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Effects of carotenoid supplementation on colour expression, oxidative stress and body mass in rehabilitated captive adult kestrels (*Falco tinnunculus*)

David Costantini · Carlo Coluzza · Alberto Fanfani · Giacomo Dell'Omo

Abstract Carotenoids are considered a limited resource for animals because they are not synthesised by the body. Birds use carotenoids, mainly xanthophylls, for physiological functions, such as anti-oxidant activity, and for colour expression; hence, they need to shunt carotenoids between competitive demands. Recent studies suggest that the anti-oxidant role of xanthophylls might not be as important as previously thought and that at high concentrations they may, in fact, acquire pro-oxidant properties. In this work, we studied the effects of a moderate xanthophyll supplementation (115 mg of carotenoids/kg diet/day; 4 weeks) on serum carotenoids, serum concentration of reactive oxygen metabolites (ROMs), serum anti-oxidant capacity (OXY), the degree of oxidative stress (OS; ROMs/OXY $\times$ 1,000), body mass, and skin colour, in rehabilitated captive adult Eurasian kestrels (*Falco tinnunculus*). The supplementation caused increased levels of serum carotenoids ($\sim$90%), ROMs ($\sim$82%), OS ($\sim$115%) and an immediate loss of body mass ($\sim$6.2%), but it did not affect OXY and tarsi skin hue. The red ($\sim$16%) and yellow ($\sim$15%) colorimetric components were increased after the first week of supplementation and the effect persisted during the rest of the experiment. Two months after the end of supplementation, serum carotenoids, OS and ROMs returned to baseline levels, however the body mass did not. Our findings suggest that, above a certain physiological threshold, carotenoids can cause detrimental effects. This is relevant for the trade-off between expression of sexual signals and the costs of maintaining/producing them.

Keywords Oxidative stress · Free radicals · Anti-oxidant capacity · Carotenoids · Pro-oxidant activity

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Introduction

The trade-off in the allocation of limited resources among competing demands is a pivotal topic in life-history theory (Roff 1992; Stearns 1992). Natural selection is actually predicted to favour individuals able to deploy limited resources in a way that maximises their survival and reproductive success.

The trade-off that occurs in the exploitation of dietary carotenoids is known and documented (Blount 2004). The availability of carotenoids is suggested to be a limiting factor for birds since carotenoids can only be acquired from food and the carotenoid content varies according to the different food types (Goodwin 1984). Dietary differences may actually explain the inter-population variation in both circulating carotenoids and skin pigmentation in different animal groups [e.g., fish in Grether et al. (1999); birds in Negro et al. (2000); reptiles in Costantini et al. (2005b)].

In birds, these pigments are responsible for the yellow to red colouration of the skin or plumage (Brush 1990); they

are also involved in many physiological functions, such as immunostimulation (Blount et al. 2003; Faivre et al. 2003) and the anti-oxidant activity. Furthermore, the carotenoid-based colourations are thought to convey the health status of the male, i.e., its ability to cope with parasites (Lozano 1994) and oxidative stress (von Schantz et al. 1999; Hartley and Kennedy 2004), and its sperm quality (Blount et al. 2001; Peters et al. 2004).

The anti-oxidant protection mediated by maternally transferred carotenoids is well documented for embryo and hatchling models (Surai 2002; McGraw et al. 2005). Nevertheless, recent evidence suggests that the anti-oxidant role of xanthophylls in nestling or adult birds may not be as important as was previously thought [*Falco tinnunculus* in Costantini et al. (2006, 2007); Costantini and Dell'Omo (2006a); *Carduelis chloris* in Hõrak et al. (2006); *Parus major* in Tummeleht et al. (2006)]. Further, recent studies suggest that, at high concentrations, carotenoids such as lutein, lycopene and β-carotene can lose their anti-oxidant effect and acquire pro-oxidant properties in vitro and in vivo systems (Young and Lowe 2001; El-Agamey et al. 2004; Siems et al. 2005).

There is a common idea that wild birds are generally in need of carotenoids (Blount 2004), although the amount of carotenoids consumed daily by free-living birds is not well known (Partali et al. 1987). Thus far, many studies document a turnover of carotenoids (e.g., during parasite infections) suggesting that the level of need for carotenoids may vary from one situation to another (see review in Møller et al. 2000). However, the idea that the diet can limit the anti-oxidant defences of birds deserves careful empirical study because we do not have any knowledge of the levels at which carotenoids might lose their beneficial properties or whether carotenoids are really crucial in decreasing oxidative stress.

In the present study, we evaluated the effects of a moderate supplementation of carotenoids in captive adult Eurasian kestrels (*Falco tinnunculus*), a raptor displaying sexual dimorphism in the carotenoid-based colouration of the skin (Casagrande et al. 2006). More specifically, we measured the response of the following variables to the supplementation of carotenoids: (1) serum carotenoids and tarsi skin colour, (2) serum reactive oxygen metabolites (ROMs; marker of early oxidative damage), (3) serum anti-oxidant capacity (OXY), and (4) body mass. The aims were to evaluate (1) whether carotenoids help maintain redox homeostasis, i.e., the balance between pro-oxidant production and anti-oxidant protection, and (2) in what way carotenoids are allocated among competing demands such as skin colouration, which is suggested to work as sexual advertisement, and anti-oxidant activity in this species.

