Testosterone and oxidative stress: the oxidation handicap hypothesis

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Secondary sexual traits (SST) are usually thought to have evolved as honest signals of individual quality during mate choice. Honesty of SST is guaranteed by the cost of producing/maintaining them. In males, the expression of many SST is testosterone-dependent. The immunocompetence handicap hypothesis has been proposed as a possible mechanism ensuring honesty of SST on the basis that testosterone, in addition to its effect on sexual signals, also has an immunosuppressive effect. The immunocompetence handicap hypothesis has received mixed support. However, the cost of testosterone-based signalling is not limited to immunosuppression and might involve other physiological functions such as the antioxidant machinery. Here, we tested the hypothesis that testosterone depresses resistance to oxidative stress in a species with a testosterone-dependent sexual signal, the zebra finch. Male zebra finches received subcutaneous implants filled with flutamide (an anti-androgen) or testosterone, or kept empty (control). In agreement with the prediction, we found that red blood cell resistance to a free radical attack was the highest in males implanted with flutamide and the lowest in males implanted with testosterone. We also found that cell-mediated immune response was depressed in testosterone-treated birds, supporting the immunocompetence handicap hypothesis. The recent finding that red blood cell resistance to free radicals is negatively associated with mortality in this species suggests that benefits of sexual signalling might trade against the costs derived from oxidation.

Keywords: anti-androgen; evolutionary trade-offs; free radicals;
immunocompetence handicap hypothesis; oxidative stress; sexual selection

1. INTRODUCTION

Testosterone has different functions, which include the morphological development of muscles and reproductive organs and spermatogenesis, as well as the expression of conspicuous sexual behaviours and ornaments (e.g. Wingfield *et al*. 2001). Indicator models of sexual selection postulate that secondary sexual traits (SST) reflect the phenotypic/genetic quality of their bearers (Zahavi 1975; Hamilton & Zuk 1982), the reliability of the information being ensured by the cost paid to produce and/or maintain the signal (Grafen 1990). In a seminal paper, Folstad & Karter (1992) put forward a novel hypothesis to explain the honesty of testosterone-based sexual signals. The immunocompetence handicap hypothesis is based on the assumption that high circulating levels of testosterone, necessary for the full development of the signal, can also entail a cost due to the higher risk of mortality derived from the immunosuppressive properties of this steroid. Thus, only individuals with a high-quality immune system, able to fight off parasitic infections, could afford to pay the cost of sexual signalling.

The immunocompetence handicap hypothesis has attracted a lot of attention and an impressive amount of studies have been published on this topic (reviews in Owen-Ashley *et al*. 2004; Roberts *et al*. 2004; Muehlenbein & Bribiescas 2005). However, a recent meta-analysis has shown that convincing experimental evidence supporting the assumption that testosterone is immunosuppressive is rather meagre (Roberts *et al*. 2004). There are several possible reasons for the inconsistency of the results that have been reported so far. Among them, the trade-off between immune functioning and testosterone-dependent sexual signals might reflect the differential allocation of energy/resources to the two functions, instead of a direct immunosuppressive effect of testosterone (Wedekind & Folstad 1994; Casto *et al*. 2001; Wikelski & Ricklefs 2001). This suggests that the reliability of testosterone-dependent sexual signals might be based on other costs and might involve other physiological functions than the immune system.

Oxidative stress is the result of an imbalance between the production of reactive oxygen species (ROS) and antioxidant defences (Finkel & Holbrook 2000). ROS are unstable and very reactive by-products of normal metabolism, causing damaging effects on the principal biomolecules (Finkel & Holbrook 2000). Since testosterone usually enhances the metabolic rate (e.g. Feuerbacher & Prinzinger

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1981; Fryburg *et al.* 1997; Buchanan *et al.* 2001), one could expect that high testosterone levels, necessary to the production of sexual ornaments, might alter the balance between ROS production and antioxidant defences, resulting in an enhanced risk of oxidative stress. In addition, some testosterone-dependent ornaments are produced by pigments with antioxidant properties (e.g. Stoehr & Hill 2001; Jayasooriya *et al.* 2002; Alvarez *et al.* 2005). The allocation of these antioxidant pigments (i.e. carotenoids) to colourful traits (i.e. many red–yellow traits) would reduce the capacity to fight off ROS and might therefore further compromise the oxidative balance.

von Schantz *et al.* (1999) were the first to suggest a role for oxidative stress in the trade-off between the benefits of sexual signalling and the costs derived from sustaining high testosterone levels. This hypothesis is based on the finding that testosterone has been shown to generate an oxidative stress in mammalian tissues (i.e. Chainy *et al.* 1997; Zhu *et al.* 1997). Following von Schantz *et al.* (1999), several other studies have postulated that testosterone has pro-oxidant properties (e.g. Royle *et al.* 2001; Gil *et al.* 2004; Rutkowska *et al.* 2005), even though a closer scrutiny of available data reveals a more complex pattern. High testosterone levels produce oxidation in rat and rabbit testicular tissues (Chainy *et al.* 1997; Aydilek *et al.* 2004; but see Peltola *et al.* 1996), rat muscles (Pansarasa *et al.* 2002) and female human placenta (Zhu *et al.* 1997), but testosterone also has antioxidant properties in the human prostate (Tam *et al.* 2003) and rat nervous system (e.g. Ahlbom *et al.* 2001; Calderón Guzmán *et al.* 2005). These findings therefore indicate that the pro-oxidant effect of testosterone is tissue- and sex-dependent.

