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Variation of a carotenoid-based trait in relation to oxidative stress and endocrine status during the breeding season in the Eurasian kestrel: A multi-factorial study

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ABSTRACT

Carotenoid-based skin colorations vary seasonally in many bird species and are thought to be honest sexually selected signals. In order to provide more insight in the potential signal function and underlying mechanisms of such colorations we here quantified patterns of variation of leg coloration in adult male and female Eurasian kestrels (*Falco tinnunculus tinnunculus*) over the breeding season, and evaluated the relationship between coloration and levels of carotenoids, androgens and estrogens, oxidative damage and plasma non-enzymatic antioxidant capacity. We studied both reproducing wild and non-reproducing captive birds to test for the effect of diet and breeding effort. Males were more colored than females only during mating, and independently of diet, suggesting that leg-color is a sexually selected trait. Seasonal variation in leg color was associated with circulating carotenoids, but concentrations of these molecules were not related to antioxidant capacity, body condition or oxidative damage. These results indicate that carotenoid-based colorations may not be an honest signal of health status in this species. Production of carotenoid rich eggs coincided with low levels of circulating carotenoids in females, indicating that carotenoids might be a limited resource for laying female kestrels. Finally, young rearing males had higher levels of oxidative damage than females, and wild birds of both sexes had higher levels of these parameters than captive birds. These results may indicate that parental effort and physical activity are costly, independently from hormonal status. Since androgens did not explain carotenoid variation we suggest that multiple interacting factors can regulate carotenoid levels along the season.

1. Introduction

Many animal species exhibit conspicuous color patterns that are generated using carotenoids (Blount and McGraw, 2008). Carotenoids are fat-soluble pigments that animals must obtain through their diet, and that can have important immunoenhancing (Chew and Park 2004) and antioxidant properties (Alves-Rodrigues and Shao, 2004). Carotenoid dependent color signals are often more intense in males than in females suggesting that these traits are shaped by sexual selection (Lozano, 1994; Badyaev and Hill, 2000). Such traits are assumed to constitute an honest signal of individual quality because they are costly to produce ("handicap principle"; Zahavi, 1975). Since carotenoids are a limited resource (Blount, 2004), costs of coloration manifest because animals trade-off the allocation of these pigments between signaling and health maintaining processes (von Schantz et al., 1999; Alonso-Alvarez et al., 2004). However, studies on the role of carotenoids as beneficial antioxidants have given mixed evidence and this topic remains highly disputed (reviewed in Costantini and Møller, 2008; Pérez-Rodríguez, 2009). Developing a general explanation for the role of carotenoid dependent signals requires a better understanding of their regulatory mechanisms. However, the processes involved in generating color signals and in controlling carotenoid concentrations are complex. Several factors are likely to be important among which gonadal hormones, body reserves, health status, and oxidative stress. There is experimental evidence that androgens stimulate the expression of avian non-plumage carotenoid-based traits (Eens et al., 2000; Blas et al., 2006; McGraw, 2006; McGraw et al., 2006; Mougeot et al., 2007; Mougeot et al., 2009; Casagrande et al., 2011). However, the natural covariation between carotenoids and androgens in untreated birds is far from clear. For example, the skin color of the American kestrel *Falco sparverius* varies with sex, season and age (Bortolotti et al., 1996; Negro et al., 1998) as do the androgen levels of most bi-parental species (Kimball, 2006), but the coloration was not associated with androgen levels (Bortolotti et al., 1996). Similar results were achieved studying leg and bill color variation in the free ranging white ibis *Eudocimus albus* (Heath and Frederick, 2006), where the expression of non-plumage coloration peaks during the pre-breeding phase and decreases after mating, but in males it is was not associated with androgen levels (Heath and Frederick, 2006).

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This discrepancy could be explained by a multifactorial regulation of signals and carotenoid allocation, in which the multiple factors may affect each other, as well. For example, testosterone (T) can affect the physiological conditions of the signaler by lowering its immune competence (Folstad and Karter, 1992; Roberts et al., 2004) and immune activation can affect testosterone (T) production (Boonekamp et al., 2008). Testosterone can also promote pro-oxidant processes (Buchanan et al., 2001; Reckelhoff,2005; Alonso-Alvarez et al., 2007), and oxidative stress and immune activation may affect the trade-off in carotenoid allocation to the signal. Evidence for pro-oxidant action of T has been demonstrated both in mammals (Chainy et al., 1997; Zhu et al., 1999; Aydilek et al., 2004) and birds (Alonso-Alvarez et al., 2004; 2007). T can increase the production of reactive chemical species indirectly, by increasing the metabolic rate (Lynn et al., 2000; Buchanan et al., 2001) or directly, by activating NADPH oxidase (Reckelhoff, 2005). Carotenoids in turn can buffer the pro-oxidant effect of T (Alonzo-Alvarez et al., 2008).

By investigating changes in relevant parameters such as hormonal and oxidative status we may get better insight in regulatory processes and function of carotenoid dependent signals. The complex relationships between androgen secretion and oxidative status physiology have been investigated mainly in short-lived avian species (e.g., zebra finch in Alonso-Alvarez et al., 2007; red-legged partridge in Alonzo-Alvarez et al., 2008) maintained in captivity under controlled conditions and so it is unclear if such findings also apply to species with different life-history strategies and that live under natural conditions.

The relationship between reproductive hormones and oxidative status is further complicated by changes in workload sustained by parents during the reproductive cycle. A recent meta-analysis of avian research indicated that reproductive effort promotes blood parasitaemia and lowers immune responsiveness (Knowles et al., 2009). Moreover, another study demonstrated that an increased workload, such as brood rearing, affects antioxidant enzymes in pectoral muscles (Wiersma et al., 2004). It has also been shown that an increase in metabolic rate, such as that occurring under periods of increased physical activity, is may be accompanied by an increase in oxidative stress (Costantini et al., 2008a; Powers and Jackson, 2008). However, how these previously identified processes relate to carotenoid allocation to sexual signals, and to oxidative damage caused by reproductive effort, is as yet not known, although these physiological changes may underlie the trade-off between current and future reproductive success that has been demonstrated in many species (e.g., Dijkstra et al., 1990; Deerenberg et al., 1995; Daan et al., 1996; Visser and Lessells, 2001).

This paper explores the inter-relationships between gonadal hormones, oxidative damage, plasma non-enzymatic antioxidant capacity, body condition and reproductive effort and their relation with circulating carotenoids and a carotenoid dependent signal. To this end we made use of the strong changes in coloration in the course of the reproductive season in a wild bird species, the Eurasian kestrel *Falco tinnunculus tinnunculus* and we measured concurrent changes in the parameters mentioned above. Both sexes display a carotenoid yellow coloration of bare skin on the bill and legs, with males being more strongly colored than females (Casagrande et al., 2006). Parental care is unequally divided between the breeding pair allowing us to correlate differences in parental effort with measured indicators of oxidative stress and performance.

