded on the 31 July 2006 and 11 May 2007.

Immune challenges

PHA-induced immune response

This immune challenge (often described as cell-mediated) stimulates the proliferation of multiple immune cells and involves both the innate and adaptive elements of the immune system (Martin et al., 2006) and has been widely used in avian behavioural ecology (e.g. Duffy et al., 2000; Casto et al., 2001; Buchanan et al., 2003; Roberts et al., 2007a; Roberts et al., 2007b). Each male was injected with the mitogen phytohaemagglutinin (PHA-P, Sigma Chemical Co., St Louis, MO, USA) intradermally into the left wing web (Lochmiller et al., 1993) on the 11 September 2006 and in the following spring on the 11 May 2007. Each male received 50 µl of a suspension of 0.50 mg PHA-P in 0.1 ml phosphate buffer saline (1×PBS) (Lochmiller et al., 1993). A spessimeter was used to measure the wing web before injection (as a control measurement) and at 24 h after injection to measure the wing web swelling in response to the mitogen, because little further swelling occurs after this time point (Martin et al., 2006). We did not include individuals in the analyses in which any evidence of leakage from the injection site occurred. The males had been injected once with PHA before the start of the treatments (10 July 2006), and no differences existed in PHA response between the treatments (all $P>0.05$).

Humoral response

Challenge by SRBC injection provokes an antibody response, thereby testing the humoral component of immune defence (Evans et al., 2000; Peters, 2000; Ardia et al., 2003; Buchanan et al., 2003; Hanssen et al., 2004). Each male was immunised with 50 µl of a 10% solution of thrice-washed fresh SRBC in PBS (Fiebig Nährstofftechnik, Idstein, Hesse, Germany) by intraperitoneal injection on the 23 August 2006 and on the 18 April 2007 to stimulate the secondary antibody response. As with the PHA injections, we did not include individuals in the analyses in which any evidence of leakage from the injection site occurred. The primary response had been tested in the same way as described in the present study before the start of the treatments for the purposes of another study on the 26 and 27 June 2006 (mean

antibody titre ± s.e.m.: 0.9±0.2); there was no significant difference in primary response between the treatment groups (all $P>0.05$). Control samples were taken before the first and second injections; only one sample had heterologous antibodies (score of 1) before the primary injection. The secondary response measured during the moult in August 2006 was calculated as the primary response subtracted from the difference between the total secondary response and the antibody titre measured in the secondary control blood sample. This was done partly because the treatments began between the primary and secondary injections and we wished to test the effects of the treatments specifically on the secondary antibody response itself (therefore we needed to control for the primary response), and partly because the secondary control titres were much higher than expected, even higher than the primary response titres. We have no satisfactory explanation for this; possibly there was a general infection affecting our birds or the commencement of the treatments had physiological effects that we had not anticipated. However, by calculating the response from the second injection in this way we are confident that only the provoked response to the second injection was analysed. For ethical reasons (we tried to avoid non-essential invasive procedures during spring 2007) we did not take a pre-injection blood sample for the third injection; therefore, the response was determined simply as the antibody titre obtained eight days after injection without any corrections.

Eight days after each injection, blood samples were taken and subsequently tested for cross-reaction to SRBC. The antibody response was assayed (using the same sheep blood sample as for immunisation) using a standard haemagglutination technique (Hay and Westwood, 2002). Briefly, 20 µl plasma was serially diluted in 20 µl PBS across one 12-well row of a V-form microtitre plate after first being heat-treated at 56°C for 30 min in a water bath. A 20 µl sample of 2% SRBC was then added to all of the wells of the tray and the tray was incubated at 37°C for 1 h. Haemagglutination is evident when the antibodies in the plasma form a thin film of blood cells that covers the surface of the well. The most dilute titre of plasma exhibiting agglutination was recorded. The trays were left at room temperature for 24 h to ensure the process had been completed, and photographs were then taken of the trays to record agglutination.