In this study, we tested the hypothesis that testosterone generates measurable costs in terms of oxidative stress in a bird species, the zebra finch, whose males harbour a testosterone-dependent ornament. Male zebra finches have orange to red beaks. Beak colour is due to carotenoids in this species (McGraw *et al.* 2002; Alonso-Alvarez *et al.* 2004a) and has been shown to be used by females for mate choice (Burley & Coopersmith 1987; Blount *et al.* 2003; but also see Collins & ten Cate 1996). Testosterone also regulates the expression of the trait, probably by upregulating the synthesis of lipoproteins that carry the pigment (Cynx & Nottebohm 1992; McGraw *et al.* 2006). To investigate the cost of testosterone, we randomly allocated male zebra finches to one of the three groups. In one group, males were implanted with silastic tubes filled with crystalline testosterone (T), in another group, males were implanted with empty tubes and served as a control (C), and the final group received implants filled with an anti-androgen (flutamide, F). Flutamide is an androgen receptor antagonist that prevents endogenous testosterone from binding to androgen receptors (e.g. Schwabl & Kriner 1991; Van Roo 2004).

This experimental design allowed us to assess the effect of testosterone on sexual signalling (beak colour), body mass, T-cell mediated immune response and red blood cell resistance to a free radical attack. We predicted that testosterone-implanted males should have redder beaks than other groups and that controls should have redder beaks than F-implanted males. Similarly, if testosterone is immunosuppressive, we should expect immune response to be the strongest in F-implanted males and the weakest in T-males. Finally, if testosterone generates oxidative stress, we expect red blood cell resistance to free radicals to be the highest in F-males and the lowest in T-males.

2. MATERIAL AND METHODS

(a) *General procedure*

This study was carried out on a captive population of zebra finches housed in an indoor aviary. Forty-two adult males were maintained in individual cages ($0.3 \times 0.4 \times 0.4$ m) for the entire duration of the experiment (four weeks) under constant photoperiod (13 L : 11 D) and temperature ($21 \pm$ s.d. 1°C). Water and food (a commercial mix; Benelux NV, Belgium) were provided *ad libitum*. At the beginning of the experiment (day 0), birds were randomly allocated to one of the following experimental groups. Fourteen birds per group received a 10 mm subcutaneous implant (1.47 mm i.d., 1.96 mm o.d.; Silastic laboratory tubing, Dow Corning) filled with the crystalline testosterone (SIGMA, ref. T1500), flutamide (SIGMA ref. F9397) or left empty. Implants were sealed at both the ends with 1 mm silicon glue (Nusil ref. MED1000, Nusil Technology, Canada). Therefore, the actual portion filled approximated 8 mm. Same-sized implants, filled with T and F, have been successfully used in studies on zebra finches and other passerines (Schwabl & Kriner 1991; Adkins-Regan *et al.* 1994; Williams *et al.* 2003; Van Roo 2004; McGraw *et al.* 2006). The androgen-receptor antagonist activity of flutamide in this species is supported by the results from a recent study (Tomaszycki *et al.* 2006). T- and F-implants were not completely empty at the end of the experiment.

The implants were inserted under the skin between the shoulder and the neck. In spite of the use of a surgical adhesive strip (Steri-Strip Adhesive Skin Closures, 3M) to seal the wound, some birds lost the implants (7 F, 2 C and 2 T) during the course of the experiment. These individuals were of course removed from the statistical analyses (hence $n = 7$, 12 and 12, for F-, C- and T-males, respectively).

(b) *Sampling protocol*

A blood sample (*ca* 150 μl) was taken, and body mass (± 0.1 g) and beak colour measured when birds were implanted (day 0) and 28 days later (day 28). Blood was collected from the brachial vein using heparinized capillaries. A 20 μl aliquot was immediately transferred to another tube containing 730 μl of KRL buffer (150 mM Na$^+$, 120 mM Cl$^-$, 6 mM K$^+$, 24 mM HCO$_3^-$, 2 mM Ca^{2+}, 340 mosm, pH 7.4). The KRL-diluted samples were stored at 4°C before analysis, which occurred within 24 h of collection. The remaining blood sample was centrifuged, and plasma separated and stored at -20°C.

(c) *Circulating testosterone levels*

Testosterone concentration was determined by radioimmunoassay (RIA). Protocols were developed and validated for birds at the CEBC (Centre d'Etudes Biologiques de Chizé; CNRS) as detailed in Chastel *et al.* 2003. Testosterone was extracted from a 40 μl plasma sample in 3 ml diethyl ether and assayed in duplicate in one single assay for all the samples. The lowest concentration detectable was 0.1 ng ml^{-1} and the intra-assay coefficient of variation was 5%. Cross reactivities were less than 15% and 2% between testosterone and dihidrotestosterone, and between testosterone and androstenodione, respectively.

Recovery rates were higher than 90%. Testosterone levels of some samples were below the detection threshold and were assigned to the minimum detection limit ($0.1 \, \mathrm{ng \, mL^{-1}}$) for statistical purposes (i.e. initial sampling: $n=6$, final sampling: $n=2$).

Initial testosterone concentration did not differ between treatments (mean $\pm$ s.e., range; T: 0.75 ± 0.28, $0.1–3.2 \, \mathrm{ng \, ml^{-1}}$; C: 1.58 ± 0.43, $0.1–3.7 \, \mathrm{ng \, ml^{-1}}$; F: 0.94 ± 0.44, $0.1–3 \, \mathrm{ng \, ml^{-1}}$; $F_{2,28} = 0.50$, $p=0.61$). At the end of the experimental period (day 28), T-implanted males clearly had higher values of plasma testosterone than C- and F-males (mean $\pm$ s.e., range; T: 24.7 ± 2.54, $10.5–35 \, \mathrm{ng \, ml^{-1}}$; C: 1.15 ± 0.61, $0.16–7.66 \, \mathrm{ng \, ml^{-1}}$; F: 1.21 ± 0.61, $0.1–4.77 \, \mathrm{ng \, ml^{-1}}$; $F_{2,28} = 60.99$, $p < 0.001$), whereas the difference between these two last groups was not statistically significant (least significant difference (LSD) *post hoc* test: $p=0.95$). This is expected because flutamide does not cause overproduction of testosterone by blocking hypothalamic receptors (Schwabl & Kriner 1991; Van Roo 2004).