In wild birds seasonal changes in diet might be partly responsible for seasonal changes in coloration and carotenoid levels. Carotenoids must be taken up from the diet, while diet and body condition can also affect the amount of circulating lipoproteins (the carotenoid carrier) which can greatly affect the amount of carotenoid both in the blood and associated with skin pigmentation (McGraw and Parker, 2006). Moreover, body condition, which is a proxy for nutritional status, can explain a large part of carotenoid variation in certain avian species (Blas et al., 2006). To account for this potentially confounding factor in our analyses we also studied seasonal changes in the same parameters in a captive colony of the same species, provided with a constant diet. Since these birds did not reproduce, we took advantage of a more experimental approach to test the effect of reproductive effort and gonadal hormones on coloration by comparing the physiology of captive versus wild birds. We tested the following predictions on both wild and captive kestrels in controlled conditions: (a) If leg color is a sexually selected trait we predict that males are more colored (redder) during mating than females and that this difference disappears after egg laying (thus after the phase of the mate choice); (b) If color expression honestly signals health status we predict that color (and carotenoids) vary positively with lipoproteins (the carotenoid carriers and index of nutritional status, Navarro and González-Solís, 2007), antioxidant barrier, and body condition and negatively with oxidative damage; (c) If both color and circulating carotenoids are up-regulated by androgens we predict that these variables vary positively with androgen levels; (d) If diet is partly responsible for seasonal changes in leg coloration we predict that in both sexes leg color fluctuates less over the season in captive birds kept under a constant diet than in wild birds; (e) If carotenoids are a limited resource for the wild female kestrels, we predict that laying females will show lower levels of coloration and circulating carotenoids during the phase of egg production than their captive non-reproducing counterparts; (f) If parental effort is costly in terms of oxidative stress and color expression, we predict that during the chick-rearing period, males have lower levels of color expression and carotenoids, and higher levels of oxidative damage than females, and wild birds of both sexes have lower levels of these parameters than captive non-reproducing birds.

2. Materials and methods

2.1. Ethics statement

The study was authorized by the Distrectual Authority (Provincia di Parma prot. n. 865/08.03.2006) and by the Superior Institute for Environmental Protection and Research (ISPRA, prot. N. 1612/T-A31; 01.03.2006).

2.2. Model species

The Eurasian kestrel (*Falco tinnunculus tinnunculus*) is a socially monogamous bi-parental species (Village, 1990). Incubation of eggs and brooding of newly hatched chicks are predominantly accomplished by females, while males hunt to provide both the female and chicks with prey (Village, 1990). The species is dimorphic for size (males are smaller than females), plumage color (males have a gray head and tail and a reddish back, while females are brown all over), and skin color during courtship (males have a redder cere, eye ring and tarsi than females, Casagrande et al., 2006). Such skin coloration is produced by deposition of lutein and zeaxanthin in the integument without any metabolic transformation (Casagrande et al., 2006).

2.3. Wild bird study

The diet of the studied population is based mainly on voles (Casagrande et al., 2008), poor in carotenoids (Casagrande et al., 2006), but also of invertebrates, which are rich in carotenoids (1062 mg/kg; Olson, 2006) and represent the most important carotenoid source for kestrels in our study area (Casagrande et al., 2006; 2007).

During two consecutive years (2006–2007), we studied a wild population of kestrels breeding in nest boxes attached to the pylons of utility lines in Parma, Italy. The area is a plain characterized by grasslands, large agriculture fields with irrigation streams, artificial and natural river banks and scattered fallow fields and farmhouses.

Kestrel reproductive activity was followed using binoculars 8×20 and telescope 20×20–60. Reproductively active birds were trapped once during the pre-laying phase (from mid March to mid April; 6–20 days before egg laying) or during young rearing (from mid May to mid June; 6–15 days after hatching) using traps placed on the ground (bal-chatri) or in the nest (sliding door controlled remotely). We trapped each bird only once because it was impossible to re-trap the same individual, even when using different trapping systems. Moreover, we could trap only one individual of each pair, such that all samples are independent. We succeeded in trapping 9 females (5 in 2006 and 4 in 2007) and 10 males (5 in 2006 and 5 in 2007) during mating and 9 females (4 in 2006 and 5 in 2007) and 9 males (5 in 2006 and 4 in 2007) during rearing.

Within 10 min after trapping a sample of blood (800 μL) was taken from the brachial vein with a heparinized syringe and samples were kept in a cool thermos (2–4 °C for a maximum of 10 h) until centrifugation (at 1400 *g* for 5 min). Plasma was stored at −20 °C until analyses. The length of the right tarsus was measured with a caliper to the nearest 0.1 mm and the body mass was measured with a Pesola balance to the nearest 1 g.

2.4. Captive bird study

The study of captive adult kestrels was carried out from the 1st of April to 1st of June 2007. Eleven male and ten female birds were randomly paired and housed at the beginning of March (one male alone) 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) starting one month before the beginning of the study. 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 conditions). The birds were maintained on a constant diet providing daily two one-day-old chicks during the study. At the beginning of April and June birds were bled and measured using the same protocol as for wild birds yielding repeated measurements on the same birds.

2.5. Color measurements

The yellow coloration of the right tarsus skin was measured with a portable digital color meter X-Rite Color Digital Swatchbook (X-Rite®, Grandville, MI, USA), registering the whole human visible spectrum (400–700 nm) under CIE Illuminant D65 (for further details see Casagrande et al., 2006; 2009). Standard colorimetric variables (L—lightness: color variation from black (0) to white (300); a*—red component: color variation from green negative values to red positive values; b*—yellow component: color variation from blue negative values to yellow positive value) were obtained with the software ColorShop 2.5 (X-Rite®) in the uniform color space CIELAB (CIE, 1978) from measured reflectance spectra. A mean value obtained from three sequential readings per individual was used for statistical analysis because measures were highly repeatable (intraclass correlation coefficients: all $r>0.92$, all $p<0.01$; Lessels and Boag, 1987). Although kestrels can perceive the UV component of coloration, our approach provides a valid measure of the variation of carotenoid content in the skin since the carotenoid-based traits reflect primarily in the human-visible light range and have a subtractive action of short waves light, like UV and blue (Andersson and Prager, 2006). In addition, some studies have shown that CIELAB variables are reliable proxies of the amount of deposited carotenoids in tissues (Rønsholdt, 2005; Velando et al., 2006; Melendez-Martinez et al., 2007). So, we refer to La*b* system for variation of carotenoids deposited in the skin. In addition, skin color of Eurasian kestrel has been already described using visible range spectrum (Casagrande et al., 2006; 2007; 2008; 2009).

