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Sex and testosterone effects on growth, immunity and melanin coloration of nestling Eurasian kestrels

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Summary

1. Sex differences in testosterone levels and sex-biased sensitivity to testosterone are the basis of some ideas postulated to account for sex-linked environmental vulnerability during early life. However, sex variation in circulating testosterone levels has been scarcely explored and never manipulated at post-natal stages of birds in the wild.

2. We measured and experimentally increased circulating testosterone levels in nestling Eurasian kestrels *Falco tinnunculus*. We investigated, possible sexual differences in testosterone levels and the effect of this hormone on growth (body mass and tarsus length) and cell-mediated immunity in males and females. We also explored testosterone effects on rump coloration, a highly variable melanin-based trait in male nestlings. We analysed data on circulating testosterone levels of nestlings in 15 additional bird species.

3. Increased levels of testosterone tended to negatively affect body condition, reduced cell-mediated immune responses in male and female nestlings and also diminished the expression of grey rump coloration in male nestlings. No sex differences were observed in testosterone levels in either control or increased testosterone group nestlings, and no interactions were found between sex and treatment. However, male nestlings showed a lower cell-mediated immune response than females in both groups.

4. Our results indicate first, that a high level of testosterone in all nestlings in a brood entails costs, at least in terms of immunity, coloration and probably growth. Secondly, sex differences in post-natal cell-mediated immunity, and consequently in the capacity to prevent diseases, cannot be explained by sex differences in circulating testosterone levels. Finally, by comparing published data at an interspecific level, contradictory sex patterns in circulating testosterone levels have been found, supporting the idea that circulating testosterone might not be a proximate factor causing sex-dependent vulnerability in bird species.

Key-words: androgen, immunocompetence, rump, sex allocation.

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Introduction

Environmental conditions during early development that affect survival can differentially affect male and female offspring (McClure 1981; Clutton-Brock 1991; Kruuk *et al*. 1999), and thereby define the reproductive value of the sexes (Trivers & Willard 1973; Clutton-Brock 1991). In birds, post-natal sex-related vulnerability to nest environment has been described (e.g. Clutton-Brock, Albon & Guinness 1985; Bortolotti 1986). In most studies, sexual size dimorphism, producing different energy demands and/or competitive abilities, has been found to be a mayor cause of sex-specific sensitivity to environmental conditions in early

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life (e.g. Velando 2002; Kalmbach, Furness & Griffiths 2005; Fargallo *et al*. 2006). Aside from size, another idea suggests that inherent characteristics in sex phenotype, such as the high levels of testosterone in males, oestrogen in females (Grossman 1985; Owens & Short 1995; Olsen & Kovacs 1996) or different sensitivity to similar levels of hormones (Müller *et al*. 2005a) can determine differential vulnerability between sexes. This idea has been proposed to explain why males are generally disfavoured in the development of immune function (Fargallo *et al*. 2002; Müller, Dijkstra & Groothuis 2003; Tschirren, Fitze & Richner 2003), or they are more susceptible to parasite infection (Potti & Merino 1996; Tschirren *et al*. 2003; but see Bize *et al*. 2005).

One aspect that complicates the above-mentioned ideas is the association between testosterone and aggressive behaviour. Aggressiveness benefits individuals by optimizing social competitiveness (Wingfield *et al*. 1990; Groothuis & Meeuwissen 1992; Ketterson *et al*. 1996). However, testosterone can also impair fat deposition, moult, growth or begging behaviour (see Ketterson *et al*. 1996; Groothuis *et al*. 2005a). If agonistic social encounters occur for short periods in life, then long-term elevated levels of testosterone could inflict costs, for which it would be advantageous, that testosterone increases (above the baseline level) only in situations of agonistic encounters (Wingfield *et al*. 1990). Recent studies have found support for this hypothesis in scramble sibling competition at the nestling stage (Tarlow, Wikelski & Anderson 2001; Ferree, Wikelski & Anderson 2004). For example, the high baseline testosterone levels found in the less competitive B-chicks of Nazca boobies *Sula grantii* compared with A-chicks has been proposed to be due to either greater upregulation by the B-chick itself in response to a competitor, or to manipulation by the mother via the egg contents (Ferree *et al*. 2004). If so, the less competitive smaller sex in sexually size-dimorphic species could similarly benefit from higher levels of testosterone during post-natal stages.

