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Published in:
Behavioral Ecology and Sociobiology

DOI:
10.1007/s00265-012-1387-3

Behav Ecol Sociobiol (2012) 66:1319–1331
DOI 10.1007/s00265-012-1387-3

ORIGINAL PAPER

Differential effects of testosterone metabolites oestradiol and dihydrotestosterone on oxidative stress and carotenoid-dependent colour expression in a bird

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Received: 15 May 2012 / Revised: 3 July 2012 / Accepted: 4 July 2012 / Published online: 20 July 2012

Abstract Despite extensive research, the potential costs that keep secondary sexual traits honest and evolutionary stable remain somewhat elusive. Many carotenoid-based signals are regulated by testosterone (T), which has been suggested to impose a cost to the signaller by suppression of the immune system or an increase in oxidative stress. Results are, however, inconsistent, which may be due to the fact that T can be metabolised to both 5α-dihydrotestosterone (DHT, a potent androgen) and oestradiol (E2, a potent oestrogen). To evaluate for the first time the independent effect of these testosterone metabolites on oxidative status, circulating carotenoids and a carotenoid-dependent sexual signal, we administered DHT and E2 to captive non-breeding adult kestrels *Falco tinnunculus* of both sexes. E2 increased oxidative damage and down-regulated the antioxidant barrier without affecting colouration or circulating carotenoids. In contrast, DHT did not affect oxidative status, but increased skin redness, again without affecting circulating carotenoids. No sex-specific effects were found. These results suggest that the pro-oxidant activity of T could be induced indirectly by its metabolite, E2, whereas the other metabolite, DHT, stimulates signal expression. Finally, the study shows that changes in oxidative damage or antioxidant status of plasma were not correlated with either skin redness or circulating carotenoids.

Keywords Antioxidant defence · Oxidation handicap hypothesis · Raptor · Reactive oxygen metabolites · Skin

Communicated by J. A. Graves.

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Introduction

The identification of the proximate mechanisms regulating the expression of secondary sexual traits (SST) is crucial for understanding how such traits are regulated, and which selective pressures may affect their function and evolution. In many cases, the expression of SSTs is in part regulated by testosterone (T), which in turn has been postulated to lower the physiological condition of the signaller, representing a cost that is required for producing reliable sexual signals ("handicap principle", Zahavi and Zahavi 1997). Almost two decades ago, it was proposed that one such cost was immune suppression (Folstad and Karter 1992), but evidence for this is still equivocal (e.g. Roberts et al. 2004). More recently, it was proposed that the elevation of T would

increase oxidative stress (oxidation handicap hypothesis, Alonso-Alvarez et al. 2007), which is generally considered an important factor underlying cellular and reproductive senescence and ageing (Halliwell and Gutteridge 2007; Costantini 2008; Metcalfe and Alonso-Alvarez 2010; Costantini et al. 2010). T can act as a pro-oxidant by increasing the production of reactive chemical species (e.g. free radicals), mostly by increasing the metabolic rate (Buchanan et al. 2001) or activating NADPH oxidases (Reckelhoff 2005). Evidence for the effect of T on oxidative stress and its underlying pathway needs, however, more experimental evidence. For example, T-treated red-legged partridges *Alectoris rufa* controlled oxidative stress levels by upregulating the intra-cellular antioxidant defence (Alonzo-Alvarez et al. 2008), while males zebra finches *Taenopygia guttata* treated with T showed a lower red blood cell resistance to free radicals compared to males treated with a T blocker (Alonso-Alvarez et al. 2007).

Both of these studies considered species exhibiting carotenoid-based sexual signals. Indeed, another potential cost of T-dependent SST is related to the fact that T also regulates the deposition of carotenoids (reviewed in Kimball 2006), responsible for the colouration of many SSTs (reviewed in McGraw 2006). It has been proposed that carotenoid-based colouration can honestly signal the capacity of the bearer to cope with oxidative stress since carotenoids would have antioxidant properties and their allocation to the signal must then be traded off between health and communication functions (von Schantz et al. 1999; Hartley and Kennedy 2004; Bertrand et al. 2006; Alonzo-Alvarez et al. 2008; Mougeot et al. 2009), although it has also been suggested that the increase in carotenoid availability promoted by T might buffer the pro-oxidant activity of T itself (Alonzo-Alvarez et al. 2008). The contribution of carotenoids to in vivo antioxidant defences in birds is, however, still controversial, and much evidence suggests that their effect is either low or very specific (Costantini and Møller 2008; Isaksson and Andersson 2008; Pérez et al. 2008; Cohen and McGraw 2009; but see Pérez-Rodríguez 2009).

The relationships between androgen secretion, oxidative status and carotenoid-based colouration are further complicated by the fact that T is the precursor of both the androgen 5α-dihydrotestosterone (DHT), and the oestrogen oestradiol (E2). Although information about the effect of the former on oxidative status is scarce (but see Pathak et al. 2008), DHT can be considered relevant as its action is exerted by binding to the same androgen receptor to which T binds, but with an even higher affinity. E2 binds to two specific oestrogenic receptors (E2 α and β). E2 can enhance antioxidant defences (Halifeoglu et al. 2003) and reduce reactive chemical species production (Borrás et al. 2010), but it is also known to be a potent carcinogenic agent due to its pro-oxidative activity (Han and Liehr 1994; Cavalieri et al. 2000; Karbownik et al. 2001). Indeed, it has been ascertained that oestradiol can have both a direct and an indirect stimulatory effect on the production of reactive oxygen species (ROS). Firstly, the molecule itself can be oxidised by cytochrome P450 enzymes forming hydroxylated products such as 2-, 4-, and 16-hydroxyoestradiol (e.g. Weisz et al. 1992). These metabolites contain hydroxyl groups in a vicinal position, which predisposes them to further oxidation to semiquinones, and to quinines, with the formation of superoxide anion radicals ($O_2^{-}\cdot$) (Bui and Weisz 1988; Bunyagidj and McLachlan 1988). Quinones and semiquinones are capable of redox cycling as long as there is molecular oxygen available, and, therefore, even a small amount of E2 may cause substantial ROS production and subsequent cellular damage (Cavalieri et al. 2000). Oestrogens can generate ROS by peroxidatic metabolism as well, by producing phenoxyl radicals in the lactoperoxidase-catalyzed reaction, leading to the formation of other radical species such as GS· and NAD· (Sipe et al. 1994). Moreover, E2 can be pro-oxidant for its immune stimulatory activity as at physiologic doses, it potently induces interleukin IL-1, a cytokine that can initiate a cascade of factors involved in the inflammatory reaction with consistent production of ROS and reactive nitrogen species (Cutolo et al. 1995). Oestrogens also stimulate the function of leukocytes, neutrophils and granulocytes that produce copious amounts of ROS when activated. Strikingly, E2 may even induce inactivated cells to stimulate the generation of oxidants in the absence of pathogens (Jansson 1991). This oestradiol-mediated action causes HOCl/OCl formation ensuing oxidative cell damage, even in the absence of the proper targets. For these reasons, the T metabolite E2 could play a relevant role in mediating the relationships between endocrine system and physiological condition, and so should be taken into account when manipulating T level in birds.