(d) Colour measurement
Beak colour was assessed using a Dulux Trade Colour chart (Dulux, France) under the same light conditions (i.e. Bertrand *et al.* 2006). The following specific scale ranging from less red to redder colours was used: 1 (69YR 34/780), 2 (56YR 28/778), 3 (44YR 26/756), 4 (34YR 20/708), 5 (31YR 18/648), 6 (16YR 16/594), 7 (19YR 13/558), 8 (09YR 11/475), 9 (14YR 10/434); the first number and letters indicate the hue, the numerator is the brightness and the denominator is the saturation (Blount *et al.* 2003; Bertrand *et al.* 2006). Beak colour measurements were always performed by the same person (SB) blindly with respect to the treatments.

(e) Assessment of red blood cell resistance to free radicals
Resistance to free radicals was assessed as the time needed to haemolyse 50% of red blood cells exposed to a controlled free radical attack. We used the KRL test (Brevet Spiral V02023, Couternon, France; http://www.nutriteck.com/sunyatakrl. html) adapted to bird physiological parameters (osmolarity and temperature; Alonso-Alvarez *et al.* 2004a,b; Bertrand *et al.* 2006).

Eighty micro litres of the whole blood diluted in the KRL buffer were added to each well of a 96 well microplate. We subsequently added to each well 136 µl of a solution of 2,2'-azobis-(amidinopropane)hydrochloride (646 mg of 2,2'-azobis-(amidinopropane)hydrochloride diluted in 20 ml of KRL buffer), a free radical generator (Rojas Wahl *et al.* 1998). The microplate was then read with a titrator (iEMS Reader MF, Kirial SA, Couternon, France) at 40°C. The rate of haemolysis is determined by the change in optical density measured at 540 nm. Previous work has shown that, if at least one component of the antiradical detoxification system is impaired, the haemolysis curve shows a shift towards shorter times (Blache & Prost 1992; Girard *et al.* 2005). This test therefore provides an assessment of total antioxidant defences, since all families of antioxidants present in the blood are used to fight off the oxidant attack (Blache & Prost 1992; Pieri *et al.* 1996, Girodon *et al.* 1997; Lesgards *et al.* 2002; Stocker *et al.* 2003. Girard *et al.* 2005). Finally, since the rate of peroxidation of lipids of the red blood cell membrane determines the capacity to resist the free radical aggression (e.g. Brzezinska-Slebodzinska 2001; Nagasaka

et al. 2004), this assay would also reflects the degree of oxidation suffered in the recent past. Since the average lifespan of erythrocytes in birds is about 30 days (Sturkie & Griminger 1986), the second blood sampling (28 days from the treatment start) could assess the oxidative damage suffered throughout the experiment.

(f) T-cell mediated immune response
At day 21 of the experiment, T-cell mediated immune response was assessed in all birds. A delayed-type hypersensitivity test was used following the simplified protocol of Smits *et al.* (1999). Birds were injected subcutaneously in the wing web with 50 µg of PHA-P (SIGMA, ref. L-8754) diluted in 20 µl of phosphate-buffered saline (PBS). The thickness of the wing web was measured with a spessimeter (± 0.01 mm) at the injection site just prior to and 24 h after challenge. Swelling of the wing web was the difference in thickness of the wing web prior to and 24 h after injection. The repeatability (Lessells & Boag 1987) of our triplicated measurements was high ($r=0.80$, $p < 0.001$) and hence mean values were used for analyses.

(g) Statistical analyses
Differences in initial values among treatments were analysed using one-way ANOVAs. T-cell mediated immune response was analysed with ANCOVA models including body mass at the sampling date and resistance to free radicals as covariates. Changes in body mass and red blood cell resistance to free radicals during the course of the experiment were analysed by running ANCOVA models with final values as dependent variables and initial values as covariates. Changes in body mass during the course of the experiment and T-cell immune response were also included as covariates in the ANCOVA model that analysed changes in resistance to the free radicals. Body mass, red blood cell resistance to free radicals and T-cell mediated immune response met the homocedasticity (F-Levene tests) and normality assumptions (Shapiro–Wilk test).

To assess the effect of the hormonal treatment on beak colour, we used an ordinal model for multinomial data with a cumulative logit link function (PROC GENMOD in SAS software; SAS Institute 2001).

3. RESULTS
Initial values of body mass, red blood cell resistance to free radicals and beak colour were statistically indistinguishable among treatments (body mass: $F_{2,28} = 2.17$, $p = 0.133$; red blood cell resistance to free radicals: $F_{2,28} = 0.56$, $p=0.579$; beak colour: $\chi_2^2 = 1.36$, $p=0.506$; LSD *post hoc* tests: $p > 0.120$).

The hormonal treatment did not affect changes in body mass (treatment: $F_{2,27} = 0.35$, $p=0.708$; initial body mass as a covariate: $F_{1,27} = 171.3$, $p < 0.001$).