2.6. Plasma analyses

2.6.1. Hormone determination

For each bird we assessed the level of T, E2 and DHT by radio-immuno-assay (RIA). Samples were extracted twice adding to the measured volume of plasma (200 μL) 4 mL of petroleum ether/diethylether (30–70%) allowing steroids to pass from the watery phase to the organic one. The extracts were 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 dried and dissolved in 185 μL of PBSG-buffer. T was assayed from 50 μL of plasma, using DSL-4000 Active Testosterone Coated-Tube Radio immunoassay Kit (Diagnostic System Laboratories, Inc., Webster) and the concentration was expressed in ng/mL. DHT was assayed from 25 μL of plasma using DSL-96100 Dihydrotestosterone Radio immunoassay Kit (DSL-Diagnostic) following the protocol provided by DSL. Estradiol was assayed in 50 μL of plasma using DSL-4400 Estradiol Radioimmunoassay Kit (DSL-Diagnostic) following the DSL protocol. The concentrations of DHT and E2 were expressed in pg/mL. Recovery rates were 84.66% for E2 and 85.23% for DHT and 85.7% for T. Intra-assay CV for these hormones were 5.1%, 2.25% and 2.20% respectively, while inter-assay CV were 6.9%, 2.21% and 4.61%. Cross reactivity with steroids others than the target of the kit was very low (Testosterone kit: 5.8% with 5α-dihydrotestosterone, 2.3% with androstenedione, 0% with estrogens; 5α-dihydrotestosterone kit: androstandiol: 3.3%, testosterone: 0.6%, 0% with estrogens; 17β-estradiol kit: 3.40% with estrone and 0% with androgens).

2.6.2. Carotenoid assay

It is known that Eurasian kestrels can absorb dietary lutein and zeaxanthin, 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, UK) spectrophotometer at 446 nm. The carotenoid concentration was estimated as $\mu g\ mL^{-1}$ of plasma using the standard absorbance curve of lutein (Sigma-Aldrich).

2.6.3. Cholesterol analysis

To evaluate the concentration of carotenoid carriers (lipoproteins) in the peripheral blood, we measured the total amount of cholesterol, which has been used as proxy of lipoproteins in birds (McGraw and Parker, 2006). We diluted 10 μL of plasma with 1 mL of the reagent kit Nobiflow Cholesterin (Hitado Diagnostic System, Möhnesee-Delecke, Germany) reading the sample with the spectrophotometer Pharmacia Biotech Ultrospec (Pharmacia) at 500 nm. The concentration of cholesterol was calculated in $mmol\ L^{-1}$ referring to Nobical Cholesterin (Hitado Diagnostic System, Möhnesee-Delecke, Germany) as standard. The kit is sensitive to both LDL-cholesterol (low density lipoproteins) and HDL-cholesterol (high density lipoproteins).

2.6.4. Plasma oxidative status

Plasma hydroperoxides (ROMs; marker of oxidative damage) were measured by the d-ROMs test (Diacron International, Grosseto, Italy) based on previous studies (e.g., Costantini et al., 2006; 2007b; Costantini and Dell'Omo, 2006). Briefly, 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-Reaserach.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 mM of H_2O_2 equivalents. The plasma antioxidant

capacity, including the contribution of both exogenous and endogenous non-enzymatic antioxidants, was measured by the OXY-Adsorbent test (Diacron International, Grosseto, Italy) based on previous studies (e.g., Costantini et al., 2006; 2007b; Costantini and Dell'Omo, 2006). 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 oxidized by the residual HOCl and transformed into a pink derivative. The intensity of the colored complex is inversely related to OXY. The intensity of the colored complex was measured with a spectrophotometer at 505 nm. Measurements are expressed as mM of HOCl neutralized. We then calculated the ratio between ROMs and OXY (×1000) and used it as an 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 oxidized molecules than antioxidant compounds. We also calculated another OS index from the difference between standardized values of ROMs and OXY (Vassalle, 2008). Since the values calculated for the two different OS indices were highly correlated ($r = 0.93$), we used only the first index of OS, which has been previously applied to the study of OS in birds (Costantini et al., 2006).

2.7. Data analyses

All analyses were performed with STATISTICA 7.0 (StatSoft 2004, Tulsa, OK, USA) using parametric statistics. Normality was tested with Shapiro–Wilk and E2 concentrations were log transformed as they did not reach the normal distribution. A body condition index (BC) was calculated for wild and captive birds separately as the residuals of a linear regression of body mass on tarsus length (wild birds: $F_{(1,35)} = 29.57$, $p < 0.0001$, $R^2 = 0.46$; captive birds $F_{(1,40)} = 12.69$, $p = 0.001$, $R^2 = 0.24$).

We first analyzed patterns of seasonal changes for each variable separately. To this end a one-way ANOVA with year as predictor was performed to check for any between-year differences for all the variables measured in wild kestrels. Given that years did not differ (ANOVA year effect, BC: $F_{(1,35)} = 0.68$, $p = 0.41$; T: $F_{(1,35)} = 0.39$, $p = 0.54$; DHT: $F_{(1,35)} = 0.04$, $p = 0.84$; E2: $F_{(1,35)} = 0.53$, $p = 0.47$; cholesterol: $F_{(1,35)} = 0.03$, $p = 0.87$; ROMs: $F_{(1,35)} = 1.59$, $p = 0.22$; OXY: $F_{(1,35)} = 0.37$, $p = 0.53$; a*: $F_{(1,35)} = 1.99$, $p = 0.19$; b*: $F_{(1,35)} = 1.15$, $p = 0.29$; $L^* = F_{(1,35)} = 2.50$, $p = 0.11$; blood carotenoids $F_{(1,35)} = 1.08$, $p = 0.30$), we pooled the data for both years. The variation of the variables describing skin color (L*, a*, b*), plasma carotenoids, steroids, cholesterol, body condition and biomarkers of plasma oxidative status in wild kestrels was assessed using ANOVA GLM with sex, breeding phase and their interaction as fixed factors. The variation of the same dependent variables was assessed in captive birds with a GLM repeated measure ANOVA, including the same predictors. Post-hoc tests for evaluating differences between sexes for each time in the season separately, or for differences between phases for each sex, were conducted with Fisher LSD tests. Differences between wild and captive birds were tested per sex and season with independent t-tests.