Materials and methods

Animals and housing

The experiment was carried out from May 28th to June 25th, 2005, in a dedicated structure in Valmontone, 30 km south of Rome. Ten males and ten females adult kestrels were obtained from local rehabilitation centres. The birds had stabilized bone fractures in the wings and could not be released in the wild, but they were in good conditions, as shown by the baseline values of body mass (mean $\pm$ SE and range males 203.7 ± 3.2 g, 188–223 g; females 222.9 ± 6.1 g, 180–251 g) which fall within the range (range of monthly averages males 187–210; females 220–276) of values recorded in previous studies using the same species (Dijkstra 1988; Dijkstra et al. 1988). A visual inspection of the birds showed that all the specimens were also free of ectoparasites. Finally, no specimen showed any behavioural aberrations.

The birds were randomly housed in pairs in a row of outdoor aviaries ($1 \times 1.7 \times 2$ m; w $\times$ l $\times$ h) equipped with nest boxes and perches to allow the kestrels to move freely throughout the aviary. Each aviary was separated from the others by a shade net to prevent pairs from seeing each other. The birds were in the facility for about one year before the experimental manipulations started and were maintained on a diet of one-day-old cockerels according to Negro et al. (1998).

Experimental design and data collection

The pairs were randomly assigned to two groups which were alternately housed in adjacent aviaries: supplemented and control birds. For 28 days, the supplemented birds received chicks enriched with 45 µl of a safflower oil solution every day (Kemin Foods, L.C., FloraGLO Lutein, Des Moines, Iowa, USA), with 20% lutein and 0.8% zeaxanthin, corresponding to a dose of about 8 mg of xanthophylls derived from oleoresins extracted from marigold flowers (*Tagetes erecta*). According to the literature, such dose of carotenoids can be considered moderate. To avoid degradation and photo-oxidation of the carotenoids, the safflower oil solution was stored cold and in the dark.

Considering a mean body mass of 204 g for males and 223 g for females ($n = 10$ each; weight recorded a few hours after feeding), a dose of 35.8–39.2 mg of carotenoids/kg of body mass/day (115 mg of carotenoids/kg diet/day) was supplemented. Control birds received 45 µl of oil only. Lutein and zeaxanthin are the only carotenoids identified in the blood and skin of the Eurasian kestrel, in which they are present at a ratio of 9–1 (Casagrande et al. 2006). The oil was injected into the mouth of the chicks because kestrels

begin to eat cockerels from the head (personal observation). Chicks were always eaten within a few minutes by all individuals. After eating one chick enriched with carotenoids, the kestrels were given other non-enriched chicks (total: 1.5 chicks/individual/day; ~70 g/individual/day). The dose was determined on the basis of the available information about the prey xanthophyll content of kestrels (Czeczuga 1978, 1979; Casagrande et al. 2006). It is difficult to determine the precise range of carotenoid amounts ingested by the kestrels since the kestrels in our study-region have a wide dietary composition and can display individual feeding habits (Costantini et al. 2005a). In addition, we suggested that carotenoid absorption in kestrels can also be affected by a physiological constraint at the intestinal level (Casagrande et al. 2007). Consequently, the carotenoid amount daily ingested is expected to vary greatly from one kestrel to another. In a previous study, we used a dose of 265 mg of carotenoids/kg of body mass which might be about 10–30 times higher than a dose assumed daily by kestrels in the wild (Casagrande et al. 2007) and is around sevenfold higher than the dose used in the present study. The dose used in Casagrande et al. (2007) did not have any detrimental effect on body mass of nestling kestrels. However, the fact that nestlings and adults may require different quantities of carotenoids since nestlings are still growing and have immature detoxifying systems should be taken into account.

A blood sample was taken from each individual just before the beginning of the treatments and at 7-day intervals during the whole treatment period, for a total of five samplings: pre-treatment (Pre; baseline levels), and weeks 1, 2, 3 and 4. To evaluate the recovery of all the physiological variables and the body mass, we carried out a further sampling two months later (week 12). Blood was always taken between 8.00 and 11.00 a.m., before feeding the animals. The blood (400 µl) was drawn from the brachial vein and kept in ice until centrifugation, which occurred within a few hours. The serum was stored in three separate aliquots at −20°C until laboratory analyses. Body mass was measured to the nearest 1 g at the time of the bleeding procedures.

Measurement of carotenoids and skin colour

The serum (30 µl) was diluted with absolute methanol (1:20) and the flocculent proteins were precipitated by centrifugation at 12,000g for 5 min. Carotenoids were quantified with a Beckman DU 7400 spectrophotometer at 476 nm. The carotenoid concentration was estimated as µg ml^{-1} of serum using the standard absorbance curve of lutein (Sigma-Aldrich; Costantini et al. 2006). The tarsus skin colour was measured with a hand-held spectrophotometer (Minolta CM-2600d, Minolta Italia S.p.A., Milan, Italy) operating in a wavelength range of 360–740 nm. The measurements were relative and developed in reference to a standard white reference tile and to darkness. The diameter of the spectrophotometer's measurement aperture was set to 3 mm to fit the tarsus size accurately. Before the measurement, the skin was cleaned with water and air-dried. Skin colour was quantified through its hue because it reflects colour variation in the Eurasian kestrel (a lower hue value stands for a higher degree of skin redness; Casagrande et al. 2006, 2007). Hue is one of the three main attributes of perceived color, in addition to lightness and chroma (or colorfulness). We also considered the colorimetric parameters $a*$ and $b*$ in order to evaluate how the components of hue responded to carotenoid supplementation (hue = arctan $b*/a*$). $a*$ and $b*$ are opponent color axes of the L*$a*b*$ color space. A plot of $a*$ versus $b*$ values for a colour sample can be used as an accurate measure of where that sample lies in colour space, i.e., what its hue is. The parameter $a*$ indicates the position of the sample between green and red (negative values indicate green while positive values indicate red) while the parameter $b*$ indicates the position of the sample between blue and yellow (negative values indicate blue and positive values indicate yellow). Samples for which $a* = b* = 0$ are achromatic. All the measurements were carried out only during the supplementation period. All colorimetric variables were calculated using SpectraMagic 3.6 software supplied with the CM-2600d instrument. The mean value of three sequential measurements per individual was used in the data analysis (intraclass correlation coefficient: all $r > 0.96$, all $P < 0.001$; Lessells and Boag 1987).

