

Patterns of serum carotenoid accumulation and skin colour variation in kestrel nestlings in relation to breeding conditions and different terms of carotenoid supplementation

Stefania Casagrande · David Costantini ·
Alberto Fanfani · James Tagliavini ·
Giacomo Dell’Omo

Received: 9 May 2006 / Revised: 3 October 2006 / Accepted: 5 October 2006 / Published online: 8 November 2006
© Springer-Verlag 2006

Abstract Carotenoids are pigments synthesised by autotrophic organisms. For nestlings of raptorial species, which obtain carotenoids from the consumption of other heterotrophic species, the access to these pigments can be crucial. Carotenoids, indeed, have fundamental health maintenance functions, especially important in developing individuals as nestling kestrels. The aim of this study was to investigate how body carotenoid levels and skin pigmentation vary in kestrel nestlings (*Falco tinnunculus*) in relation to nesting parameters. Furthermore, we experimentally altered carotenoid availability (short- medium- and long-term) for nestlings and investigated skin and serum variance.

The skin colour variance of 151 nestlings was explained by nest of origin, age and by the body condition (body mass corrected by age), older nestlings with higher body condition being redder. No difference in skin colour was detected between sexes. Differences in hue (skin “redness”) between treatments did not emerge during the first week, but did occur 15 days after administration between long-term supplemented and control chicks. In contrast, the serum carotenoid concentration showed a treatment-dependent increase after 5 days from the first carotenoid administration and at least after two supplemented feedings. In general, hue but not serum carotenoids, was correlated with the body condition of nestlings. Based on the increased skin pigmentation of nestling kestrels in the long-term experimental group, we suggest carotenoid availability to be limited for colour expression. The small increase of serum carotenoids due to supplementation is consistent with the hypothesis that there is a physiological constraint on these pigments, as well as an environmental limitation. The presented results are useful for the understanding of carotenoid uptake and accumulation by a wild raptorial species, located at the top of the food web, highlighting that carotenoids are a limited resource for kestrel nestlings.

Communicated by G. Heldmaier.

S. Casagrande
Department of Behavioural Biology,
University of Groningen, Kerklaan 30, 9751NN,
Haren, Groningen, The Netherlands

S. Casagrande (✉) · J. Tagliavini
Dipartimento di Biologia Evolutiva e Funzionale,
Università di Parma, Parco Area delle Scienze 11/A,
43100 Parma, Italy
e-mail: casagrande@biol.unipr.it

D. Costantini · A. Fanfani
Dipartimento di Biologia Animale e dell’Uomo,
Università La Sapienza, Viale Università 32,
00185 Rome, Italy

D. Costantini
Dipartimento dell’Ambiente e Prevenzione Primaria,
Unità di Chimica Tossicologica, Istituto Superiore di Sanità,
Viale Regina Elena 299, 00161 Rome, Italy

G. Dell’Omo
Ornis italica, Piazza Crati 15, 00199 Rome, Italy

Introduction

The brilliant yellowish-red colouration produced by carotenoids in the skin and feathers of birds is the result of a trade-off between the storage of these pigments and their utilization. In fact, carotenoids are used to deal with negative physiological conditions, such as immune system activation (Blount et al. 2003;

Faivre et al. 2003; Kilpimaa et al. 2004), bad nutritional status (Hill 2000), endoparasites (Brawner et al. 2000; Hōrak et al. 2001), and bacterial infection (Fenoglio et al. 2004).

Nestlings, particularly, need carotenoids because they have an undeveloped immune system and they can experience undernourishment due to hatching asynchrony or low-quality parental care (Hōrak et al. 2000; Fitze et al. 2003). During development nestlings face up to high rates of body growth and cell proliferation with a consequent exposition to free radicals action (Surai et al. 2001), although in kestrel nestlings serum carotenoid concentration has not shown any relationship with pro-oxidants, antioxidants or degree of oxidative stress (Costantini et al. 2006; Costantini and Dell’Omo 2006).

Birds, like all vertebrates (e.g. Costantini et al. 2005a), acquire carotenoids exclusively from the diet because they are unable to synthesise them *de novo* (Brush 1990). However, the environmental availability of these pigments is thought to be limited and only skilled foragers can be conspicuously coloured (carotenoid-limitation hypothesis, Endler 1983; Hill 1992; Blount et al. 2004). Consequently, the expression of carotenoid-based colouration is also limited by the dietary intake, especially for birds at the top of the food web, such as exclusive carnivores like raptors (Tella et al. 2004). Most studies analysing the administration and accumulation of carotenoids and the effects on colour expression in birds have been conducted in captivity (Hill 1992, 2000; McGraw et al. 2003; Navara and Hill 2003; Alonso-Alvarez et al. 2004). The few studies carried out in the field did not manipulate the access to carotenoid sources (Hill et al. 2002), except three cases in which the variation of plumage colour in the great tit *Parus major* (Fitze et al. 2003; Tschirren et al. 2003) and blue tit *P. caeruleus* (Biard et al. 2005) were considered. Moreover, no study has used an experimental approach to evaluate the variation of skin colouration in the wild.