Next we analyzed the factors accounting for variation in sexual signal coloration (color) and circulating levels of carotenoids using general linear models. Because of the different data structure in both studies we did so for the wild and captive birds separately. We also did this for both sexes separately, since adding sex and the interaction between sex and all other predictors would have resulted in over-parameterization of the model (Hawkins, 2004). Since this was an explorative study, interactions and factors were sequentially removed from the models when non significant ($p > 0.05$) following a standard backward procedure, analyzing color and circulating carotenoids as dependent variables in two separate models. In all models for wild birds, we included BC, DHT, T, E2, ROMs and OXY as covariates. For analyzing color variation, we added carotenoid concentration as a covariate. We used the same covariates for the data of captive birds but, since captive birds were sampled twice, we included individual identity as random factor in a mixed model. Since season may affect the dependent variables independently of the above listed covariates, for example by affecting carotenoid metabolism, we included season (pre-breeding mating phase and chick rearing phase) as a fixed factor in all models. Collinearity was checked performing a linear regression between the dependent variables and all covariates within each groups and a threshold of 5.0 for the variance inflation factor (VIF) was applied.

Finally we analyzed variation in oxidative stress (OS), calculated as the ratio of OXY divided by ROMs (×1000), in similar models. We used as covariates circulating carotenoids, body condition and the hormones DHT and E2. In these analyses T was removed as a predictor because of its collinearity with DHT (VIF > 14) and we therefore considered just T metabolites (DHT and E2).

3. Results

We will first describe the seasonal patterns of leg color, body condition and the plasma concentrations of cholesterol, hormones, and oxidative stress parameters, separately for each sex and for wild versus captive birds. The outcomes of the statistical tests are presented in Tables 1–2 and the data in Figs. 1–3. For each variable we will first focus on seasonal changes and subsequently describe difference between captive and wild birds testing potential effects of diet and reproductive effort and physical activity. Statistics for the latter are presented in Figs. 1–3. Finally we will describe the results of the GLM models explaining variation in color, carotenoids and oxidative stress (Table 3) and the correlation matrix of all variables (Appendix 1).

Table 1
Sources of variation of the dependent variables considered in the study of wild kestrels.

Dependent variable	Source of variation	$F_{(1,32)}$	p-values
Color (L)	Sex	0.22	0.64
	Season	1.04	0.32
	Sex × season	2.08	0.17
Color (a*)	Sex	2.07	0.16
	Season	0.94	0.34
	Sex × season	8.99	0.005
Color (b*)	Sex	0.14	0.71
	Season	0.001	0.98
	Sex × season	0.005	0.82
Carotenoids	Sex	0.60	0.44
	Season	3.89	0.06
	Sex × season	12.12	0.001
BC	Sex	20.68	<0.001
	Season	11.19	0.002
	Sex × season	8.44	0.007
Cholesterol	Sex	0.10	0.76
	Season	0.46	0.50
	Sex × season	0.19	0.67
DHT	Sex	0.11	0.75
	Season	26.56	<0.0001
	Sex × season	0.77	0.39
T	Sex	0.32	0.57
	Season	32.06	<0.0001
	Sex × season	0.92	0.34
E2	Sex	4.59	0.04
	Season	1.10	0.30
	Sex × season	1.13	0.29
ROMs	Sex	4.98	0.03
	Season	9.53	0.004
	Sex × season	3.67	0.06
OXY	Sex	10.37	0.003
	Season	1.37	0.25
	Sex × season	6.44	0.02
OxS	Sex	14.06	0.0007
	Season	3.27	0.08
	Sex × season	7.04	0.01

Table 2
Sources of variation of the dependent variables considered in the study of captive kestrels.

Dependent variable	Source of variation	$F_{(1,19)}$	p-values
Color (L)	Sex	1.10	0.31
	Season	0.01	0.91
	Season × sex	0.60	0.45
Color (a*)	Sex	5.52	0.03
	Season	56.87	<0.001
	Season × sex	0.27	0.61
Color (b*)	Sex	1.66	0.21
	Season	2.65	0.12
	Season × sex	0.45	0.51
Carotenoids	Sex	0.10	0.76
	Season	359.77	<0.001
	Season × sex	4.53	0.046
BC	Sex	33.57	<0.0001
	Season	128.65	<0.001
	Season × sex	0.06	0.81
Cholesterol	Sex	0.23	0.64
	Season	1.23	0.28
	Season × sex	0.29	0.59
DHT	Sex	8.55	0.009
	Season	13.03	0.002
	Season × sex	8.55	0.009
T	Sex	7.67	0.01
	Season	2.82	0.11
	Season × sex	0.004	0.95
E2	Sex	NA	NA
	Season	NA	NA
	Season × sex	NA	NA
ROMs	Sex	0.66	0.43
	Season	0.004	0.93
	Season × sex	0.07	0.80
OXY	Sex	0.06	0.80
	Season	1.57	0.22
	Season × sex	1.03	0.31
OxS	Sex	0.30	0.59
	Season	0.04	0.85
	Season × sex	0.01	0.92

3.1. Carotenoid-based coloration

Of the three colorimetric variables, only the red component of hue (a*) showed an effect of season, either alone (captive birds) or in interaction with sex. In wild birds, as expected, a* decreased over time in males, while in females it did not change over time and was present in lower levels than males during the pre-breeding season (Fig. 1A). In captive birds both males and females showed a decrease in coloration with season but captive pre-breeding females also had less pronounced color levels than males (Fig. 1B). Wild and captive males did not differ in color at any stage throughout the study but wild females were less strongly colored than captive females but during pre-breeding.

3.2. Circulating carotenoids

Plasma carotenoid concentrations showed a similar trend as that for color a*: in wild birds circulating levels decreased in males throughout the season but not in females (significant effect of season times sex). Overall, males had higher levels of these pigments than females during pre-breeding, and lower levels during chick rearing (Fig. 1C). In captive birds of both sexes, despite their constant diet, carotenoid levels decreased over time and this effect was somewhat stronger for males than for females (interaction between season and sex, Fig. 1D) although the post hoc tests for sex were not significant. Overall, prior to breeding captive birds of both sexes showed higher pigment levels than wild birds except during the post-breeding phase when captive females showed lower levels than wild females.

3.3. Body condition index

Similar to the results for coloration and carotenoids levels, BC decreased during the reproductive season in wild males that were responsible for supplying food to chicks, but not in wild females (significant effect of phase × sex, Fig. 1E). Males had the same BC of females during pre-breeding but were significantly in worse condition during rearing. In captivity, without the need for parental care, both males and females showed a decrease in BC (significant main effect of time, Fig. 1F) following similar trends to those observed for coloration and carotenoids. These birds did not differ in BC in April, like the wild birds, but also not in June, in contrast to wild birds where the male had to take care for food provisioning. There were no statistical significant differences between wild and captive birds when analyzed per sex and season.

3.4. Plasma cholesterol

Plasma cholesterol concentrations did not vary according to season or sex in either wild or captive birds (Fig. 1G,H). However, in contrast to body condition, captive birds tended to have higher levels than wild birds.