Measurement of reactive oxygen metabolites

The serum concentration of ROMs (primarily hydroperoxides ROOH; marker of early oxidative damage) was measured by the d-ROMs test (Diacron, Grosseto, Italy). The serum (20 µl) was 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 first; it was then incubated for 75 min at 37°C. The acidic pH favours the release of iron and copper from serum proteins. These metals catalyse the cleavage of ROOH, leading to the generation of two highly reactive and histolesive pro-oxidants, namely the alkoxyl (R–O$^\bullet$) and alkylperoxyl (R–OO$^\bullet$) radicals. When these compounds react with an alkyl-substituted aromatic amine (A-NH$_2$) solubilized in the chromogen, they produce a complex whose colour intensity (pink) is directly proportional to their concentration. After incubation, the absorbance was read with a spectrophotometer (Microplate Reader Model 550) at 490 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$_2$O$_2$

equivalents (repeatability $r = 0.98$, $P < 0.001$; for further details see Costantini et al. 2006; Costantini and Dell'Omo 2006a, b).

Measurement of the serum anti-oxidant capacity

The serum anti-oxidant capacity quantifies the activity of both exogenous and endogenous anti-oxidants. It was measured by the OXY-Adsorbent test (Diacron, Grosseto, Italy). This dedicated kit uses a colorimetric determination to quantify the ability of the anti-oxidant barrier to cope with the oxidant action of hypochlorous acid (HOCl; oxidant of pathologic relevance in biological systems). The serum (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 serum for 10 min at 37°C. Then, 5 μl of the chromogen solution used for the determination of the ROMs was added. An alkyl-substituted aromatic amine solubilized in the chromogen is oxidized by the residual HOCl and transformed into a pink derivative. The intensity of the coloured complex, which is inversely related to OXY, was measured with the same spectrophotometer at 490 nm. Measurements are expressed as mM of HOCl neutralised (repeatability $r = 0.86$, $P < 0.001$; for further details see Costantini et al. 2006; Costantini and Dell'Omo 2006a, b).

Statistical analyses

Repeated-measures ANOVA was performed including Treatment and Gender as fixed factors. Tukey's HSD test was used for post-hoc comparisons (results reported in the figures). Main effects, two- and three-way interactions were considered. The response variables are serum carotenoid concentration, ROMs, OXY, the ratio ROMs/OXY × 1,000 as an index of oxidative stress (OS), body mass, and tarsi skin colour (hue, $a*$, $b*$).

Repeatability of measures was calculated according to Lessells and Boag (1987).

In general, the use of ratios is suggested when both variables are significantly correlated (Hayes and Shonkwiler 1996). However, pro-oxidant and anti-oxidant compounds may or may not be significantly correlated. In fact, their relationship can be affected by the ability of the organism to cope with pro-oxidant production or by the time needed to mount an effective anti-oxidant response. A recent meta-analysis revealed that most anti-oxidants exhibit a non-monotonic relationship with other markers of OS, with all markers correlating only under severe pathological conditions (Dotan et al. 2004). For example, nestling kestrels show increased levels of ROMs and a decreased anti-oxidant barrier during an immune challenge (Costantini and Dell'Omo 2006a). In the present case, ROMs and OXY were significantly correlated only at week 12 ($r = -0.51$,

$n = 18$, $P = 0.032$). Therefore, a second model for oxidative stress was run including ROMs as response variable and OXY as changing covariate.

In a first ANOVA, we included the measurements recorded before the treatment (Pre) and those recorded during the supplementation period (from week 1 to 4). In a second ANOVA, we included the measurements collected at Pre and week 12 in order to evaluate the recovery to pre-treatment values. From a statistical point of view, the members of a pair are not independent since they share the same environment (i.e., the cage) and this can cause pseudoreplication. However, we did not include cage identity in any of the models performed because birds in different aviaries did not differ with respect to pre-treatment values of all the variables measured (cage effect one-way ANOVA: all P-values ≥ 0.11).

The haemolysis of some sera forced us to discard two control individuals from the models for ROMs and OXY because lysed erythrocytes release pro-oxidants and anti-oxidant enzymes, increasing the blood levels of both. All the variables met the normality and homoscedasticity assumptions. The statistical analyses were performed with STATISTICA (Version 6, StatSoft 2001, Tulsa, USA).

Results

Serum carotenoids increased in supplemented individuals (Tr $F_{1,16} = 8.92$, $P = 0.008$; Tr × Time $F_{4,64} = 2.73$, $P = 0.036$; Fig. 1a). No further significant effects or interactions were found (all P-values ≥ 0.37). Over the supplementation period, the values showed a significant intra-individual repeatability ($r = 0.50$, $P < 0.001$). At week 12, serum carotenoids recovered to the pre-treatment values (Tr × Time $F_{1,16} = 0.55$, $P = 0.47$).