Hormone assay

Blood samples were taken during the moult (23, 24 and 25 August 2006) and during the following spring (26 and 27 March 2007 and 11 May 2007). A small blood sample (*ca*. 75 µl) was taken from each bird by puncturing the brachial vein, kept vertically on ice, centrifuged at 1512 ***g*** for 5 min and the plasma was then harvested and stored at –70°C until assay. Sufficient plasma for T assay was obtained for a total of 24 males during moult 2006, for 35 males during March 2007 and for 32 males in May 2007. Plasma T levels were assayed by direct radioimmunoassay (RIA) following Goymann et al. (Goymann et al., 2006) [for details see Kurvers et al. (Kurvers et al., 2008)]. Briefly, after extraction and estimation of individual extraction recoveries, duplicate aliquots of each sample were analysed by direct competition RIA. The lower detection limit of the assay was determined as the first value outside the 95% confidence intervals for the zero standard (B_{max}) and was 0.006 ng ml^{-1}. The mean recovery rate was 87% and the intra-assay c.v. was 9.6%.

Statistical analyses

We used the restricted maximum likelihood (ReML) procedure to test for effects of T and dietary treatments on PHA responses, T levels and condition. Body condition was estimated as body mass corrected for skeletal size by including tarsus length in these models. The maximal fixed models consisted of T treatment × diet interaction with bird family as the random term. In addition to experimental differences between treatment groups, we also performed a correlational analysis of the effects of actual T levels, body mass and their interaction on immune responses. For this we always used mass as recorded at the time of immunisation; bird family was again included as a random term. For the moult immune challenges, we used tarsus and T levels recorded during moult. For the spring immune challenges, we used tarsus measurements taken in spring and T levels from March (SRBC) and May (PHA). For the analysis across seasons, individual bird nested within family was the random model. The residuals of the models were checked for homoscedasticity and normality. The number of agglutinated wells out of the total number was treated as the antibody response to SRBC injection; as these data were proportions, a General Linear Mixed Model (GLMM) was employed with a binomial distribution, except for the response during moult. Because this was calculated as the difference between the observed response, the antibody titre immediately before injection, and the primary response, this variable was treated as non-proportional so a ReML was used for this analysis. T levels were natural log transformed and PHA responses were square root transformed for the moult analyses. One outlier that had a T level (log value: 0.80) over 2 s.d.'s from the mean for its treatment group (C males: log group mean=–1.75, s.d.=0.82) was excluded from the March T and antibody analyses. In addition, an individual weighing over 14.0 g was excluded from the March condition analyses, as it was over 2 s.d.'s from the overall mean (mean=11.86, s.d.=0.73). One individual that exhibited a much higher primary response (eight positive wells) than secondary response (two positive wells) was omitted from the moult analysis as its inclusion as an outlier (over 2 s.d.'s from the mean) resulted in the residuals of the model being non-normal and therefore violating the assumptions of the model despite transformation. Inclusion or removal of these individuals made no qualitative difference to the results. Minimal models were derived by stepwise deletion of non-significant terms ($P>0.05$). We present Wald statistics from the final models (that followed a chi-squared distribution with d.f.=1 in all cases). Results are presented as raw means ± s.e.m. unless stated otherwise. For all statistical analyses we used Genstat 8.1 (VSN International, Hemel Hempstead, Hertfordshire, UK).

RESULTS

Moult 2006

The T treatment groups differed significantly in T levels during the moult, with the T-implanted males having higher levels than the placebo-implanted controls [T (N=12), 3.70±1.15 ng ml^{-1}; C (N=12), 0.71±0.15 ng ml^{-1}; Wald=11.43, $P<0.001$)]. Males fed the E diet were in significantly better condition than those on the S diet during the SRBC injections on 23 August (Wald=4.00, P=0.046; predicted means ± s.e.m. E, 12.25±0.16 g; S, 11.93±0.16 g, controlling for tarsus length). There was no difference between diet groups in mass during the PHA injections on 11 September (Wald=1.18, P=0.28). There was no difference between T-groups in body mass during the SRBC injections (Wald=2.03, P=0.15) or during the PHA injections (Wald=0.68, P=0.41). There was no difference between dietary groups in T levels (Wald=1.11, P=0.29). There were no significant T × diet interactions on mass or T levels (all $P>0.05$). Mean T levels of the control as well as the T-implanted experimental birds were much higher than those of wild male blue tits from the local

area during post-juvenile moult (late August–early September 2004 mean, 0.10 ng ml^{-1}; range, 0.06–0.23 ng ml^{-1}) (A.P., unpublished). The body masses of the experimental birds fell in the natural range of moulting male juvenile blue tits captured in the local area at that time of year (mass, 12.1±0.2 g; range, 11.5–12.7) (A.P., unpublished).