In this study, we tested the effects of supplemental carotenoids on both blood carotenoid concentration and carotenoid-based skin colouration in wild Eurasian kestrel nestlings (*Falco tinnunculus*) in the Mediterranean region. It is known from previous studies that kestrels absorb only two xanthophylls (i.e. oxygenated carotenoids), lutein (~90% of the total xanthophylls) and zeaxanthin, which are deposited in the bare parts (skin of lores, cere and tarsi) without any transformation (Casagrande et al. 2006) and producing a yellow–orange colouration. In northern Europe, kestrels feed mainly on small mammals (Korpimäki 1986), which are a poor source of xanthophylls (Bortolotti et al. 2000).

Therefore, the birds are likely to have low levels of carotenoids in their body. In the few areas of Italy with subcontinental climatic conditions, the kestrel’s preferred prey are still voles and mice (Casagrande, data unpublished). However, in more typical Mediterranean areas, the diet is much wider and includes lizards, small birds and many insects, which are a better source of carotenoids (Olson and Owens 1998; Costantini et al. 2005b). Therefore, this potentially high availability of pigments allows us to investigate the carotenoid-limitation hypothesis under less constrictive circumstances in terms of bioavailability of carotenoids in the diet. Regardless of whether carotenoid-based skin colouration in nestlings does or does not play a role in intra-specific communication, the study of carotenoid variation in young kestrels can lead to a better understanding of the carotenoid-based signal expressed by adults, since the intensity of the colouration can be permanently influenced by growth conditions later in life, as the beak colour of the zebra finch (McGraw et al. 2005).

The aims of this study were (1) to analyse the natural variation of serum carotenoids and carotenoid-based skin colouration in kestrel nestlings in relation to age, sex, body condition, laying date, brood size, and nest of origin; (2) to carry out a dietary supplementation study with xanthophylls to determine the effects of short- versus medium- and long-term administration of xanthophylls uptake and recovery in serum and skin during the treatment and after the end of the supplementation. If environmental carotenoid availability is limited, we expect to find differences in body carotenoid levels between treatment groups.

Materials and methods

Study area

The study was carried out near Rome, central Italy, in an area of about 1,200 km² characterized mainly by cultivated and set-aside fields. The cultivations included olive plantations, vineyards and cereal fields, whereas the set-asides were dry pastures interspersed with typical Mediterranean scrub vegetation. Chicks hatched in nest-boxes mounted on the utility lines of two local electric power companies. The study encompassed three successive breeding seasons and included two phases: in the first phase (2002), we quantified the natural variability of serum xanthophylls in relation to various factors in a large sample of chicks during their development; in the second phase (2003–2004), we carried out a dose-response study to evaluate the time

course of the effects of supplemented xanthophylls on serum carotenoid levels and skin colour.

Natural variation of skin and serum xanthophyll levels

The first study was conducted during the 2002 breeding season. Starting from March, the nest-boxes were visited every 2 weeks to assess occupation and to collect breeding and reproductive data. When nestlings were 5–31 days old, a 0.4 ml blood sample was taken from the brachial vein, kept cool (0–5°C) for a few hours, centrifuged, and the serum stored at –20°C until the laboratory analyses. At the time of blood sampling, measurements of wing length (chord, nearest 1 mm) and body mass (nearest 1 g) were also taken. Hatching dates were determined by visits to the nests in the perihatching period and were supported by growth curves (error: ± 1 day). The wing length was used as an indicator of age. For body condition analysis we considered that, since the covariation between the dependent variable and each single covariate is corrected for the covariation with all the others, the covariation with body mass actually mirrors that with body condition, that is body mass corrected for age-differences (see García-Berthou 2001). For graphical representation of the body condition index we used the residuals of a regression of body mass on the cube of the wing chord ($r = 0.71$, $F_{1,158} = 158.31$, $P < 0.001$).

The skin colour of the right tarsus was measured with a hand-held spectrophotometer operating at wavelengths of 400–700 nm (Oracolor Corob, Modena). The colour was quantified by the hue value (Hörak et al. 2001) and since the spectrophotometer assigns lower hue value to redder skin we consider the inverse of the original value. We took three consecutive measures per individual and, after checking for repeatability (hue: $r = 0.80$, $P < 0.001$; Lessels and Boag 1987), we used the mean value for the data analysis. The data obtained with the spectrophotometer were analysed with Corob Quality 1.0 software (Corob, Modena). The skin of the tarsus was cleaned with water and air-dried before the measurements.

Dose-response study with xanthophylls

The second study was conducted during the 2003–2004 breeding seasons. The experimental broods varied from four to six chicks as any effect of brood size on carotenoid-based colouration or on blood carotenoids concentration was observed during the study conducted in 2002. In particular, there were seven clutches of four, three of five and one with six chicks

for a total of 49 chicks. Only four chicks of each brood were randomly assigned to one of four treatments, namely controls (0 mg, $n = 11$), short-term (36 mg/subject, $n = 11$), medium-term (36 mg/subject given twice on alternate days, $n = 11$) and long-term (36 mg/subject given three times every other day, $n = 11$). Starting when chicks were 1 week old (weight 135.12 ± 5.25 g), they were supplemented three times every other day with 1-day-old dead laboratory mice containing either 200 mg of corn oil with 20% lutein and zeaxanthin in a 9:1 ratio (corresponding to a dose of about 265 mg of carotenoids/kg, Kemin Foods L.C., FloraGlo Lutein, Des Moines, Iowa) or corn oil alone. These doses were selected on the basis of the available information about animals body xanthophylls content (Czczuga 1978, 1979; Casagrande et al. 2006). To avoid photo-oxidation of the carotenoids, we filled the mice with the corn oil in a dark room and kept them cool in a refrigerated bag until they were given to the chicks (a few hours after their preparation).