One more important effect described for androgens is that on the expression of melanin-based coloration (Groothuis & Meeuwissen 1992; Haase, Ito & Wakamatsu 1995; Ros 1999). Melanin pigments (eumelanin and phaeomelanin) are the most common basis of coloration in animals. Higher concentrations of phaeomelanin relative to eumelanin result in reddish-brown and brown coloration. On the contrary, higher concentrations of eumelanin relative to phaeomelanin are responsible for grey and black coloration (Haase *et al*. 1992, 1995; Jawor & Breitwisch 2003). Melanin pigments are synthesized from nutritionally dispensable amino acids (e.g. tyrosine; Meister 1965), but the biosynthesis of phaeomelanin and eumelanin pigments follow different biochemical pathways (Wakamatsu & Ito 2002) that appear to be differentially sensitive to levels of circulating sex steroids in certain species (Haase *et al*. 1995; Kimball 2006). This aspect is of special relevance for understanding ornament expression as the different phaeomelanin : eumelanin ratios produce the whole spectrum of melanin coloration in vertebrates including birds (McGraw 2006).

There is growing scientific literature on the effect of testosterone at early stages of development, but in general, current knowledge about the effect of androgens on nestlings in the wild comes only from studies on embryonic development (see Groothuis *et al*. 2005a). Higher androgen levels in eggs (Schwabl 1993) have, in general, beneficial effects on the development of the embryo, post-natal growth and competitiveness in both nestling and juvenile offspring (Groothuis *et al*. 2005a; but see Sockman & Schwabl 2000; Rubolini *et al*. 2006). However, some researchers have found recently that the pre-natal exposure to elevated levels of androgens negatively affects immunity in nestlings (Müller *et al*. 2005b; Groothuis *et al*. 2005b), although some other studies have failed to corroborate this finding (Tschirren *et al*. 2005; Rubolini *et al*. 2006). Furthermore, it is little known if the concentration of yolk androgens are correlated with corresponds to that in chicks. To our knowledge the only study investigating this aspect did not find such a correlation (Gil *et al*. 2006). There are studies describing the relationship between circulating testosterone of nestlings or young birds and physiological and behavioural characteristics in wild species (e.g. Williams *et al*. 1987; Núñez-de la Mora, Drummond & Wingfield 1996; Silverin & Sharp 1996; Tarlow *et al*. 2001; Goodship & Buchanan 2006). However, the effect of testosterone on nestlings has only been studied in poultry (e.g. Gause & Marsh 1986; Al-Afaleq & Homeida 1998; Ottinger, Pitts & Abdelnabi 2001) or captive species (e.g. Groothuis & Meeuwissen 1992; Ros 1999) where experimental manipulations of circulating androgens have been carried out. Nevertheless, levels of circulating testosterone can differ between free-living and captive individuals (Wingfield *et al*. 1990).

We investigated the effect of testosterone in nestlings of the Eurasian kestrel *Falco tinnunculus* (hereafter kestrel), a sexually dimorphic species in plumage and size (females are 20% heavier than males). We experimentally increased the circulating levels of testosterone in all the nestlings of the brood and, comparing with a control group, we examined the effect on cell-mediated immune (CMI) response and growth. We first checked if male nestlings show lower CMI response (Fargallo *et al*. 2002, 2003) and analysed whether this potential difference can be explained by differences in circulating testosterone levels or by sex-linked sensitivity to testosterone. If the former is true, we predict high levels of plasma testosterone in males, and if the latter is true we predict an interaction between sex and experimental treatment in CMI response. Higher levels of circulating testosterone could also be expected in male nestlings if high baseline levels of this hormone increase success in sibling competition. We do not have clear predictions about the effect of testosterone on growth as previous

bird studies (see above) have found both beneficial and detrimental effects. Secondly, the amount of grey vs. brown coloration in black-barred rumps in nestlings is a melanin character associated with the environmental quality in which male kestrels grow. A cross-fostering experiment showed that nestlings with larger grey area in rumps are produced in better quality nests, as deduced from body mass of mothers (see Fargallo *et al*. 2007). We investigated whether the expected modulatory effect of testosterone on the expression of melanin-based coloration affects this character in male nestlings. Finally, by incorporating data from nestlings of 14 bird species, we assessed whether sexual differences in circulating testosterone levels could be the basis of sexual differences in immune function at the nestling stage of several bird species.

