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Yolk testosterone shapes the expression of a melanin-based signal in great tits: an antioxidant-mediated mechanism?

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SUMMARY

Conspicuous traits produced by melanin deposition in integuments are often involved in visual communication. The information content of melanin-based signals is unclear as their expression is tightly controlled by genes and, apparently, is less dependent on individual condition. In birds, high heritabilities have been attributed to melanin-based plumages, often on the basis of egg-swapping manipulations (cross-fostering experiments). However, it is well known that female birds can differentially transfer testosterone to the egg yolk. Furthermore, high testosterone levels have been related to high oxidative stress. As we recently found that oxidative stress experienced during development influences the expression of melanin-based traits, here we manipulated the level of yolk testosterone in great tits (*Parus major*) to assess the influence of this maternal effect on the expression of the black breast stripe, a well-known melanin-based signal. We predicted that fledglings hatched from eggs with high testosterone levels will not only show larger black stripes but also experience changes in their antioxidant machinery. Indeed, the size of the black stripe of great tits hatched from testosterone-injected eggs was almost double that of controls. Furthermore, the same individuals showed a trend to higher levels of circulating antioxidants, which suggests an adaptive response against some testosterone-induced oxidative challenge.

Key words: maternal effect, melanin, oxidative stress.

INTRODUCTION

Phenotypic traits generated by melanin deposition in integuments are often involved in animal communication, acting as honest signals of quality (McGraw, 2008). The information content of melanin-based signals has remained unclear owing to the belief that the expression of these traits is almost exclusively under genetic control (Griffith et al., 2006), whereas any signal of quality needs to be both environment dependent and heritable to be honest (Hasson, 1997). However, environmental factors such as the availability of certain metals and melanin precursors seem to affect melanin production (McGraw, 2008). Recently, exogenous oxidative stress has been highlighted as an important factor modulating plumage melanization (Galván and Alonso-Alvarez, 2009). Moreover, melanin synthesis (Slominski et al., 2004) is regulated by a number of different hormones that could potentially be sensitive to environmental influences. Among these hormones, the positive effect exerted by testosterone (T) on the expression of melanin-based traits of vertebrates at both intraspecific (Stokkan, 1979; Diaz et al., 1986; González et al., 2001) and interspecific (Bókony et al., 2008) levels has been demonstrated.

In birds, heritability has often been estimated after experiments involving egg swapping (cross-fostering experiments) to avoid confounding parental effects acting across incubation or chick-rearing behaviours. Although such experiments have shown a high heritability for melanin-based traits (Roulin and Dijkstra, 2003) (but see Griffith et al., 2006), the fact that females can differentially transfer T to the egg yolk (Müller and Eens, 2009) could have led to overestimated heritabilities of melanin-based traits in the past. In the house sparrow (*Passer domesticus*), an artificial increase of yolk T levels led to an increase in the expression of a melanin-based plumage signal that is only present in males [i.e. the black bib (Strasser and Schwabl, 2004)]. Such a result opened a new perspective in the context of alternative mechanisms modulating the expression of melanic signals. However, it is not known whether this mechanism could be present in other species and whether this mechanism interacts with other factors affecting melanization such as oxidative stress.

We manipulated yolk T levels in eggs of great tits (*Parus major* Linnaeus) and determined the effect of this on the expression of the melanin-based black breast stripe, a trait present in both sexes that is positively related to social dominance and reproductive success (Järvi and Bakken, 1984; Norris, 1990; Carrascal et al., 1998; Quesada and Senar, 2007). We have previously found that the development of large stripes in great tit fledglings requires low levels of a key intracellular antioxidant [i.e. glutathione (GSH)] as well as compensatory mechanisms to avoid oxidative stress (i.e. mobilization of alternative antioxidants) (Galván and Alonso-Alvarez, 2008). Furthermore, T might increase susceptibility to oxidative stress in birds (Alonso-Alvarez et al., 2007) and might decrease GSH levels in humans (Prudova et al., 2007). Therefore, if yolk T increases the expression of the black breast stripe, we predict that this should be associated with a decrease in GSH levels and a compensatory increase in plasma antioxidants.