T-cell mediated immune response significantly differed among hormonal groups ($F_{2,28} = 8.04$, $p=0.002$), with T-implanted males having the weakest response and F-males the strongest one (figure 1). T-males had a significantly lower cell-mediated immune response when compared with controls (LSD *post hoc* test: $p=0.028$), whereas the difference between controls and F-males was only close to the significance threshold ($p=0.060$). None of the covariates (body mass and red blood cell resistance
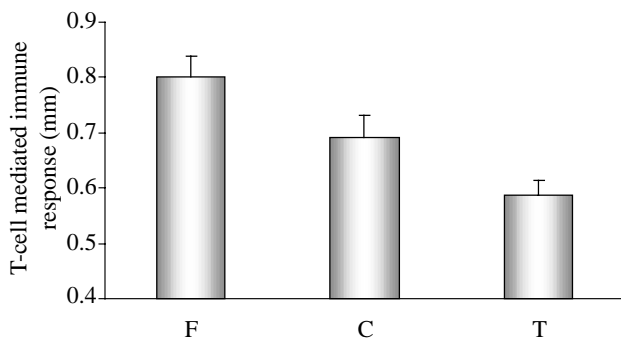
Figure 1. T-cell mediated immune response for male zebra finches treated with anti-androgen (F; flutamide), control (C) or testosterone (T) implants during a 21-day period. Values are means ± SE.
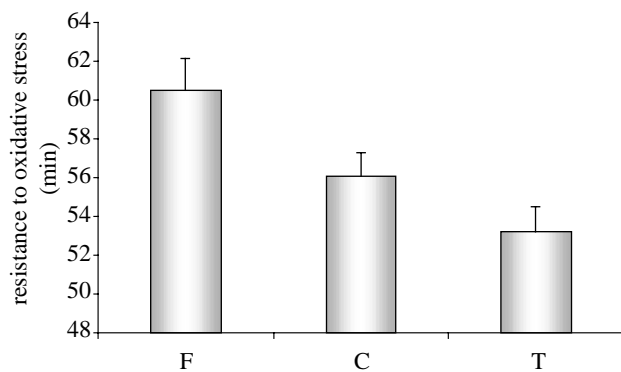

Figure 2. Final values of resistance to oxidative stress for male zebra finches treated with anti-androgen (F; flutamide), control (C) or testosterone (T) implants during a 28-day period. Values are least square means ± SE from an ANCOVA model including initial values of resistance to oxidative stress and body mass at sampling date as covariates.

to free radicals) was correlated with T-cell mediated immune response (all p-values > 0.35).

Beak colour was not affected by the treatment (treatment: $\chi^2_2 = 1.00$, $p = 0.608$; initial beak colour: $\chi^2_1 = 10.75$, $p = 0.001$).

Red blood cell resistance to free radicals was significantly affected by the hormonal treatment (treatment: $F_{2,26} = 5.64$, $p = 0.009$; initial resistance: $F_{1,26} = 8.22$, $p = 0.008$; body mass at the sampling time: $F_{1,26} = 6.85$, $p = 0.015$). Body mass at the sampling time was negatively correlated with the red blood cell resistance to free radicals ($r = -0.49$, $p = 0.005$). T-cell mediated immune response was not significantly correlated with the red blood cell resistance to free radicals ($p = 0.301$). In agreement with the prediction, red blood cell resistance to free radicals was the highest in the group of males implanted with F and the lowest in T-males (figure 2; LSD *post hoc* test: $p = 0.003$). Pairwise comparisons showed that red blood cell resistance to free radicals was significantly higher in F-males when compared with controls ($p = 0.048$), whereas the difference between controls and T-males was not statistically significant ($p = 0.188$).

4. DISCUSSION

In line with our predictions, we found that experimental manipulation of testosterone levels, affected both red blood cell resistance to free radicals and T-cell mediated

immune response. These effects were independent of each other as the T-cell mediated immune response and the red blood cell resistance to free radicals were not correlated. Male zebra finches whose testosterone receptors were blocked by the anti-androgen (flutamide) showed the strongest resistance to free radicals and the strongest immune response, whereas T-implanted males had the weakest resistance to free radicals and the weakest immune response. Contrary to our predictions, the hormonal treatment did not affect beak colour.

von Schantz *et al.* (1999) were the first to suggest a role for oxidative stress in the trade-off between the benefits of sexual signalling and the costs derived from sustaining high testosterone levels. Their hypothesis was based on the finding that testosterone can generate an oxidative stress in testes (Chainy *et al.* 1997) and female placenta (Zhu *et al.* 1997). Later work has, however, shown that the relationship between testosterone and oxidative stress can be more complex than previously thought, as it is tissue- and gender-dependent. For instance, testosterone has been shown (i) to impair the activity of three main antioxidant enzymes in human testes (catalase, superoxide dismutase and glutation peroxidase; Chainy *et al.* 1997), (ii) to upregulate catalase activity in the brain (Ahlbom *et al.* 2001), (iii) to downregulate the activity of pro-oxidant enzymes (i.e. NADPH-oxidase) in the prostate (Tam *et al.* 2003), and (iv) to reduce lipid peroxidation (Calderón Guzmán *et al.* 2005).

In this study, we experimentally tested the hypothesis that high testosterone levels necessary for the expression of secondary sexual traits can generate a cost in terms of oxidative stress. To manipulate testosterone, we used implants filled with this steroid and flutamide, a testosterone antagonist that binds to testosterone receptors. Although we used implants of the same size as those used in studies involving zebra finches, as well as other passerine species, we found that birds implanted with testosterone showed a substantial increase in their levels of circulating testosterone. To assess the relevance of the induced levels of T, we compared them to those reported in other studies. The highest value reported for unmanipulated male zebra finches is 8.2 ng ml^{-1} (McGraw *et al.* 2006), although untreated captive males may reach values higher than 20 ng ml^{-1} (E. Adkins-Regan 2006, personal communication). In this study, the highest value for control birds (with empty implants) was 7.66 ng ml^{-1}, whereas the highest value for T-individuals was 35 ng ml^{-1}. We do not know whether this high value could have induced a pharmacological effect. To assess the sensitivity of the results to these extreme values, we also ran the analyses excluding birds that had testosterone values higher than 20 ng ml^{-1}. Even when restricted to this reduced subsample of birds, the results showed that the androgen manipulation significantly affected both red blood cell resistance to free radicals and T-cell mediated immune response ($p < 0.01$ and $p < 0.05$, respectively), but not beak colour. We are therefore confident that the observed results are not an artefact due to high testosterone levels.