Materials and methods

STUDY AREA AND PROCEDURES

The study was conducted during the breeding season of 2004 in Campo Azálvaro (central Spain), where most kestrels breed in nest boxes mounted on poles and trees (Fargallo *et al*. 2001). Only nests placed in nest boxes were considered for the experiment. Nests were visited every 2 days until the first egg in the population was laid. Then, we randomly assigned nests with similar laying dates into control and testosterone groups. The mean ± SD brood size in our study was 4·6 ± 0·9 chicks.

Kestrel nestling stage in our population is 28–30 days long. At 10 days of age, all the nestlings in each brood of the testosterone group were implanted with testosterone in order to carry out between-nest comparisons. Each testosterone group nestling (T-nestling) received one subcutaneous silastic tubing implant (11-mm length; 1·95-mm outer diameter and 1·47-mm inner diameter; Dow Corning, www.dowcorning.com) filled with 5 mm of crystalline testosterone propionate (Sigma, www.sigmaaldrich.com). Both extremes of the silastic tube were closed with nylon stoppers (nylon line 1·5-mm diameter, 3 mm in length). The propionate increases the half life of testosterone (72 h), for which it has frequently been used in animal studies (e.g. Veiga *et al*. 1998; Groothuis & Ros 2005). Empty tubes were inserted in two chicks of control nests (sham implanted). We inserted the implant in the scapular body region by making a small cut in the skin. Then, we closed the skin with a surgical suture while the bird was under anaesthesia (Forane, Abbott, www.abbott.com). At the age of 25 days of the chicks, we took body measurements and a blood sample (1 mL extracted from the brachial vein with a syringe). The day after, rump coloration and the immune response were measured (see below), and both testosterone implants and control silastic tubes were removed. In 60 testosterone implants, we measured the residual (unabsorbed) testosterone propionate after removing them. To do this, we pushed one of the nylon stoppers until the stopper no longer advanced, and then measured the remaining testosterone propionate in the silastic tube with a digital calliper (0·01 mm).

The sex of chicks was determined from blood samples with molecular methods as described by Fridolfsson & Ellegren 1999) applied on kestrels (Fargallo *et al*. 2002). Cell-mediated immune (CMI) response of chicks was measured using the common assay of intradermal injection of the T-cell mitogen phytohaemagglutinin-P (0·3 mg of PHA dissolved in 0·1 mL of phosphate-buffered saline; see Fargallo *et al*. 2002; for procedures).

TESTOSTERONE ASSAY

Plasma concentration of testosterone in blood was determined by radioimmunoassay. Testosterone was extracted from 200 µL plasma with cyclohexane and ethyl acetate. Aliquots of samples and testosterone standards were mixed with ^{3}H-testosterone and serum antitestosterone and free and bound fractions were subsequently separated with a solution of Norit A (Serva Co., Heidelberg, Germany) and Dextrane (Sigma). The ovine serum antitestosterone was kindly provided by the Unit 'Physiologie de la Reproduction et des Comportements' (INRA, Nouzilly, France). The cross-reaction with different steroids other than testosterone was never more than 1%. Serial dilutions of pooled plasma samples from kestrels with high concentrations of immunoreactive testosterone gave inhibition curves parallel to those generated for testosterone in buffer. Low and high testosterone control samples were included in the assay. The detection limit of the assay was 0·05 ng mL^{-1}. The samples were analysed in a single assay, and the intra-assay coefficient of variation was 13%. The mean extraction recovery was 80 ± 3%. Only six individuals showed lower concentrations than 0·05 ng mL^{-1}.

RUMP COLORATION

The rump of male nestlings was measured at the age of 26 days of the chicks by digitally photographing the rump (camera: Nikon D70, objective: 18–70 mm AF-S Nikkor DX). We then imported the images into the ImageJ 1·33u program developed at the US National Institutes of Health, USA (http://rsb.info.nih.gov/ij/ Java 1·3·1–03) to determine the grey area (number of pixels) in the rump (see Fargallo *et al*. 2007 for procedures). Repeatability of measurements was estimated from separate photos of 20 birds that were taken 20 s apart. Measurements of grey coloration areas were highly repeatable ($r = 0{\cdot}98$, $F_{19,20} = 92{\cdot}2$, $P < 0{\cdot}001$).