T-treated males produced a significantly higher antibody response to SRBC injection than C males (Wald=5.56, P=0.026) (Fig. 1). There was no significant difference in antibody response between the dietary groups (Wald=0.02, P=0.89). Interestingly, there was a significant difference in antibody titre between the dietary treatments as measured from the control sample for the secondary injection. The birds on the E diet had significantly more antibodies in their blood plasma than birds on the S diet (S, 1.61±0.28; E, 2.71±0.31; Wald=8.80, P=0.007). There was no difference between T groups in the number of antibodies present in the blood pre-secondary injection (Wald=1.42, P=0.23). There was no difference between either dietary groups (Wald=0.01, P=0.94) or T groups (Wald=0.01, P=0.92) in PHA response. All interactions between dietary groups and T groups for all response variables were non significant (P>0.05). For all means and s.e.m.'s, see Table 1.

The correlational analysis showed that there was a significant interaction between body mass and T levels on PHA response (Wald=8.02, P=0.005) (Fig. 2): males that were heavier and had higher T levels produced a greater PHA-induced swelling. Analysis of the relationship between mass and T levels on antibody response was not possible because the iterative process did not converge, probably due to the small sample size.

Spring 2007

Males that had been T-treated during the previous moult still had significantly higher T levels than previously control-implanted males during March (T, 0.61±0.20 ng ml^{-1}; C, 0.18±0.04 ng ml^{-1}; Wald=5.19, P=0.02) as well as during May (T, 0.17±0.06 ng ml^{-1}; C, 0.08±0.01 ng ml^{-1}; Wald=6.29, P=0.01). No difference existed in body mass between the moult dietary treatment groups either in March (Wald=0.48, P=0.49) or in May (Wald=1.55, P=0.21). No significant differences existed between the T groups in body mass (March: Wald=0.30, P=0.58; May: Wald=0.39, P=0.53), and no differences were found between diet groups in T levels in March (Wald=0.27, P=0.60) or May (Wald=2.51, P=0.11).

The males that had been in the T treatment group in the previous year had significantly higher antibody responses than the C males (Wald=3.94, P=0.047) (Fig. 3). The dietary treatment groups did not differ in antibody response (Wald=0.46, P=0.50). There was no significant difference in PHA response between the T groups (Wald=0.33, P=0.56) or between the dietary groups (Wald=0.37, P=0.55). All interactions between former dietary and T groups for all response variables were non significant (P>0.05). For all means and s.e.m.'s, see Table 1.

Again, there was a significant interaction between body mass and T levels on PHA response, such that males with the highest T levels and poorer body condition responded greatest (Wald=6.74, P=0.009) (Fig. 4). There was no effect of T and body mass on antibody response either alone (T: Wald=0.05, P=0.83; condition: Wald=0.24, P=0.63) or in interaction (Wald=0.14, P=0.71). There was a significant, positive correlation between March and May T levels (correlation coefficient=0.507, P=0.004) but this was found only within the previously T-treated males (C males: correlation coefficient=0.061, P=0.82; T males: correlation coefficient=0.690, P=0.009). There was no correlation between moult and March T (correlation coefficient=0.177, P=0.31; within groups: C males: correlation coefficient=0.131, P=0.68; T males: correlation coefficient=–0.254, P=0.54) or between moult and May T (correlation coefficient=0.190, P=0.45; within groups: C males: correlation coefficient=0.198, P=0.56; T males: correlation coefficient=–0.227, P=0.67).

The moult T levels of the males used in this study were relatively high compared with levels in wild moulting blue tits, and spring T levels were relatively low compared with wild birds (Peters et al., 2006). This was the case not only for the T-treated males but also for the C males. To check whether the control implants contained T, we assayed them: T levels were below the detection limit, suggesting that this was not the cause of high T levels in the controls during moult. Although the moult T levels were high compared with wild birds, the relative differences in T between the C and T males were within the physiological range.