As mentioned above, testosterone has been reported to have a tissue-dependent pro-oxidant effect. To overcome the limitation of this potential tissue-dependent effect of T, we used an assay that is supposed to reflect the organismal antioxidant status. This assay measures the capacity of red blood cells to resist a free radical attack, by mobilizing a

range of antioxidant molecules. Low availability of antioxidants results in rapid cell lysis. In agreement with the prediction, we found that the red blood cell resistance to free radicals was the strongest in birds implanted with an anti-androgen and the weakest in birds implanted with T. The effect of testosterone on resistance to free radicals might involve different mechanisms. First, testosterone can affect metabolic rate (e.g. Feuerbacher & Prinzinger 1981; Fryburg *et al.* 1997; Buchanan *et al.* 2001; but see Buttemer & Astheimer 2000). High metabolic rate might lead to increased free radical production and oxidative damage (Finkel & Holbrook 2000). Moreover, testosterone can also increase locomotor activity as a consequence of changes in behaviour (Wikelski *et al.* 1999; Lynn *et al.* 2000) and a high muscle activity might enhance oxidative stress (e.g. Finaud *et al.* 2006 and references therein). Alternatively or additionally, the effect could be mediated by the role of androgens in the regulation of blood pressure. Androgens increase angiotensin II production, which regulates vasoconstriction by producing peroxides from NADH- and NADPH-oxidase (Reckelhoff 2001). The increase of circulating ROS due to any of these mechanisms could have increased red blood cell susceptibility to free radicals.

Whatever the physiological mechanism behind the observed results, our findings support the idea that the honesty of testosterone-based sexual signals might be reinforced by multiple, possibly additive, costs. This is also in agreement with recent studies (Buchanan *et al.* 2001; Duckworth *et al.* 2001; see also Mougeot *et al.* 2004), which have shown that the cost of high testosterone concentration is not exclusively based on immunosuppression, as proposed by the immunocompetence handicap hypothesis (Folstad & Karter 1992). On the other hand, testosterone-dependent immunossuppression could also be the consequence of an 'indirect pathway' (Owen-Ashley *et al.* 2004) mediated by the pro-oxidant properties of this androgen, as firstly proposed by von Schantz *et al.* (1999). Oxidative stress can impair immune function (e.g. van der Ven & Boers 1997; Cemerski *et al.* 2002), as it seems to occur during aging (immunosenescence; e.g. Daynes *et al.* 2003). Our findings show that T affected independently red blood cell resistance to free radicals and T-cell mediated immune response; nevertheless, only experiments assessing immunity after manipulating both testosterone and oxidative stress levels might disentangle the effects of T on the antioxidant machinery and the immune system.

Contrary to our expectations and to findings from other studies on zebra finches (i.e. Cynx & Nottebohm 1992; McGraw *et al.* 2006), testosterone did not affect the expression of beak colour, a sexually selected trait in this species. It is difficult to say why we could not replicate the results previously obtained in other studies. Cynx & Nottebohm (1992) found a change after only one week post-treatment. However, the study is not comparable because they previously castrated the males, thus inducing the decolouration of the beak (feminization). Meanwhile, McGraw *et al.* (2006) detected a change in zebra finch beak colour eight weeks after the onset of the testosterone treatment, whereas our experiment lasted only for four weeks. We cannot, therefore, exclude that we might have found the same result if the experiment had lasted for eight weeks. Alternatively, it could be that birds experienced optimal conditions in our aviaries and that they already

were harbouring their optimal sexual trait. Nevertheless, in principle, this should apply to any study based on captive birds. In addition, it is also possible that the small sample size used in our study (although similar to the sample size used in the two cited articles), with the associated low statistical power, prevented us from rejecting the null hypothesis that androgen manipulation affects bill colour (with this sample size we would only have been able to detect quite large effect sizes: $f > 0.64$; Cohen 1988). In any case, we should bear in mind that beyond the possible effect of testosterone on beak colour, this androgen has also been repeatedly reported to affect other sexual displays in male zebra finches such as song rate (e.g. Cynx *et al.* 2005) and courtship behaviour (e.g. Springer & Wade 1997).

Physiological costs of testosterone can confer the honesty of sexual signalling only if these costs are associated with fitness components such as survival and reproduction. We have previously found that red blood cell resistance to free radicals decreases with age in zebra finches (Alonso-Alvarez *et al.* 2006). Moreover, red blood cell resistance to free radicals is also positively correlated with survival in this species (Alonso-Alvarez *et al.* 2006), suggesting that the fitness cost of testosterone-induced increase in oxidative stress might be substantial.

REFERENCES

Adkins-Regan, E., Mansukhani, V., Seiwert, C. & Thompson, R. 1994 Sexual differentiation of brain and behavior in the zebra finch: critical periods for effects of early estrogen treatment. *J. Neurobiol.* **25**, 865–877. (doi:10.1002/neu.480250710)

Ahlbom, E., Prins, G. S. & Ceccatelli, S. 2001 Testosterone protects cerebellar granule cells from oxidative stress-induced cell death through a receptor mediated mechanism. *Brain Res.* **892**, 255–262. (doi:10.1016/S0006-8993(00)03155-3)

Alonso-Alvarez, C., Bertrand, S., Devevey, G., Gaillard, M., Prost, J., Faivre, B. & Sorci, G. 2004a An experimental test of dose-dependent effect of carotenoids and immune activation on sexual signals and antioxidant activity. *Am. Nat.* **164**, 651–659. (doi:10.1086/424971)

Alonso-Alvarez, C., Bertrand, S., Devevey, G., Prost, J., Faivre, B. & Sorci, G. 2004b Oxidative stress as a proximate cost of reproduction. *Ecol. Lett.* **7**, 363–368. (doi:10.1111/j.1461-0248.2004.00594.x)

Alonso-Alvarez, C., Bertrand, S., Devevey, G., Prost, J., Faivre, B., Chastel, O. & Sorci, G. 2006 An experimental manipulation of life history trajectories and resistance to oxidative stress. *Evolution* **60**, 1913–1924. (doi:10.1554/05-644.1)

Alvarez, F., Sanchez, C. & Angulo, S. 2005 The frontal shield of the moorhen: sex differences and relationship with body condition. *Ethol. Ecol. Evol.* **17**, 135–148.

