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Male ornament size in a passerine predicts the inhibitory effect of testosterone on macrophage phagocytosis

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Summary

1. The immunocompetence handicap hypothesis (ICHH) proposes that androgen-induced immunosuppression is the mechanism that restricts the expression of exaggerated male ornaments to superior males. Numerous tests of this hypothesis have been conducted on the humoral and cell-mediated components of immunity, with mixed results. Surprisingly, no study so far has addressed whether macrophage phagocytosis, a basic immune function, plays a role in the ICHH.

2. We tested whether the ornament size of male spotless starlings (*Sturnus unicolor*) is a predictor of in vitro macrophage phagocytosis. We found that a moderate physiological concentration of testosterone (T) induced strong phagocytic inhibition. We found no relationship between ornament size and phagocytic activity in basal conditions.

3. Basal phagocytosis was not significantly predicted by ornament size or original testosterone levels. Contrary to expectations, phagocytosis under a moderate T concentration was negatively related to ornament size. Furthermore, a nonsignificant trend for original T concentration to negatively affect T-medium phagocytosis was also found.

4. Our results provide support to the ICCH and suggest that males with exaggerated ornaments and high T concentrations may counteract the inhibitory action of testosterone by some compensatory mechanism. Possible candidates include the presence of immunoenhancing substances, such as melatonin or antioxidants, or differential receptor activity. These mechanisms should be evaluated when testing the reliability of the ICCH in wild populations.

Key-words: handicap, immunocompetence, immunosuppression, ornament, phagocytosis, sexual selection, spotless starling, *Sturnus unicolor*, testosterone

Introduction

In the last decade, a large body of evidence has accumulated, suggesting that exaggerated secondary sexual characteristics in males, also called ornaments, are reliable indicators of condition (Zahavi 1975, 1977; Grafen 1990; Andersson 1994). Females have been shown to obtain direct benefits and/or good genes by selecting males bearing large ornaments as mates (Welch, Semlitsch & Gerhardt 1998; Møller & Alatalo 1999a).

However, there is still debate about the mechanism by which ornaments acquire the additive genetic variance that is required by a honest signalling process when females do not obtain direct benefits (Pomiankowski & Møller 1995; Rowe & Houle 1996). One of the possibilities that has received most support is parasite-driven sexual selection (Hamilton & Zuk 1982; Andersson 1994; Møller, Christe & Lux 1999b), coupled with a process of condition-dependent ornamentation called the immunocompetence handicap hypothesis (ICHH) (Folstad & Karter 1992).

According to the ICHH, high testosterone (T) levels required to express large ornaments (or back up their presence) act as a handicap, restricting which males can withstand the immune depression that is imposed by T. This process is expected to lead to positive covariance between immune function, parasite resistance, T levels and ornament expression (Folstad & Karter 1992).

In birds, many studies have found evidence consistent with the ICHH (e.g. Saino & Møller 1996; Møller, Christe & Lux 1999b; Peters 2000; Casto, Nolan & Ketterson 2001; Loyau *et al.* 2007). However, this result is by no means universal (e.g. Hasselquist *et al.* 1999) and depends on the taxa being

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examined (Roberts, Buchanan & Evans 2004). Several modifications of the original hypothesis have been suggested to account for these discrepancies. For instance, it has been proposed that the immunosuppressive effect of T implants is actually due to a parallel increase in the levels of corticosterone (CORT) or reactive oxygen species (Alonso-Alvarez *et al*. 2007), thus suggesting an indirect route for the process (Owen-Ashley, Hasselquist & Wingfield 2004). Furthermore, the immune system comprises many different components that need not covary positively among each other (e.g. Palacios *et al*. 2007). An additional level of complexity is the redistribution of immune resources through the body, which suggests the need for holistic measures of immunocompetence (Braude, Tang-Martinez & Taylor 1999).