MATERIALS AND METHODS

Experimental design

The study was carried out during May–July 2008 in a deciduous forest of Pyrennean oak (*Quercus pyrenaica*) in Miraflores de la

Sierra, Sierra de Guadarrama, Madrid Community, central Spain [40°49′N, 03°46′W, 1352 m above sea level (a.s.l.)]. Frequent checks of nest-boxes allowed us to determine laying dates and clutch sizes. Breeding failure was attributed to nest desertion when the clutch was present but cold (i.e. no incubation) and to predation when the entire clutch was absent or only egg remains were present. For breeding pairs in the T-group, all eggs in the clutch were injected with T within 24 h after clutch completion. Pure crystalline testosterone (ref. 132832; Steraloids, NewPort, RI, USA) was injected into the yolk, using 26 ng of T in 5 µl of sesame oil. Eggs from control pairs received 5 µl of sesame oil. The injection protocol was based on that described by Schwabl (Schwabl, 1996) and Strasser and Schwabl (Strasser and Schwabl, 2004). Injections were made through the small pole of the egg with a microliter syringe (26s G needle; Hamilton Bonaduz, Switzerland), using illumination (5-LED lantern) from underneath to ascertain that the tip of the needle had penetrated the yolk membranes. The hole in the shell was then patched with cyanoacrylate. Treatments were alternatively assigned to 42 nests (22 control and 20 T-injected nests) and randomly distributed in the forest. Both predation and nest desertion reduced the sample to 13 controls and 6 T-injected nests, containing 157 eggs and finally producing 84 fledglings. These figures show a trend to a higher hatching failure among nests with T-injected eggs (χ^2_1=3.58, P=0.06). Nevertheless, the T dose used in our study is similar to the natural T content in egg yolks of great tits (Tschirren et al., 2004) and is below the dose used by other authors (i.e. 30 ng), who did not find hatching failure associated with T injections (Tschirren et al., 2007). Moreover, the probability of hatching failure was less clear among nonpredated nests (χ^2_1=2.93, P=0.09), with the probability of predation not being different between treatments (χ^2_1=0.84, P=0.36). Among those nests producing fledglings, the proportion of hatchlings in a clutch was not significantly different among the treatment groups (Mann–Whitney U-test: Z=–1.22, P=0.219). Finally, tarsus length, body mass and body condition (residuals of body mass regressed against tarsus length; R^2=19.3, P<0.001) did not differ between fledglings of both groups (all P>0.46; nest identity as a random factor), which suggests that no difference in individual quality between groups was present. On day 15, nestlings were weighed, their tarsus length measured and a digital photograph of the breast in a standardized position was taken. In our study site, 15-day-old nestlings have most body feathers fully developed and are close to leaving the nest (Galván and Alonso-Alvarez, 2008). A blood sample was also collected from the brachial vein with a heparinized capillary tube and kept cool until centrifugation for 5 min at 1125 ***g*** within 5 h of collection. Cell and plasma fractions were stored separately at –80°C.

Photograph analysis

The assessment of the melanized breast stripe surface was done by means of Adobe Photoshop CS from the digital photographs of 15-day-old nestlings, which included a graph paper as a reference of surface. The entire black area of the plumage of nestlings was selected from the throat to the belly, as this method ensures a high repeatability in the particular case of great tits (Figuerola and Senar, 2000) (see also Galván and Alonso-Alvarez, 2008). The number of pixels in a square of the graph paper allowed us to calculate the surface covered by the black breast stripe.

Testosterone assays

Plasma testosterone concentration was determined using an enzyme immunoassay kit from DRG International (Mountainside, NJ, USA). These kits have been successfully used in birds, showing recovery rates greater than 95% (Wilhelms et al., 2005). Sensitivity is established at 0.08 ng ml^{-1}. Repeatability calculated from a subset of samples measured twice (N=30) in the same session (intra-assay repeatability) or in different sessions (inter-assay repeatability) was high (r=0.94 and 0.91, respectively). Treatments and nests were randomly distributed among plates. The testosterone concentration could not be determined in 23 samples owing to there being an insufficient plasma volume to perform analyses.