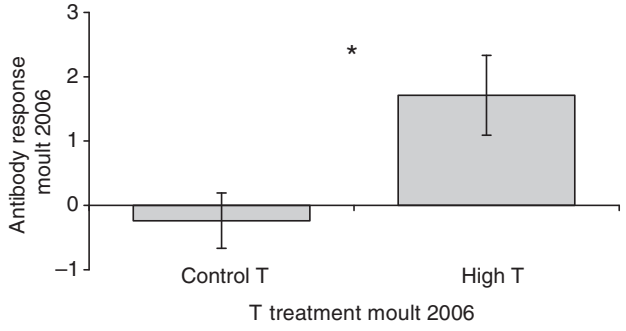

Fig. 1. Secondary antibody response of juvenile male blue tits to sheep red blood cell (SRBC) injection during moult 2006 according to testosterone (T) treatment (±s.e.m.). Response was calculated as the difference between the secondary response and secondary control, and the primary response (T males N=17; control males N=17). Asterisk indicates P<0.05.

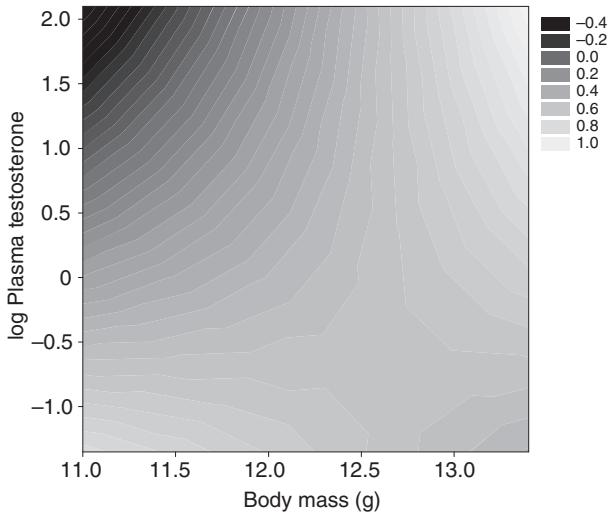

Fig. 2. High testosterone (T) levels and good condition during moult were associated with a high phytohaemagglutinin (PHA) response. Shown are the results of a fitted model of the relationship between log testosterone titre, body mass and PHA response in juvenile male blue tits during moult 2006. Lighter shades represent higher PHA responses. The interaction was significant (P<0.01; N=15).

Table 1. Means and standard errors of immune responses of male blue tits by dietary and testosterone treatment during moult 2006 and in spring 2007

		Moult 2006			
		Dietary treatment		Testosterone (T) treatment	
		Enhanced	Standard	High T males	Control males
Moult 2006	Anti-PHA response (mm)	0.32±0.04	0.33±0.04	0.32±0.05	0.33±0.04
	Anti-SRBC antibody response (corrected values)	0.63±0.64	0.83±0.54	1.71±0.62	–0.24±0.43
Spring 2007	Anti-PHA response (mm)	0.65±0.05	0.70±0.05	0.68±0.04	0.66±0.06
	Anti-SRBC antibody response	5.1±0.40	4.6±0.50	5.5±0.50	4.3±0.40

For details of statistical tests, see text. PHA, phytohaemagglutinin; SRBC, sheep red blood cell; T, testosterone.

Seasonal effects on immune response

The birds' SRBC antibody and PHA responses were significantly higher during the spring than during the previous moult period (SRBC: Wald=13.5, $P<0.001$; PHA: Wald=56.63, $P<0.001$). There were no significant interactions between diet, T treatment group or season (all $P>0.05$). There was no relationship between moult and May PHA responses (correlation coefficient=0.179, $P=0.33$) but there was a significant, positive relationship between moult and March SRBC responses (correlation coefficient=0.384, $P=0.033$).