On day 1, the chicks were bled (0.2 ml), measured (wing length and body mass) and the skin colour of the tarsus was measured with the portable spectrophotometer. Then, they were fed with a 1-day-old mouse containing either no xanthophylls (controls) or 36 mg xanthophylls (all the other treatment groups). On day 3, the chicks underwent the same bleeding and measurement procedures except that short-term supplemented chicks, like controls, received no more xanthophylls in the mice. On day 5, only the long-term supplemented chicks received xanthophylls whereas all the other groups received mice with corn oil. All chicks were bled and measured again on day 7 and 16, without receiving any xanthophylls.

Laboratory analyses

Carotenoids were quantified with a Beckman DU 7400 spectrophotometer at 476 nm (Kemin Foods L.C., Des Moines, Iowa). The serum was diluted with methanol (1:8) and the flocculent proteins were precipitated by centrifugation to obtain the serum. The xanthophyll concentration was estimated as $\mu\text{g ml}^{-1}$ of serum using the standard absorbance curve of lutein (alpha-carotene-3,3'-diol; Sigma–Aldrich), the predominant carotenoid in kestrels (Bortolotti et al. 2000; Casagrande et al. 2006). The sex of the nestlings was determined by amplification of the CHDW and CHDZ genes (Fridolfsson and Ellegren 1999).

All procedures performed in this study were consistent with the European directive for the protection of vertebrate animals used for experimental and

other scientific purposes (86/609/EEC). The study was approved by the Istituto Superiore di Sanità (Approval no.: Fasc. 43 /1994 ISS and subsequent updates).

Data analysis

The data were analysed with the STATISTICA package (Version 5.1, StatSoft, '97, Padua, Italy). We used a mixed model ANCOVA to determine which factors accounted for the variation in skin hue and serum carotenoid concentration. We considered brood size as fixed factor and laying date, body condition and age as covariates; to avoid pseudoreplication, nest was included as random factor (Hulbert 1984). To analyse the effect of xanthophyll provisioning, we used a mixed model ANOVA for repeated measures, considering treatment as fixed factor and nest as random factor. A mixed model ANOVA was used to evaluate the effect of the year on the dependent variables considered in the experiment (skin hue, xanthophyll concentration and body condition), with year as fixed factor and nest as random factor. We used the Mann–Whitney *U* Test to compare hue (samples of 2002) and carotenoid concentration (samples of 2003) between male and female chicks. Normality was checked with the Kolmogorov–Smirnov test. Values of parametric results are shown as mean (+se), while non-parametric results are shown as median with upper and lower quartiles.

Results

Natural variability of serum xanthophylls and skin colour

Data were collected for 151 nestlings from 38 broods, specifically 11 with 3, 17 with 4 and 10 with 5 chicks. The skin colour variance was explained by the nest of origin (Table 1), the age and the body condition, older nestlings and with a higher index being redder

Table 1 Full model with the factors explaining skin hue variation in nestling kestrels (38 nests, 151 nestlings)

Factors	<i>df</i>	<i>F</i>	<i>P</i> -values
Laying date	1, 33.66	0.16	0.69
Age	1, 37.67	25.95	<0.0001
Body mass	1, 43.19	12.83	<0.001
Xanthophyll concentration	1, 48.44	0.71	0.41
Brood size	2, 34.16	0.32	0.73
Nest (random factor)	35, 109	5.14	<0.0001

Statistically significant *P*-values are shown in bold type

(Table 1, Fig. 1). The hue did not covary with the amount of xanthophylls detected in the serum. There was no sex difference in skin colour (Table 2) ($U = 0.64$, $n = 16$, $p > 0.05$). The xanthophyll variation in serum was explained only by the nest (Table 3). Again, males and females had similar serum xanthophyll concentrations ($U = 1,854.5$, $n = 134$, $P > 0.05$) (Table 2).

Experimental study

Data were collected from 44 nestlings from 11 nest-boxes. In the measurements taken immediately before treatment, the individuals assigned to different treatments did not show any difference in skin colour (One-way Anova: $F_{3,40} = 0.23$, $P = 0.87$), serum carotenoid

