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# Carotenoid availability does not limit the capability of nestling kestrels (*Falco tinnunculus*) to cope with oxidative stress

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## Summary

**It is recognized that carotenoids are useful anti-oxidants in embryo and hatchling avian models. However, recent evidence suggests that the anti-oxidant role of carotenoids in nestling or adult birds may not be as important as previously thought. The aim of the present work was to investigate if supplemental carotenoids decreased the level of oxidative damage (by reactive oxygen metabolites, ROMs) and increased the serum anti-oxidant capacity (OXY) in nestling Eurasian kestrels *Falco tinnunculus*. Circulating carotenoids in supplemented nestlings increased about 1.5-fold compared to the control and pre-treatment levels at the end of the supplementation period. There was no effect on ROMs, OXY or the level of oxidative stress (ratio between ROMs and OXY), however, or on body mass or body condition of nestlings. ROMs and OXY decreased with age, but this pattern varied across the nests. Our results show that (i) in general, younger nestlings actually have to cope with a high free radical production, and (ii) the ability of wild nestling kestrels to cope with oxidative stress is not affected by carotenoid availability.**

Key words: antioxidants, free radicals, life history, metabolism, oxidative damage.

## Introduction

To maintain redox homeostasis, aerobic organisms have evolved mechanisms to neutralize the oxidative effects of oxygen and its reactive metabolites. Specifically, organisms exploit a wide array of anti-oxidant chemicals, i.e. any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly inhibits or delays a pro-oxidant initiated oxidation of that substrate (Halliwell and Gutteridge, 1989). The anti-oxidant machinery is basically based on endogenous (e.g. enzymes) and exogenous (e.g. dietary anti-oxidants) chemicals (e.g. Yu, 1994). When the anti-oxidant defence system is unable to cope with the anti-oxidant capacity, or when pro-oxidants exceed it, oxidative stress can arise because of an imbalance toward pro-oxidants. Oxidative stress is actually suggested to be the main proximate mechanism underlying degenerative processes such as aging (Harman, 1956; Harman, 1972; Beckman and Ames, 1998; Finkel and Holbrook, 2000).

In birds, several factors have been shown to shift the redox balance toward pro-oxidants, hence generating oxidative stress. In zebra finches *Taeniopygia guttata*, increased rearing effort depletes the anti-oxidant capacity of parents (Alonso-Alvarez et al., 2004b; Wiersma et al., 2004); zebra finches injected with lipopolysaccharides have a weaker resistance of red blood cells to a free radical attack than controls (Bertrand et al., 2006a); in nestling Eurasian kestrels *Falco tinnunculus*, T-cell-mediated immune response causes increased and decreased levels of oxidative damage and anti-oxidant capacity, respectively (Costantini and Dell'Omo, 2006a); in barn swallows *Hirundo rustica* and garden warblers *Sylvia borin*, oxidative stress is higher in individuals with lower energy stores after a sustained flight across the sea, during spring migration (Costantini et al., 2007).

Carotenoids are fat-soluble pigments capable of scavenging pro-oxidants (e.g. Møller et al., 2000; Surai, 2002; Krinsky and Yeum, 2003). Since animals are unable to synthesise carotenoids *de novo*, they must rely on dietary sources (Brush, 1990). Moreover, the complex absorption of carotenoids from the intestinal tract also seems to put a physiological constraint on their availability to wild birds (e.g. Casagrande et al., 2007). In this light, carotenoids are suggested to be in limited supply for reproduction, health-related functions, or the expression of sexual colouration (Blount, 2004).

Life-history theory states that an increase in the amount of limited energy or chemicals devoted to one process might result in a decreased allocation to other processes (Roff, 1992; Stearns, 1992). This is because life-history traits cannot evolve independently from one another. The trade-off among

competing demands of limited resources such as carotenoids is therefore a pivotal issue, since organisms might trade the gains in increasing allocation to one fitness component against losses in reducing allocation to another one. Natural selection is actually predicted to favour individuals able to deploy limited resources in a way that maximises their survival and reproductive success.

Whilst it is well supported that maternally transferred carotenoids are useful anti-oxidants in embryo and hatchling models (Surai, 2002; McGraw et al., 2005), recent evidence suggests that the anti-oxidant role of xanthophylls (i.e. oxygenated carotenoids) is less important than previously thought in nestling or adult birds [Eurasian kestrel (Costantini et al., 2006; Costantini and Dell'Omo, 2006a); greenfinch *Carduelis chloris* (Hõrak et al., 2006); great tit *Parus major* (Tummeleht et al., 2006)]. In addition, recent studies suggest that, at high concentrations, carotenoids such as lutein and β-carotene can lose their anti-oxidant effectiveness and acquire pro-oxidant properties *in vitro* or *in vivo* systems (Young and Lowe, 2001; El-Agamey et al., 2004; Siems et al., 2005; Costantini et al., in press).