DISCUSSION

In the present study we found that moulting male blue tits implanted with T had higher antibody responses than C males. Neither diet nor T manipulation affected PHA responses. When comparing immune responses between the moult treatment groups in the following spring, we found that males that had been T-treated (and still had higher T levels than males that had been control treated) again had enhanced humoral immunity. When actual T titres and body mass were used as response variables (rather than treatment groups), we found evidence to suggest that T-mediated effects on immune responses can be condition-dependent, at least in relation to PHA challenge. The interaction between T levels and condition on PHA response was also dependent upon season, with high T associated with high responses during the moult in individuals in good condition; the lowest responses were by males with high T and which were in relatively poor condition. This pattern was reversed during the breeding season, however, with the lowest PHA responses being exhibited by males that were both relatively heavy and had high T levels; the highest responses were by males with low body mass that had high T levels.

During moult, and in the following spring, males that had been T-treated during moult showed significantly higher antibody responses than controls, although there was no direct correlation between actual plasma T and antibody production. In the spring this may have been because blood samples for T assay were taken several weeks before SRBC injection, masking any direct association between plasma T and antibody concentrations. Unfortunately it was not possible to run the same analysis for the moult antibody response but no relationship was discernable from simple graphical investigation of the variables. Therefore, it may be that although T males had higher antibody responses than C males, actual T titres may not have been related to antibody response. It is possible that an unknown side-effect of increasing T caused the enhancement of humoral immunity rather than levels of T *per se*. Although several studies have found an immunosuppressive effect of T (see Roberts et al., 2004), others have also found no effect of T on immunity or a positive relationship between T and immunity (e.g. Hasselquist et al., 1999; Evans et al., 2000; Peters, 2000; Westneat et al., 2003; Greenman et al., 2005; Navara et al., 2006; Roberts et al., 2007a). It seems that high T levels can, directly or indirectly, enhance immunocompetence in the right circumstances. The challenge is to understand when T acts in an immunoenhancing way and when it is immunosuppressive.

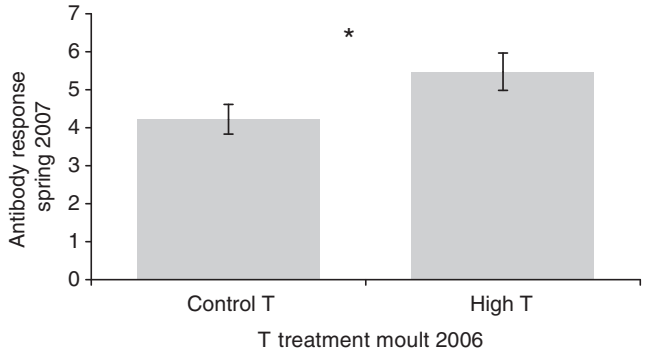

Fig. 3. Secondary antibody response (±s.e.m.) to sheep red blood cell (SRBC) immunisation in March 2007 according to testosterone (T) treatment during the previous moult (T males $N=17$; control males $N=18$). Asterisk indicates $P<0.05$.

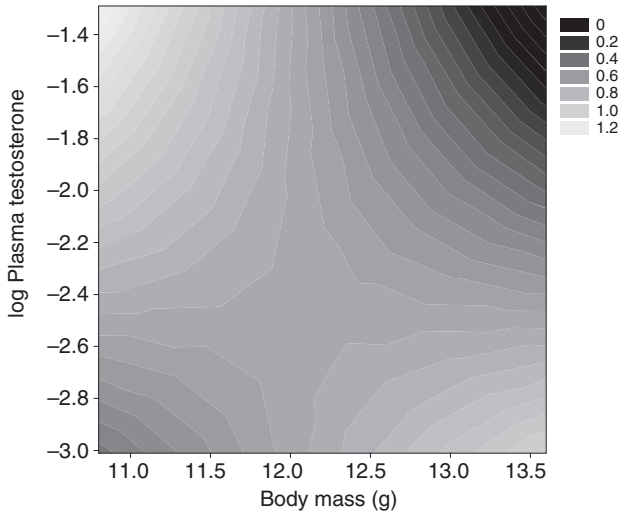

Fig. 4. Males in good condition and with high testosterone (T) levels in the spring had the lowest phytohaemagglutinin (PHA) responses. Shown are the results of a fitted model of the relationship between log testosterone titre, body mass and PHA response in juvenile male blue tits in May 2007. Lighter shades represent higher PHA responses. The interaction was significant ($P<0.01$; $N=26$).