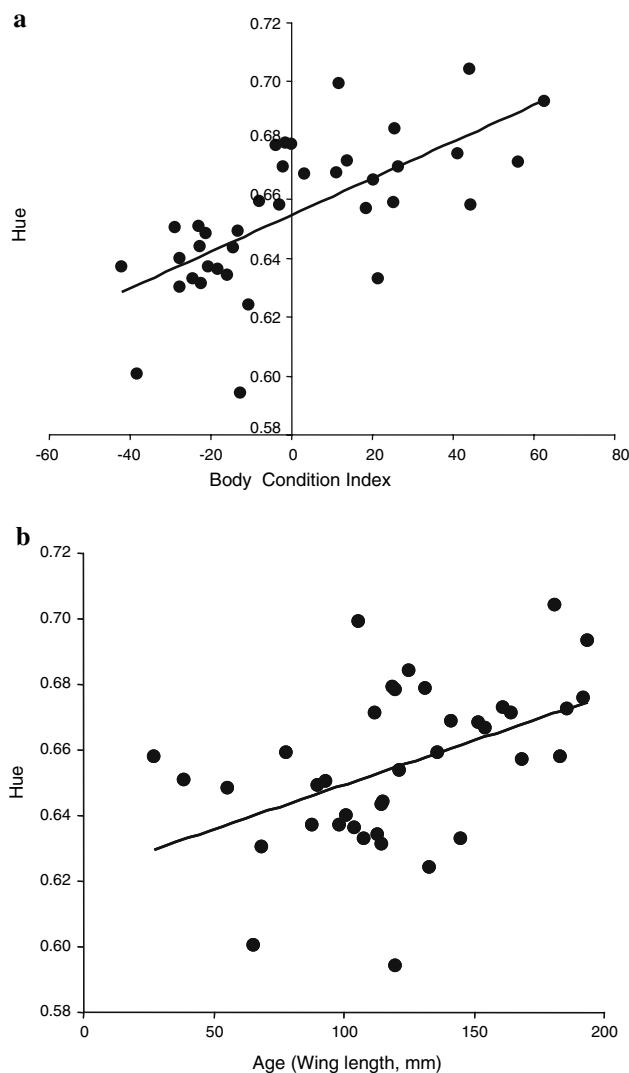


Fig. 1 Relationship between skin colour hue and **a** body condition index and **b** age as expressed by the wing chord. Mean nest values ($n = 38$) are presented to avoid pseudoreplication

Table 2 Tarsal skin hue values in young and adult kestrels

	Males	Females
Hue		
Young	0.65, 0.66, 0.67	0.65, 0.66, 0.66
Adults ^a	0.81, 0.82, 0.85	0.76, 0.77, 0.77
Blood xanthophylls ($\mu\text{g ml}^{-1}$)		
Young	14.36, 18.58, 22.21	15.38, 20.65, 27.99
Adults ^a	25.10, 34.78, 46.47	21.64, 32.51, 40.94

The three values represent, respectively, the lower quartile, median and upper quartile

^a Reported in Casagrande et al. (2006)

Table 3 Full model with the factors explaining serum xanthophyll variation in nestling kestrels (38 nests, 151 nestlings)

Variables	df	F	P-values
Laying date	1, 32.31	0.14	0.71
Age	1, 49.63	1.05	0.31
Body mass	1, 62.72	0.23	0.63
Skin hue	1, 52.47	0.76	0.39
Brood size	2, 33.36	0.13	0.88
Nest (random factor)	35, 109	2.68	<0.0001

Statistically significant P-values are shown in bold type

concentration (One-way Anova: $F_{3,31} = 0.04$, $P = 0.99$) or body condition (One-way Anova: $F_{3,40} = 0.02$, $P = 1.00$; Fig. 2a–c). Moreover, there was no year effect on hue ($F_{1,6,29} = 3.56$, $P = 0.11$), xanthophyll concentration ($F_{1,6,75} = 0.27$, $P = 0.62$) or body condition ($F_{1,6,84} = 0.001$, $P = 0.97$). Therefore, in the subsequent analyses, we pooled the data collected in 2003 and 2004.

Carotenoid-based colouration

Skin hue significantly increased from day 1 (PRE) to day 16, independently of the treatment (Table 4; Fig. 2a). The treatment alone did not explain the change in skin colour of the nestlings, as there was an effect of the supplementation term (Fig. 2a, Table 4). The hue value did not vary significantly from PRE to day 7 (Fig. 2a), and no differences between treatment groups emerged in this period (all $P > 0.05$, LSD Fisher post hoc test). The difference among treatments became evident only on day 16, nine days after the last xanthophyll administration, with long-term supplemented chicks having a higher hue than the control ($P < 0.05$, LSD Fisher post hoc test) and short-term supplemented chicks ($P < 0.05$, LSD Fisher post hoc test) (Fig. 2a).

Serum xanthophyll concentration

In this analysis, two nests were excluded because it was not possible to bleed the nestlings. In contrast to skin