Aydilek, N., Aksakal, M. & Karakilcik, A. Z. 2004 Effects of testosterone and vitamin E on the antioxidant system in rabbit testis. *Andrologia* **36**, 277–281. (doi:10.1111/j.1439-0272.2004.00618.x)

Bertrand, S., Alonso-Alvarez, C., Devevey, G., Faivre, B., Prost, J. & Sorci, G. 2006 Carotenoids modulate the trade-off between reproduction and resistance to oxidative stress in zebra finches. *Oecologia* **147**, 576–584. (doi:10.1007/s00442-005-0317-8)

Blache, D. & Prost, M. 1992 Free radical attack: biological test for human resistance capability. In *A lunar-based chemical analysis laboratory* (eds C. Ponnamperuma & C. W. Gehrke), pp. 82–98. Hampton, VA: A. Deepak Publishing.

Blount, J. D., Metcalfe, N. B., Birkhead, T. R. & Surai, P. F. 2003 Carotenoid modulation of immune function and sexual attractiveness in zebra finches. *Science* **300**, 125–127. (doi:10.1126/science.1082142)

Brzezinska-Slebodzinska, E. 2001 Erythrocyte osmotic fragility test as the measure of defence against free radicals in rabbits of different age. *Acta Vet. Hung.* **49**, 413–419.

Buchanan, K. L., Evans, M., Goldsmith, A. R., Bryant, D. M. & Rowe, L. V. 2001 Testosterone influences basal metabolic rate in male house sparrows: a new cost of dominance signalling? *Proc. R. Soc. B* **268**, 1337–1344. (doi:10.1098/rspb.2001.1669)

Burley, N. & Coopersmith, C. B. 1987 Bill color preferences of zebra finches. *Ethology* **76**, 133–151.

Buttemer, W. A. & Astheimer, L. B. 2000 Testosterone does not affect basal metabolic rate or blood parasite load in captive male white-plumed Honeyeaters *Lichenostomus penicillatus*. *J. Avian Biol.* **31**, 479–488. (doi:10.1034/j.1600-048X.2000.310407.x)

Calderón Guzmán, D., Mejía, G. B., Vázquez, I. E., García, E. H., del Angel, D. S. & Olguín, H. J. 2005 Effect of testosterone and steroids homologues on indolamines and lipid peroxidation in rat brain. *J. Steroid Biochem. Mol. Biol.* **94**, 369–373. (doi:10.1016/j.jsbmb.2004.11.006)

Casto, J. M., Nolan Jr, V. & Ketterson, E. D. 2001 Steroid hormones and immune function: experimental studies in wild and captive dark-eyed juncos (*Junco hyemalis*). *Am. Nat.* **157**, 408–420. (doi:10.1086/319318)

Cemerski, S., Cantagrel, A., Van Meerwijk, J. P. & Romagnoli, P. 2002 Reactive oxygen species differentially affect T cell receptor-signaling pathways. *J. Biol. Chem.* **277**, 19 585–19 593. (doi:10.1074/jbc.M111451200)

Chainy, G. B. N., Samantaray, S. & Samanta, L. 1997 Testosterone-induced changes in testicular antioxidant system. *Andrologia* **29**, 343–349.

Chastel, O., Lacroix, A. & Kersten, M. 2003 Pre-breeding energy requirements: thyroid hormone, metabolism and the timing of reproduction in house sparrows *Passer domesticus*. *J. Avian Biol.* **34**, 298–306. (doi:10.1034/j.1600-048X.2003.02528.x)

Cohen, J. 1988 *Statistical power for the behavioural sciences*, 2nd edn. Hillsdale, NJ: Erlbaum.

Collins, S. A. & ten Cate, C. 1996 Does beak colour affect female preference in zebra finches? *Anim. Behav.* **52**, 105–112. (doi:10.1006/anbe.1996.0156)

Cynx, J. & Nottebohm, F. 1992 Testosterone facilitates some conspecific song discriminations in castrated zebra finches (*Taeniopygia guttata*). *Proc. Natl Acad. Sci. USA* **89**, 1376–1378. (doi:10.1073/pnas.89.4.1376)

Cynx, J., Bean, N. J. & Rossman, I. 2005 Testosterone implants alter the frequency range of zebra finch songs. *Horm. Behav.* **47**, 446–451. (doi:10.1016/j.yhbeh.2004.11.018)

Daynes, R. A., Enioutina, E. Y. & Jones, D. C. 2003 Role of redox imbalance in the molecular mechanisms responsible for immunosenescence. *Antioxid. Redox Signal.* **5**, 537–548. (doi:10.1089/152308603770310185)

Duckworth, R. A., Mendonça, M. T. & Hill, G. E. 2001 A condition dependent link between testosterone and disease resistance in the house finch. *Proc. R. Soc. B* **268**, 2467–2472. (doi:10.1098/rspb.2001.1827)

Feuerbacher, I. & Prinzinger, R. 1981 The effects of the male sex-hormone testosterone on body temperature and energy metabolism in male Japanese quail (*Coturnix coturnix japonica*). *Comp. Biochem. Physiol.* **70A**, 247–250. (doi:10.1016/0300-9629(81)91454-7)

Finaud, J., Lac, G. & Filaire, E. 2006 Oxidative stress—relationship with exercise and training. *Sports Med.* **36**, 327–358. (doi:10.2165/00007256-200636040-00004)