STATISTICAL PROCEDURES

General Linear Mixed Models (GLMM) in SAS statistical software (SAS 1989–96 Institute Inc., Cary, NC, USA). The models used Randomized Complete Block Design (RC; Littell *et al*. 1996), where nests were

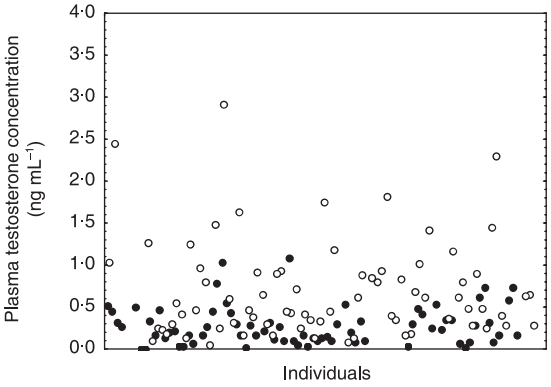

Fig. 1. Levels of circulating testosterone in control (●) and testosterone-implanted (○) Eurasian kestrel nestlings.

considered as blocks (and random effects) nested within treatment. We analysed between-group differences in the measured variables. Treatment and sex were included as fixed factors and other variables, such as body measurements, were included as covariates when required. Testosterone plasma concentration did not show a normal distribution (K-S, $d = 0{\cdot}17$ $P < 0{\cdot}01$). However, residuals from the model in which testosterone plasma concentration was used as response variable showed a normal distribution (K-S, $d = 0{\cdot}09$, $P = 0{\cdot}15$), for which it was also analysed using a nested GLMM. The proportion of grey coloration on the rump was arcsine transformed. To assess the similarity of testosterone values between sham-implanted and nonimplanted siblings we used mean values of testosterone levels of both subgroups to examine whether they were intercorrelated and then estimated the repeatability of all non- and sham-implanted nestlings (Lessells & Boag 1987) within each nest. Both measures were highly correlated ($r_s = 0{\cdot}91$, $P < 0{\cdot}001$, $n = 15$) and repeatable ($r = 0{\cdot}67$, $F_{14,15} = 5{\cdot}04$, $P = 0{\cdot}002$). Furthermore, as expected, within the control group, nonimplanted nestlings did not differ from sham-implanted nestlings in the other variables analysed (body mass, tarsus length, body condition, CMI response or rump coloration; GLMM, all $P > 0{\cdot}2$). Hence, we grouped all control group nestlings (C-nestlings). In total we measured 75 T-nestlings and 71 C-nestlings from 17 testosterone and 15 control experimental nests that did not differ in laying date, clutch size or number of fledged young (GLM, all $P > 0{\cdot}36$).

Results

TESTOSTERONE

Plasma testosterone levels in T-nestlings were negatively correlated with the remaining of testosterone propionate in removed implants ($r_s = -0{\cdot}50$, $P < 0{\cdot}001$, $n = 60$). Testosterone uptake did not differ between sexes and was unrelated to body mass (GLMM, sex: $F_{1,44} = 0{\cdot}95$, $P = 0{\cdot}33$; body mass $F_{1,44} = 0{\cdot}11$, $P = 0{\cdot}73$). Plasma testosterone concentration was significantly higher in T-nestlings (mean ± SD, $0{\cdot}69 \pm 0{\cdot}54$ ng mL^{-1}, range = 0·058–2·92, $n = 75$) than in C-nestlings (mean ± SD, $0{\cdot}28 \pm 0{\cdot}23$ ng mL^{-1}, range < 0·05–1·08, $n = 71$; GLMM, $F_{1,30} = 28{\cdot}15$, $P < 0{\cdot}001$). Only 13 T-nestlings (17·3%) showed T-values over the two maximum values found in the natural (control) range (Fig. 1). Males and females did not differ in average testosterone levels (GLMM, sex: $F_{1,112} = 0{\cdot}72$, $P = 0{\cdot}40$), although the statistical power of the test was low (14%). The experimental treatment did not affect this similarity between sexes (GLMM, sex × treatment: $F_{1,112} = 0{\cdot}69$, $P = 0{\cdot}41$).