We recently showed that carotenoids may be considered a limited resource for skin colour production in nestling kestrels (Casagrande et al., 2007). In addition, we proposed that the current idea that carotenoid availability might limit the effectiveness of pro-oxidation retardation or inhibition should be reconsidered (Costantini et al., in press). In this light, our work aimed at investigating if carotenoids are really useful anti-oxidants in wild birds, by a supplementation experiment. We tested this hypothesis in nestling Eurasian kestrels, a raptor species for which both the carotenoid and redox systems are sufficiently known (see references herein). If carotenoids actually limit the anti-oxidant capacity of the organism, we would expect to find supplemented nestlings showing lower levels of oxidative damage and higher levels of anti-oxidant capacity than controls.

## Materials and methods

### *Field study and experimental design*

The experiment was carried out during the 2006 breeding season in a 1200 $km^2$ area around Rome using nestling Eurasian kestrels *Falco tinnunculus* L. Nest boxes were visited regularly during the pre-hatching period in order to record the hatching date. The supplementation started when nestlings were 7–8 days old, in order to be sure that all chicks had hatched and avoided disturbance during the hatching span, which could provoke stress. Each bird was supplemented with 22.5 μl of a safflower oil solution (Kemin Foods, LC, FloraGLO Lutein, Des Moines, IA, USA), with 20% lutein and 0.8% zeaxanthin, corresponding to a dose of about 4 mg of xantophylls derived from oleoresins extracted from marigold *Tagetes erecta* flowers. Lutein and zeaxanthin are the only carotenoids identified in the blood and skin of the Eurasian kestrel, in which they occur at a ratio of 9:1 (Casagrande et al., 2006). The solution was orally administered every other day for a total of five administrations using a 1 ml syringe (Fig. 1). Control birds received 22.5 μl of oil only. To avoid degradation and photo-oxidation of carotenoids, the safflower oil solution was stored cold and in the dark. The dose was selected on the basis of the available information about the xanthophyll content of the species preyed on by kestrels (Czeczuga, 1978; Czeczuga, 1979; Goodwin, 1984; Casagrande et al., 2006) and of our previous supplementation studies to wild nestling (Casagrande et al., 2007) or captive adult (Costantini et al., in press) kestrels.

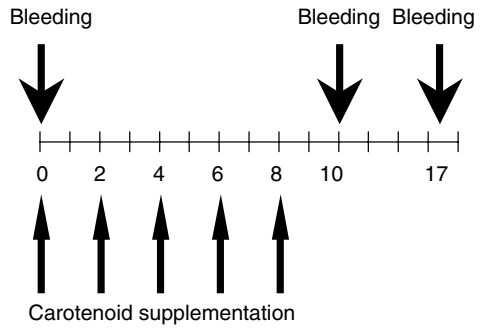


Fig. 1. Sketch of the timing of different parts of the experiment. Numbers indicate the day. At day 0, nestling kestrels were 7–8 days old.

A blood sample (400 μl) was drawn from the brachial vein just before the beginning of the experiment (pre-treatment values), 10 days later (2 days since the end of the supplementation; day 10), and 1 week later (day 17). Blood samples were kept cool (0–5°C) until centrifugation, which occurred within a few hours, and the serum was stored at –20°C. At the time of bleeding, wing length (mm) and body mass (g) of each individual were also recorded.

In total, 61 nestlings (30 controls and 31 supplemented) from 12 nests were included in the experiment. Specifically, there were three nests with four chicks, five with five chicks, and four with six chicks. Within each brood (except the five-chick one), half of the nestlings were randomly assigned to the two treatment groups. In the five-chick broods, two nestlings were assigned to the controls and three to the supplemented or *vice versa* in order to get a balanced design.

### *Measurement of carotenoids*

The serum (100 μl) was diluted with absolute methanol (1:8) and the flocculent proteins were precipitated by centrifugation at 12 000 ***g*** 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, Milano, Lombardia, Italy) (Costantini et al., 2006).