Whether T acts via the androgenic or oestrogenic pathway is as yet unclear, may depend on the context, and may determine the relation between signalling and oxidative status and therefore the cost of signalling. Moreover, several studies investigated the role of T in regulating body carotenoids (e.g. Blas et al. 2006; McGraw et al. 2006; Mougeot et al. 2009; Alonso-Alvarez et al. 2007), but none considered the role of T metabolites. In this study, we analysed the effects of the androgenic and oestrogenic pathways on the expression of carotenoid-based SST and plasma oxidative status in male and female common kestrels (*Falco tinnunculus*).

The common kestrel is a long-lived bird of prey in which both sexes display carotenoid-dependent colouration on bare skin of the bill, lores and legs (see "Methods" section for more details). We studied how DHT (androgenic pathway) and E2 (oestrogenic pathway) affect parameters of plasma oxidative status, skin colour and circulating

carotenoids by manipulating DHT and E2 plasma concentrations of male and female captive kestrels outside the breeding season, when gonadal steroid levels are at their minimum (Meijer and Schwabl 1989). Both T and DHT bind to the same androgen receptor and have the same effects (Roya et al. 1998) in target tissues, whereas E2 binds specifically to E2 receptors (Levin 2005). In contrast to T, DHT cannot be converted to E2 and has a higher affinity to the androgen receptor than T.

These two metabolites mediate a substantial fraction of the behavioural and physiological effects of T at the cellular level (Ball and Balthazart 2008). E2 can be produced by the enzyme P450-aromatase, either directly from testosterone or via the production of the intermediate estrone from androstenedione, the precursor of T, while DHT is produced from T by the enzyme 5α-reductase. Therefore, T exerts its tissue-specific action partly through the activity of its metabolites, E2 and DHT (Hau 2007). From the detection of aromatase at local level, it has been shown that most part of oestradiol in birds is produced in the brain (Schlinger and Arnold 1991), skin (Somes et al. 1984), bone (Deng et al. 2010), inner ear (Noirot et al. 2009), liver (Silverin 2000) and in the gonads of both females (Armstrong 1984) and males (Tanabe et al. 1986). A broader expression of avian aromatase in other peripheral tissues is, however, plausible since in other vertebrates the enzyme aromatase is present in the cellular endoplasmic reticulum of almost all tissues (Carreau et al. 1999). The distribution of 5α-reductase is not well known in birds, except for some studies that have detected this enzyme in the brain and skin (Schlinger et al. 1989; Tramontin et al. 2003). However, the presence of two androgens, DHT and T, with different affinities to the same receptor suggests the existence of different mechanisms regulating T action at very local level, as shown in other vertebrates than birds by the tissue-specific expression of 5α-reductase, such as in the skin of humans (Gao and Dalton 2007) or in the bone and muscle of mice (Windahl et al. 2011).

The manipulation of E2 and DHT allowed us to evaluate the effect of gonadal steroids, disentangling the androgenic and oestrogenic pathway, on oxidative balance, circulating carotenoids and signal expression. Since we studied captive birds after their reproductive season, kept in cages where locomotor activity was limited, the effect of steroids could be assessed independently from steroid-dependent breeding and workload. In addition, we studied to what extent carotenoid expression in the signal might be mediated by the upregulation of their carrier, lipoproteins, as both androgens (McGraw and Ardia 2007) and oestrogens (Kudzma et al. 1979; Chapman 1980; Dashti et al. 1983; Casagrande et al. 2011a) can regulate the redistribution of lipoproteins.

Methods

Study species and housing

The common kestrel is a social monogamous bi-parental species, sexually dimorphic for size (males smaller than females), plumage colour (males have grey head and tail and reddish back, while females are all brownish) and bare skin (cere, eye ring and tarsi) during courtship (males' skin hue redder than females; Casagrande et al. 2006). Yellow-orange bare parts are produced by deposition of the oxy-carotenoids lutein and zeaxanthin in the integument without any metabolic transformation (Casagrande et al. 2006). Both colour and blood carotenoids are almost entirely environmentally determined (Casagrande et al. 2009).

This study was carried out from 1 June to 1 August 2007 on a captive population settled at the beginning of March. Twelve male and 12 female adult kestrels were randomly housed in individual pairs in outdoor aviaries (1×1.7×2 m; w×l×h) located 30 km south of Rome and managed by Ornis italica (www.ornisitalica.com). Each aviary was equipped with a nest box (30×30×60 cm), two perches and water ad libitum and was separated from the others by a shade net to prevent pairs from seeing each other (see Costantini et al. 2007a for further details on housing condition). The birds were maintained on a constant diet of 1-day-old chicks of *Gallus gallus domesticus*. Due to the death of one female (of unknown causes) by the end of June, sample sizes are 11 females and 12 males.