3.5. Plasma steroids

Although, as expected, androgens mostly decreased over the season, they showed a slightly different pattern than observed for color and carotenoids. In wild birds, DHT and T concentrations decreased across the breeding season in both sexes (significant effect of season only, Fig. 2A,C). During mating, males had higher levels of these androgens than females, while they did not differ between sexes during rearing. Similarly, DHT decreased in captive males, showing higher levels than females only during pre-breeding, while females maintained low levels of DHT both in April and June (significant interaction time × sex, Fig. 2B). T did not change with season in captive birds that did not have to rear young, and again males had higher levels than females (Fig. 2D).

In wild birds, as expected, females had pre-breeding, but not during chick rearing, higher levels of E2 than males, although the change with season was not significant for both sexes (only significant effect of sex, Fig. 2E). E2 levels were always undetectable in non-reproducing captive birds. (Fig. 2F). During pre-breeding, wild (and reproducing) females tended to have higher levels of androgens and estradiol than captive (and non-reproducing) females. Wild males had lower levels of T during chick rearing than captive males that did not need to care for chicks.

3.6. Plasma oxidative status

While color, carotenoids and androgen levels decreased over season, especially in males (see above), the level of ROMs and OS increased in wild males but not in wild females (interaction between season and sex, Fig. 3A,E). As a consequence, there was no sex difference in these pre-breeding parameters, but during the period of hard work for the male in the chick rearing phase, wild males had significantly higher levels than wild females. In contrast, oxy levels increased for females but not for males (significant interaction between season and sex), yielding significant higher levels for females during chick rearing (Fig. 3C). Surprisingly, in captive birds, despite their changes in coloration and carotenoid levels, ROMs, OXY and OS did not differ between sexes or breeding phases (Table 2; Fig. 3B,D,F) and were generally lower than those of wild birds. OS values were comparable for all the groups, except for wild males during rearing, which had higher levels of OS than non-breeding captive males.

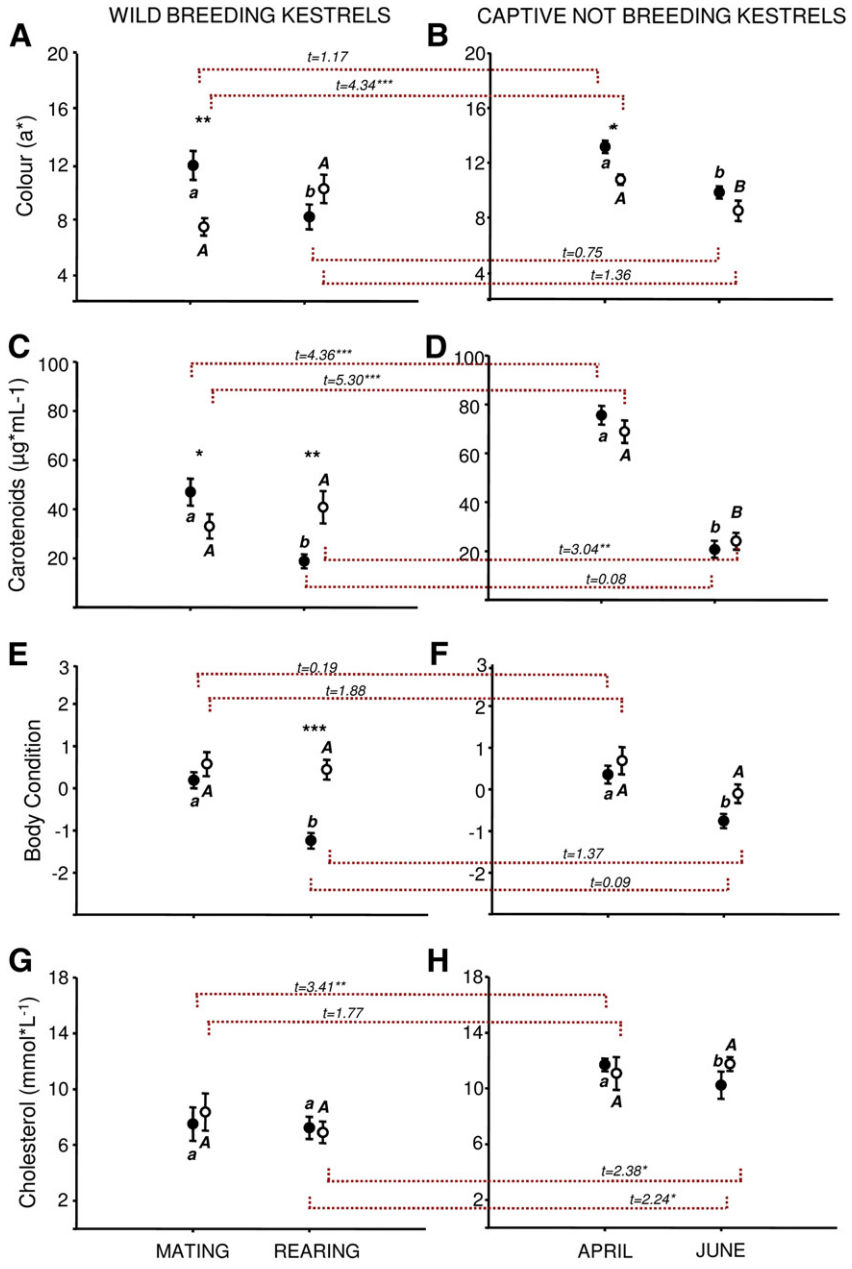

Fig. 1. Seasonal variation of the red component of color (A, B), carotenoids circulating in the blood (C, D), body condition index (E, F), cholesterol (G, H) for wild (right panels) and captive (left panels) kestrels of both sexes. Full circles are males, open circles are females (means and st. err). Asterisks refer to sex differences within a season. Fisher LSD post-hoc results: $p<0.05$ (*); $p<0.1$ (**); $p<0.01$ (***). Letters (small for males and capital for females) indicate differences between seasons for each sex separately. T-test between wild and captive birds within sexes are also reported indicating significance as describe above (d.f. = 1,19 in all *t*-test except for females' comparison during pre-breeding: d.f = 1.17).

3.7. Models explaining color and circulating carotenoids

As expected for a carotenoid dependent signal, the final explanatory models for color variation yielded a strong positive effect of carotenoid plasma concentrations for all 4 categories of birds (Table 3A1,2; for direction and size of effects see Appendix 1 reporting correlations between variables), and positive effects of season, oxy, and body condition for wild females (Table 3A1; see Appendix 1 for correlations between variables). Variation in carotenoid levels in turn was also explained by season in all birds except wild females for which none of the predictors significantly explained carotenoid variation (Table 3B1,2). In addition, testosterone, in wild males, and body condition, in captive males, were significant predictors of carotenoid variation (Table 3B1,2; Appendix 1).