### *Measurement of reactive oxygen metabolites*

The serum concentration of reactive oxygen metabolites (ROMs; primarily hydroperoxides, ROOH) was measured by the d-ROMs test (Diacron, Grosseto, Italy). Hydroperoxides

are primary products of the oxidative cascade derived from the oxidation of organic compounds (mainly lipids) (e.g. Porter et al., 1995; Moore and Roberts, II, 1998). The serum (20 μl) was first diluted with 200 μl of a solution containing 0.01 mol $l^{-1}$ 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. 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 ($R\text{-}O^{-}$) and alkylperoxyl ($R\text{-}OO^{-}$) radicals. When these compounds react with an alkyl-substituted aromatic amine ($A\text{-}NH_2$) 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, Tokyo, Japan) 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 mmol $l^{-1}$ of $H_2O_2$ equivalents (for details, see Costantini et al., 2006; Costantini and Dell'Omo, 2006a; Costantini and Dell'Omo, 2006b).

### *Measurement of the serum anti-oxidant capacity*

The serum anti-oxidant capacity (OXY) quantifies the activity of both exogenous and endogenous anti-oxidants. It was measured by the OXY-Adsorbent test (Diacron, Grosseto, Italy). This 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 same chromogen solution used for the ROMs determination 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 mmol $l^{-1}$ of HOCl neutralised (for details, see Costantini et al., 2006; Costantini and Dell'Omo, 2006a; Costantini and Dell'Omo, 2006b).

### *Statistical analyses*

ANOVA for repeated measures (RM) was performed to evaluate the effects of the treatment on serum carotenoids, ROMs, OXY, OS (index of oxidative stress evaluated as ROMs/OXY×1000) (see Costantini et al., 2006), body mass and body condition (residuals of a linear regression of body mass on the wing length, calculated for each sampling separately). Treatment (Tr) was included as fixed factor and nest as random factor to avoid pseudoreplication. Two- and three-way interactions were included in all the models. *Post-hoc* comparisons were performed by the Tukey test (results are shown in the figures). Log- or square-root transformations were applied where appropriate. All the statistical analyses were performed with STATISTICA 6.0 (StatSoft 2001, Tulsa, OK, USA).

## Results

The treatment schedule with carotenoids caused increased levels of circulating carotenoids compared to controls as measured on day 10, 2 days after the last supplementation (Tr: $F_{1,11}$=14.88, $P$=0.003; Tr×RM: $F_{2,22}$=13.22, $P$<0.001). One week later (day 17), serum carotenoids returned to pre-treatment values and to control levels (see Fig. 2A). The change of circulating carotenoids over time was different from one nest to another (nest×RM: $F_{22,74}$=5.52, $P$<0.001). The effect of supplemental carotenoids did not shown any differences among nests (nest×Tr: $F_{11,37}$=0.92, $P$=0.53). Finally, the nest of rearing accounted for most of the carotenoid variance ($F_{11,37}$=11.32, $P$<0.001), carotenoid concentration did not vary over time in either group ($F_{2,22}$=2.50, $P$=0.11), and the interaction among nest, Tr and RM were not significant ($F_{22,74}$=1.14, $P$=0.33).

Circulating ROMs were unaffected by the treatment with surplus carotenoids (Tr: $F_{1,11}$=0.21, $P$=0.65; Tr×RM: $F_{2,22}$=0.15, $P$=0.86; Fig. 2B), and no significant nest×Tr ($F_{11,37}$=0.64, $P$=0.78) or nest×Tr×RM interaction ($F_{22,74}$=1.24, $P$=0.24) emerged. The nest of rearing accounted for most of the ROMs variance ($F_{11,37}$=2.06, $P$=0.05) and for the negative relationship between ROMs and age of chicks (RM: $F_{2,22}$=4.99, $P$=0.016; nest×RM: $F_{22,74}$=5.85, $P$<0.001).

Supplemental carotenoids did not increase OXY (Tr: $F_{1,11}$=0.28, $P$=0.61; Tr×RM: $F_{2,22}$=0.11, $P$=0.89; Fig. 2C), although a significant interaction nest×Tr×RM emerged ($F_{22,74}$=2.05, $P$=0.012). The nest of rearing significantly accounted for OXY variance (nest: $F_{11,37}$=2.79, $P$=0.009) and for the negative relationship between OXY and age of chicks (RM: $F_{2,22}$=6.53, $P$=0.006; nest×RM: $F_{22,74}$=1.86, $P$=0.026). The interaction between nest and Tr was not significant ($F_{11,37}$=0.87, $P$=0.58).