Total glutathione assays

Total glutathione (tGSH) levels in red blood cells were determined by following the method described by Griffith (Griffith, 1980), with some particular modifications. Briefly, the blood pellet was thawed and the red blood cells were pipetted while avoiding the pellet surface (i.e. the buffy coat containing white blood cells). Erythrocytes were immediately diluted (1:10 w/v) and homogenized in a stock buffer (0.01 mol l^{-1} PBS and 0.02 mol l^{-1} EDTA), always working on ice to avoid oxidation. Three working solutions were made up in the same stock buffer as follows: (I) 0.3 mmol l^{-1} NADPH, (II) 6 mmol l^{-1} DTNB and (III) 50 units of glutathione reductase per millilitre. An aliquot (0.5 ml) of homogenate of blood cells was vortexed with 0.5 ml of diluted trichloroacetic acid (10% in H_2O) three times for 5 s each bout within a 15 min period. Meanwhile, samples were protected from light and refrigerated to prevent oxidation. Afterwards, the mixture was centrifuged (1125 ***g***, 15 min, 6°C) and the supernatant removed. The next steps were carried out in an automated spectrophotometer (A25-Autoanalyzer; Biosystems SA, Barcelona, Spain). Solutions I and II were mixed at a ratio of 7:1 by volume, respectively. 160 µl of this new mixture was automatically added to 40 µl of sample (supernatant, see above) in a cuvette. Afterwards, 20 µl of solution III was added after 15 s, and the absorbance at 405 nm was monitored after 30 and 60 s. The change in absorbance was used to determine the total glutathione concentration in red blood cells by comparing the output with the results from a standard curve generated by serial dilution of glutathione from 1 mmol l^{-1} to 0.031 mmol l^{-1}. Results are given as µmol per gram of pellet.

Total antioxidant status

The total antioxidant status (TAS) of plasma was assessed by means of commercial kits (Randox Laboratories, Crumlin, UK) adapted to an automated spectrophotometer (A25-Autoanalyzer; Biosystems SA, Barcelona). Briefly, plasma samples were incubated for 15 s with a chromogen comprising metmyoglobin and 2,2-azino-di-(3-ethylbenzthiazoline sulphonate) (ABTS). Then, hydrogen peroxide (H_2O_2) was added and the sample was incubated for 195 s. Addition of H_2O_2 induces the production of the radical cation ABTS, which generates a blue–green colour. Colour is measured at 600 nm before and after H_2O_2 addition to determine the change in colour. Antioxidants in the plasma sample cause suppression of this colour change to a degree that is proportional to their concentration. Results are given as mmol/l of total antioxidants. All samples were measured in the same assay.

Sex determination

Nestlings were molecularly sexed from a subsample of the red blood cell fraction. DNA from the sex chromosomes (Z and W) was amplified by PCR using the primers P2 and P8 (see Griffiths et al., 1998).

Statistical analyses

The factors affecting blood variables and breast stripe surface were tested by general linear mixed models, including treatment, sex and their interaction as fixed factors. Nest identity was included as a random factor (using the Satterthwaite method to calculate degrees of freedom), although it never showed a significant effect (always $P>0.10$; except in the model for tGSH, $P=0.001$). Body condition and brood size were tested as covariates. In the model testing breast stripe variability, tarsus length was tested as a covariate instead of body condition to control for individual body size. Starting from the saturated model, a backward stepwise procedure was used to remove terms with $P>0.10$. The normality assumption was confirmed by checking the residuals of the models. All analyses were made using SAS software. Final models after the stepwise procedure are shown.

RESULTS

Variability in plasma T levels was only explained by a positive effect of nestling body condition ($\beta=0.28$, $F_{1,58}=4.82$, $P=0.032$). The treatment did not show a significant effect on this variable ($F_{1,56}=0.67$, $P=0.415$; mean±s.e.m., T-injected: 0.77±0.08 ng ml^{-1}, control: 0.64±0.06 ng ml^{-1}). By contrast, nestlings from T-injected eggs showed lower mean tGSH values than those of controls (Fig. 1A), although the difference was not significant ($F_{1,16.3}=1.49$, $P=0.239$). The opposite pattern was detected in TAS variability (Fig. 1B), in this case the effect being marginally nonsignificant ($F_{1,76}=3.60$, $P=0.061$). A negative relationship between TAS and brood size was present ($\beta=-0.24$, $F_{1,76}=4.23$, $P=0.043$).