Carotenoid supplementation caused increased levels of ROMs (Tr $F_{1,14} = 7.43$, $P = 0.016$; Tr × Time $F_{4,56} = 6.76$, $P < 0.001$; Fig. 2b). No further significant effects or interactions were found (all P-values ≥ 0.36). Over the supplementation period, the values showed a significant intra-individual repeatability ($r = 0.60$, $P < 0.001$). At week 12, the levels of ROMs recovered to the pre-treatment values (Tr × Time $F_{1,14} = 2.86$, $P = 0.11$).

Supplemental carotenoids did not affect OXY during the treatment period (Tr $F_{1,14} = 0.03$, $P = 0.87$; Tr × Time $F_{4,56} = 1.36$, $P = 0.26$; Fig. 1c) and at week 12 (Tr × Time $F_{1,14} = 0.61$, $P = 0.45$). In general, males showed higher OXY values ($F_{1,14} = 6.81$, $P = 0.021$). Over the supplementation period, the values showed a low but significant intra-individual repeatability ($r = 0.30$, $P < 0.001$).

OS was increased by the supplementation (Tr $F_{1,14} = 9.81$, $P = 0.007$; Tr × Time $F_{4,56} = 2.67$, $P = 0.041$; Fig. 1d). In general, females showed higher levels of OS
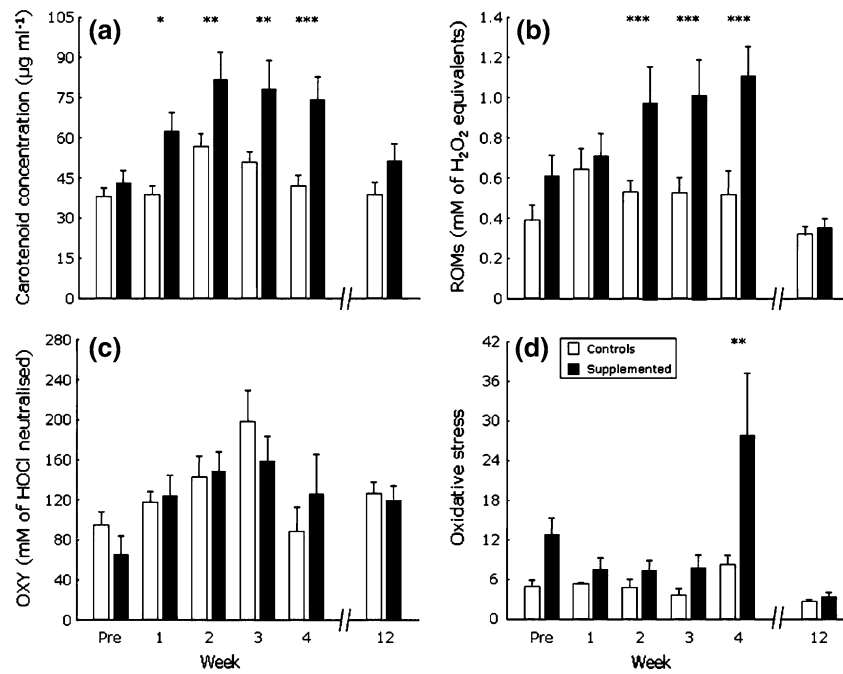
Fig. 1 Effect of carotenoids supplemented daily for 4 weeks to captive adult kestrels (mean ± SE; Tukey test *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$): **a** serum carotenoid concentration increased rapidly and reached a physiological threshold 2 weeks after the beginning of the supplementation period. The highest carotenoid concentrations in supplemented males and females were 151.41 µg ml^{-1} (week 2) and 123.28 µg ml^{-1} (week 3), respectively. **b** The oxidative damage (i.e. ROMs) induced by carotenoid supplementation increased progressively but returned to baseline levels two months after the end of treatment. **c** Carotenoid supplementation did not significantly increase the total serum antioxidant capacity (OXY). **d** The degree of oxidative stress as evaluated by the ratio ROMs/OXY × 1,000 was increased in supplemented individuals at week 4, but returned to baseline levels 2 months after the end of treatment
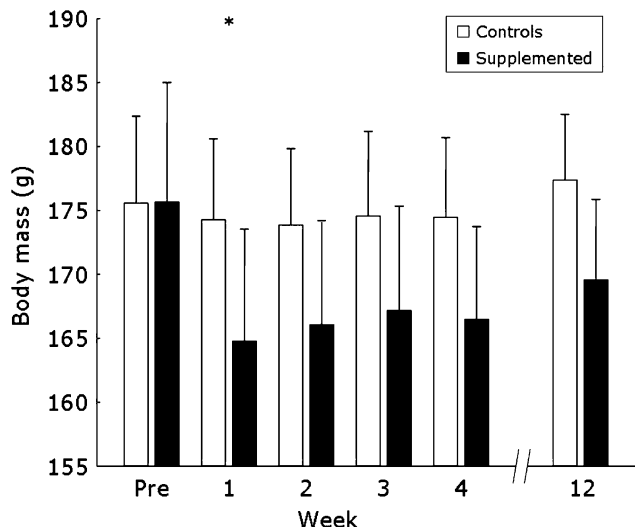

Fig. 2 Effect of carotenoids supplemented daily for 4 weeks on the body mass of captive adult kestrels (mean ± SE; Tukey test *$P < 0.05$). The body mass of supplemented individuals decreased after the first week of carotenoid provisioning and had not recovered at week 12

($F_{1,14} = 7.33$, $P = 0.017$). The interaction between Tr and gender approached the level of significance ($F_{1,14} = 4.00$, $P = 0.065$). At week 12, OS was lower than pre-treatment values (Tr × Time $F_{1,14} = 7.97$, $P = 0.014$).