Manipulation of sexual steroids

Hormones were provided implanting birds with two silicon capsules (i.d.=1.50 mm, o.d.=2.0 mm; length, 24 mm) filled with crystalline 17β-oestradiol (E2, Sigma, St. Louis, product number E2758) or with 5α-dihydrotestosterone (DHT, product number 10300, Sigma, St. Louis). Controls were implanted with empty tubes of the same size. Implants were embedded for 24 h into phosphate-buffered saline before implantation. Birds were locally anesthetised with lidocaine (Xylocaine, AstraZeneca BV, Zoetermeer), and the implant was inserted under the skin after making a small cut in the right flank. The cut was sealed with surgical glue (Hansaplast-Beiersdorf, Hamburg). We randomly assigned individuals to two groups respectively: an experimental group (both sexes) receiving two sequential treatments (E2 and DHT) and a non-manipulated group (both sexes), which served as controls twice sequentially. On 1 June (day 0), all birds were bled for determination of hormones, carotenoids and oxidative stress-related parameters (see below), and colour and morphometrical measurements were taken. Next, six males and six females were treated with 17β-oestradiol, while six males and five females served as controls. Kestrels

were housed in pairs (one male and one female) with one treated bird and one control per cage. After 30 days (1 July, day 30), they were again blood sampled and measured, and the E2 implant was removed and replaced with 5α-dihydrotestosterone implants. Birds were sampled and measured again after 30 days (1 August, day 60).

Colour measurements

The yellow colouration of the right tarsus skin was measured with a portable digital colour meter (for further details, see Casagrande et al. 2011b). Standard colorimetric variables [L-lightness (or brightness); a* (hereafter referred as "red")—red component; and b* (hereafter referred as "yellow")—yellow component)] were obtained with the software ColorShop 2.5 (X-Rite®, Grandville, MI) in the uniform colour space CIELAB (CIE 1978). A mean value obtained from three sequential colorimetric readings per individual was used for statistical analysis because measures were highly repeatable (intraclass correlation coefficients calculated following Lessells and Boag (1987): all $r>0.92$, all $p<0.01$). Although kestrels can perceive UV component of colouration, we think that our approach is valuable to assess the variation of carotenoid content in the skin since the carotenoid-based traits reflect primarily in the human-visible range and absorb light of short wavelength, i.e. UV and blue (Andersson and Prager 2006). In addition, it has been shown that these CIELAB variables are reliable proxies of the amount of deposited carotenoids in tissues (Butler et al. 2011). Moreover, the skin colour of the common kestrel has already been described using the visible range spectrum (Casagrande et al. 2006, 2007, 2009; Costantini et al. 2007b). We therefore use the L*a*b* system for estimating variation of carotenoids deposited in the skin.

Blood sampling and morphometrical measurements

A sample of blood (800 μL) was taken from the brachial vein with a heparinised syringe within 10 min after trapping and samples kept in a cool thermos (2–4 °C) until centrifugation (8 h maximum) at 1,400×*g* for 5 min. The length of the tarsus, wing chord and the body mass was measured for each individual with a calliper at the nearest 0.1 mm, the wing length chord was measured with a ruler at the nearest 1.0 mm, and the body mass was measured with a Pesola balance at the nearest 1 g.

Laboratory analyses

Radioimmunoassay

For each bird, we assessed the level of T, E2 and DHT by radioimmunoassay. A detailed protocol used during this study has been reported in Casagrande et al. (2011a, b) and Casagrande and Groothuis (2011). Samples were extracted twice adding to the plasma (200 μL) 4 mL of petroleum ether/diethylether (30–70 %), to allow steroids to pass from the watery phase to the organic one. The extraction was dried under nitrogen stream and then dissolved in 90 % ethanol, dried again under nitrogen stream, dissolved in 70 % methanol and placed at −20 °C overnight. The solution was then dried and dissolved in 185 μL of PBSG buffer. T was assayed from 50 μL of plasma using the DSL-4000 Active Testosterone Coated-Tube Radioimmunoassay Kit (Diagnostic System Laboratories, Inc., Webster), with the concentration expressed in nanograms per millilitre. DHT was assayed from 25 μL of plasma using DSL-96100 Dihydrotestosterone Radio immunoassay Kit (DSL-Diagnostic System Laboratories, Inc., Webster) following the protocol provided by DSL. Oestradiol was assayed in 50 μL of plasma using DSL-4400 Estradiol Radioimmunoassay Kit (Diagnostic System Laboratories, Inc., Webster) following the DSL protocol. The concentrations of DHT and E2 were expressed in picograms per millilitre. Recovery rates were 84.66 % for E2, 85.23 % for DHT and 85.7 % for T. Intra-assay CV for these hormones were 5.1, 2.3 and 2.2 %, respectively, while inter-assay CV were 6.9, 2.2 and 4.6 %. Cross-reactivity with steroids other than the target of the kit was very low (testosterone kit, 5.8 % with 5α-dihydrotestosterone, 2.3 % with androstenedione, 0 % with oestrogens; 5α-dihydrotestosterone kit, androstandiol, 3.3 %; testosterone, 0.6 %, 0 % with oestrogens; 17β-oestradiol kit, 3.40 % with estrone and 0 % with androgens).

Carotenoid and cholesterol analysis

It is known that common kestrels can absorb lutein and zeaxanthin from the diet and that these pigments are deposited unaltered in the skin (Casagrande et al. 2006). To measure the amount of carotenoids circulating in the blood, the plasma (20 μL) was diluted with absolute methanol (1:25), and the flocculent proteins were precipitated by centrifugation at 12,000×*g* for 5 min. Carotenoids were quantified with a Pharmacia Biotech Ultrospec (Pharmacia, Cambridge) spectrophotometer at 446 nm. The carotenoid concentration was estimated as micrograms per millilitre of plasma using the standard absorbance curve of lutein (Sigma-Aldrich). To evaluate the concentration of carotenoid carriers (lipoproteins) in the peripheral blood, we measured the total amount of cholesterol (McGraw and Parker 2006). We diluted 10 μL of plasma with 1 ml of the reagent kit Nobiflow Cholesterin (Hitado Diagnostic System, Möhnesee-Delecke) reading the sample with the Pharmacia Biotech Ultrospec (Pharmacia, Cambridge) spectrophotometer at 500 nm. The concentration of cholesterol was calculated in milligrams per decilitre referring to Nobical

Cholesterin (Hitado Diagnostic System, Möhnesee-Delecke) as standard. The kit is sensitive to both low-density lipoprotein cholesterol and high-density lipoprotein cholesterol.