Table 1. Mean ± SE body mass and tarsus length of nestling Eurasian kestrels in control and testosterone treatments

	C-females	T-females	C-males	T-males
Body mass	224 ± 4·6	211 ± 4·3	200 ± 4·6	190 ± 4·5
Tarsus length	49·0 ± 2·9	48·3 ± 2·7	48·5 ± 2·9	47·9 ± 2·8

NESTLING GROWTH

Controlling for sex (GLMM, $F_{1,112} = 101{\cdot}15$, $P < 0{\cdot}001$), there was a nonsignificant trend for body mass of T-nestlings to be lower than that of C-nestlings (GLMM, $F_{1,30} = 3{\cdot}68$, $P = 0{\cdot}065$; Table 1). The sex × treatment interaction was not significant ($F_{1,112} = 0{\cdot}20$, $P = 0{\cdot}65$). Also controlling for sex (GLMM; $F_{1,112} = 6{\cdot}35$, $P = 0{\cdot}013$), T-nestlings showed a nonsignificant trend toward shorter tarsi than C-nestlings (GLMM; $F_{1,30} = 3{\cdot}78$, $P = 0{\cdot}063$; Table 1). The sex × treatment interaction was not significant (GLMM, $F_{1,112} = 0{\cdot}08$, $P = 0{\cdot}78$). Body condition, measured as the effect of body mass when tarsus length was included in the model to correct for size, was not significantly lower for testosterone than for C-nestlings (GLMM, tarsus length: $F_{1,111} = 13{\cdot}41$, $P < 0{\cdot}001$; sex: $F_{1,111} = 89{\cdot}21$, $P < 0{\cdot}001$; treatment: $F_{1,30} = 2{\cdot}56$, $P = 0{\cdot}12$). The sex × treatment interaction was not significant (GLMM, $F_{1,111} = 0{\cdot}14$, $P = 0{\cdot}71$).

CELL-MEDIATED IMMUNE RESPONSE

CMI response was unrelated to body mass, tarsus length or the residuals of body mass on tarsus length (GLMM, all $P > 0{\cdot}24$) and therefore these variables were excluded from the model. Testosterone treatment had a significant effect on CMI response: testosterone implants caused a significantly lower immune reaction to PHA antigen in both males and females than birds in the control group (GLMM, $F_{1,30} = 5{\cdot}67$, $P = 0{\cdot}024$; Fig. 2). Males also showed lower CMI responses than females (GLMM, $F_{1,112} = 17{\cdot}46$, $P < 0{\cdot}01$; Fig. 2). No significant effect of the interaction sex × treatment was found (GLMM, $F_{1,112} = 0{\cdot}49$, $P = 0{\cdot}48$).

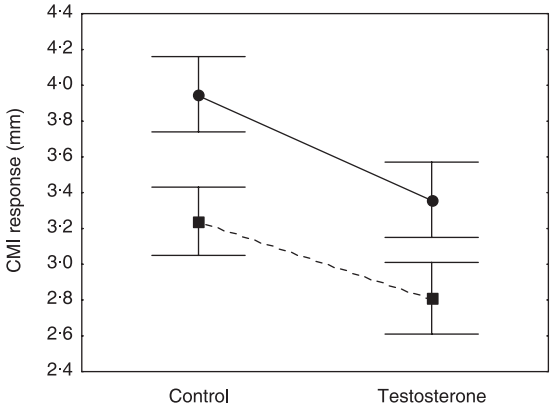

Fig. 2. Treatment differences in cell-mediated immune response of male (squares) and female (dots) nestling Eurasian kestrels. Points and whiskers represent mean and SE values, respectively.