Similar results were obtained including in the model ROMs as dependent variable and OXY as covariate. The redox balance was actually shifted toward pro-oxidants by the supplementation (Tr $F_{1,13} = 9.16$, $P = 0.01$; Tr × Time $F_{4,52} = 3.76$, $P = 0.009$). The effect of gender, however, did not emerge ($F_{1,13} = 1.38$, $P = 0.26$; Tr × Sex $F_{1,13} = 1.40$, $P = 0.26$). At week 12, oxidative stress was lower than pre-treatment values (Tr × Time $F_{1,13} = 6.04$, $P = 0.029$).

Body mass showed an obvious gender-related difference, being higher in females ($F_{1,16} = 45.50$, $P < 0.001$). Also, body mass showed an immediate loss in supplemented individuals (Tr × Time $F_{4,64} = 3.19$, $P = 0.019$) which was more pronounced in the first week of treatment (Fig. 2). No further significant effects or interactions were found (all P-values ≥0.26). At week 12, body mass recovered in treated birds to pre-treatment values (Tr × Time $F_{1,16} = 1.78$, $P = 0.20$).

The effect of carotenoid supplementation on a^* was more pronounced at the end of the experiment with supplemented individuals showing higher values (Time $F_{4,64} = 4.20$, $P = 0.004$; Fig. 3a). No further significant effects or interactions were found (all P-values ≥0.23). The variation in b^* was significantly explained by Time ($F_{4,64} = 5.41$, $P = 0.001$) and Tr × Time ($F_{4,64} = 5.32$, $P < 0.001$; Fig. 3b). No further significant effects or interactions were
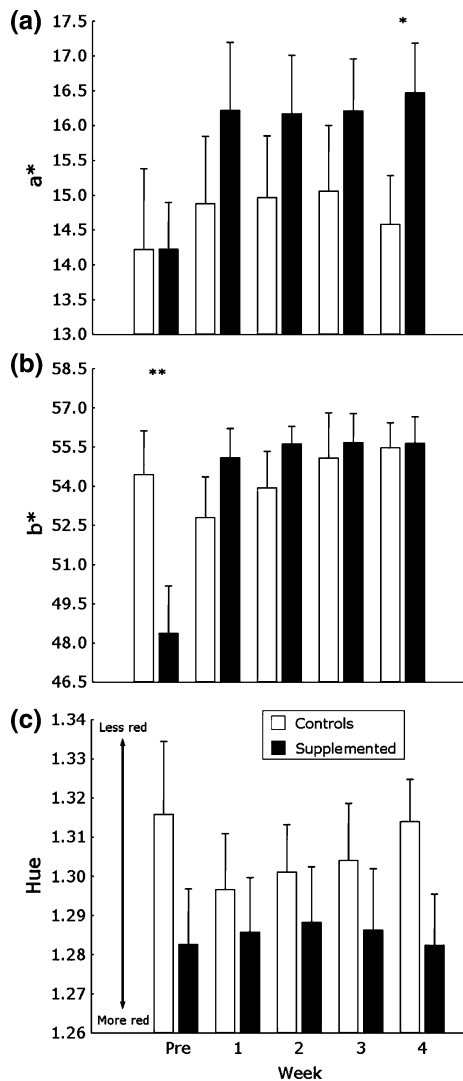
Fig. 3 Effect of carotenoids supplemented daily for 4 weeks on the skin colour (*a* negative values* indicate green whilst *positive values* indicate red; *b* negative values* indicate blue whilst positive values indicate yellow; hue) of captive adult kestrels (mean ± SE; Tukey test *P < 0.05, **P < 0.01)

found (all *P*-values ≥0.13), No significant effects were detected on the tarsi skin hue (all *P*-values ≥0.16; Fig. 3c). No between-gender differences emerged for any of all the colorimetric variables investigated.

Discussion

It could be argued that the birds used in the present study are poor models for physiological study since they had stabilized bone fractures in the wings that prevented their release in the wild. In fact, flight increases metabolism and perhaps free radical production. This raises the possibility that the negative effects of the treatment could be an arte-

fact of the birds having been rehabilitated specimens and thus unable to fly. However, it should be noted that the injuries did not prevent birds from flying freely throughout the aviary. Therefore, the limits of our model species are similar to those of any other bird species kept in captivity.

It is known that carotenoid supplementation in birds increases blood carotenoids and the redness of their colouring until a plateau is reached (Alonso-Alvarez et al. 2004). In our study, carotenoid supplementation caused increased levels of serum carotenoids, which reached their peak two weeks after the beginning of the treatment. The red and yellow colorimetric components were increased after the first week of supplementation and the effect remained during the rest of the experiment. However, the tarsi skin hue did not show any response. The reason why the red and yellow colorimetric components responded to supplementation whilst hue did not is not entirely clear. Hue is defined by the arctan of the ratio of those components, hence our results suggest that *a** (redness–greeness) and *b** (yellowness–blueness) changed in a way that did not vary their ratio. The mechanisms underlying this pattern are not known and so are warranted of future investigation.

Baseline levels of serum carotenoids measured in the present study (95% confidence interval 34.96–46.48 µg ml^{-1}) were within the range of those recorded in wild adult kestrels measured during the courtship phase (95% confidence interval 27.81–46.80 µg ml^{-1}; Casagrande et al. 2006) or slightly higher than those in nestling kestrels (95% confidence interval 20.20–22.22 µg ml^{-1} in Costantini et al. (2006) and 34.04–38.75 µg ml^{-1} in Casagrande et al. (2007). This does not necessarily entail that captive and wild kestrels have a similar carotenoid intake. In fact, carotenoids are stored in liver and fat (e.g. Negro et al. 2001; Surai 2002); therefore experimental birds could have diverted part of circulating carotenoids to those tissues.