Birds from T-injected eggs developed stripes of greater size than those of controls ($F_{1,75}=85.77$, $P<0.0001$: tarsus length: $F_{1,75}=0.80$, $P=0.374$; Fig. 1C). The model also revealed a marginally nonsignificant effect of sex (mean±s.e.m., males: 3.88±0.27 cm^2; females: 3.65±0.20 cm^2; $F_{1,75}=2.83$, $P=0.097$). Yolk T increased the size of the stripe in both sexes, as a non-significant interaction sex × treatment ($P=0.441$) indicated that the treatment effect dif not differ between sexes. The surface area of the breast stripe of birds from T-injected eggs (Fig. 1C) was very similar to that reported in a previous study (Galván and Alonso-Alvarez, 2008) in the same population in great tit nestlings that were injected with a specific inhibitor of GSH levels (i.e. buthionine sulfoximine, BSO; 5.57±0.18 cm^2; *t*-test: $t_{166}=0.465$, $P=0.642$). tGSH levels of birds hatched from T-injected eggs (Fig. 1A) were also similar to those found in birds treated with a high dose of BSO [i.e. 2.28±0.15 µmol g^{-1} (Galván and Alonso-Alvarez, 2008); $t_{69}=0.957$, $P=0.342$)].

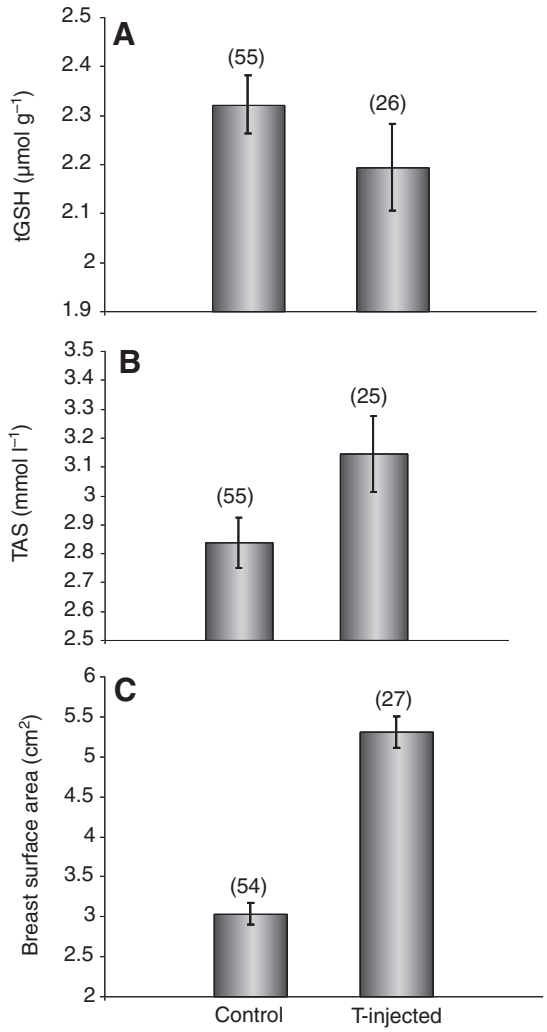

Fig. 1. Total glutathione level (tGSH) in erythrocytes (A), total antioxidant status (TAS) in plasma (B) and breast stripe surface (C) of great tits hatched from control or testosterone-injected eggs. Bars represent least-square means ± s.e.m. The number of nestlings per treatment is indicated in parentheses on each bar. *P*-values for the differences were 0.239, 0.061 and <0.001 for tGSH, TAS and breast surface area, respectively.

DISCUSSION

We showed that the breast stripe of great tits can be strongly affected by yolk testosterone. The size of the breast stripe of birds hatched from T-injected eggs was almost double that of birds hatched from control eggs. In addition to the recently described influence of endogenous oxidative stress (see Galván and Alonso-Alvarez, 2008), the present result amplifies the perspective on the potential mechanisms involved in the expression of this signal. Together with recent findings that show the effect of the availability of melanin precursors and some metals in the development of melanic traits (Poston et al., 2005; McGraw, 2008), these results reinforce the view of melanin-based signals as being highly dependent on environmental factors instead of being mainly under a tight genetic control (Griffith et al., 2006). Nevertheless, breast stripe size also has a heritable component in great tits (Norris, 1993), and high heritability values have been found in maternal T in another passerine bird species [i.e. the collared flycatcher (*Ficedula albicollis*) (Tschirren et al., 2009)]. Thus, the exact contribution of environmental *versus* genetic influences to the expression of melanin-based traits through maternal effects will only be determined by future studies that manipulate both components of phenotypic variation.