The variance in OS was explained only by the nest ($F_{11,37}$=2.22, $P$=0.035) and by the interaction between nest and RM ($F_{22,74}$=3.60, $P$<0.001), all $P$-values of the other terms being ⩾0.12.

Body mass and body condition were unaffected by the supplementation (all $P$-values ⩾0.18). Body mass showed a significant increase over time (RM: $F_{2,22}$=1369.42, $P$<0.001).

## Discussion

Circulating carotenoids in supplemented nestlings increased about 1.5-fold compared to the control and pre-treatment levels at the end of the supplementation period. Such an increase is within the physiological range of circulating carotenoids previously measured in free-living nestling or adult kestrels (Casagrande et al., 2006; Casagrande et al., 2007; Costantini et al., 2006), hence we can exclude any pharmacological effect of the administered dose.

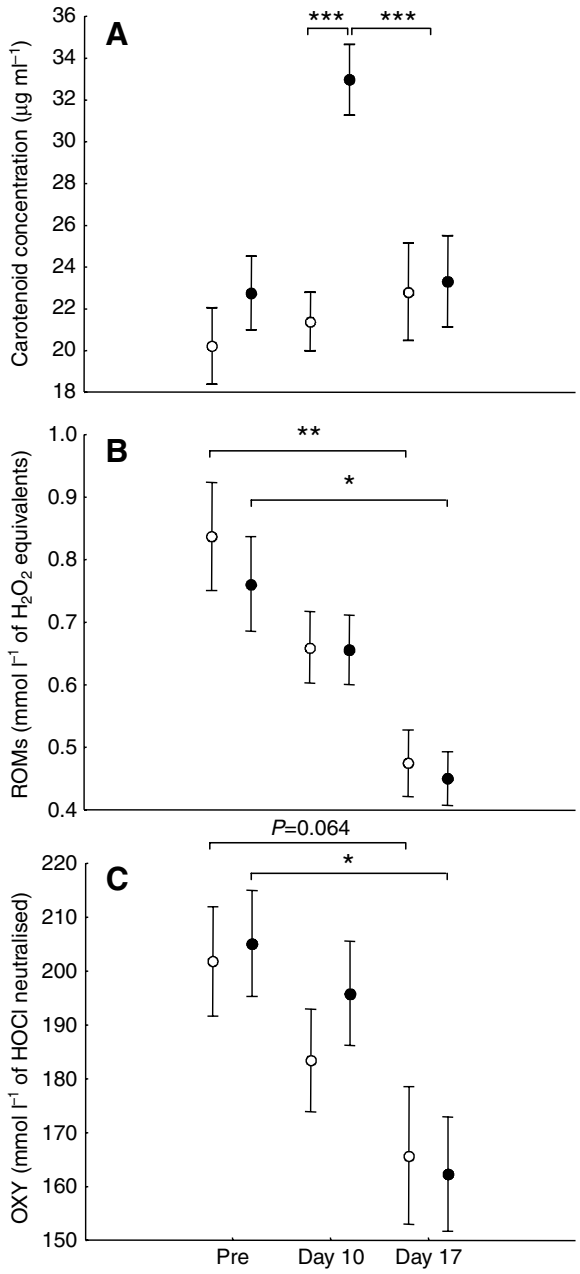


Fig. 2. The sample included 12 nests for a total of 61 nestling kestrels (open circles, controls, *N*=30; filled circles, supplemented, *N*=31). (A) Circulating carotenoids increased in supplemented nestlings, but returned to basal levels at the end of the experiment; (B) supplemental carotenoids did not affect the level of oxidative damage as measured by serum reactive oxygen metabolites (ROMs); (C) supplemental carotenoids did not increase the serum anti-oxidant capacity (OXY). Values are shown as mean ± s.e.m. Tukey *post-hoc* comparisons: **P*<0.05; ***P*<0.01; ****P*<0.001.

Baseline levels of serum carotenoids measured in the present study are lower than those in wild adult kestrels measured during the courtship phase (Casagrande et al., 2006) and are twofold higher than those measured in captive nestling American kestrels *Falco sparverius* (Negro et al., 1998). If compared to a larger dataset (25 families, eight orders) (Tella et al., 2004), baseline levels of nestling kestrels are within the physiological range of plasma carotenoids measured in 80 species, which varies from a mean concentration of 0.47 μg ml$^{-1}$ to 53.17 μg ml$^{-1}$.