On the contrary, circulating carotenoids of supplemented individuals (95% confidence interval, e.g. at day 2 56.56–82.14 µg ml^{-1}) were about twofold higher than the levels recorded in wild birds (see above). However, they should not be considered as unnatural concentrations because wild kestrels can reach levels of circulating carotenoids of up to 80 µg ml^{-1} (Casagrande et al. 2007). Therefore, these concentrations might represent the highest levels under physiological conditions in kestrels. These results suggest the possibility that some individuals within a wild kestrel population might have access to high carotenoid sources. For example, in central Italy, free-living kestrels show consistent individual differences in feeding habits which are maintained from one year to the next (Costantini et al. 2005a). These may result in differences in carotenoid intake since carotenoid content may vary greatly among prey groups (Goodwin 1984). Finally, we should consider the

possibility that some variation in the response of captive kestrels to carotenoid supplementation may reflect a physiological constraint (e.g., differential intestinal absorption; Blount et al. 2003; Casagrande et al. 2007).

The tarsi hue colour baseline values of all captive kestrels fell approximately within the same range as those measured in wild ones (95% confidence interval 1.21–1.27), although they were slightly higher (less bright colouration) in captive males (95% confidence interval 1.24–1.32) than in wild ones (95% confidence interval 1.18–1.23; Casagrande et al. 2006). These findings suggest that the expression of colour intensity in wild adult kestrels may not be strictly limited by carotenoid availability only. Above a certain carotenoid intake, the visual signal could be modulated by other factors, such as sex hormones. It is recognized that some endocrine mechanisms can control the colour expression (Owens and Short 1995). For example, a study on captive male and female American kestrels (*Falco sparverius*) maintained on the same diet showed that the males displayed higher blood carotenoids and brighter colouration than the females during mating and laying (Negro et al. 1998). Recently, it has been shown that testosterone increases both circulating carotenoids and colour in red-legged partridges *Alectoris rufa* (Blas et al. 2006) and in zebra finches *Taeniopygia guttata* (McGraw et al. 2006). Hence, in wild males, testosterone could have allowed depositing dietary carotenoids in the legs, whereas captive males were not able to divert these pigments to this trait. The absence of gender differences we found in both colour and carotenoid concentration could be related to the phase of the reproductive cycle. It is known that male and female captive American kestrels are highly sexually dimorphic only during mating and laying (Negro et al. 1998). The present experiment was actually carried out toward the end of the normal kestrel breeding season in central Italy. However, this result deserves further attention because of the small sample size.

Our study shows the first evidence that carotenoid supplementation can increase the individual oxidative stress as a consequence of increased levels of oxidative damage without a parallel increase in serum anti-oxidant capacity in a bird species. This latter result does not depend upon the method used to quantify the anti-oxidant capacity since it is widely recognized that HOCl reacts with carotenoids (Handelman et al. 1991; Siems et al. 2000).

The imbalance in the relative concentrations of anti-oxidants could explain the increased oxidative damage caused by the carotenoid supplementation. For example, the synergistic protection provided by carotenoids and vitamins C and E depends upon a balance among these compounds (Palozza 1998). It is also known that an increase in the concentration of one of them can disturb the balance, causing a decrease in the effectiveness of the anti-oxidant (Wefers and Sies 1988). For example, an increase in carotenoid concentration may cause the formation of carotenoid radicals or adducts at a level that impedes the tocopherol/ascorbate pool from coping with oxidative stress successfully, leading to pro-oxidant effects (Young and Lowe 2001). Moreover, carotenoids at high concentration tend to aggregate or crystallize out of solution (Gruszecki 1999). Such aggregates have been directly observed in cell membranes, and they are thought to affect the properties of the membrane profoundly by increasing its fluidity and permeability, ultimately causing pro-oxidant effects.

The changes in body mass in kestrels reflect the accumulation or loss of body reserves (mainly fat; see Chap. 5 in Village 1990). It is known that body mass shows seasonal variations in wild kestrels in relation to food availability and reproduction (Dijkstra et al. 1988). It is also recognized that the maintaining of body reserves may help individuals to cope with adverse conditions (Dijkstra 1988; Dijkstra et al. 1988; Village 1990). In the present study, we recorded an unexpected immediate loss of body mass which was more pronounced in the first week of carotenoid administration. This impairment persisted throughout the supplementation period and was still evident 2 months after treatment had ceased. To our knowledge, this is the first evidence of a potentially negative effect of carotenoids on body mass in a bird species. Previous studies, in which a carotenoid supplementation was carried out in an avian species, did not describe any effect on body mass or any other physiological dysfunction [e.g., Leghorn chicks supplemented with 100 mg/kg diet in Woodall et al. (1996); zebra finches supplemented with 12.5–200 µg ml^{-1} of drinking water in Alonso-Alvarez et al. (2004); kestrel nestlings supplemented with different terms in Casagrande et al. (2007). Moreover, a recent work found a gain in mass in zebra finches supplemented with 100 µg ml^{-1} of drinking water (Bertrand et al. 2006).

The loss in mass could have been due to pervasive oxidative damage in vital organs such as the liver or the kidney. A massive lipid per-oxidation in these tissues could have affected the body homeostasis negatively and more quickly leading, e.g. to an impaired nutrient assimilation from food. The accentuated formation of potentially damaging molecules, such as pro-oxidant compounds, in conditions of high carotenoid concentration can itself lead to further oxidative damage to fat stores (Gruszecki 1999). Therefore, the loss in the body mass could have been due to the dysfunction of liver or kidney caused by pervasive oxidative damage and secondarily to per-oxidation of fat reserves.