In order to analyze more specifically to what extent variation in color might signal health status, we implemented a model analyzing color variation including, as predictors, only antioxidant barrier, oxidative damage and body condition, and including individual (random) for the captive birds. In wild birds none of the predictors was useful in explaining color variation (Full GLM: Females, BC, $F_{(1,14)} = 0.78$, $p = 0.39$; ROMs, $F_{(1,14)} = 2.62$, $p = 0.13$; OXY, $F_{(1,14)} = 2.25$, $p = 0.16$; Males, BC, $F_{(1,15)} = 0.51$, $p = 0.49$; ROMs, $F_{(1,15)} = 0.68$, $p = 0.42$; OXY, $F_{(1,15)} = 0.03$, $p = 0.87$). In contrast, coloration of captive birds was explained by BC in both males (final GLM: $F_{(1,10)} = 19.66$, $P = 0.001$, individual, $F_{(11,10)} = 2.61$, $p = 0.07$) and females (final GLM: $F_{(1,9)} = 10.34$, $P = 0.01$, individual, $F_{(11,9)} = 6.81$, $p = 0.005$).

Finally we investigated which factors accounted for OS (Oxy/ROMs 1000) in each subgroup, but none of the predictors (carotenoids,

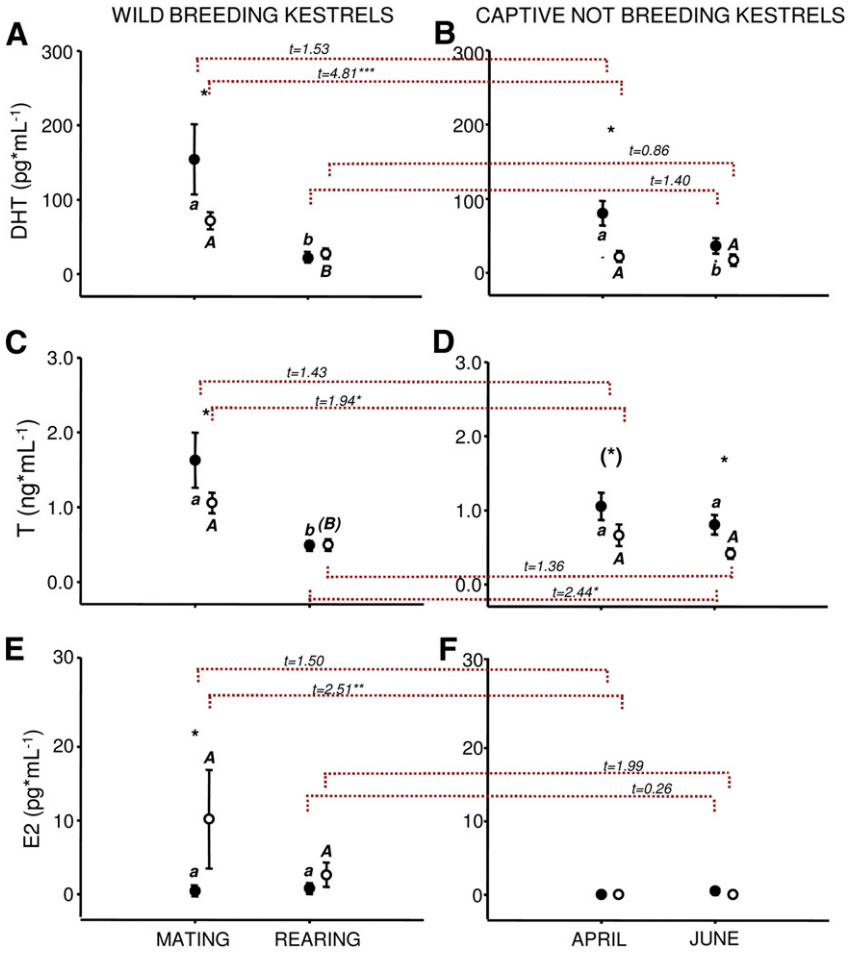

Fig. 2. Variation of DHT (A, B), T (C, D), E2 (E, F). We used log transformed value of E2 to perform GLM, while we have reported original data in the graphs. Legend as in Fig. 1.

body condition or the hormones DHT and E2) contributed significantly to the model (all p-values >0.15).

4. Discussion

We analyzed seasonal changes in a carotenoid dependent signal coupled with seasonal changes in the potential physiological factors that drive changes in carotenoid levels with the aim of elucidating the mechanisms that underlie variation in sexual signals. We included both wild reproducing birds and captive non-reproducing birds in this investigation, in order to detect a potential influence of diet and reproductive effort. Based on data and hypotheses presented in the literature, we postulated six expectations that are discussed in turn below.

(a) Males are more colored during the mating phase than females and this difference disappears after egg laying, suggestive for leg color being a sexually selected trait. Indeed, both in wild and captive birds male skin coloration was more intense than in females during mating, but not during young rearing.

(b) Color expression and carotenoids vary positively with lipoproteins, antioxidant barrier, and body condition and negatively to oxidative damage, suggestive for honestly signaling health status.

Contrary to this expectation, we have found no evidence for either of these predictions. As expected, plasma concentration of carotenoids showed the same seasonal changes as coloration patterns did for all four categories of birds (wild males and females, captive males and females), supporting the idea that color expression is dependent on circulating carotenoids. Moreover, carotenoid variation was the only significant predictor for color variation based on the GLM analyses. However, circulating levels of cholesterol did not correlate with carotenoids and color expression, undermining the hypothesis that circulating lipids may be a limiting factor in carotenoid dependent signal expression (McGraw et al., 2006) in this species.

Moreover, parameters related to oxidative stress did not correlate with the signal expression. This is because oxidative stress markers were not associated with circulating carotenoids and skin coloration, except for an association between skin color and OXY in females. Recent studies showed that carotenoids have a minor role in mediating oxidative stress in birds (reviewed in Hartley and Kennedy, 2004; Costantini and Møller, 2008). For kestrels more specifically it has also been shown that carotenoid availability does not limit the capabilities of wild nestlings (Costantini et al., 2007a) or captive adults under both non-stressful (Costantini et al., 2007b) or stressful conditions (Costantini et al., 2008b) to cope with oxidative stress. Given the results of the present study, such a scenario may also hold for wild male breeding kestrels. While carotenoid availability for color production may be limited, this "limited resource hypothesis" does not hold for other functions in which carotenoids are involved (Hudon, 1994; Thompson et al., 1997; Isaksson et al., 2007; Costantini and Møller, 2008, but see Pérez-Rodríguez, 2009). Therefore, future studies should identify which functions are actually constrained by carotenoid availability and therefore compete with color production (see also below: prediction "e").