It has recently been shown that carotenoids may be a limited resource for colour expression in nestling kestrels (Casagrande et al., 2007). Nestlings supplemented with different doses of carotenoids show a quick increase in circulating xanthophylls and a slow intensification of the skin colour of tarsi. The present study, using a lower dosage and a different timing of supplementation, corroborates the observation that carotenoid availability limits the circulating levels of carotenoids in nestling kestrels. Yet the significance and the effects of such limitation seem to vary largely between nests. Indeed, a large inter-nest variance in serum carotenoid concentration can be observed in kestrels (Casagrande et al., 2006; Casagrande et al., in press). Circulating carotenoids harbour a high level of environmental variance, which is mainly diet-dependent (Bortolotti et al., 2000; Casagrande et al., in press). In our study region, kestrels show consistent differences in feeding habits, even when sharing the same hunting habitat (Costantini et al., 2005). Such feeding styles, together with habitat differences in prey availability, might affect the carotenoid intake in free-living kestrels, since the carotenoid content may vary greatly amongst prey groups (e.g. Goodwin, 1984). Yet, it is suggested that such variance may also be related to a physiological constraint during the absorption in the gut (Casagrande et al., 2007). Finally, *in ovo* carotenoid exposure in chickens *Gallus gallus* is important later in life for subsequent absorption, metabolism and/or tissue deposition of diet-derived carotenoids (Koutsos et al., 2003). Further study is needed to disentangle the importance of all these components potentially underlying carotenoid variance in free-living birds.

The absence of a nest-related effect of treatment and of a treatment *per se* on both markers of oxidative stress rule out the possibility that such inter-nest variation in diet affects the levels of oxidative stress by shifting the carotenoid supplies or demands of chicks. Indeed, our study shows that carotenoids are minor anti-oxidants in nestling kestrels more than 1-week-old. Whilst carotenoids represent a limited resource for colour expression, they seem not to be a limiting factor for the maintenance of redox homeostasis. In fact, nestlings did not benefit from the increased intake of carotenoids in terms of reduction in oxidative damage and increase in serum anti-oxidant capacity. Therefore, the recent proposal that carotenoid availability might not limit the effectiveness of coping with oxidative stress in kestrels (Costantini et al., in press) is additionally supported by the present work.

It is known that maternally transferred carotenoids are useful anti-oxidants in developing embryos. It is also suggested that carotenoids are likely to be especially important during the immediate post-hatching period (Surai, 2002; Karadas et al., 2005; McGraw et al., 2005). Indeed, the hatching and early neonatal stages represent a critical period for birds in terms of oxidative stress, due to the exposure of the chick to atmospheric

oxygen, the shift to pulmonary respiration, and the increase in metabolic rate (Freeman and Vince, 1974; Vleck and Bucher, 1998). For example, circulating ROMs are negatively related to age in nestling kestrels (Costantini et al., 2006). This observation is supported by the present work. In addition, the serum anti-oxidant capacity is negatively correlated with age. In both cases, however, this pattern seems to differ amongst nests. Finally, the level of oxidative stress as defined by the balance between ROMs and OXY decreased with age, as found in a previous study (Costantini et al., 2006), but in the present study this trend was not significant. Taken together, these results suggest that younger nestlings have to cope with higher free radical production than older ones. This in turn might mediate the overexpression of the anti-oxidant response in order to balance pro-oxidants (for reviews, see Dröge, 2002; Scandalios, 2005). In contrast, nestlings about to fledge suffer from lower oxidative damage and do not need to maintain a high anti-oxidant defence.

The inter-nest differences in the relationship between age and ROMs or serum anti-oxidant capacity might reflect, to some extent, the expression of different genetic polymorphisms and differences in dietary uptake of anti-oxidants, respectively, as we recently suggested in this species (Costantini et al., 2006b; Martin et al., 1996).

Carotenoid-supplemented females of free-living lesser black-backed gulls *Larus fuscus* had eggs with lower susceptibility to lipid peroxidation, as measured by malondialdehyde (Blount et al., 2002a), and higher plasma anti-oxidant activity (Blount et al., 2002b). Lutein supplementation to captive zebra finches had no direct effect on resistance to oxidative stress, as measured by the time needed to haemolyse 50% of the red blood cells exposed to a controlled free radical attack (Alonso-Alvarez et al., 2004a). In the same experiment, however, the birds with the highest increase in plasma carotenoids (as a result of the supplementation) showed the highest resistance to the free radical attack. In a subsequent paper, the same authors suggested that carotenoid availability might modulate the trade-off between reproduction and resistance to oxidative stress in zebra finches (Bertrand et al., 2006b).