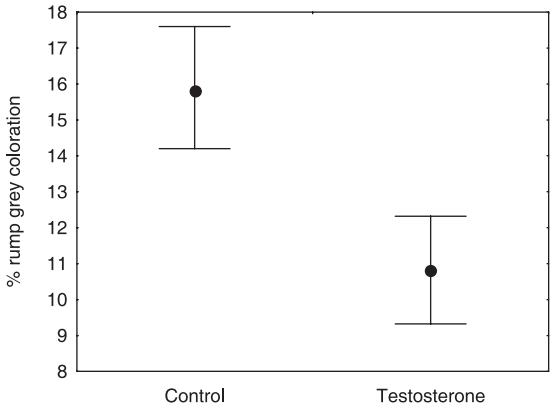

Fig. 3. Mean differences in the proportion of grey coloration in the rump of male nestling Eurasian kestrels. Points and whiskers represent mean and SE values, respectively.

RUMP COLORATION IN MALE NESTLINGS

The area of grey coloration in the rump of male nestlings was significantly smaller in the T- than in C-nestlings (GLMM, $F_{1,28} = 5{\cdot}34$, $P = 0{\cdot}028$; Fig. 3) and was not significantly affected by their body mass (GLMM, $F_{1,33} = 0{\cdot}02$, $P = 0{\cdot}88$). However, there was also a significant interaction between weight and treatment (GLMM, $F_{1,33} = 4{\cdot}63$, $P = 0{\cdot}039$), with the heaviest T-males displaying rumps with larger grey areas. This trend was not observed in C-males. Considering only male nestlings and controlling for treatment, the rump coloration was not correlated with CMI response (GLMM, $F_{1,34} = 0{\cdot}60$, $P = 0{\cdot}44$).

Discussion

TESTOSTERONE CONCENTRATION

Although testosterone implants resulted in circulating levels 2·5 times that of control chicks, 83% of the values found in testosterone group chicks were within the range observed in un-manipulated chicks (Fig. 1). The action of hormones depend on the sensitivity or concentration of receptors in target organs (Wingfield & Farner 1993). In addition, most steroids circulate bound to proteins, such as albumins or sex steroids binding globulins. When bound to the high-affinity binding systems, steroid hormones are though to be biologically inactive (Wingfield & Farner 1993). Consequently, the difference in circulating levels of testosterone do not necessarily mean the same scale of differences in its effect. For these reasons, we think that the results obtained in this study can be interpreted from an ecological point of view.

NESTLING GROWTH AND IMMUNE RESPONSE

Experimentally increased physiological testosterone levels were associated with a reduction of mass and size in both male and female nestlings. However, this trend was not statistically significant. Furthermore, this increase led to a reduction in the ability of chicks to mount a CMI response. This result lends support to the idea that the immune function (at least the cell-mediated component measured in the current study) may be impaired as a consequence of increased physiological levels of testosterone, as predicted. Our results, thus, support that elevated levels of androgens can involve important costs as the CMI is essential for nestlings at a time when the naïve immune system is still developing in chicks (Apanius 1998). Previous evidence has shown a positive relationship between CMI response in early life and survival (Hõrak *et al.* 1999; Tella *et al.* 2000; Müller *et al.* 2003). We also have to consider that an increase in testosterone circulating levels may have caused changes in corticosterone levels, a stress hormone able to produce immunodepression in elevated concentrations (Evans, Goldsmith & Norris 2000). The effect of testosterone on corticosterone levels could be direct because of interactions between both steroid pathways, or indirect because testosterone changes competitive interactions that might then affect corticosterone levels. The immunosuppressive effect of testosterone has been suggested to occur via corticosterone (Møller 1995; Evans *et al.* 2000). However, in our kestrel population no association between circulating testosterone and corticosterone concentrations in chicks was found (J. Martínez-Padilla, D. Gil & J. Viñuela, unpublished manuscript).

Unexpectedly, we found no significant correlations between body mass, size or condition and CMI response. We have no clear explanations for this result as other studies have shown these correlations, including in Eurasian kestrels (Fargallo *et al.* 2002, 2003; Laaksonen *et al.* 2004) and in the same population (Martínez-Padilla 2006). However, the same discrepancy has been found in other bird species, such as white storks *Ciconia ciconia* in which the correlation between body condition and CMI has been found in some years but not in others (Jovani *et al.* 2004). Interannual and

interpopulation environmental conditions on CMI response may contribute to this variation.

