

# Carotenoids in nestling Montagu's harriers: variations according to age, sex, body condition and evidence for diet-related limitations

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**Abstract** Carotenoids are colored pigments forming the basis of many avian social traits. Before their utilization carotenoids must be acquired through diet and mobilized for specific uses. The relationships between carotenoid-based coloration, circulating carotenoids and body condition have been well studied in adult birds, but little is known in nestlings. Here, we investigated variations in carotenoid-based coloration in a raptor nestling, the Montagu's harrier (*Circus pygargus*), both in captivity and in natural conditions, and within a vole (poor-carotenoid source and cyclic prey) specialist population. We studied these variations according to nestling age and sex, and possible limitations in carotenoid availability by comparing years of contrasted prey abundance and using carotenoid supplementation experiments. Captive nestlings, fed only with mice, were strongly carotenoid limited. Wild nestlings were also carotenoid limited, especially in a year of high vole abundance. Nestlings were in better condition but less colored during a peak vole abundance year than during a

low vole abundance year, when harriers targeted more alternative preys (birds, insects). Thus, variation in vole abundance resulted in a de-coupling of body condition and carotenoid-based coloration in this population. This suggested that the positive relation between the body condition and carotenoid-based traits, typically found in adult birds, could be restricted to adults or nestlings of species that feed on carotenoid-rich food. Our results should stimulate more work on the functions and mechanisms of carotenoid-based traits in nestlings, which deserve more attention and most likely differ from those of adult birds.

**Keywords** Carotenoid-based coloration · Condition dependence · Montagu's harrier · *Circus* · Vole

## Introduction

Carotenoids are fat-soluble pigments that determine the bright yellow-red colors of many traits involved in social interactions (Goodwin 1984), but also serve important health-related physiological functions (Bendich and Olson 1989; Chew and Park 2004; but see also Costantini and Møller 2008). As the basic forms of carotenoids originate from plants, algae or fungi, vertebrates cannot synthesize these pigments de novo, but have to acquire them from their diet. Carotenoids access might thus be limited by food resource through environment (Brush 1981; Goodwin 1984; Olson and Owens 1998), hence suggesting potential life-history trade-offs between these dual functions (Blount 2004). The potential of carotenoid-based traits to act as honest signals has generated considerable attention recently, with special emphasis on the signals of adult birds within a sexual selection framework (Blas et al. 2006a;

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Lozano 1994; McGraw and Ardia 2003; von Schantz et al. 1999). Carotenoid-based traits can reveal the foraging ability (Endler 1983; Hill and Montgomerie 1994), and the nutritional (Endler 1980; Hill et al. 2002) and immune status of their bearers (Faivre et al. 2003; Lozano 1994; McGraw and Hill 2000; von Schantz et al. 1999). For instance, carotenoid traits might be indicators of foraging efficiency because only good foragers would be able to acquire more carotenoids than their less efficient conspecifics, and therefore express more intense coloration (Bostrom and Ritchison 2006; Endler 1983; Hill and Montgomerie 1994). To a larger extent, carotenoid-based colorations are widely known to be condition-dependent traits, providing information about the condition of the bearer (Hill 2006).

Surprisingly, there is still limited evidence to date that carotenoids may be a limiting resource for adults in nature, and even fewer studies have addressed this issue on nestlings (Biard et al. 2006; Casagrande et al. 2007; Costantini et al. 2007; Tschirren et al. 2003), despite the supposed importance of carotenoids for offspring fitness (Biard et al. 2005; Biard et al. 2006; Blount et al. 2002; Karadas et al. 2005; Royle et al. 2001; Rubolini et al. 2006; Saino et al. 2003; Surai et al. 2003). Similarly, functions and condition dependence of carotenoid-based coloration remain little studied in nestlings (but see Casagrande et al. 2007), even if many species already exhibit carotenoid-based colored traits as nestlings. In addition, nestling carotenoid status has been shown to affect carotenoid pigmentation later in life, as adult: for instance in zebra finches (*Taeniopygia guttata*), females incorporating high concentrations of carotenoids into eggs produced sons which exhibited more sexually attractive traits when adults (McGraw et al. 2005). Lastly, age and sex differences in relation to carotenoid requirements or in the physiological ability to absorb, convert, circulate and deposit carotenoids in colored traits (McGraw 2006; Negro et al. 2000) have also been understudied in nestlings, even in sexually dimorphic species that express carotenoid-based colorations at a very young age.