Plasma oxidative status

Plasma hydroperoxides [reactive oxygen metabolites (ROMs); marker of oxidative damage] and total antioxidant capacity (OXY; including the contribution of both exogenous and endogenous antioxidants) were measured as in previous studies (e.g. Costantini et al. 2006; Costantini and Dell'Omo 2006; Casagrande et al. 2011b). Briefly, ROMs were measured by the d-ROMs test (Diacron International, Grosseto, Italy). The plasma (20 μL) was first diluted with 200 μL of a solution containing 0.01 M acetic acid/sodium acetate buffer (pH 4.8) and *N*,*N*-diethyl-*p*-phenylenediamine as chromogen and then incubated for 75 min at 37 °C. After incubation, the absorbance was read with a tissue plate spectrophotometer (Banderini; www.AB-Research.it) at 505 nm, and the concentration of ROMs was calculated by comparison with a standard curve obtained by measuring the absorbance of a standard solution. ROMs are expressed as millimolars of H_2O_2 equivalents. The plasma antioxidant capacity was measured by the OXY-Adsorbent test (Diacron International, Grosseto, Italy). The plasma (10 μl) was diluted 1:100 with distilled water. A 200-μl aliquot of a titred HOCl solution was incubated with 5 μl of the diluted plasma for 10 min at 37 °C. Then, 5 μl of the same chromogen solution used for the ROMs determination was added. An alkyl-substituted aromatic amine dissolved in the chromogen is oxidised by the residual HOCl and transformed into a pink derivative. The intensity of the coloured complex, which is inversely related to OXY, was measured at 505 nm using a spectrophotometer. OXY was expressed as millimolars of HOCl neutralised. We then calculated the ratio of ROMs to OXY (×1,000) and used it as index of overall plasma oxidative status (OS; see Costantini et al. 2006 for further details), with higher values indicating that the plasma contains a greater concentration of oxidised molecules than antioxidant compounds [see Costantini et al. (2006, 2007) and van de Crommenacker et al. (2012) for further details on OS]. This index does not reflect all the complexity of the redox system, but, as shown by extensive literature in ecological, veterinary and clinical research, it is sensitive to several kinds of stressors and marks well a condition of oxidative stress. We use this ratio to summarise and complement information obtained from the two biomarkers ROMs and OXY, respectively.

Data analyses

All analyses were performed with STATISTICA 7.0 (StatSoft 2004, Tulsa, OK, USA). A body condition index was calculated as the residuals of a linear regression of body mass on tarsus length ($F_{(1,19)}$=13.98, p<0.01, R^2=0.39). To analyse the effect of hormone manipulation on the variation in skin colour, plasma carotenoids, cholesterol, body condition and biomarkers of plasma oxidative status, we performed a repeated measures ANOVA [three levels: day 0 (before the start of the experiment), day 30 (after 30 days of E2 administration) and day 60 (after 30 days of DHT administration)] for all the variables considered before and with sex (two levels) and hormone treatment (two levels, yes or no) as fixed factors. We initially included all interactions in the models but removed those not significant. We were especially interested in the interaction between time and treatment, or time, treatment and sex, indicating an effect of hormone treatment and a sex-specific effect of this treatment, respectively. We only evaluated main effects where interactions were not significant. When the interaction between time and treatment was significant, we performed post hoc tests to determine which hormone treatment was effective, by testing the differences between experimental and control animals for each of the 3 days of sampling separately by means of independent *t* tests. Since interactions including sex were all nonsignificant, the post hoc tests were performed with the sexes pooled. We also used, where relevant, paired *t* tests to test for changes within individuals over time for each group separately. Finally, the relation between plasma T concentrations and measurements of oxidative status was analysed by means of Pearson correlations. Normality was tested using the Shapiro–Wilk test; values are reported as mean±SE, and E2 concentrations data were subsequently log transformed to normalise the distribution.

Results

Plasma hormone concentrations

Both E2 and DHT implants were in both sexes effective in increasing their plasma concentration as in both cases the interaction between time and hormone treatment was highly significant (Table 1) and independent of sex. Post hoc *t* tests for each day separately indicated a treatment effect for E2 only at day 30, after implantation of E2 ($t_{(21)}$=8.11, p< 0.0001), and for DHT only at day 60, after implantation of DHT, ($t_{(21)}$=4.68, p<0.0001; see Fig. 1a, b; all other time points p>0.23).

Since carotenoids can be regulated by T, we measured plasma T concentrations as well. These were not significantly affected by the hormone treatment (Table 1). The interaction between time and sex (Table 1), and the post hoc tests revealed that over time, T decreased in males (post hoc paired *t* tests: day 0 vs. day 30, p=0.04; day 0 vs. day 60,

Table 1 Finals outcomes of repeated measures ANOVA models assessing the effect of sexual steroid manipulation on hormones levels

Dependent variables	Factors	*df*	*F*	*p*
E2	Hormone treatment	1,21	84.23	<0.0001
	Time	2,42	96.73	<0.0001
	Time×hormone treatment	2,42	12.88	<0.0001
DHT	Hormone treatment	1,21	9.58	0.005
	Time	2,42	5.68	0.007
	Time×hormone treatment	2,42	9.92	<0.0001
T	Sex	1,21	9.96	0.005
	Time	2,42	5.95	0.005
	Time×sex	2,42	4.73	0.01

Day 30 gives E2 effects, while day 60 DHT effects

p=0.007), but not in females, whose T levels remained stable during the experiment (post hoc paired t tests, all p>0.25; Fig. 1c). T on day 0 was not correlated with the parameters describing oxidative stress (all p>0.16) also considering each sex separately (all p>0.42 except for OS in males, yielding a lower but still not significant p value: r=−0.52, p=0.09, n=12). The same was found for T on day 30 (all p>0.68 except for OXY, r=0.35, p=0.10, n=24) and on day 60 (all p>0.29).