RUMP COLORATION OF MALES

Increased levels of testosterone promoted a reduction in grey coloration in the rumps of male nestlings, a character associated with the quality of the environment in which the chicks matured (Fargallo *et al*. 2007). Our results suggest that increases in physiological testosterone levels promote a reduced expression of a melanin-based signal in concordance with CMI response results in this study. In other cases, such as in the house sparrow *Passer domesticus*, testosterone can enlarge the area of the black bib of males, a melanin-based sexual trait (Evans *et al*. 2000; González *et al*. 2001; Strasser & Schwabl 2004). According to the immunocompetence handicap hypothesis, it is expected that the expression of a secondary sexual character, such as the sparrow bib, increases with testosterone (Folstad & Karter 1992). In our case, grey coloration on the black-barred rump has been suggested to be a character that could indicate social status within the fledgling age-class out of sexual selection context (Fargallo *et al*. 2007), which might explain why it is not enhanced by the administration of testosterone. Although there is evidence that melanocytes have cell surface receptors for androgens, it is not clear what effect these hormones have on melanogenesis (Hearing 1998). One of the effects described is that testosterone treatment stimulates phaeomelanogenesis, but not eumelanogenesis (Haase *et al*. 1995). The grey colour of kestrel feathers stems from the deposition of three times more eumelanin than phaeomelanin. Brown coloration of feathers, on the contrary, is due to the presence of seven times more phaeomelanin than eumelanin (Fargallo *et al*. 2007). If testosterone stimulates phaeomelanogenesis, more brown than grey coloration should be found in male nestling rumps, as observed. Thus, our results could indicate: (1) an effect *per se* of testosterone as a modulator in the different pathways of melanin biosynthesis, or (2) a reflection of inferior health condition (CMI response) promoted by the increased levels of testosterone. In either cases, elevated levels of testosterone seem to have adverse consequences for male nestling coloration. Also, in black-headed gulls *Larus ridibundus*, a sexually monochromatic species, testosterone treatment modified melanin-based plumage coloration, giving the chicks a more adult-like appearance than control chicks due to the exhibition of more black in the head mask or narrower black-tail bars (Groothuis & Meeuwissen 1992; Ros 1999). This result has been interpreted as an acceleration of plumage maturation. More work is needed on the proximate mechanisms through which androgens interact with melanin deposition in the melanocytes of feather follicles.

We do not have clear conclusions about why grey rump coloration is positively correlated with body mass in T-nestlings but not in C-nestlings. Nestling body mass has varied in our population from 204 g to 221 g in an 11-year period (1995–2005). The mean body mass in 2004 in the control group was 213 g, indicating that our study year could be characterized as more of a good year than a bad year for kestrels. In a year of good food conditions, the variation of a character expressing environmental quality is expected to be lower than in a bad year where differences between offspring of good and poor quality pairs are more prominent. Under more stressful situations, such as that provoked in the testosterone group, only good quality chicks might be able to produce the character.

SEX AND TESTOSTERONE

No sex differences were observed in circulating levels of testosterone, either in C-nestlings or T-nestlings, indicating that testosterone uptake was similar in both sexes, as also shown by the analysis of the remaining testosterone propionate in implants. As in previous studies (Fargallo *et al*. 2002), we also found sex differences in CMI response. The absence of sexual differences in testosterone levels and the unexpected nonsignificant interaction between sex and treatment in CMI response do not support the idea that postnatal sexual vulnerability observed in CMI response, is associated with sexual differences in levels of or sensitivity to circulating testosterone, at least in this species. Nevertheless, the pulsatile nature of testosterone in plasma and its variation in concentration over the period of nestling growth, do not allow confirmation of the absence of differences in testosterone levels in any phase of growth (Adkins-Regan *et al*. 1990; Silverin & Sharp 1996) that could later have affected the CMI response.

The smaller size of males in reverse sexually size-dimorphic species can impair their competitive capacity against larger females (see Introduction). In this scenario, it may be adaptive that the smaller sex (males) have increased baseline levels of testosterone to better compete with larger nestmates (females) for resources. On the other hand, elevated levels of testosterone may be a consequence of greater levels of stress due to the disadvantage in sibling competition (Ferree *et al*. 2004). Our study does not provide support to either of these ideas in kestrels.