The fact that males that were previously in the high T treatment group (and still had significantly higher T than males that had been in the control group) exhibited higher antibody responses than controls during the moult and in the spring suggests that they were more immunocompetent than C males, at least in terms of humoral immunity. In a separate study on the same experimental subjects, we found that males from the high T group had higher crown UV chroma (a sexually selected signal) in the spring than C males (Roberts et al., 2009). The combination of superior quality sexual signals as well as a robust antibody response suggest that high T during the breeding season is indicative of high quality, because only high T males can both fully express their sexual signals and maintain a fully responsive humoral immune system. This result agrees with work carried out on superb fairy-wrens (*Malurus cyaneus*) by Peters, who found that males with naturally elevated levels of T during the breeding season were also more likely to respond to SRBC injection than males with basal levels of T (Peters, 2000). These results suggest that males that have the highest quality sexual signals during the breeding season (at least party due to high T levels) are advertising their superior immunocompetence to females [*sensu* Hamilton and Zuk (Hamilton and Zuk, 1982)]. But as our present study demonstrates, the effects of T on immunocompetence depend upon the measure of immunity tested and on what condition the individual is in.

The males on the E diet had a significantly greater number of circulating antibodies during the moult than those on a C diet, and the same males were in better condition than the control diet males; however, actual responses did not differ between dietary groups and they were not associated with actual individual condition, contrary to other studies (Birkhead et al., 1998) [but see Møller and Petrie (Møller and Petrie, 2002)]. Some studies have found a positive effect of both supplemental proteins (Smith et al., 2007) [but see Gonzalez et al. (Gonzalez et al., 1999)] and carotenoids (McGraw and Ardia, 2005) on humoral immunocompetence and these may have been the factors that increased antibody titres in the E diet males relative to the control diet males. Contrary to other studies (Lochmiller et al., 1993; Gonzalez et al., 1999; Blount et al., 2003; McGraw and Ardia, 2005) [but see McGraw et al. (McGraw et al., 2006)], we found no effect of extra protein or carotenoids on the PHA response. We also found no evidence of any interaction between T and carotenoids on immunity that has been suggested in other studies (Blas et al., 2006; McGraw and Ardia, 2007) but we did not measure plasma carotenoids and therefore cannot rule out a possible link between T and carotenoid levels. In addition, our study was not designed to investigate the effects of carotenoids *per* se, only to manipulate condition; therefore, it is possible that the E diet had insufficient concentrations of carotenoids to directly affect PHA response. We also found no evidence that T either reduces condition or is related to increased condition, contrary to several earlier studies (Duckworth et al., 2001; Mougeot et al., 2004; Owen-Ashley et al., 2004; Roberts et al., 2007a).

Our experimental T manipulation during moult appeared to have caused a permanent alteration in male phenotype, creating males with higher T, higher antibody responses and more UV crown colour [a sexual signal (Roberts et al., 2009)]. The birds were around nine weeks of age when they were implanted with T or control implants – much older than other avian studies that have found an organisational effect of exogenous T (Strasser and Schwabl, 2004; Eising et al., 2006), although there is evidence in mammals that T can have organisational effects during the juvenile stage of development (Abitbol et al., 1999; Eichmann and Holst, 1999; Sisk and Zehr, 2005). Our present study shows that even at such a relatively old age birds can also still be receptive to organisation by T. The implants themselves could not have caused the differences observed in T between the T groups in the spring, as they had dissolved by November of the previous year, at least five months before the spring immune tests were undertaken (18 of April, 11 of May). Therefore, we have to presume that T manipulation at the fledgling stage does have long-term organisational effects (including producing relatively high levels of T in the breeding season) in this species, although we cannot say if this effect would also be apparent or of importance in free-living blue tits that have much higher T levels.