Taken together, our results suggest that carotenoids can be costly at high concentrations in terms of increased oxidative stress and loss of body mass. This is relevant for the trade-off between expression of sexual signals and the costs of maintaining/producing them (Stearns 1992). Detrimental

effects of carotenoids have been proposed as an alternative explanation of how the expression of carotenoid-based ornaments is limited and why they are costly to produce or maintain (Zahavi and Zahavi 1997; Olson and Owens 1988). In this light, we would have expected a decrease in colour in carotenoid-treated individuals since oxidation alters or destroys their colour. However, supplemented birds were still redder. These results suggest three possible explanations: treated birds were able to sustain such costs and so to maintain redder colourations; the detrimental effect on the skin colour could emerge over a longer period of time than that of the present study; carotenoid-based colourations no longer convey the health status at high carotenoid concentrations. These explanations, however, deserve further study because the sample size and the experimental design do not allow to draw strong conclusions concerning these hypotheses.

Finally, we suggest that given these and previous results (Costantini et al. 2006, 2007; Costantini and Dell'Omo 2006a), the current idea that the carotenoid availability can limit the effectiveness of pro-oxidation retardation or inhibition be reconsidered.

References

Alonso-Alvarez C, Bertrand S, Devevey G, Gaillard M, Prost J, Faivre B, Sorci G (2004) An experimental test of the dose–dependent effect of carotenoids and immune activation on sexual signals and antioxidant activity. Am Nat 164:651–659

Bertrand S, Alonso-Alvarez C, Devevey G, Faivre B, Prost J, Sorci G (2006) Carotenoids modulate the trade-off between egg production and resistance to oxidative stress in zebra finches. Oecologia 147:576–584

Blas J, Pérez-Rodríguez L, Bortolotti GR, Viñuela J, Marchant TA (2006) Testosterone increases bioavailability of carotenoids: insights into the honesty of sexual signaling. Proc Natl Acad Sci USA 103:18633–18637

Blount JD (2004) Carotenoids and life-history evolution in animals. Arch Biochem Biophys 430:10–15

Blount JD, Møller AP, Houston DC (2001) Antioxidants, showy males and sperm quality. Ecol Lett 4:393–396

Blount JD, Metcalfe NB, Birkhead TR, Surai PF (2003) Carotenoid modulation of immune function and sexual attractiveness in zebra finches. Science 300:125–127

Brush AH (1990) Metabolism of carotenoid pigments in birds. FASEB J 4:2969–2977

Casagrande S, Csermely D, Pini E, Bertacche V, Tagliavini J (2006) Skin carotenoid concentration correlates with male hunting skill and territory quality in the kestrel (*Falco tinnunculus*). J Avian Biol 37:190–196

Casagrande S, Costantini D, Fanfani A, Tagliavini J, Dell'Omo G (2007) Patterns of serum carotenoid accumulation and skin color variation in nestling kestrels in relation to breeding conditions and different terms of carotenoid supplementation. J Comp Physiol B 177:237–245

Costantini D, Casagrande S, Di Lieto G, Fanfani A, Dell'Omo G (2005a) Consistent differences in feeding habits between neighbouring breeding kestrels. Behaviour 142:1409–1421

Costantini D, Dell'Omo G, Casagrande S, Fabiani A, Carosi M, Bertacche V, Marquez C, Snell H, Snell H, Tapia W, Gentile G (2005b) Inter-population variation of carotenoids in Galápagos land iguanas (*Conolophus subcristatus*). Comp Biochem Physiol B 142:239–244

Costantini D, Dell'Omo G (2006a) Effects of T-cell-mediated immune response on avian oxidative stress. Comp Biochem Physiol A 145:137–142

Costantini D, Dell'Omo G (2006b) Environmental and genetic components of oxidative stress in wild kestrel nestlings (*Falco tinnunculus*). J Comp Physiol B 176:575–579

Costantini D, Casagrande S, De Filippis S, Brambilla G, Fanfani A, Tagliavini J, Dell'Omo G (2006) Correlates of oxidative stress in wild kestrel nestlings (*Falco tinnunculus*). J Comp Physiol B 176:329–337

Costantini D, Fanfani A, Dell'Omo G (2007) Carotenoid availability does not limit the capability of nestling kestrels (*Falco tinnunculus*) to cope with oxidative stress. J Exp Biol 210:1238–1244

Czeczuga B (1978) Carotenoids in the skin of certain species of birds. Comp Biochem Physiol B 6:107–109

Czeczuga B (1979) Carotenoids in some parts of certain species of lizards. Comp Biochem Physiol B 65:755–757

Dijkstra C (1988) Reproductive tactics in the kestrel *Falco tinnunculus*. Ph.D. thesis, University of Groningen

Dijkstra C, Daan S, Meijer T, Cavé A.J, Foppen RPB (1988) Daily and seasonal variations in body mass of the kestrel in relation to food availability and reproduction. Ardea 76:127–140

Dotan Y, Lichtenberg D, Pinchuck I (2004) Lipid peroxidation cannot be used as a universal criterion of oxidative stress. Progr Lip Res 43:200–227

El-Agamey A, Lowe GM, McGarvey DJ, Mortensen A, Phillip DM, George Truscott T, Young AJ (2004) Carotenoid radical chemistry and antioxidant/pro-oxidant properties. Arch Biochem Biophys 430:37–48