Our results also show that yolk T increases the expression of the melanin-based signal in both sexes. The same mechanism was demonstrated in house sparrows (Strasser and Schwabl, 2004) and black-headed gull (*Larus ridibundus*) nestlings (Eising et al., 2006). However, the nestlings of this latter species do not present the definitive adult phenotype, as opposed to the breast stripe of great tits that is fully developed in fledglings (Cramp and Perrins, 1993).

With regard to the antioxidant protection markers, the difference between treatments in mean TAS values was in the predicted direction (i.e. higher in T-injected birds), although the difference was not significant. By contrast, Tobler and Sandell (Tobler and Sandell, 2009) found lower plasma antioxidant levels in male, but not in female, zebra finches (*Taeniopygia guttata*) hatched from T-injected yolks. These opposite results might be due to disparities in the methods used to assess antioxidants. Alternatively, sex-related differences in the antioxidant metabolism of both species might arise owing to the

different rearing conditions of nestlings, as, while our study was conducted with wild great tits, Tobler and Sandell (Tobler and Sandell, 2009) studied zebra finches in captivity, which could result in the availability of antioxidants being drastically different between the studied populations. Tobler and Sandell (Tobler and Sandell, 2009) suggested that yolk T could have programmed the nestling metabolism, leading to a higher T production in males, and ultimately, to a weaker TAS. Here, however, circulating T did not differ between treatments and/or sexes. The different direction of both results could also be explained by changes in blood dynamics of antioxidants throughout development, which might initially decline when fighting oxidative stress and subsequently increase, as an adaptive mechanism to endure a long-term challenge (Dimova et al., 2008). In fact, the T effect on zebra finches was detected 10 days after hatching, but not at 34 days (Tobler and Sandell, 2009), whereas great tits were assessed when 15 days old (i.e. at the fledging age). In any case, both studies support the idea that yolk T might challenge the antioxidant machinery of developing birds.

It is intriguing that the surface of the breast stripe of great tit nestlings hatched from T-injected eggs was very similar to that reported in great tit nestlings injected with a specific inhibitor of GSH levels (Galván and Alonso-Alvarez, 2008). tGSH levels of birds hatched from T-injected eggs were also similar to those found in great tit nestlings treated with a high dose of this GSH inhibitor (Galván and Alonso-Alvarez, 2008). These findings could support the idea that the larger breast stripe size detected in T-treated birds could have been due to oxidative stress promoted by this hormone (Alonso-Alvarez et al., 2007), which would decrease GSH levels (Prudova et al., 2007). However, the difference in tGSH levels between birds hatched from T-injected and control eggs was clearly nonsignificant, and it must be mentioned that the expression of phenotypic traits can vary between years in the same population. Nonetheless, we should note that tGSH levels of great tits hatched from control eggs were lower than tGSH levels detected in control birds described in Galván and Alonso-Alvarez (Galván and Alonso-Alvarez, 2008). Furthermore, the breast stripe size of control birds seems to be subtly higher than that reported in the study of Galván and Alonso-Alvarez (Galván and Alonso-Alvarez, 2008) also for control individuals. In other words, the relatively low basal tGSH level and the small sample size could have prevented detection of a significant difference in this trait.

In great tits, the most abundant yolk androgen is the T precursor androstenedione (A4), whereas the T metabolite 5-α-dihydrotestosterone (DHT) is also present at relatively high levels (Tschirren et al., 2004). This variety of androgens can induce different gene expression patterns by means of the androgen receptor pathways, and their affinities to androgen receptors also differ (reviewed in Groothuis and Schwabl, 2008). Thus, future studies exploring androgen-mediated maternal effects should amplify their scope by considering other yolk androgens (reviewed in Gil, 2008), as well as the relative proportion at which they occur.

In summary, the main finding of this study (i.e. the larger breast stripe of birds hatched from T-treated eggs) supports the view that maternal effects, and particularly T transfer to the egg yolk, represent a non-genetic factor shaping the expression of melanin-based signals in birds. By contrast, results from antioxidant parameters, although very suggestive, did not firmly establish a link between yolk T, oxidative stress and melanin-based phenotypes, making further experimental work necessary.

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