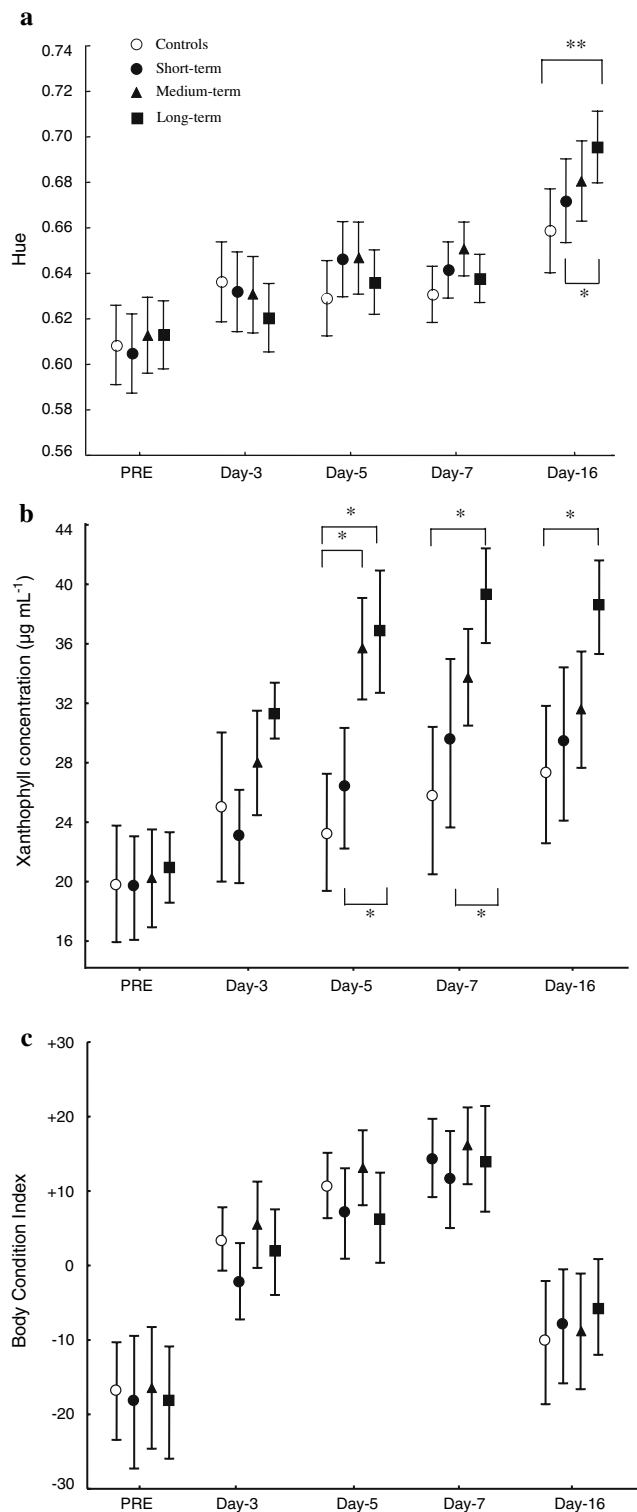


Fig. 2 Time course of the variation of skin hue (a), blood carotenoid level (b) and body condition index (c) in kestrel nestlings supplemented with xanthophylls. * $P < 0.05$; ** $P < 0.01$, LSD Fisher post hoc test. PRE refers to measures made before treatment

colour, serum carotenoid levels showed the effects of xanthophyll treatment already during the supplementation, i.e. on day 5: the long- and medium-term

Table 4 Sources of variation of the three dependent variables considered in the dose-response treatment: skin colour, xanthophyll concentration and body condition

Dependent variable	Source of variation	df	F	P-values
Skin colour	Treatment	3,30	1.41	0.26
	Time	4,40	58.45	<0.001
	Treatment × time	12,120	3.01	<0.001
Xanthophylls	Treatment	3,24	5.16	<0.001
	Time	4,32	10.24	<0.001
	Treatment × time	12,96	1.74	0.07
Body condition	Treatment	3,30	0.65	0.59
	Time	4,40	7.74	<0.001
	Treatment × time	12,120	0.59	0.85

supplemented nestlings became richer in xanthophylls than the control and short-treatment chicks (controls vs. long-term supplemented, controls vs. medium-term, short-term vs. long-term supplemented; all $P < 0.05$ LSD, Fisher post hoc test) (Fig. 2b, Table 4). On day 7, the long-term supplemented chicks had a higher carotenoid concentration than the control and short-term supplemented nestlings ($P < 0.05$, LSD Fisher post hoc test), while the difference between the medium-term and control chicks disappeared ($P > 0.05$, LSD Fisher post hoc test) (Fig. 2b). On day 16, all differences disappeared, except control vs. long-term supplemented chicks ($P < 0.05$, LSD Fisher post hoc test), since the latter still had high carotenoid levels in the blood (Fig. 2b). There were no interactions between treatment and the other factors considered, except for a marginal time-dose interaction (Table 4).

Body condition

We observed a large variation in the body condition until day 7 and then a decrease on day 16 (Table 4; Fig. 2c). This variation was not affected by the xanthophyll supplementation nor did it show a time-treatment interaction (Table 4, Fig. 2c).

Discussion

There is good evidence that the carotenoid-based colouration of nestlings is influenced by their condition, expressed by body mass (Johnsen et al. 2003; Tschirren et al. 2003). The field assessment we conducted in 2002 showed that the carotenoid-based redness of the bare skin was higher in nestlings with better condition, while there was no relationship between the blood xanthophyll concentration and body condition. Condition-dependent physiological constraints may cause differences in plumage colouration by affecting the

ability to incorporate carotenoids. Due to carotenoids positive impact on the immune system and condition, studies have suggested that it might be costly to incorporate them in the plumage (Hill 2000). So far, little has been done on skin pigmentation, but as our data suggests also skin pigmentation seem to reveal individual condition. Thus, a possible trade-off or cost between skin deposition and usage might be applicable, and also used as an honest indicator, e.g. of the ability of the bearer to find the “resource” during foraging, or to allocate carotenoids to signalling functions instead of shunting them to health maintaining activity, or to store the pigment despite their detrimental effects (review in Olson and Owens 1998 and in Møller et al. 2000). This, as well as the role played by carotenoids in the health maintaining process might explain the lack of relationship between blood carotenoids and carotenoid-based colouration.