Supplementation of carotenoids to free-living hihi *Notiomystis cincta* females had no effect on retinol or tocopherol concentrations in egg yolk, but caused decreased and increased levels of α-tocopherol and retinol in nestling plasma, respectively (Ewen et al., 2006). The decrease in plasma vitamin E was related to the reduction in the requirement of this vitamin in self-maintenance because of the concomitant increased carotenoid concentration in plasma.

Taken together, these studies suggest an important role of carotenoids as anti-oxidants in birds. However, recent studies, together with the present work, challenge this view. All these studies were carried out on nestlings older than 1 week or adult birds of several species with different life histories, and no relationships between carotenoids and several markers of oxidative stress were found. For example, circulating carotenoids were not correlated with either oxidative damage or anti-oxidant capacity in nestling kestrels (Costantini et al., 2006; Costantini and Dell'Omo, 2006a), nor to the anti-oxidant capacity in male greenfinches (Hõrak et al., 2006) or in breeding female great tits (Tummeleht et al., 2006). In our case, the absence of a relationship between circulating carotenoids and serum anti-oxidant capacity is not dependent on the method used since it is widely demonstrated that HOCl reacts with carotenoids (Handelman et al., 1991; Siems et al., 2000).

These results suggest that the systemic anti-oxidant capacity of carotenoids in birds (except for well-established protective effects on embryos and hatchlings) may not appear as important as previously thought. However, it could be argued that the activity of carotenoids in a specific tissue is not reflected at the systemic level, so that the anti-oxidant capacity in the blood is not affected. It is known that tissues may show differences in anti-oxidant distribution or susceptibility to oxidative stress (Surai et al., 1996; Surai, 1999; Karadas et al., 2005). It is also known that the relevance of a specific anti-oxidant can vary from one tissue to another. For example, zeaxanthin supplementation reduced lipid peroxidation in the liver but not in plasma, heart or pectoral skeletal muscle in male 1-day-old Leghorn chicks *Gallus gallus* (Woodall et al., 1996). Instead, canthaxanthin supplementation did not influence susceptibility to oxidative stress in any tissue examined. In addition, some anti-oxidants show a high variation across the avian orders (e.g. carotenoids) (Olson and Owens, 2005), whilst others can be synthesised or obtained by diet in a species-specific way (e.g. ascorbic acid) (Del Rio, 1997).

In the studies on oxidative stress, the recommendation is to use more than a single oxidative biochemical marker in order to get a better insight into such a complex system. Indeed, we used two different markers (ROMs and OXY), which are known to appropriately reflect oxidative stress in birds (e.g. Costantini and Dell'Omo, 2006a) and mammals (e.g. Brambilla et al., 2001). Therefore, we are confident that carotenoid availability does not limit the capability to regulate redox homeostasis in our model species.

It could be that the importance of carotenoids as anti-oxidants varies according to the species or to the specific life cycle phase investigated. For example, carotenoids may be limiting in other model systems with lower natural blood carotenoid levels than kestrels. It should also be noted that the methods used sometimes vary from one study to another, which may make comparison of the results difficult. This is because the specificity of the method to be used or its underlying biochemical rationale can be very different. For example, shortcomings can emerge in the quantification of malondialdehyde to assess lipid peroxidation in complex biological fluids or tissues because of its non-specificity (Chirico, 1994).

Finally, supplemental carotenoids did not affect body mass or body condition (i.e. energy stores). Previous studies with carotenoid supplementation to avian species (i) could not find any effect on body mass [e.g. Leghorn chicks supplemented with 100 mg $kg^{-1}$ diet (Woodall et al., 1996); zebra finches supplemented with 12.5–200 μg $ml^{-1}$ of drinking water

(Alonso-Alvarez et al., 2004a); kestrel nestlings supplemented with different doses (Casagrande et al., 2007)], (ii) found a gain of mass in zebra finches supplemented with 100 μg $ml^{-1}$ of drinking water (Bertrand et al., 2006b), or (iii) found an immediate loss of mass in captive kestrels following a supplementation of 8 mg of carotenoids/day in a 4-week study (Costantini et al., in press). This latter result was perhaps related to the high intake of carotenoids that caused the dysfunction of liver or kidney caused by pervasive oxidative damage, and secondarily to peroxidation of fat reserves.

In conclusion, our results show that the capability of wild nestling kestrels to cope with oxidative stress is not limited by carotenoid availability in the diet. This finding supports previous studies suggesting that the anti-oxidant activity of carotenoids in nestling and adult birds has been exaggerated.

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