One of the most basic components of immune defence is phagocytosis, a process that bridges innate and adaptive branches of immunity (Stuart & Ezekowitz 2005). It is surprising that no study so far has studied the possible role of phagocytosis in the ICHH in a wild population. There is a single study in domestic hens in which juvenile chickens whose testosterone levels had been increased showed reduced phagocytic ability (Al Afaleq & Homeida 1998). Phagocytosis is the first step of the immune response to a large number of pathogen species or strains (Roitt, Brostoff & Male 1996). Phagocytic cells detect microbial aggression by means of wide-spectrum receptors and then ingest and kill microbes inside the phagosome by means of various cellular weapons. Once the dead microbe is digested, its antigenic information is extracted and presented to molecules of the major histocompatibility complex which will later bind to T-cell receptors in the cell surface. This way, phagocytes coordinate the innate and adaptive branches of the immune system (for reviews see for instance Aderem 2002; Stuart & Ezekowitz 2005).

Besides its central role in immunity, phagocytosis meets several important criteria for a mechanism involved in the ICHH. Firstly, phagocytic cells can respond to androgens by a variety of means, including specific androgen receptors (AR), nongenomic cytoplasmatic processes and regulation of IgG antigen receptors (Kuhnle *et al*. 1994; Slater, Fitzpatrick & Schreck 1995; Gómez *et al*. 2000; Ahmadi & Mccruden 2006). Secondly, phagocytic cell function and survival are highly dependent on the hormonal milieu, showing immunosuppression by androgens and glucocorticoids and immunoenhancement by melatonin (Slater & Schreck 1997; Al Afaleq & Homeida 1998; Rodríguez *et al*. 2001; Benten *et al*. 2004; Singh & Haldar 2005). Thirdly, phagocytic efficiency shows senescent patterns (Butcher *et al*. 2001; Terrón *et al*. 2004), thus suggesting a cost for its maintenance, as we would expect of a costly component of the immune system (Sheldon & Verhulst 1996).

It is likely that the neglect that phagocytosis has received from evolutionary ecologists is because of the higher difficulty in measuring this response as compared with other immune functions (Grasman 2002). In compensation for this difficulty, methods for the study of phagocytic function allow researchers to perform in vitro comparisons of phagocytic function under different conditions, thus allowing a test of the ICHH in which each individual can work as its own control under different androgen levels. In this study, we implement these existing in vitro methods from ecotoxicology to measure phagocytic function in a population of spotless starlings (*Sturnus unicolor*). In this species (Fig. 1), throat feather length is an age-dependent, sexually dimorphic, sexually selected character (Hiraldo & Herrera 1974; Aparicio, Cordero & Veiga 2001). We test whether phagocytic function is reduced under physiological T levels and whether ornament size is a good predictor of this reduction. Specifically, we predict a negative relationship between T-mediated immunosuppression and throat feather length, as expected if ornaments evolve as condition-dependent handicaps (Zahavi 1977).

Fig. 1. Male spotless starling (photograph: Diego Gil).

Materials and methods

FIELD PROCEDURES

The spotless starling (*Sturnus unicolor*) is a medium-sized, facultatively polygynous passerine. Previous experimental studies show that high testosterone levels lead to increased mate attraction in this species (Veiga *et al*. 2001). The study was conducted in a mixed woodland of oak and ash with abundant open areas used by grazing cattle, in Soto del Real (Madrid). The breeding population (*c*. 200 nest boxes) has been studied since 2003. For this study, we caught males by placing additional nest boxes fitted with spring traps near their real nest boxes. Males typically explore and defend potential new nests as soon as they discover them, often depositing green material inside the box. We captured males ($N = 43$) in the first 5 h after dawn during the first fortnight of June 2006, at the time when most of the population was incubating their second clutch. Males occupy and defend new boxes until late June, because floater females can be attracted to breed throughout the breeding season. From each male, we took extensive biometrical data. Three feathers were carefully plucked from the throat, aiming at selecting those that were the longest. Previous data in this species have shown that feather length is a male secondary sexual character predicting female attraction and reproductive success (Aparicio, Cordero & Veiga 2001). The length of these feathers was measured later in the laboratory (from the tip to the base), showing a very high measurement repeatability (intraclass regression coefficient: $r_i = 0{\cdot}92$; $F_{65,132} = 33{\cdot}53$, $P < 0{\cdot}001$). A blood sample of around 0·8 mL (range 0·5–0·8) was taken from the

jugular vein and stored in a glass Vacutainer containing traces of EDTA and an equal volume of RPMI+ culture medium (see elsewhere for formulae of culture media). Tubes were gently rocked and stored in an ice bag (around 4 °C) until we arrived 4–7 h later to our field laboratory (Ventorrillo Field Station).