We found also that nestlings became more coloured with the increasing of age. We can speculate that this can be a consequence of maturation of absorption and deposition processes, or a consequence of longer time of accumulation. Alternatively, the skin colour of older nestlings could mirror, again, the condition of the individual, as immune system generally improve during ontogenesis (Hart 1997), but further investigations are needed.

We did not find any sex difference in skin colour or serum carotenoids in our nestlings. In general, the skin of adult males is redder than females (Casagrande et al. 2006; see also Negro et al. 1998 for the American kestrel). However, differences in the expression of carotenoid-based colouration between nestlings and adults could be due to sexual maturation and to an involvement of sex hormones in the modulation of carotenoid tissue deposition (Witschi 1961; Bjerkeneg et al. 1999).

The differences among treatment groups in skin colour and blood xanthophylls suggest that we tested an appropriate range of xanthophyll doses and time periods. The fixed dose administered during each treatment was about 10–30 times higher than a dose assumed daily in the wild. We decided to administer a high dose every other day instead of a lower dose every day to lower nestlings stress due to manipulation. As carotenoids absorption depends on many factors as, e.g. the lipid food content, the physical and physiological conditions of the bearer or the degree of carotenoid esterification, we hypothesised that the percentage of carotenoid absorption could not be very high. Indeed, the increase of serum carotenoids in treated birds was in accordance with a dietary increase of pigments. We have not even observed detrimental or beneficial

effects on body condition or vitality of treated birds compared to controls, which let us to exclude a pharmacological rather than a dietary effect of the administered dose (Klasing 1998; Alves-Rodrigues and Shao 2004).

The expression of blood xanthophylls and hue showed a different time course after the treatment. Xanthophylls are absorbed in the gut and pass relatively rapidly into the blood. An increase in the blood levels was recorded already 96 h after treatment, probably as a result of a release of carotenoids stored in the liver (Surai 2002). Previous dietary supplementation experiments in wild birds have shown an increase in plasma carotenoid concentration (Blount et al. 2002; Bortolotti et al. 2003), while others failed to show such an increase (Biard et al. 2005, 2006). This may be explained by the fact that plasma carotenoid level is regulated by carotenoid release from the liver and other storage tissues other than blood. By contrast, changes in hue values emerged only after 2 weeks of treatment. After the same period, the differences in blood carotenoids disappeared, indicating a quick reduction of these pigments after involvement in physiological, storing or eliminating processes.

The nestlings in the xanthophyll-supplemented groups generally developed skin colouration in accordance to the term of dietary xanthophyll supply. This indicates that the natural carotenoid-based colouration of the nestlings was below that achievable, providing indirect support for the carotenoid-limitation hypothesis and suggesting that the assimilation of carotenoids depends on individual foraging skill (Endler 1983; Hill 1992; Blount et al. 2004). However, despite the high dose of xanthophylls provisioned to the chicks, the blood carotenoid level did not increase to the same degree. These findings suggest that there could be a limitation of the ability to absorb xanthophylls. Therefore, the concept of “limited resource”, referring to the availability of these pigments to the animals, could partly reside in a differential efficiency of their absorption in the gut. Thus, a resource could be “limited” not only environmentally but also physiologically (see McGraw 2005 for a link among dietary carotenoid assimilation, colour ornamentation and phylogeny). It is known, for example, that xanthophylls need specific carriers that must be activated at the gut level before they can pass from the digestive tract into the circulation (Brush 1990). Differences in the concentrations or affinities of the molecules that take up carotenoids from food (e.g. micelles, chylomicrons) or that transport xanthophylls through the circulatory system to different tissues (e.g. lipoproteins) could also explain some of the inter-individual variation in blood carotenoids

and colour expression (Allen 1987; Mossab et al. 2001; McGraw and Parker 2006).

The hue values recorded in the long-term supplemented nestlings at the end of the experiments were lower than those measured in previous studies on wild adult kestrels (see Table 2; Casagrande et al. 2006), despite the large amount of xanthophylls ingested during the experiment. This suggests that there is still room for a further increase in the redness that can be achieved by an individual and also that a longer period might be needed for the pigments to produce an intense skin colouration. Unfortunately, we could not measure the hue after day 16 because the nestlings fledged a few days after the last sampling. Whether a maturation process is involved in the final colour expression is a topic requiring further investigation. It was recently shown that, above a certain carotenoid intake, there can be a dissociation between the circulating carotenoid levels and colour, and that both traits are limited by a physiological threshold (Alonso-Alvarez et al. 2004). In our case, even though the quantity administered was high, it did not allow us to evaluate the levels at which serum carotenoid concentration and colour reach their physiological plateau.

Furthermore, the differences between the values of long-term supplemented nestlings and the values measured previously in wild adult kestrels are consistent also with the hypothesis of a physiological limitation on the amount of carotenoids nestlings can allocate to colour their skin. Support for this “intrinsic limitation of colour expression”, and for the idea that chicks do not allocate carotenoids to signal production, comes from the observation that skin colouration probably does not yet have a communicative significance at this stage of their life. Hence, carotenoids can be allocated to other tissues and shunted for other physiological needs.

The decrease of the body condition observed on day 16 could have been due to the natural loss of weight that characterises nestlings close to fledging (e.g. Dijkstra et al. 1990, unpublished results). In fact, extra carotenoids supplied to other bird species did not affect body condition. However, it cannot be excluded the possibility that they can have a detrimental effect on the individual (e.g. El-Agamey et al. 2004; Costantini et al., submitted).