Faivre B, Grégoire A, Préault M, Cézilly F, Sorci G (2003) Immune activation rapidly mirrored in a secondary sexual trait. Science 300:103

Goodwin TW (1984) The biochemistry of carotenoids. Animals, vol II. Chapman and Hall, London

Grether GF, Hudon J, Millie DF (1999) Carotenoid limitation of sexual coloration along an environmental gradient in guppies. Proc R Soc Lond B 266:1317–1322

Gruszecki WI (1999) Carotenoids in membranes. In: Frank HA, Young AJ, Britton G, Cogdell RJ (eds) The photochemistry of carotenoids. Kluwer, Dordrecht, pp 363–379

Handelman GJ, van Kuijk FJ, Chatterjee A, Krinsky NI (1991) Characterization of products formed during the autoxidation of β-carotene. Free Rad Biol Med 10:427–437

Hartley RC, Kennedy MW (2004) Are carotenoids a red herring in sexual display? Trends Ecol Evol 19:353–354

Hayes JP, Shonkwiler JS (1996) Analyzing mass-independent data. Physiol Zool 69:974–980

Hõrak P, Zilmer M, Saks L, Ots I, Karu U, Zilmer K (2006) Antioxidant protection, carotenoids, and the costs of immune challenge in greenfinches. J Exp Biol 209: 4329–4338

Lessells CM, Boag PT (1987) Unrepeatable repeatabilities: a common mistake. Auk 104:116–121

Lozano GA (1994) Carotenoids, parasites, and sexual selection. Oikos 70:309–311

McGraw KJ, Adkins-Regan E, Parker RS (2005) Maternally derived carotenoid pigments affect offspring survival, sex ratio, and sexual attractiveness in a colorful songbird. Naturwissenschaften 92:375–380

McGraw KJ, Correa SM, Adkins-Regan E (2006) Testosterone upregulates lipoprotein status to control sexual attractiveness in a colorful songbird. Behav Ecol Sociobiol 60:117–122

Møller AP, Biard C, Blount JD, Houston DC, Ninni P, Saino N, Surai PF (2000) Carotenoid-dependent signals: indicators of foraging efficiency, immunocompetence or detoxification ability? Avian Poult Biol Rev 11:137–159

Negro JJ, Bortolotti GR, Tella JL, Fernie KJ, Bird DM (1998) Regulation of integumentary colour and plasma carotenoids in American kestrel consistent with sexual selection theory. Funct Ecol 12:307–312

Negro JJ, Tella JL, Blanco G, Forero MG, Garrido-Fernández J (2000) Diet explains interpopulation variation of plasma carotenoids and skin pigmentation in nestling white storks. Physiol Biochem Zool 73:97–101

Negro JJ, Figuerola J, Garrido J, Green AJ (2001) Fat stores in birds: an overlooked sink for carotenoid pigments? Funct Ecol 15:297–303

Olson VA, Owens IPF (1998) Costly sexual signals: are carotenoids rare, risky or required? Trends Ecol Evol 13:510–514

Owens IPF, Short RV (1995) Hormonal basis of sexual dimorphism in birds: implications for new theories of sexual coloration. Trends Ecol Evol 10:44–47

Palozza P (1998) Prooxidant actions of carotenoids in biological systems. Nutr Rev 56:257–265

Partali V, Liaaen-Jensen S, Slagsvold T, Lifjeld JT (1987) Carotenoids in food chain studies II. The food chain of *Parus* spp. monitored by carotenoid analysis. Comp Biochem Physiol B 87:885–888

Peters A, Denk AG, Delhey K, Kempenaers B (2004) Carotenoid-based bill colour as an indicator of immunocompetence and sperm performance in male mallards. J Evol Biol 17:1111–1120

Roff DA (1992) The evolution of life histories. Chapman and Hall, New York

von Schantz T, Bensch S, Grahn M, Hasselquist D, Wittzell H (1999) Good genes, oxidative stress and condition-dependent sexual signals. Proc R Soc Lond B 266:1–12

Siems WG, Sommerburg O, Hurst JS, van Kuijk FJGM (2000) Carotenoid oxidative degradation products inhibit Na^+-K^+-ATPase. Free Rad Res 33:427–435

Siems W, Wiswedel I, Salerno C, Crifò C, Augustin W, Schild L, Langhans C-D, Sommerburg O (2005) β-carotene breakdown products may impair mitochondrial functions-potential side effects of high-dose β-carotene supplementation. J Nutr Biochem 16:358–397

Stearns SC (1992) The evolution of life histories. Oxford University Press, Oxford

Surai P (2002) Natural antioxidants in avian nutrition and reproduction. Nottingham University Press, Nottingham

Village A (1990) The kestrel. T & AD Poyser, London

Tummeleht L, Mägi M, Kilgas P, Mänd R, Hõrak P (2006) Antioxidant protection and plasma carotenoids of incubating great tits (*Parus major* L.) in relation to health state and breeding conditions. Comp Biochem Physiol C 144: 166–172

Wefers H, Sies H (1988) The protection by ascorbate and glutathione against microsomal lipid peroxidation is dependent on vitamin E. Eur J Biochem 174:353–357

Woodall AA, Britton G, Jackson MJ (1996) Dietary supplementation with carotenoids: effects on α-tocopherol levels and susceptibility of tissues to oxidative stress. Br J Nutr 76:307–317

Young AJ, Lowe GM (2001) Antioxidant and prooxidant properties of carotenoids. Arch Biochem Biop 385:20–27

Zahavi A, Zahavi A (1997) The handicap principle: a missing piece of Darwin's puzzle. Oxford University Press, Oxford