Biomarkers of plasma oxidative status

Variation in ROMs, OXY and OS were significantly explained by the interaction between time and hormone treatment, independently of sex (Table 2, Fig. 2a–c), with an increase in ROM and OS and a decrease in OXY due to E2 implantation. Indeed, in all three variables the independent t tests showed an effect of treatment only after E2 treatment (day 30; ROMs, $t_{(21)}$=3.20, p=0.004; OXY, $t_{(21)}$=5.88, p<0.0001; OS, $t_{(21)}$=3.52, p=0.003), and no effect of DHT (day 60; ROMs, $t_{(21)}$=1.19, p=0.25; OXY, $t_{(21)}$=1.72, p=0.10; OS, $t_{(21)}$=0.42, p=0.68).

Skin colour, carotenoids, body mass and cholesterol

Variation in skin redness (expressed by the colorimetric variable a*, Fig. 2d) was also significantly explained by the interaction between time and hormone treatment without any interaction with sex (Table 3). Post hoc comparisons showed that the degree of redness differed significantly between the controls and experimental birds after DHT implantation (day 60, $t_{(21)}$=3.78, p=0.001), with the hormone increasing redness, and no significant effect of E2 ($t_{(21)}$=0.32, p=0.75; Fig. 2d). Overall, males displayed more redness than females (Table 3). The decrease in redness between day 0 and 30 did not differ significantly between controls and experimental birds and was likely due to an effect of season rather than hormone treatment. The other colorimetric variables changed only with time but not with hormone treatment nor with sex (Table 3) and are therefore not further analysed.

Circulating carotenoids decreased over time irrespective of hormone treatment, sex or their interaction (Table 4 and Fig. 2e). Body mass only varied with both sex and time (Table 4), and therefore, both parameters are not further analysed since we are interested in hormone effects. Variation in cholesterol (Fig. 2f) was explained by a strong significant interaction between time×hormone treatment and a marginal time by sex interaction (Table 4 and Fig. 2f). Post hoc independent t tests showed that cholesterol was significantly elevated only after implantation with E2 (day 0, $t_{(21)}$=0.23, p=0.82; day 30, $t_{(21)}$=5.15, p<0.0001; day 60, $t_{(21)}$=0.62, p=0.54).

Discussion

Secondary sexual traits are supposed to be honest signals of quality as their expression would impose costs for the signaller, although the nature of these costs is not yet clear. Such traits are often dependent on testosterone that has recently been invoked in generating oxidative stress, but data on the potential costs of this hormone are inconsistent. This might be due to the fact that this hormone can be metabolised to two other hormones with a metabolic rate that might be context specific. We therefore manipulated these two hormones independently of each other. The hormone treatments were successful in that they selectively elevated E2 and DHT respectively.

The treatment induced average peak level of DHT of around 60 pg/ml. Wild males and females in spring have on average of 150 and 75 pg/ml, (Casagrande et al. 2011b), indicating that our treatment was well within the physiological range of this species. E2-treated birds had peak E2 levels of 400 pg/ml (females) or 150 pg/ml (males), which is higher than those previously recorded in this species in the wild (being close to 0 in breeding males and about 10 pg/ml in females at the time of mating and close to 0