In conclusion, our experiment showed that only skin colour and not serum carotenoid concentration was correlated with the body condition of nestlings. The provisioning of nestlings with extra xanthophylls caused an increase in carotenoid-based colouration intensity, indicating that the hue value of skin colouration of non-provisioned birds was below the

maximum achievable and that the carotenoid content of food can be considered a limited resource for skin colour production. The limited increase of serum carotenoids compared to the amount supplied is consistent with the possibility that there is a physiological constraint on these pigments, as well as an environmental limitation.

Acknowledgments We thank two anonymous reviewers for offering insightful comments to the manuscript increasing its quality. We are also grateful to G. Di Lieto and F. Costantini for help with the data collection and to ENEL-TERNA and ACEA, which allowed the monitoring of the nest-boxes on their utility lines. In particular, we are thankful to G. Cavallari, N. Landini and A. Olivieri from ENEL, and to G. La Catena, G. Noia and C. Puliti from ACEA. Particular thanks go to E. Sodo (Kemin Food L.C.) for providing the carotenoids free of charge and to F. Chiarelli for statistical advice. P.W. Christie for improving the English. *Ornis italica*, an ornithological scientific association, sponsored part of the field work. D. Costantini was supported by a Ph.D. fellowship from the University of Rome La Sapienza.

References

- Allen PC (1987) Effect of *Eimeria acervulina* infection on chick (*Gallus domesticus*) high density lipoprotein composition. *Comp Biochem Physiol* 87B:313–319
- 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
- Alves-Rodrigues A, Shao A (2004) The science behind lutein. *Toxicol Lett* 150:57–83
- Biard C, Surai PF, Møller AP (2005) Effects of carotenoids availability during laying on reproduction in the blue tit. *Oecologia* 144:32–44
- Biard C, Surai PF, Møller AP (2006) Carotenoid availability in diet and phenotype of blue and great tit nestlings. *J Exp Zool* 209:1004–1015
- Bjerkeng B, Johnsen K, Mayer I, Storebakken T, Nilssen KJ (1999) Influence of 11-ketotestosterone, 17-estradiol, and 3,5,30-triiodo-L-thyronine on distribution and metabolism of carotenoids in Arctic charr, *Salvelinus alpinus* L. *Fish Physiol Biochem* 21:353–364
- Blount JD, Surai PF, Nager RG, Houston DC, Møller AP, Trewhy ML, Kennedy MW (2002) Carotenoids and egg quality in the lesser black-backed gull (*Larus fuscus*): a supplemental feeding study of maternal effects. *Proc R Soc Lond B* 269:29–36
- Blount JD, Metcalf NB, Birkhead R, Surai PF (2003) Carotenoid modulation of immune function and sexual attractiveness in zebra finches. *Science* 300:125–127
- Blount JD, Houston DC, Surai PF, Møller AP (2004) Egg-laying capacity is limited by carotenoid pigment availability in wild gulls *Larus fuscus*. *Proc R Soc Lond B* 271(Suppl):S79–S81
- Bortolotti GR, Tella JL, Forero MG, Dawson RD, Negro JJ (2000) Genetics, local environment and health as factors influencing serum carotenoids in wild American kestrels (*F. sparverius*). *Proc R Soc Lond B* 267:1433–1438
- Bortolotti GR, Negro JJ, Surai PF, Prieto P (2003) Carotenoids in eggs and plasma of red-legged partridge: effects of diet and reproductive output. *Physiol Biochem Zool* 76:367–374
- Brawner III WR, Hill GE, Sundermann CA (2000) Effects of coccidial and mycoplasmal infections on carotenoid-based plumage pigmentation in male house finches. *Auk* 117:952–963
- 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
- Costantini D, Dell’Omo G, Casagrande S, Fabiani A, Carosi M, Bertacche V, Marquez C, Snell H, Snell H, Tapia W, Gentile G (2005a) Inter-population variation of carotenoids in Galápagos land iguanas (*Conolophus subcristatus*). *Comp Biochem Physiol B* 142:239–244
- Costantini D, Casagrande S, Di Lieto G, Fanfani A, Dell’Omo G (2005b) Consistent differences in feeding habits between neighbouring breeding kestrels. *Behaviour* 142:1409–1421
- 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* 176:329–337
- Costantini D, Dell’Omo G (2006) Effects of T-cell-mediated immune response on avian oxidative stress. *Comp Biochem Physiol A* 145:137–142
- Czeczuga B (1978) Carotenoids in the skin of certain species of birds. *Comp Biochem Physiol* 6B:107–109
- Czeczuga B (1979) Carotenoids in some parts of certain species of lizards. *Comp Biochem Physiol* 65B:755–757
- Dijkstra C, Bult A, Bijlsma S, Daan S, Meijer T, Zijlstra M (1990) Brood size manipulations in the kestrel *Falco tinnunculus*: effects on offspring and parental survival. *J Anim Ecol* 48:158–174
- 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
- Endler JA (1983) Natural and sexual selection on colour patterns in poeciliid fishes. *Environ Biol Fishes* 9:173–190
- Faivre B, Grégoire A, Prévault M, Cézilly F, Sorci S (2003) Immune activation rapidly mirrored in a secondary sexual trait. *Science* 300:103
- Fenoglio S, Cucco M, Fracchia L, Martinetti MG, Malacarne G (2004) Shield colours of the moorhen are differently related to bacteria presence and health parameters. *Ethol Ecol Evol* 16:171–180
- Fitze PS, Tschirren B, Richner H (2003) Carotenoid-based colour expression is determined early in nestling life. *Oecologia* 137:148–152
- Fridolfsson AK, Ellegren H (1999) A simple and universal method for molecular sexing of non-ratite birds. *J Avian Biol* 30:116–121
- García-Berthou E (2001) On the misuse of residuals in ecology: testing regression residuals vs. the analysis of covariance. *J Anim Ecol* 70:708–711
- Hart BJ (1997) Behavioural defence. In: Clayton DH, Moore J (eds) Host-parasite evolution: general principles and avian models. Oxford University Press, Oxford
- Hill GE (1992) Proximate basis of variation in carotenoid pigmentation in male house finches. *Auk* 109:1–12
- Hill GE (2000) Energetic constraints on expression of carotenoid-based plumage coloration. *J Avian Biol* 31:559–566
- Hill GE, Inouye CY, Montgomerie R (2002) Dietary carotenoid predict plumage coloration in wild house finches. *Proc R Soc Lond B* 269:1119–1124