CULTURE MEDIA AND CELL SEPARATION

For cell cultures, we used RPMI+ (Lavoie & Grasman 2005). This medium was obtained by mixing hepes-supplemented Roswell Park Memorial Institute 1640 culture medium (Sigma, St. Louis, MO, USA) with 0·1% bovine serum albumin and penicillin/streptomycin/neomycin (Sigma). This mix was kept at 4 °C in the fridge and warmed to 39 °C before it was used on the samples.

We followed the protocol of Finkelstein *et al.* (2003). In the laboratory, samples containing blood plus RPMI+ were layered in a glass tube over a 1 : 1 volume of a double density gradient (Histopaque 1177 and 1119; Sigma Aldrich, St. Louis, MO, USA). Tubes were spun 700 ***g*** for 30 min at room temperature. The upper plasma plus culture medium layer was removed and stored at −20 °C for hormone analysis. Monocytes and leucocytes trapped in the buffy ring between plasma and Histopaque 1177 were carefully removed with a pipette and washed twice in warm (37 °C) RPMI+. Viable cells were counted in a hematocytometer after staining with trypan blue, and final solutions were resuspended in RPMI+ to obtain a working concentration of 5×10^{-5} cells mL^{-1}.

PHAGOCYTOSIS ASSAY

We followed a previously published protocol used in birds (Rodríguez *et al.* 2001). For additional information on phagocytosis assays, please see Supporting Information (Appendix S1). Briefly, we pipetted 0·25 mL of the cell solution in MIF plate wells and incubated them for 1 h at 39 °C. This allows macrophages to adhere to the bottom of the plates. Average cell viability as measured by trypan blue exclusion at this step was very high: mean = 98·32 (SE = 0·25). Nonadherent leucocytes were removed by gently flicking off old media from the wells. Wells were then filled with 0·25 mL of a warm RPMI+ solution containing latex beads at 0·01% (Sigma) supplemented with either no T (control solution) or 1 ng mL^{-1} T concentration diluted in the same culture medium (T medium). This concentration is well within the physiological variation in this species, the closely related European starling (*Sturnus vulgaris*) and indeed most passerine species (author's unpublished data; Duffy *et al.* 2000; Garamszegi *et al.* 2004). Plates were incubated for 30 min at 39 °C, rinsed with PBS, fixed in a methanol bath for 5 min, stained with eosin and hematoxylin (seven plunges per stain) and dried overnight.

Plates were examined at × 1000 magnification with immersion oil. We analysed 100 cells per well, recording the number of latex beads that were either engulfed or in contact with each cell (see Appendix S1 in Supporting Information). The person that evaluated phagocytosis was blind with respect to the identity of the sample. We expressed phagocytic function as percentage of cells in which at least a latex bead had been engulfed or was adhered to the cell surface (percentage of phagocytosis: Rodríguez *et al.* 2001). Identical results were obtained if we used the total number of phagocytised latex beads as a measure. Three replicates per condition were run, resulting in a total of six wells per sample. Analysis of variance of triplicate wells per treatment showed that our phagocytosis assay was moderately repeatable in both conditions (intraclass correlation coefficients derived from one-way ANOVAS: basal: $ICC = 0{\cdot}46$ ($F_{55,84} = 3{\cdot}6$, $P < 0{\cdot}001$); T medium: $ICC = 0{\cdot}51$ ($F_{55,83} = 4{\cdot}1$, $P < 0{\cdot}001$). Thus, average values were computed per sample.

HORMONE ASSAYS

Although macrophages had been separated and washed from plasma, it is possible that circulating hormones in plasma might have caused a long-term effect in the physiology of the cells (e.g. by altering receptor numbers or receptor responsiveness). We assayed T and CORT in the available samples to ascertain whether phagocytosis was affected by concentration of these steroids of the original samples and added these concentrations to ornament size in the analyses.