Although the observed PHA responses during moult fit with the expectations of a condition-dependent role for T in affecting immunity, i.e. T was only immunosuppressive in those males in poor condition, the same cannot be said for the spring PHA results. Counter-intuitively, high PHA responses in this period were associated with both high T levels and low body mass, and the lowest responses were associated with high T and high body mass. We have no satisfactory explanation for this result but one interpretation is that high quality males (those with both high T and in good condition) can tolerate some low-level damage caused by a PHA challenge without needing to mount a large immune response. If this is the case, then the assumption that greater immunocompetence is synonymous with greater immune responses has to be questioned (see Viney et al., 2005). It may be possible that high quality males are able to resist the negative effects of infection without launching a large cell-mediated immune reaction. The fact that PHA responses appear to be particularly costly (e.g. Buchanan et al., 2003; Roberts et al., 2007b) supports this supposition. The significant difference in T levels between moult and spring may account for the different relationships observed between PHA responses, T levels and condition in the different seasons. During moult, T levels, body condition and PHA responses were positively related, possibly because the birds' T levels were very high (both controls and T implanted) and possibly at such high levels T is immunosuppressive except in birds with the necessary resources (i.e. fat reserves) to be able to respond robustly to a mitogen challenge. Higher quality males may pursue a strategy of suppressing costly immune responses (such as PHA responses) in favour of diverting resources to reproductive requirements in the breeding season but invest more heavily in immune function during moult. Another possible reason the nature of the interaction between T and body mass on PHA responses differed between moult and spring is the fact that moult T levels in the T-treated males were artificially increased and so were not of endogenous origin whereas the spring T levels were. Therefore exogenous T may be immunosuppressive in individuals in poor condition (as during the moult) but naturally high T levels (as in spring) are associated with a poor immune response in individuals in good condition. The differences observed between moult and spring PHA responses in relation to T levels and body condition may therefore be dependent upon season, absolute T levels, magnitude of PHA response in general and/or whether the T was endogenous or exogenous in origin. Unfortunately it is impossible in the present study to disentangle the factors that contributed to the observed differences; we encourage future studies to take the above factors into account when investigating the relationship between T, body condition and the response to PHA injection.

Immune responses in the spring were significantly higher than during the moult. Raising a response to PHA injection [that stimulates both innate and acquired aspects of immunity (Martin et al., 2006)] may be too energetically expensive during the moult

and is consequently suppressed or alternatively it may be more important to have a robust response during the breeding season than during the moult. Anti-SRBC antibody responses were also higher in spring than during moult, again suggesting either that moult is too sensitive a period to be able to fully respond to pathogens or that such a response is more important in the spring. Regardless, our results disagree with some previous studies that have found lower immune responses during the breeding period (Greenman et al., 2005; Martin et al., 2008) [but see Møller et al. (Møller et al., 2003)]. However, the birds used in the present study were not subject to any social interactions and, in particular, were not engaged in any breeding-related activities that may trade-off with immunocompetence. This is contrary to the results obtained by Greenman et al. (Greenman et al., 2005), who showed that mere photoperiodic induction of breeding state (without any breeding activity) caused suppression of the PHA response in house sparrows. Also, our captive birds had much lower T levels than is usual for breeding blue tits (Peters et al., 2006). Possibly the lack of social interaction, both in terms of the absence of females and opportunities for male–male aggression, may have lowered T levels in our captive birds (see Wingfield et al., 1990). Maybe the relatively high immune responses our experimental birds exhibited during the breeding season were attributable to their low T levels, although Greenman et al. found that reproductive state itself, rather than T levels, affected immunity during the breeding season (Greenman et al., 2005).

In conclusion, our results show that juvenile male blue tits implanted with high T during the moult exhibited a higher antibody response to SRBC injection than C males both during moult and in the subsequent breeding season, and that responses to PHA challenge depended upon interactions between condition and T level, as well as on seasonal differences. These results do not support the ICHH; indeed the antibody responses suggest that T may enhance immunity. The PHA results do however give some support for the idea of a condition-dependent role for T in mediating immunocompetence. Future studies on the ICHH should take into account the condition of their experimental subjects, their life-history stage and what immune challenges the studies employ, because all of these variables may affect the relationship between T and immunity.

LIST OF ABBREVIATIONS

C	control males
E	enhanced diet
ICHH	immunocompetence handicap hypothesis
PHA	phytohaemagglutinin
ReML	restricted maximum likelihood
RIA	Radioimmunoassay
S	standard diet
SRBC	sheep red blood cells
T	testosterone

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