We have found that the coloration of wild female kestrels was related to OXY level. Indeed, Fig. 1 shows that skin color and OXY show the same increasing trend throughout time between mating and

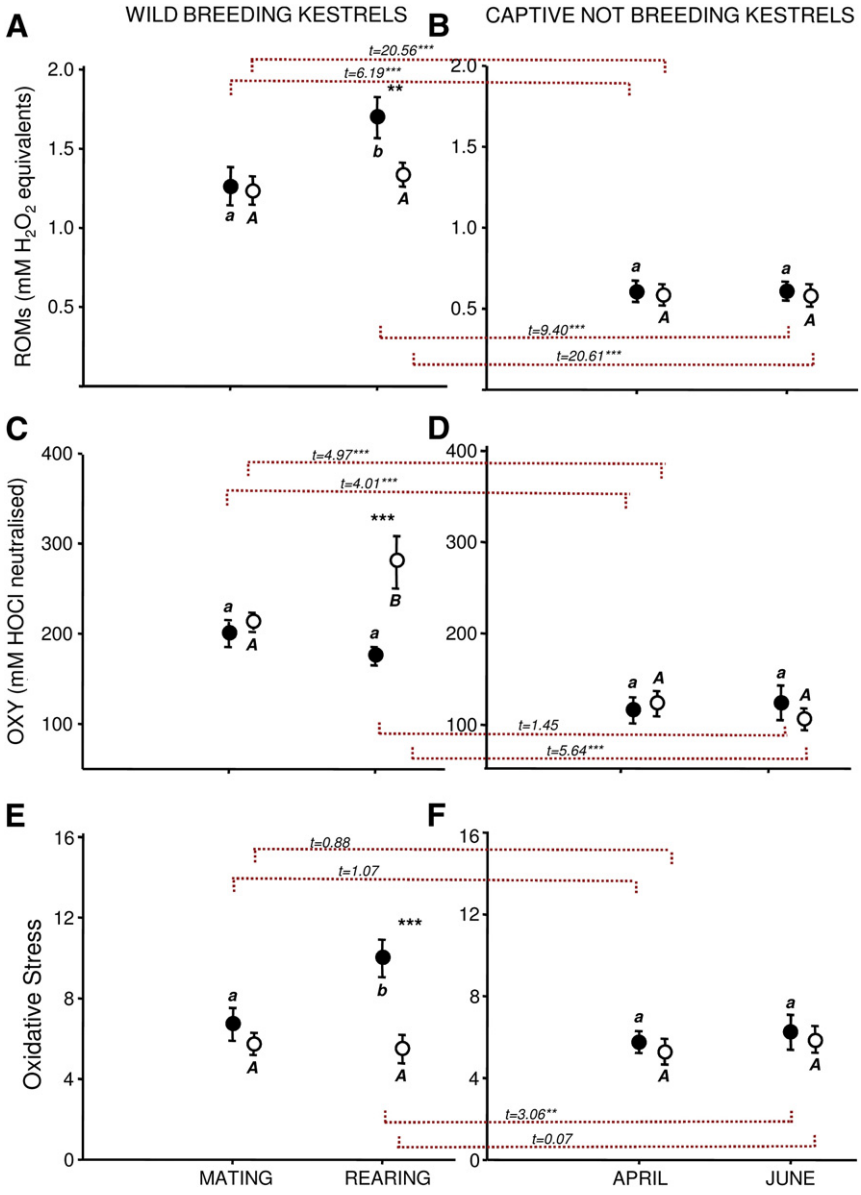

Fig. 3. Variation of ROMs (A, B), OXY (C, D), OS (E, F) in wild breeding (left) and captive not breeding (right) kestrels. Legend as in Fig. 1.

young rearing. Thus, it seems that during the chick-rearing phase female's carotenoid-based coloration may signal the antioxidant capacity of the females. However, it is difficult to explain this result in the context of the sexual selection theory since it is likely that the signaling function is less important during young rearing.

(c) Color and/or carotenoids vary positively with androgens. This prediction was based on experimental evidence from the literature that indicates that androgens up-regulate carotenoid dependent signaling (Kimball, 2006; Casagrande et al., 2011) or circulating carotenoids (Blas et al., 2006; McGraw et al., 2006; Mougeot et al., 2007; 2009). However, correlative evidence is ambiguous (Bortolotti et al., 1996; Heath and Frederick, 2006). Indeed, this is also the case in our correlative study since androgens only explained variation in carotenoids in wild males, but not of skin color. In wild males, both androgens decreased over the season similar to the change in color expression and carotenoid levels, but this similar pattern between androgens and color/carotenoids was only true for DHT in captive males. Moreover, in wild and captive females the seasonal pattern of androgens was not reflected in color or carotenoids. Since experimental evidence has demonstrated an effect of androgens on color expression or carotenoid levels in other bird species, our results indicate that the regulation of the latter two may be multi-factorial.

(d) If in both sexes leg color fluctuates less over the season in captive birds than in wild birds, dietary changes may partly be responsible for seasonal changes in circulating carotenoids and leg coloration. For the first time we can exclude that the decrease of trait redness and circulating carotenoids registered over the season in free ranging birds was due to a change in diet, as birds in captivity on the same standard diet showed a strong seasonal decline in both coloration and plasma carotenoid concentrations. This is consistent with the finding that circulating carotenoid levels declined along the season despite a constant diet in captive American kestrels (Negro et al., 1998) and captive red-legged partridges (Negro et al., 2001; Pérez-Rodríguez, 2008).

(e) Wild females show lower levels of coloration and circulating carotenoid levels during the production of carotenoid rich eggs than males and captive non-reproducing females. Indeed, during the egg laying phase pre-breeding, reproducing wild females had the lowest

Table 3
Full and final GLM for (A) color and (B) blood carotenoids concentration.