Steroids were extracted by mixing samples with 10 × diethyl ether, vortexing for 1 min and decanting the upper layer after freezing in mixture of dry ice and ethanol. The etheric phase was dried by a stream of filtered air and steroids resuspended in steroid-free serum (DRG, Marburg, Germany). Total T and CORT were assayed in duplicate by EIA kits (T: Cayman Chem, Ann Arbor, MI, USA; CORT: IDS Ltyd, Boldon, UK), following manufacturer's instructions. Within-assay coefficients of variation were as follows: 6·43% for CORT and 8·72% for T. Reported cross-reactivity of the T antiserum with two other androgens was relatively high (5α-DHT: 27%, androstenedione: 3·7%), and thus, we will refer to this measure as androgen concentration.

Because total T levels may not be a biologically relevant measure owing to variation in carrying proteins in plasma, we measured free T in a small sample of animals for which we had enough plasma. Concentrations of free T assayed by a commercial EIA kit (DRG) were highly correlated with total T, confirming that total testosterone is a reliable estimate of testosterone availability ($F_{1,9} = 106{\cdot}1$, $P < 0{\cdot}001$; $r^2 = 0{\cdot}91$). Reported cross-reactivity of the T antibody was < 0·001% for all androgen metabolites tested. Because blood samples were diluted 1 : 1 in culture media, our procedure for separating cells does not allow us to obtain pure plasma. Therefore, hormone concentrations are expressed with respect to blood volume rather than plasma volume.

STATISTICS

Parametric statistics were used throughout after log transformation of hormone concentrations. Phagocytosis scores were normally distributed. We used general linear modelling in SAS to test the contribution of several predictors to phagocytosis scores. Models were progressively reduced removing interactions and nonsignificant terms, and final models with the lowest AIC were selected. Residuals from models did not depart from normality for any analyses.

Results

The presence of T in moderate physiological levels (T medium: 1 ng mL^{-1}) inhibited cell phagocytosis (paired t test: $t = 10{\cdot}58$, d.f. = 42, $P < 0{\cdot}001$; Fig. 2). The extent of this inhibition was large: 33% fewer cells phagocytised beads in the T medium than in basal condition [basal phagocytosis: 45·31% (SE = 1·63) vs. T-medium phagocytosis: 29·9% (SE = 1·67)].

We ran separate ANCOVA models for basal and T-medium phagocytosis. The initial model for basal phagocytosis included ornament size (throat feather length), and corticosterone and testosterone levels at the time of blood sampling,

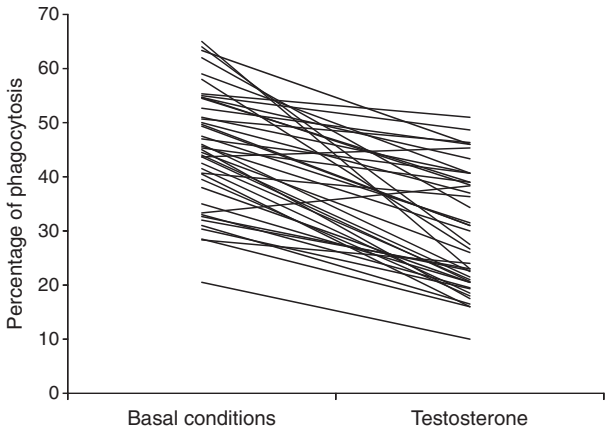

Fig. 2. Percentage of cells that phagocyte in basal conditions and in T medium in adult male spotless starlings. Lines connect mean individual values in the two treatments.

as well as first-order interactions. No significant effects were found, and interactions and terms were dropped in order of lack of significance. The final model (lowest AIC) kept, however, two nonsignificant covariates as follows: throat feather length [$F_{1,40}$ = 2·67, P = 0·11; estimate (SE) = 0·65 (SE = 0·40)] and testosterone levels [$F_{1,40}$ = 2·12, P = 0·15; estimate (SE) = −5·55 (SE = 3·81)].