The Montagu's Harrier (*Circus pygargus*) is a ground-nesting and sexually dimorphic raptor ( $281 \pm 28$  g for males,  $340 \pm 31$  g for females; Millon et al. 2008) that exhibits yellow-carotenoid pigmented bare-parts (cere and tarsi) in both adults and nestlings, like many other raptor species (see for example Bortolotti et al. 2000; Casagrande et al. 2006). In this species, carotenoid-based coloration and sexual size dimorphism are already apparent at a young age (from 10 to 20 days old, respectively; this study and Millon and Bretagnolle 2005). In adult male Montagu's harriers, cere coloration is condition-dependent, and most likely plays a role in facilitating mate appraisal and choice (Mougeot and Arroyo 2006). In our study area, Montagu's harriers specialize on a mammal prey, the common vole

*Microtus arvalis* (Salamolard et al. 2000). This species, which is energy rich but contains low carotenoid concentration (Goodwin 1984; see Casagrande et al. 2006 for data on carotenoids content in another vole species *Microtus savii*), is a cyclic prey that exhibits extreme inter-annual variation in abundance, from low abundance in crash vole years to high abundance in peak vole years (Lambin et al. 2006; Millon and Bretagnolle 2005). When voles are abundant, harriers feed almost exclusively on this prey, whereas when voles are scarce, they feed on other preys (Salamolard et al. 2000), richer in carotenoids content but poorer in caloric intake such as passerine birds and insects (Goodwin 1984). This is in sharp contrast to most other studies involving birds (mainly passerine birds): in the latter studies, food quantity (caloric intake) and quality (carotenoid intake) are usually positively correlated and confounded. In Montagu's harrier however, carotenoid-rich resources (insects, birds) are potentially traded against carotenoid-poor resources (voles), leading to potential trade-off between quantity and quality in diet provided to nestlings.

We focused therefore on the relationship between carotenoid-based coloration, circulating plasmatic carotenoid concentration and body condition of Montagu's harrier nestlings, and how these relationships varied with vole abundance. More specifically, we empirically and experimentally investigated whether (1) carotenoid pigments were limiting for nestling coloration, and (2) a trade-off between caloric intake and nutritive quality appeared under varying regimes of prey availability. As the use of carotenoids (both through coloration and circulating carotenoid concentration) and body condition may depend on sex and age in size dimorphic nestlings, these two factors were also examined. To test if carotenoids were a limited resource for wild nestlings, we first supplemented them with carotenoids during a crash vole year, and expected supplemented nestlings to increase more circulating carotenoids and bare-parts coloration than control nestlings. In addition, using captive nestlings, we were able to strictly control diet (conversely to the field situation), and fed those nestlings either with an ad libitum diet consisting exclusively of small mammals (mice *Mus musculus*) or with the same ad libitum diet enriched with carotenoids. We expected the former (mouse-only diet) to be deprived from carotenoids and consequently to show paler coloration than wild control birds. We also expected captive birds to be in better body condition than wild birds, due to the ad libitum diet they received. Finally, we predicted that in a peak vole year, as opposed to a crash vole year, nestlings would be in better body condition due to greater food availability, but would be more constrained in their access to carotenoid pigments and would therefore be less colored.

## Materials and methods

### Study site and vole abundance

We studied Montagu's harriers during two successive breeding seasons (2006 and 2007) in a central-western France agricultural area of ca 450 km<sup>2</sup> (46°15N, 0°30W). Breeding density was similar in this area during the two seasons (65 and 69 nests found in the whole area in 2006 and 2007, respectively). This cultivated area was characterized by winter cereal crops (35% of the surface), other winter crops such as rape-seed and peas (15%), spring-sown crops (sunflower and corn, 25%), pasture land and other permanent or semi-permanent crops used for live-stock rearing (15%; see Millon et al. 2008, for additional information on the study site).

Within this site, common vole abundance was assessed six times a year (from March to August) in 2006 and 2007, using 80 lines of live trapping (Millon and Bretagnolle 2005). Trap-lines consisted in a 100-m-long 51 traps transect deposited for 24 h in randomly selected fields and checked once after 24 h. Vole abundance was estimated as the number of captures per 100 traps per 24 h. In this area, common voles exhibit cyclic population dynamic over a 3-year period (see Lambin et al. 2006; Millon and Bretagnolle 2005), corresponding to a crash vole year in 2006 as compared with 2007.

Harriers' nests were searched with a constant effort, during the pre-laying period, and most of them were found during egg-laying or incubation. Nests were visited as soon as possible to assess reproduction stage (for details, see Millon et al. 2008), and checked again three to eight times during the nestling period to assess breeding success and collect reproductive data. Eggs were measured (nearest 0.1 mm) and weighed (nearest 0.1 g), which allowed to estimate hatching dates (estimated from egg density, knowing the fixed daily rate of egg mass loss) and planning future nest visits.