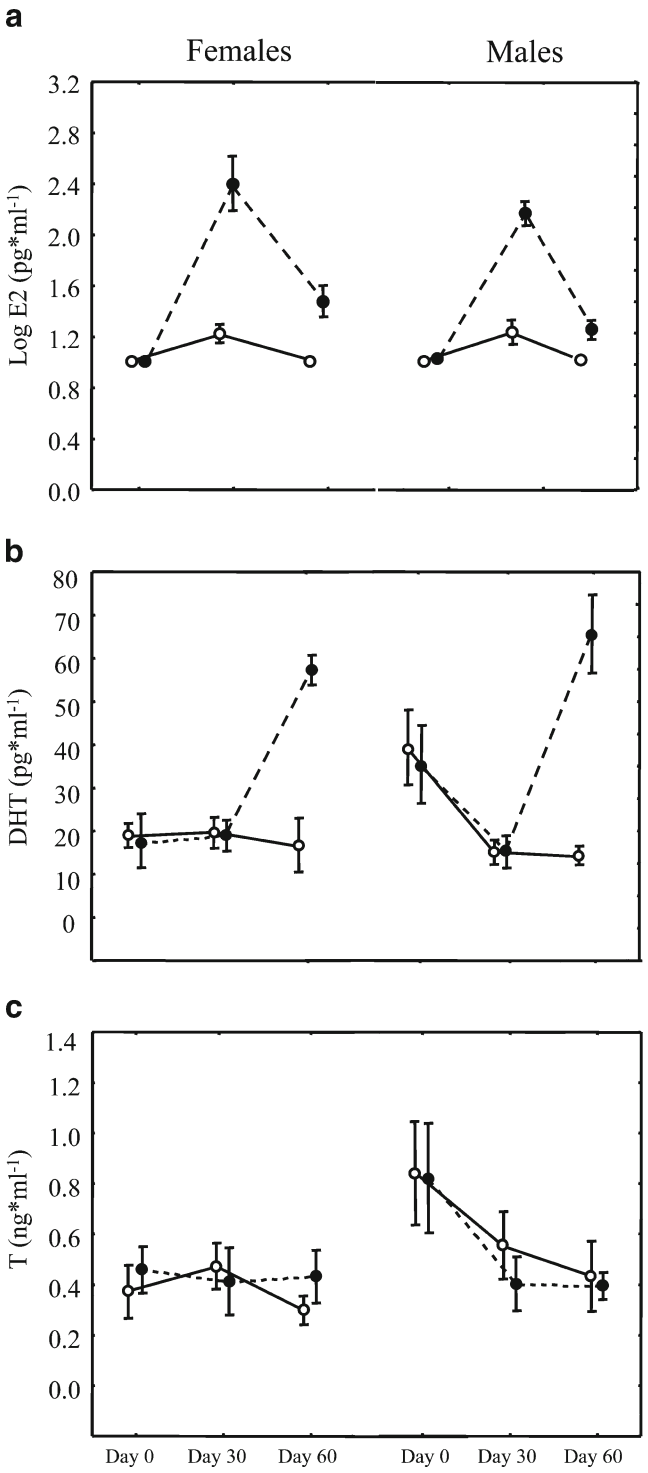

Fig. 1 Concentrations of circulating sexual steroids (**a** E2, **b** DHT, **c** T) before the start of the experiment (day 0), after manipulation of E2 (day 30) and DHT (day 60). *Open circles* represent mean and SE of control birds, *filled circles* those of experimental birds. Untransformed values of E2 measured in treated birds: day 0, 0.37±0.28 pg mL^{-1}; day 30, 306.20±143.91 pg mL^{-1}; day 60, 36.28±27.47 pg mL^{-1}

during rearing; Casagrande et al. 2011b). Nevertheless, peak levels of E2 are expected to occur during laying in females, as observed in the closely related American kestrel (*Falco sparverius* Rehder et al. 1986) and other bird species (Bluhm et al. 1983, Hunt and Wingfield 2004; Blas and Hiraldo 2010), when they can rise to levels much higher (580±60 pg ml^{-1}; Tramontin et al. 2003; 1,412± 245 pg ml^{-1}; Hunt and Wingfield 2004) than those induced in the present study without adverse effects. For this reason, we do not think that the oxidative status induced by our E2 treatment could be considered above the natural range for this species.

We found a clear pattern of variation in cholesterol that peaked on day 30 (a typical consequence of elevated E2 levels, Casagrande et al. 2011a) but not on day 60 (at the end of the DHT treatment), and of carotenoid-based colouration that peaked on day 60 (a typical effect of DHT, Casagrande et al. 2011a), but not at the end of the E2 treatment at day 30. The endocrine regulation of carotenoid-based fleshy signals in females is almost an unexplored topic until now (but see Pérez-Rodríguez and Viñuela 2008; Casagrande et al. 2011a, b). Since in the kestrel, females express similar signals as those of males, although often less elaborated, and the male, providing parental care, may also select his partner on the basis of such signals (Ketterson et al. 2005), female colour expression may, like in the male, be under sexual selection. Except for a marginal sex effect of E2 on cholesterol levels, none of the hormonal effects was sex dependent. This indicates that the hormonal regulation of oxidative status and carotenoid-related traits are similar in both sexes, at least during the non-breeding season. Interestingly, males had overall higher levels of skin colouration and, although not reaching statistical significance, also lower levels of circulating carotenoids, despite similar plasma hormone concentrations. This suggests that there are sex differences in, for example, carotenoid metabolism or deposition, perhaps due to sex differences in androgen receptor densities in the skin. Similar findings have been reported for the carotenoid-dependent eye ring colouration of the diamond dove (*Geopelia cuneata*, Casagrande et al. 2011a).

Effects of sexual steroids on oxidative status

Our results do not support the idea that testosterone affects oxidative status via the androgen receptor since DHT did not affect levels of ROMs, OXY or OS. The relationship between androgens and oxidative stress has only rarely been investigated in birds, but we know from studies on zebra finches (Alonso-Alvarez et al. 2007) and red grouse *Lagopus lagopus scoticus* (Mougeot et al. 2009) that T can be a pro-oxidant. Moreover, testosterone administration decreased red blood cell resistance to a free radical attack in zebra finches (Alonso-Alvarez et al. 2007). In mammals

Table 2 Final models of repeated measures ANOVA for assessing the effect of sexual steroid manipulation on the oxidative status

Dependent variables	Factors	*df*	*F*	*p*
ROMs	Hormone treatment	1,21	7.30	0.01
	Time	2,42	4.72	0.01
	Time×hormone treatment	2,42	6.08	0.005
OXY	Hormone treatment	1,21	2.28	0.15
	Time	2,42	6.27	0.004
	Time×hormone treatment	2,42	14.74	<0.0001
OS	Hormone treatment	1,21	7.74	0.01
	Time	2,42	6.72	0.003
	Time×hormone treatment	2,42	10.37	0.0003

Day 30 gives E2 effects, while day 60 DHT effects

(Tam et al. 2003; Calderón Guzmán et al. 2005) and birds (Alonzo-Alvarez et al. 2008; Mougeot et al. 2009), however, T can also have antioxidant properties, suggesting a context-dependent action of androgens in controlling oxidative damage related to reproduction. These equivocal results may be due to whether or not T could affect activity, resulting in an increase in energy expenditure, or to context-dependent metabolization of T to E2.

We found that the T metabolite E2 increased ROMs production and lowered antioxidant defences both in males and females. Oestrogens are thought to have strong antioxidant activity both in vitro (Behl et al. 1997) and in vivo