Finkel, T. & Holbrook, N. J. 2000 Oxidants, oxidative stress and the biology of ageing. *Nature* **408**, 239–247. (doi:10.1038/35041687)

Folstad, I. & Karter, A. K. 1992 Parasites, bright males and the immunocompetence handicap. *Am. Nat.* **139**, 603–622. (doi:10.1086/285346)

Fryburg, D. A., Weltman, A., Jahn, L. A., Weltman, J. Y., Samojlik, E., Hintz, R. L. & Veldhuis, J. D. 1997 Short-term modulation of the androgen milieu alters pulsatile, but not exercise- or growth hormone (GH)-releasing hormone-stimulated GH secretion in healthy men: impact of gonadal steroid and GH secretory changes on metabolic outcomes. *J. Clin. Endocrinol. Metab.* **82**, 3710–3719. (doi:10.1210/jc.82.11.3710)

Gil, D., Heim, C., Bulmer, E., Rocha, M., Puerta, M. & Naguib, M. 2004 Negative effects of early developmental stress on yolk testosterone levels in a passerine bird. *J. Exp. Biol.* **207**, 2215–2220. (doi:10.1242/jeb.01013)

Girard, A., Madani, S., El Boustani, E. S., Belleville, J. & Prost, J. 2005 Changes in lipid metabolism and antioxidant defense status in spontaneously hypertensive rats and Wistar rats fed a diet enriched with fructose and saturated fatty acids. *Nutrition* **21**, 240–248. (doi:10.1016/j.nut.2004.04.022)

Girodon, F., Blache, D., Monget, A. L., Lombart, M., Brunet-Lecompte, P. & Arnaud, J. 1997 Effect of a two-year supplementation with low doses of antioxidant vitamins and/or minerals in elderly subjects on levels of nutrients and antioxidant defense parameters. *J. Am. Coll. Nutr.* **16**, 357–365.

Grafen, A. 1990 Biological signals as handicaps. *J. Theor. Biol.* **144**, 517–546.

Hamilton, W. D. & Zuk, M. 1982 Heritable true fitness and bright birds—a role for parasites. *Science* **218**, 384–387. (doi:10.1126/science.7123238)

Jayasooriya, A. P., Weisinger, R. S., Weisinger, H. S., Mathai, M. L. & Sinclair, A. J. 2002 Attraction to orange: sexiness, not gluttony. *Science* **296**, 847. (doi:10.1126/science.296.5569.847b)

Lesgards, J.-F., Durand, P., Lassare, M., Stocker, P., Lesgards, G. & Lanteaume, A. 2002 Assessment of lifestyle effects on the overall antioxidant capacity of healthy subjects. *Environ. Health. Persp.* **110**, 479–487.

Lessells, C. M. & Boag, P. T. 1987 Unrepeatable repeat-abilities: a common mistake. *Auk* **104**, 116–121.

Lynn, S. E., Houtman, A. M., Weathers, W. W., Ketterson, E. D. & Nolan, V. 2000 Testosterone increases activity but not daily energy expenditure in captive male dark-eyed juncos. *Junco hyemalis. Anim. Behav.* **60**, 581–587. (doi:10.1006/anbe.2000.1510)

McGraw, K. J., Adkins-Regan, E. & Parker, R. S. 2002 Anhydrolutein in the zebra finch: a new, metabolically derived carotenoid in birds. *Comp. Biochem. Physiol. B* **132**, 813–820.

McGraw, K. J., Correa, S. M. & Adkins-Regan, E. 2006 Testosterone upregulates lipoprotein status to control sexual attractiveness in a colorful songbird. *Behav. Ecol. Sociobiol.* **60**, 117–122. (doi:10.1007/s00265-005-0135-3)

Mougeot, F., Irvine, J. R., Seivwright, L., Redpath, S. M. & Piertney, S. 2004 Testosterone, immunocompetence, and honest sexual signaling in male red grouse. *Behav. Ecol.* **15**, 930–937. (doi:10.1093/beheco/arh087)

Muehlenbein, M. P. & Bribiescas, R. G. 2005 Testosterone-mediated immune functions and male life histories. *Am. J. Hum. Biol.* **17**, 527–558. (doi:10.1002/ajhb.20419)

Nagasaka, R., Okamoto, N. & Ushio, H. 2004 Partial oxidative-stress perturbs membrane permeability and fluidity of fish nucleated red blood cells. *Comp. Biochem. Physiol. C* **139**, 259–266.

Owen-Ashley, N. T., Hasselquist, D. & Wingfield, J. C. 2004 Androgens and the immunocompetence handicap hypothesis: unraveling direct and indirect pathways of immunosuppression in song sparrows. *Am. Nat.* **164**, 490–505. (doi:10.1086/423714)

Pansarasa, O., D'Antona, G., Gualea, M. R., Marzani, B., Pellegrino, M. A. & Marzatico, F. 2002 "Oxidative stress": effects of mild endurance training and testosterone treatment on rat gastrocnemius muscle. *Eur. J. Appl. Physiol.* **87**, 550–555. (doi:10.1007/s00421-002-0668-3)

Peltola, V., Huhtaniemi, I., Ketala, T. M. & Ahotupa, M. 1996 Induction of lipid peroxidation during steroidogenesis in the rat testis. *Endocrinology* **137**, 105–112. (doi:10.1210/en.137.1.105)

Pieri, C., Moroni, F. & Marra, M. 1996 Food restriction increases the protection of erythrocytes against the hemolysis induced by peroxyl radicals. *Mech. Ageing Dev.* **87**, 15–23. (doi:10.1016/0047-6374(95)01694-5)

Reckelhoff, J. F. 2001 Gender differences in the regulation of blood pressure. *Hypertension* **37**, 1199–1208.