- Hörak P, Vellau H, Ots I, Møller AP (2000) Growth conditions affect carotenoid-based plumage coloration of great tit nestlings. *Naturwissenschaften* 87:460–464
- Hörak PL, Ots I, Vellau H, Spottiswoode C, Møller AP (2001) Carotenoid-based plumage coloration reflects hemoparasites infection and local survival in breeding great tits. *Oecologia* 126:166–173
- Hulbert SH (1984) Pseudoreplication and the design of ecological field experiments. *Ecol Monogr* 54:187–211
- Johnsen A, Delhey K, Andersson S, Kempenaers B (2003) Plumage colour in nestling blue tits: sexual dichromatism, condition dependence and genetic effects. *Proc R Soc Lond B* 270:1263–1270
- Kilpimaa J, Alatalo RV, Siitari H (2004) Trade-offs between sexual advertisement and immune function in the pied flycatcher (*Ficedula hypoleuca*). *Proc R Soc Lond B* 271:245–250
- Klasing KC (1998) Nutritional modulation of resistance to infectious diseases. *Poult Sci* 77:1119–1125
- Korpimäki E (1986) Diet variation, hunting habitat and reproductive output of the kestrel *Falco tinnunculus* in the light of the optimal diet theory. *Ornis Fennica* 63:84–90
- Lessels CM, Boag PT (1987) Unrepeatable repeatabilities: a common mistake? *Auk* 104:116–121
- McGraw KJ (2005) Interspecific variation in dietary carotenoids assimilation in birds: links to phylogeny and color ornamentation. *Comp Biochem Physiol* 142B:245–250
- McGraw KJ, Parker RS (2006) A novel lipoprotein-mediated mechanism controlling sexual attractiveness in a colour songbird. *Physiol Behav* 87:103–108
- McGraw KJ, Gregory AJ, Parker RS, Adkins-Regan E (2003) Diet, plasma carotenoids, and sexual coloration in the zebra finch (*Taeniopygia guttata*). *Auk* 120:400–410
- McGraw KJ, Adkins-Regan E, Parker RS (2005) Maternally derived carotenoid pigments affect offspring survival, sex-ratio, and sexual attractiveness in a colourful songbird. *Naturwissenschaften* 92:375–380
- 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
- Mossab A, Guillaumin S, Lessire M, Milliat F, Hermier D (2001) Plasma protein distribution in the turkey (*Meleagris gallopavo*). *Comp Biochem Physiol* 130B:227–235
- Navara KJ, Hill GE (2003) Dietary carotenoid pigments and immune function in a songbird with extensive carotenoid-based plumage coloration. *Behav Ecol* 14:909–916
- Negro JJ, Bortolotti GR, Tella JL, Fernie KJ, Bird DM (1998) Regulation of integumentary colour and plasma carotenoids in American kestrels consistent with sexual selection theory. *Funct Ecol* 12:307–312
- Olson VA, Owens IPF (1998) Costly sexual signals: are carotenoids rare, risky or required? *Trends Ecol Evol* 13:510–514
- Surai PF, Speake BK, Sparks NHC (2001) Carotenoids in avian nutrition and embryonic development. 1. Absorption, availability and levels in plasma and egg yolk. *Poult Sci* 38:1–27
- Surai PF (2002) Natural antioxidants in avian nutrition and reproduction. Nottingham University Press, Nottingham
- Tella JL, Figuerola J, Negro JJ, Blanco G, Rodríguez-Estrella R, Forero MG, Blázquez MC, Green AJ, Hiraldo F (2004) Ecological, morphological and phylogenetic correlates of interspecific variation in plasma carotenoid concentration in birds. *J Evol Biol* 17:156–164
- Tschirren B, Fitze PS, Richner H (2003) Proximate mechanisms of variation in the carotenoid-based plumage coloration of nestling great tits (*Parus major* L.). *J Evol Biol* 16:91–100
- Witschi E (1961) Sex and secondary sexual characteristics. In: Marshall AJ (ed) *Biology and comparative physiology of birds*. Academic, New York, pp 115–168