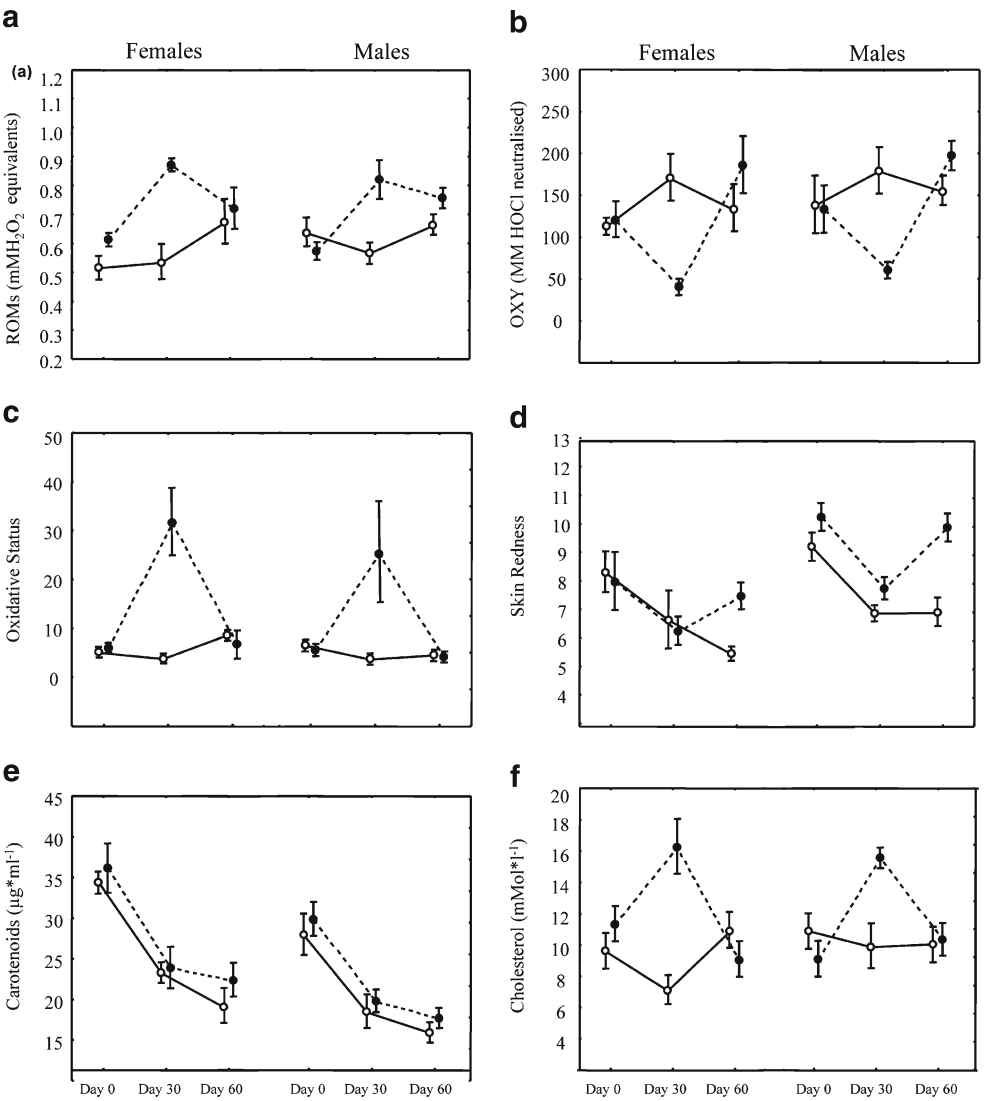

Fig. 2 Variation in skin colour (**a**, a*), circulating carotenoids (**b**), body mass (**c**), cholesterol (**d**), ROMs (**e**), OXY (**f**) and oxidative status (**g**) before the starting of the experiment (*day 0*), after manipulation of E2 (*day 30*) and DHT (*day 60*) *Open circles* represent mean of control birds, *filled circles* refer to experimental birds. *Bars* are SE

Table 3 Final models of repeated measures ANOVA for assessing the effect of sexual steroid manipulation on skin colour

Dependent variables	Factors	*df*	*F*	*p*
Redness	Sex	1,20	16.86	<0.0001
	Hormone treatment	1,20	8.42	0.009
	Time	2,40	10.66	<0.0001
	Time×hormone treatment	2,40	3.83	0.03
	Time×sex	2,40	0.68	0.51
Yellowness	Sex	1,21	6.56	0.02
	Time	2,42	1.50	0.23
	Time×sex	2,42	0.52	0.60

All factors explaining the variation of lightness were not significant. Day 30 gives E2 effects, while day 60 DHT effects

(Halifeoglu et al. 2003; Razmara et al. 2007). A study that tested the antioxidant activity of E2 compared red-legged partridges treated with a T inhibitor and birds treated with both a T blocker and a inhibitor of the aromatization of T in E2 (Alonzo-Alvarez et al. 2008), and did not find any difference in total antioxidant activity, intra-cellular antioxidant barrier or lipid peroxidation between the two groups (Alonzo-Alvarez et al. 2008). On the other hand, oestrogens are well known for their capacity for being carcinogenic in their target organ (Henderson and Feigelson 2000) by promoting oxidative stress (Bhat et al. 2003). Moreover, increased levels of ROMs have been associated with oestradiol administration in calves and are used as biomarker of oestrogen treatment exposure (Brambilla et al. 2003; see "Introduction" section for a detailed description of the pro-oxidant activity of oestradiol). This evidence, together with that provided by our study, suggests that E2 should be measured when manipulating T level in birds as many physiological and behavioural patterns occurring in male birds are achieved by the conversion of testosterone into oestradiol by the enzyme P450 aromatase (reviewed in Balthazart and Ball 1998). However, the occurrence and rate of aromatase activity can vary greatly between species (Fusani et al. 2001, 2003) or with testosterone concentration (Fusani et al. 2000, 2001) and/or time of year and sexual context (Hutchison et al. 1986; Sharp et al. 1986; Foidart et al. 1998; Soma et al. 1999). This context-dependent variability of the aromatase activity could explain the inconsistency in the effects of T manipulation on oxidative status. In accordance with recent studies showing that aromatase occurs in several peripheral tissues (see "Introduction" section), further investigation is now required to determine the presence of aromatase and E2 receptors in tissue relevant for affecting oxidative damage and antioxidant defences.