### Carotenoid supplementations

Before starting carotenoid supplementations, we identified Montagu's harrier carotenoids circulating through blood and integumentary carotenoids by high-performance liquid chromatography (HPLC; see Stradi et al. 1995, for a detailed description of the method). Nestlings circulated mainly two xanthophyll pigments, zeaxanthin and particularly lutein (~90% of the total carotenoids), and at a lesser extent  $\beta$ -cryptoxanthin and carotene (~10%). Results were similar for integumentary carotenoids. Consequently, we supplemented nestlings with a liquid carotenoid solution containing these two xanthophylls [Oro Glo liquid, 11 mg/ml lutein and zeaxanthin (20:1, w/w); Kemin France SRL, Nantes].

To avoid photo-oxidation of pigments, the solution was kept in a refrigerated bag and in opaque container until given to chicks. Chicks less than 10 days old were considered too young to receive a treatment. Nestlings were supplemented one to four (average 3 times; of all nestlings, 5, 13, 26 and 1 nestlings received 1, 2, 3 and 4 supplementations, respectively). Supplementations were given once every 5 days, at ~15, ~20 and ~25 days, nestlings receiving increasing amounts of carotenoids of 11, 22 and 33 mg, respectively, with increasing age class. Due to field work constraints and nestlings' status (i.e. crop content), the average total dose received per nestling was  $60 \pm 19$  mg. Little is known about daily quantities of carotenoids consumed by birds in their natural diets, and average blood carotenoid concentrations in free-living individuals has been determined for relatively few raptor species (Bortolotti et al. 1996; Casagrande et al. 2007; Negro et al. 2002). Therefore, we chose doses following a previous study conducted by Casagrande et al. (2007) on nestling Eurasian Kestrel (*Falco tinnunculus*), which has a similar body mass and circulates carotenoid pigments similar to those of our study species.

### Field experimental design

To test whether these pigments were diet-limited, carotenoid supplementations were conducted in 2006. This crash vole year was expected to be less 'carotenoid-constrained' than 2007, because harriers should have fed with more diversified preys than exclusively voles (Salamolard et al. 2000), the former being richer in carotenoids content. To control for possible differences in carotenoid availability for nestlings through parental food provisioning, and thus observe true carotenoid supplementation effects, we used a within-brood design. Within a given brood, treatments were randomly assigned starting with the oldest chick (either control or supplemented). Then other treatments were sequentially assigned to the remaining nestlings according to their rank within the brood (alternating treatment and control with decreasing rank). Thus, within each brood we had control (i.e. non-supplemented) and supplemented nestlings, the treatments being homogenized by brood rank, irrespective of nestling sex.

For each visit at nests, we measured nestlings' body mass (with a scale, nearest 1 g), wing length (with a ruler, nearest 1 mm), tarsus length (with a calliper, nearest 0.1 mm) and bare-parts coloration (with a colorimetric chart, see below), and gave the carotenoid supplementation. Until ringed, chicks were head-marked with non-toxic colored pen to allow identification from one visit to the next. Blood samples were taken from the brachial vein using heparinized capillaries. Blood was kept refrigerated (0–5°C) for a few hours, and then centrifuged. The plasma

was subsequently stored at  $-20^{\circ}\text{C}$  until laboratory analyses. All nestlings were ringed and wing-tagged just before fledging, at 30 days old and genetically sexed following Fridolfsson and Ellegren (1999).

### Aviary experimental design

For this experiment, we used 30 Montagu's harrier nestlings originating from nests that were rescued and would have otherwise been destroyed during harvests operations. Eggs were collected and incubated. Nestlings were then reared in captivity before being released in the wild at fledging age (15 in 2006 and 15 in 2007; see Amar et al. 2000 for additional information on rearing and releasing procedures). After hatching, nestlings were housed in separate cages according to their age in 'brood-like' groups and fed with 1-day-old dead chickens until the start of the experiment ( $\sim 5$  days old) to ensure early stage of growth. During the whole experiment (i.e. when nestlings were  $\sim 5$  to  $\sim 25$  days old), chicks were fed either with an ad libitum dead laboratory mice (*Mus musculus*) diet or with the same ad libitum diet enriched with carotenoids (see Casagrande et al. 2007). Captive nestlings were randomly allocated the same carotenoid treatment as described for field experiment and were supplemented with the same liquid carotenoid solution as nestlings in the field (on average  $72 \pm 23$  mg given  $2.8 \pm 1.7$  times). Laboratory mice contained almost no carotenoids (G. R. Bortolotti personal communication) and we are therefore confident that control captive nestlings had strictly limited access to carotenoids during the whole experiment period. Every 3 days, captive nestlings were measured (same protocol as wild birds) and had their bare-parts coloration measured both with a colorimetric chart and a hand-held reflectance spectrophotometer (see below). Blood samples were also taken. All captive nestlings were ringed and wing-tagged just before release, at  $\sim 30$  days old and genetically sexed.