In the initial ANCOVA model for T-medium phagocytosis, we included the following: basal phagocytosis, throat feather length, testosterone and corticosterone levels as covariates, as well as all first-order interactions. In the final model with the lowest AIC, all interactions had been dropped, and the following terms were kept: basal phagocytosis [$F_{1,39}$ = 28·6, P < 0·0001; estimate (SE) = 0·64 (SE = 0·11)], throat feather length ($F_{1,39}$ = 4·48, P < 0·05; estimate (SE) = −0·66 (SE = 0·31); Fig. 3a) and a nonsignificant trend for T levels ($F_{1,39}$ = 3·75, P = 0·06; estimate (SE) = −5·73 (SE = 2·96); Fig. 3b). In other words, birds with long throat feathers had a weaker T-medium phagocytosis than birds with short throat feathers. There was also a tendency for birds with high T values to be worse at phagocyting in a T medium than birds with low T levels.

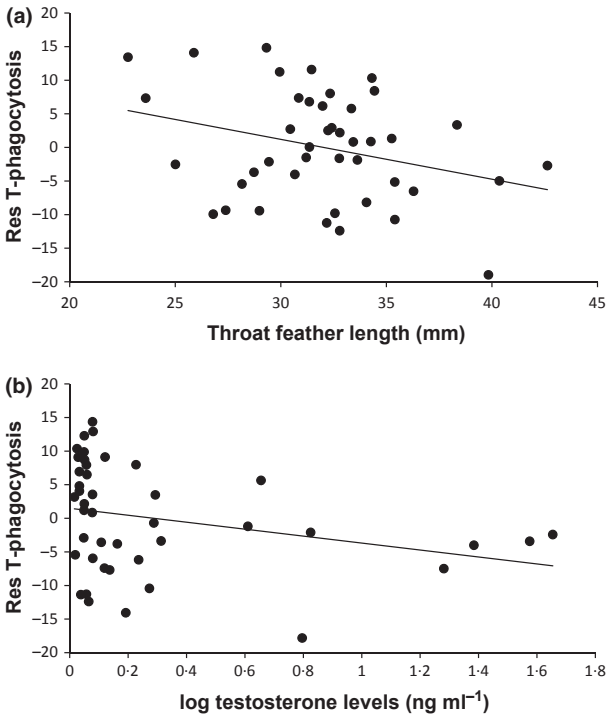

Fig. 3. T-medium phagocytosis (corrected for basal phagocytosis) covaries negatively with throat feather length (a) in adult male spotless starlings and shows a marginally nonsignificant trend in the same direction with T levels (b).

Discussion

In agreement with the previous studies showing androgen suppression of innate immune response, our data show that phagocytic function in the spotless starling is strongly inhibited under moderate physiological concentrations of T (Slater & Schreck 1997; Al Afaleq & Homeida 1998; Schneider *et al*. 2003). Previous studies have shown that androgens can modulate macrophage function by at least the following three different specific mechanisms: AR binding, nongenomic signalling or IgG receptor regulation (Gómez *et al*. 2000; Benten *et al*. 2004; Ahmadi & Mccruden 2006).

Phagocytosis is a fundamental component of the immune system, involved in direct killing and destruction of pathogens (innate immunity) and also in providing T cells with specific antigen information to allow a faster response to pathogens (adaptive or acquired immunity) (Stuart & Ezekowitz 2005). Although basal phagocytosis was not significantly explained by feather length or T levels, nonsignificant trends for these covariates suggest that larger sample sizes may show that heterogeneity in basal phagocytosis is related to ornament size or age. Large sample sizes are necessary because of the low measurement precision for the phagocytosis assay. Our results for T-medium phagocytosis imply that T will cause male birds to suffer a certain degree of immunosuppression, as expected by the ICHH (Folstad & Karter 1992). It is to be expected that such a decrease in phagocytic function would impair both primary pathogen clearance and also the formation of specialized T cells against these pathogens. An additional requirement of the ICHH would be a direct relationship between T levels and pairing success (Folstad & Karter 1992). Previous studies in the spotless starling have shown that males with experimentally increased T levels are able to hold more nesting sites and for a longer period than control males (Veiga *et al*. 2001, 2002). Thus, our data on inhibition of phagocytic function are in line with previous studies that have identified humoral and T-cell immune suppression in response to high T levels and exaggerated sexual ornamentation in birds (e.g. Saino, Møller & Bolzern 1995; Duffy *et al*. 2000; Peters 2000; Casto, Nolan & Ketterson 2001; Mougeot *et al*. 2004).