Roberts, M. L., Buchanan, K. L. & Evans, M. R. 2004 Testing the immunocompetence handicap hypothesis: a review of the evidence. *Anim. Behav.* **68**, 227–239. (doi:10.1016/j.anbehav.2004.05.001)

Rojas Wahl, R. U., Zeng, L., Madison, S. A., De Pinto, R. L. & Shay, B. J. 1998 Mechanistic studies on the decomposition of water soluble azo-radical-initiators. *J. Chem. Soc. Perk T 2* **9**, 2009–2018. (doi:10.1039/a801624k)

Royle, N. J., Surai, P. F. & Hartley, I. R. 2001 Maternally derived androgens and antioxidants in bird eggs: complementary but opposing effects? *Behav. Ecol.* **12**, 381–385. (doi:10.1093/beheco/12.4.381)

Rutkowska, J., Cichon, M., Puerta, M. & Gil, D. 2005 Negative effects of elevated testosterone on female fecundity in zebra finches. *Horm. Behav.* **47**, 585–591. (doi:10.1016/j.yhbeh.2004.12.006)

SAS Institute 2001 *SAS/STAT Software: changes and enhancements, Version 8.2.* Cary, NC: SAS Publishing.

Schwabl, H. & Kriner, E. 1991 Territorial aggression and song of male European robins (*Erithacus rubecula*) in autumn and spring: effects of antiandrogen treatment. *Horm. Behav.* **25**, 180–194. (doi:10.1016/0018-506X(91)90049-N)

Smits, J. E., Bortolotti, G. R. & Tella, J. L. 1999 Simplifying the phytohemagglutinin skin-testing technique in studies of avian immunocompetence. *Funct. Ecol.* **13**, 567–572. (doi:10.1046/j.1365-2435.1999.00338.x)

Springer, M. L. & Wade, J. 1997 The effects of testicular tissue and prehatching inhibition of estrogen synthesis on the development of courtship and copulatory behavior in zebra finches. *Horm. Behav.* **32**, 46–59. (doi:10.1006/hbeh.1997.1406)

Stoehr, A. M. & Hill, G. E. 2001 The effects of elevated testosterone on plumage hue in male house finches. *J. Avian Biol.* **32**, 153–158. (doi:10.1034/j.1600-048X.2001.320208.x)

Stocker, P., Lesgards, J. F., Vidal, N., Chalier, F. & Prost, M. 2003 ESR study of a biological assay on whole blood: antioxidant efficiency of various vitamins. *Biochima Biophysica Acta* **1621**, 1–8.

Sturkie, P. D. & Griminger, P. 1986 Body fluids: blood. In *Avian physiology* (ed. P. D. Sturkie), pp. 102–129. New York, NY: Springer.

Tam, N. N. C., Gao, Y., Leung, Y. K. & Ho, S. M. 2003 Androgenic regulation of oxidative stress in the rat prostate—involvement of NAD(P)H oxidases and anti-oxidant defense machinery during prostatic involution and regrowth. *Am. J. Pathol.* **163**, 2513–2522.

Tomaszycki, M. L., Banerjee, S. B. & Adkins-Regan, E. 2006 The role of sex steroids in courtship, pairing and pairing behaviors in the socially monogamous zebra finch. *Horm. Behav.* **50**, 141–147. (doi:10.1016/j.yhbeh.2006.02.005)

van der Ven, A. J. & Boers, G. H. 1997 Oxidative stress in immunodeficiency. *Eur. J. Clin. Invest.* **27**, 731–732. (doi:10.1046/j.1365-2362.1997.1890739.x)

Van Roo, B. L. 2004 Exogenous testosterone inhibits several forms of male parental behavior and stimulates song in a monogamous songbird: the blue-headed vireo (*Vireo solitarius*). *Horm. Behav.* **46**, 678–683. (doi:10.1016/j.yhbeh.2004.06.011)

von Schantz, T., Bensch, S., Grahn, M., Hasselquist, D. & Wittzell, H. 1999 Good genes, oxidative stress and condition-dependent sexual signals. *Proc. R. Soc. B* **266**, 1–12. (doi:10.1098/rspb.1999.0597)

Wedekind, C. & Folstad, I. 1994 Adaptive or nonadaptive immunosuppression by sex-hormones. *Am. Nat.* **143**, 936–938. (doi:10.1086/285641)

Wikelski, M. & Ricklefs, R. E. 2001 The physiology of life histories. *Trends Ecol. Evol.* **16**, 479–481. (doi:10.1016/S0169-5347(01)02279-0)

Wikelski, M., Lynn, S., Breuner, C., Wingfield, J. C. & Kenagy, G. J. 1999 Energy metabolism, testosterone and corticosterone in white-crowned sparrows. *J. Comp. Physiol. A* **185**, 463–470. (doi:10.1007/s003590050407)

Wingfield, J. C., Lynn, S. E. & Soma, K. K. 2001 Avoiding the "costs" of testosterone: ecological bases of hormone–behavior interactions. *Brain Behav. Evol.* **57**, 239–251. (doi:10.1159/000047243)

Williams, H., Connor, D. M. & Hill, J. H. 2003 Testosterone decreases the potential for song plasticity in adult male zebra finches. *Horm. Behav.* **44**, 402–412. (doi:10.1016/j.yhbeh.2003.06.005)

Zahavi, A. 1975 Mate selection—a selection for a handicap. *J. Theor. Biol.* **53**, 205–214. (doi:10.1016/0022-5193(75)90111-3)

Zhu, X. D., Bonet, B. & Knopp, R. H. 1997 17β-estradiol, progesterone, and testosterone inversely modulate low-density lipoprotein oxidation and cytotoxicity in cultured placental trophoblast and macrophages. *Am. J. Obstet. Gynec.* **177**, 196–209. (doi:10.1016/S0002-9378(97)70462-9)