The differential effects of E2 and DHT might theoretically be due to a sequence effect of the hormone treatments. However, this sequence effect is unlikely as in birds, the half time of E2 is only 10.9±1.9 min (Tsang 1984) and because it has been shown that by 24 h, 79.4 % of radiolabel oestradiol is usually excreted in birds (Tell 1997), showing that E2 levels will drop to control levels very quickly after removing the implants. Indeed, E2 levels were not different at the time the effect of DHT was established. This is supported by the low E2 levels and lack of a significant difference in E2 levels between controls and experimental groups on day 60. Nevertheless, although far from significant, E2 values of the experimental group registered on day 60 were higher than the ones registered on the same day in controls. For this reason, we cannot completely exclude that low, but not baseline, levels of E2 have interacted with DHT in affecting the physiological status of birds. However, although a potential carry over effect of oestradiol cannot be excluded, we think also that it is not very likely. As can be seen from Fig. 2, all the variables that were affected by E2 at the end of the E2 implantation period

Table 4 Final models of repeated measures ANOVA for assessing the effect of sexual steroid manipulation on carotenoids, cholesterol and body mass

Dependent variables	Factors	*df*	*F*	*p*
Carotenoids	Sex	1,21	3.53	0.07
	Time	2,42	67.48	<0.0001
	Time×sex	2,42	0.58	0.56
Cholesterol	Hormone treatment	1,21	5.72	0.03
	Time	2,42	4.19	0.02
	Time×hormone treatment	2,42	15.34	0.00001
Body Mass	Sex	1,21	84.97	<0.0001
	Time	2,42	7.86	0.001
	Time×sex	2,42	0.81	0.45

Day 30 gives E2 effects, while day 60 DHT effects

(day 30) were back to control levels when the effect of DHT was established (day 60). Moreover, those variables that were affected by DHT were not affected by the previous E2 treatment. Finally, the effects of the two hormones may differ since they were applied in a slightly different part of the season. DHT and T tended to decrease in controls from day 0 to day 30, and then stayed stable until day 60. Therefore, DHT implants were applied when DHT was at a low level compared to the beginning of the experiment. This is important since otherwise the controls would have been no proper controls, and the experimental animals may have ended with supraphysiological DHT levels. Since E2 levels do not fluctuate over the season so much, the timing of the E2 implantation might be less relevant. Therefore, it is unlikely that a DHT treatment, early in the season, if at all possible in an adequate manner, might have had another effect. Potentially an E2 treatment late in the season, when endogenous DHT levels were low, might have yielded a different result from the treatment earlier in the season. However, since late in the season E2 levels are not elevated, this might generate an ecologically irrelevant result.

Effects of sexual steroids on body carotenoids, body condition and cholesterol

Neither DHT nor E2 affected circulating carotenoids, despite the fact that E2 increased ROMs and decreased OXY. We found a strong decrease over the season in circulating carotenoid levels, irrespective of treatment. Our data, therefore, suggest that carotenoids did not contribute to antioxidant defences. Moreover, the decrease in carotenoids over time is not at all paralleled by the pattern over time in cholesterol or body condition, indicating that they might not be involved in the regulation of carotenoids in this species, in contrast to what has been suggested for other species (body mass, Blas et al. 2006; Siitari et al. 2007; lipoproteins, McGraw et al. 2006). As suggested elsewhere (Casagrande et al. 2011b), circulating carotenoid levels may be partly associated with periodic processes under photoperiodic control, such as moult, that could affect carotenoid uptake or metabolism.

Regulation and evolution of carotenoid-dependent signals

While E2 had strong effects on oxidative status, it did not influence colour expression, whereas DHT affected colour expression but not oxidative status. Moreover, the increase in ROMs was not reflected in fading of the colour signal. This indicates that carotenoids were not used for fighting oxidative stress, which is in accordance with what has been found in other studies (reviewed in Costantini and Møller 2008). One could argue that the birds were on an unnatural carotenoid-rich diet, while in the field carotenoids are limiting (Casagrande et al. 2011b). This unnaturally rich diet would enable them to keep the signal up despite deterioration of oxidative status. It is also important to highlight that our conclusions about effects on redox physiology are limited to plasma only. Although compounds like plasma hydroperoxides may reflect levels in other tissues, our results should perhaps not be generalized to all kinds of tissues and molecules involved in oxidative status changes. Therefore, further studies are needed to evaluate the effects of sexual hormones on oxidative stress physiology, including effects on other tissues and biomarkers of oxidative damage and antioxidant status. We found that DHT increased colour but not circulating carotenoids, suggesting that this androgen is active in the integument, but not in other tissues involved in the upregulation of carotenoids in the blood. Since DHT is converted from T by 5α-reductase and information about the expression of this enzyme in avian peripheral tissues is really scarce, further studies should investigate whether the tissue of the signal, in this case the integument of the leg, contains not only androgen receptors but also the reductase for this conversion.

Conclusions

In conclusion, our study showed that androgens affected carotenoid-dependent colour expression in the skin independently of circulating carotenoid levels, and with a slight sex-specific sensitivity, but do not seem to affect oxidative stress through a direct action. In contrast, oestradiol increased oxidative stress, but did not affect colour expression or carotenoid levels. In addition, variation in body condition or cholesterol did not explain colour expression, carotenoid levels or oxidative stress. Since T can be converted to both E2 and DHT, our result opens the possibility that T mediates the trade-off between colour expression (DHT) and oxidative stress (E2). Further studies will be needed to ascertain if the pro-oxidant action of T comes through only indirectly through oestradiol, or if the contribution of the T metabolites E2 and DHT is context- or species-dependent.

Acknowledgments We thank Neil Metcalfe for helpful suggestions based on an earlier version of the manuscript, the associate editor and two anonymous reviewers for providing valuable comments that helped us to improve the presentation of our study. We are grateful to Bonnie de Vries for help with hormone assays, to Alberto Fanfani for supporting the cage building, and to Nadia Macciocchi, Fernando and Serena Costantini for logistic support during the study. S.C. was funded by a MarieCurie Fellowship (FP6-MC-EIF-025369-HormColor).

Ethical standards The study was authorized by the Distrectual Authority of Parma (prot.865/08.03.2006) and by the Superior Institute for Environmental Protection and Research (ISPRAprot.1612/T-A31/01.03.2006).

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