INTERSPECIFIC COMPARISONS

Results from studies of other bird species (Table 2) reported variable results. Of 15 species we reviewed (including kestrels), three showed that growing males had more circulating testosterone (testosterone + dihydrotestosterone) levels than females. In great tits *Parus major*, this was only true for the first 2 days after hatching, but the differences disappeared afterwards. In two species, females showed higher levels of testosterone, and in the case of zebra finches *Taeniopygia guttata* both trends (male > female and female > male) were observed in different studies, while a third study did not

Table 2. Bird studies at nestling stage on plasma androgen levels in relation to the sex

	T	DHT	T + DHT	Reference
Sula nebouxii	no detectable	–	–	1
Falco tinnunculus	no	–	–	2
Gallus domesticus	m	–	–	3
Coturnix japonica	–	–	f	4
Larus ridibundus	no	–	–	5
Larus fuscus	no	–	–	6(a)
Centropus grillii	–	–	m	7
Erithacus rubecula	–	no	–	8
Ficedula hypoleuca	no	–	–	9
Parus major	m	–	–	10(b)
Hirundo rustica	no	–	–	11
Sturnus vulgaris	–	–	no	12
Sturnus unicolor	–	–	no	13
Serinus canaria	f	no	–	14(c)
Taeniopygia guttata	no	no	–	15
	–	–	m (n.s.)	16
	–	–	f	17

T, testosterone; DHT, dihydrotestosterone; m, higher levels in males; f, higher levels in females; no, no sexual differences (n.s.), no significant differences.
(1) Núñez-de la Mora *et al*. (1996); (2) this study; (3) Corbier *et al*. (1992); (4) Ottinger *et al*. (2001); (5) Groothuis & Meeuwissen (1992); (6) Verboven *et al*. (2003); (7) Goymann, Kempanaers & Wingfield (2005); (8) Schwabl & Lipar (2002); (9) Goodship (2006); (10) Silverin & Sharp (1996); (11) Gil *et al*. (2006); (12) Williams *et al*. (1987); (13) D. Gil, P. Celis & E. Bulmer (umpublished data); (14) Weichel *et al*. (1986); (15) Schlinger & Arnold (1992); (16) Naguib *et al*. (2004); (17) Adkins-Regan *et al*. (1990). (a) Individuals sampled at hatching; (b) sex differences only during the 2 days after hatching, but not later; (c) Individuals sampled as fledglings (35–55 days after hatching).

find sexual differences in circulating testosterone. In the nine remaining species no sex differences were found. These data together with our results do not seem to support the idea that sexual differences in immunity or condition in early life can be determined by sexual differences in circulating testosterone levels. However, rates of growth and development differs substantially among species and, as mentioned above, differences can be produced at different stages of nestling growth (Silverin & Sharp 1996) or embryonic development. Furthermore, although our study did not reveal between sex differences in the response of the characters we measured to circulating levels of testosterone, it may be possible that males are more sensitive to testosterone increases during embryogenesis, when the process of sexual differentiation takes place (Müller *et al*. 2005a).

This is the first experiment in the wild demonstrating the effects of elevated levels of circulating testosterone on nestlings. High levels of testosterone tended to lower nestling body condition, significantly decreased CMI response in both sexes and reduced the expression of a melanin-based character in males. Although recent works have found negative consequences of elevated levels of testosterone on immunity and growth (e.g. Müller *et al*. 2005b; Rubolini *et al*. 2006), beneficial effects of androgens on important traits potentially determining survival (Gil *et al*. 1999; Groothuis *et al*. 2005a). It is possible that testosterone has positive effects only when some individuals possess higher levels compared with nestmates, increasing their ability to compete for resources (begging display or aggressiveness), as the majority of experimental studies has shown. When testosterone is increased in all nestlings the expected advantage of increasing competitive capacity could be inhibited. Furthermore, our results suggest that if sexual differences in immunity, and possibly in parasite infection, during post-natal stages are linked to sex phenotype, then circulating testosterone does not seem to be a clear causal factor responsible for that difference. Other aspects related to testosterone (yolk concentrations or sex-linked sensitivity) should be explored. Also, other hormones, such as oestrogen (Owens & Short 1995), or the competitive disadvantage of the smaller sex in sexually dimorphic species, could explain sex-linked environmental vulnerability in early life.

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