		Males				Females			
		Full		Final		Full		Final	
	d.f.	F-values	p-values	F-values	p-values	F-values	p-values	F-values	p-values
A. Color (a)*									
1. Wild									
Season	1	0.60	0.46	Out		8.21	0.02	9.76	0.008
Carotenoids	1	3.01	0.11	16.05	0.001	7.79	0.02	16.22	0.001
DHT	1	0.42	0.53	Out		0.83	0.39	Out	
T	1	0.40	0.54	Out		1.17	0.31	Out	
E2	1	1.64	0.23	Out		2.84	0.13	Out	
BC	1	0.59	0.46	Out		2.66	0.14	9.92	0.008
Roms	1	0.18	0.68	Out		0.10	0.76	Out	
Oxy	1	0.06	0.81	Out		13.42	0.005	12.82	0.003
Error		10		17		9		13	
2. Captive									
Season	1	1.04	0.37	Out		2.53	0.21	Out	
Carotenoids	1	2.55	0.19	46.90	<0.0001	1.71	2.89	24.31	0.001
Individual	11	2.20	0.23	6.06	0.004	5.27	0.10	3.77	0.03
DHT	1	0.80	0.42	Out		16.53	0.03	Out	
T	1	1.37	0.31	Out		4.09	0.14	Out	
BC	1	1.20	0.34	Out		11.40	0.04	Out	
Roms	1	0.01	0.99	Out		6.70	0.08	Out	
Oxy	1	0.05	0.83	Out		0.84	0.43	Out	
Error		4		10		3		9	
B. Carotenoid									
1. Wild									
Season	1	8.61	0.01	25.87	0.0001	0.09	0.77	Out	
DHT	1	0.05	0.83	Out		1.82	0.21	Out	
T	1	0.58	0.46	4.89	0.04	0.88	0.37	Out	
E2	1	1.17	0.70	Out		0.70	0.42	Out	
BC	1	0.03	0.86	Out		2.50	0.15	Out	
Roms	1	0.01	0.93	Out		0.84	0.38	Out	
Oxy	1	0.95	0.35	Out		0.02	0.89	Out	
Error		11		17		10		Out	
2. Captive									
Season	1	50.25	0.001	93.92	<0.0001	9.16	0.04	124.12	<0.0001
Individual	11	3.68	0.81	4.34	0.02	0.79	0.66	2.24	0.12
DHT	1	0.007	0.94	Out		0.02	0.89		
T	1	0.26	0.63	Out		0.80	0.42		
BC	1	4.58	0.09	10.79	0.009	0.17	0.70		
Roms	1	0.13	0.73	Out		0.60	0.48		
Oxy	1	0.99	0.36	Out		0.79	0.42		
Error		5		9		4		9	

levels of color expression of all categories of birds, and significantly lower plasma carotenoid concentrations than captive non-reproducing females. This indicates that carotenoids may be a limited resource for laying female kestrels and that carotenoid deposition in the egg, which is important for the embryo development (Surai et al., 1999), may be physiologically costly for the mother. The ovary is one of the most important organs of deposition of carotenoids (Nys, 2000), as females deposit carotenoids in the eggs (Blount et al., 2000) possibly because they can contribute to mitigate oxidative damage generated during embryo development and hatching process (Surai et al., 1999). For example, hen jungle fowls *Gallus gallus* in pre-laying period allocate as much as 50% of total body zeaxanthin and as much as 80% of total body canthaxanthin to the ovaries (Nys, 2000). This carotenoid allocation to eggs may account for the sex difference in carotenoid levels during pre-breeding as such a sex difference was not the case in non-reproducing captive birds in that part of the season. However, leg coloration did show a pre-breeding sex difference also in captive birds. Interestingly, this was the time the sexes showed a sex difference in androgen levels. This suggests that androgens may still play a role in carotenoid deposition.

(f) During rearing of the young males have lower levels of color expression and carotenoids, and higher levels of oxidative damage than females, and wild birds of both sexes have lower levels of these parameters than captive birds, suggestive that parental effort and physical activity is costly. Whereas color expression and carotenoids were higher in males than in females during the pre-breeding period, they were lower in males than in wild females during the chick-rearing period, which was highly significant for carotenoids. This sex time season effect was not apparent in captive birds, possibly because captive males did not have to face with a foraging cost. In addition, body condition decreased sharply over the season in wild males, having the lowest body condition index of all categories of birds during chick rearing. Moreover, only in wild male birds, levels of ROMs and of oxidative stress sharply increased, resulting in significantly higher levels in males than females during chick rearing, a trend absent in captive birds. Male birds of prey have to cope with an intense foraging activity during the breeding phase and, in particular, during the chick-rearing phase because they provide food to both the female and nestlings (Newton, 1978). The relevance of physical activity as a factor affecting the plasma oxidative status is evident from the increased levels of ROMs found in males while chick rearing. A high investment in current reproduction may reduce future reproductive output. Oxidative stress resulting from reproductive effort could be an important mechanism mediating the trade-off between reproductive investment and lifespan (Wiersma et al., 2004). Moreover, our study is the first to provide evidence that the division of labor within the pair, which is a quite common pattern among raptors, may explain the plasma oxidative stress condition of free ranging

breeding birds. Previous studies on captive zebra finches suggested that breeding effort may deplete antioxidant defenses, potentially exposing parents to oxidative stress (Alonso-Alvarez et al., 2004; Wiersma et al., 2004). A more recent study on captive pigeons showed that individuals losing more body mass while chick feeding had also a slightly higher increase in oxidative damage (Costantini et al., 2010).

Physical activity may have affected the condition of the birds in another way. In both seasons, wild males and females displayed higher levels of ROMs than captive birds. Wild kestrels experienced higher levels of physical activity, which may have increased their metabolic activity and generation of pro-oxidants (Di Meo and Venditti, 2001; Costantini, 2008). In addition, the hormonal status of breeding birds could contribute to modulate the plasma oxidative status. Testosterone, whose levels were higher in wild males early in the season, may have enhanced the metabolic rate (e.g. Buchanan et al., 2001), altering the balance between reactive chemical species and antioxidant defenses, as shown in mammals (e.g. Chainy et al., 1997; Aydilek et al., 2004). However, since these hormones did not differ between males and females during chick rearing, they might not be responsible for the relative high levels of ROMs in wild birds during that time.

5. Conclusions

The study has not supported the hypothesis that the carotenoid-based secondary sexual trait in the kestrel reflects their health and hormonal status but suggests that regulation of the signal may be multi-factorial. The data indicate that physical activity and reproductive effort may be causally related to oxidative stress and that egg production limits levels of circulating carotenoids in females. Interestingly, independently of physical activity, reproductive effort and diet, there was a strong effect of season on both color expression and circulating carotenoid levels. This suggests an independent role of, for example, the light-dark schedule on metabolism and deposition of carotenoids. For example, it is known that abiotic environmental factors can change the capacity of gut wall to absorb nutrients in the Atlantic salmon *Salmo salar,* a species for which carotenoids are positively correlated to temperature (Ytrestøyl et al., 2005). Birds can dramatically adjust resource absorption and allocation in accordance to specific seasonal events such as molt (Dietz et al., 1992; Laucht et al., 2010). We are confident that captive kestrels did not change the amount of food intake during the season, as we have not observed chickens left uneaten in the cages. However, we found a decrease in body condition and circulating carotenoids in captive kestrels, suggesting a change in energy balance. This might be associated with periodic processes, such as molt, that could have affected carotenoid absorption. This aspect has as yet hardly been studied and needs further investigation.

Supplementary materials related to this article can be foundonline at doi:10.1016/j.cbpa.2011.04.011.

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