Heterogeneity in T-mediated phagocytic suppression was related to differences in ornament size and T concentration. Males with longer throat feather and higher T levels had the strongest T-mediated immunosuppression. This is actually

the opposite pattern of what the revealing handicap version of the ICHH would predict (Saino, Møller & Bolzern 1995; Saino & Møller 1996; Peters 2000; Loyau *et al.* 2007), because high-quality males would be expected to be least affected by the immunosuppressive effects of T. The interpretation of this pattern is not straightforward and confronts proximal with evolutionary explanations. Our data could be explained physiologically, by assuming differences in the number or activity of ARs. A higher androgen sensitivity of macrophages should bring about a stronger immunosuppression (Larsen *et al.* 2003). Evidence in humans indeed shows greater numbers of ARs in males than in females and in individuals with high endogenous androgen levels (Mccrohon *et al.* 2000; Sader *et al.* 2005). Thus, higher androgen sensitivity would be expected in the macrophages of highly ornamented, high T males, and these would probably lead to higher T-mediated immunosuppression.

This scenario would also call for an evolutionary explanation based on a pure epistasis handicap (Zahavi 1975), in which males with high T levels and large ornaments would pay a higher cost of high T and thus present lower immunocompetence. Models have since long dismissed this possibility as a likely explanation of ornament evolution (Davis & O'donald 1976), although some empirical data show evidence that could be explained in this direction (Zuk, Johnsen & Maclarty 1995; Greives *et al.* 2006; Mills *et al.* 2009). However, this hypothesis would predict a negative relationship in our data between ornament size and basal phagocytosis, which was not found. Rather, the trend was in the opposite direction, thus suggesting that ornaments in this species do not constitute a direct handicap in males. However, it needs also to be considered that our study was performed in the middle of the breeding season, and it is possible that the patterns that we found may change if we studied the prebreeding period, when birds are still fighting for boxes and higher levels of T may impose different selection regimes on the birds.

Finally, a likely possibility to explain our data and to reconcile the results with the original revealing handicap hypothesis is that the methodology used in the phagocytic assay could have removed cost-reducing mechanisms present in the plasma. Highly ornamented, high T males would be expected to present adaptations to counteract the effects of high T levels, similar to other cost-reducing mechanisms that have been found for other ornaments (Møller 1996; Oufiero & Garland 2007). As our phagocytosis assay was performed in vitro, after removal of all plasma components, cells would have been exposed to androgens outside of the full plasma matrix adapted to buffer these effects. A number of plasma components have been shown to work as immune enhancers and counteract the levels of T suppression. These substances would be expected to covary positively with T levels and ornamentation (Blas *et al.* 2006). Candidates would include different antioxidants (Blas *et al.* 2006; Mcgraw & Ardia 2007) or melatonin (Singh & Haldar 2005; Terrón *et al.* 2005), substances whose concentrations would be expected to be higher in males with high T levels.

To conclude, our study provides unique evidence showing how a basal immune component such as phagocytosis is affected by androgen-mediated immunosuppression, as predicted by the ICHH. At the same time, we suggest that several physiological mechanisms may explain the unexpected positive relationship between T-mediated immunosuppression and ornament size that we found. Our study stresses the need to address the role of cost-reducing mechanisms in the evolution of ornaments (Møller 1996; Oufiero & Garland 2007). In the case of the ICHH, a greater coordination between evolutionary and physiological perspectives becomes increasingly necessary (Ketterson & Nolan 1992).

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Supporting Information

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Appendix S1. In vitro phagocytosis techniques.

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