### Coloration measurements

Bare-parts (cere and tarsi) coloration was assessed by direct comparison with a colorimetric chart made available by Yolk Color Fan Roche (Neuilly-sur-Seine, France). Scores ranged from 0 (very pale yellow) to 15 (orange). The selected colors were characterized by tristimulus values of the CIE-1931-standard colorimetric system (Faivre et al. 2001). We took three consecutive measures per individual, which were highly repeatable (cere:  $r = 0.94$ ,  $F = 45.79$ ,  $P < 0.0001$ ,  $N = 315$  and tarsi:  $r = 0.92$ ,  $F = 36.68$ ,  $P < 0.0001$ ,  $N = 315$ ; Lessells and Boag 1987), so we used the average score for subsequent analyses. Although spectrophotometry would have been a more objective method of measuring color than colorimetric

charts, it was unpractical because of field work and species constraints. However, in a subsample of chicks (the captive nestlings), we were able to compare the chart method to spectral measures of bare-parts coloration obtained with a hand-held reflectance spectrophotometer operating at wavelengths of 300–700 nm (USB2000, Ocean Optics). Hue, chroma and brightness were then computed, between 400 and 700 nm (Endler 1990). Hue is indicative of 'color' while chroma is a measure of the 'purity' or 'saturation' of a color (Endler 1990). Comparison of color scores obtained with colorimetric chart and spectrophotometry indicated slight positive and significant correlations for both cere and tarsi scores with yellow hue measurement (Pearson correlation: cere:  $r = 0.25$ ,  $P = 0.001$ ,  $N = 165$  and tarsi:  $r = 0.49$ ,  $P < 0.0001$ ,  $N = 333$ ) and with yellow chroma (cere:  $r = 0.78$ ,  $P < 0.0001$  and tarsi:  $r = 0.48$ ,  $P < 0.0001$ ). Thus, a higher score was indicative of a more yellow-orange and more saturated color, for both cere and tarsi.

### Laboratory analyses

To determine circulating carotenoid concentration in blood, 20  $\mu\text{l}$  of plasma was diluted in 20  $\mu\text{l}$  of absolute ethanol. The dilution was mixed in a vortex during 1 min, and the flocculent protein was precipitated. Hexane (0.01% BHT, 500  $\mu\text{l}$ ) was then added to the mixture for carotenoids extraction. The hexane phase, containing carotenoids was separated by centrifugation and collected. The extraction was repeated twice. Hexane extracts were combined, evaporated and re-dissolved in a mixture of ethanol (see Bertrand et al. 2006; Eraud et al. 2007 and Karadas et al. 2005 for more details). The supernatant was examined in a spectrophotometer and the optical density of the carotenoid peak at 450 nm was determined. Carotenoids concentration was estimated from a standard curve of lutein (alpha-carotene-3,3'-diol; Sigma), the main pigment found circulating in Montagu's harrier.

### Statistical analyses

All statistical analyses were performed using SAS 9 (SAS 2001). We tested for the effects of year, age, sex and treatment both on nestling circulating carotenoids, coloration and body condition. To estimate body condition index, we used the residuals of a General Linear Model (GLM procedure, SAS 2001) of log-transformed body mass on log-transformed data for wing and tarsus length; these were calculated for each sex separately, given the size sexual dimorphism and different growth strategies between sexes. Color scores and circulating carotenoids were log-transformed and were fitted to models using a normal error distribution and identity link function. Nestlings were

measured several times during the experiment (i.e. coloration, biometry and circulating carotenoids) and we analyzed two different types of data sets. The first data set included all the repeated measures performed on the same individuals at different ages; for these, we used General Linear Mixed Models (Mixed procedure, SAS 2001) that included individual nested with the “nest of origin” as random effects for analyses. The second data set included only one measure per nestling, at the oldest age prior to fledging. In this case, we used General Linear Mixed Models that only included the “nest of origin” as a random effect. Because supplemented nestlings received different doses of carotenoids, and because the effects of supplementation on circulating carotenoids and color can be dose-dependent (i.e. increase proportional to the dose received; e.g. Alonso-Alvarez et al. 2004), we included dose as a covariate in our models testing for treatment level effects (supplemented vs. non-supplemented nestlings). All data are expressed as arithmetic mean  $\pm$  SD and all tests are two-tailed.

**Results**

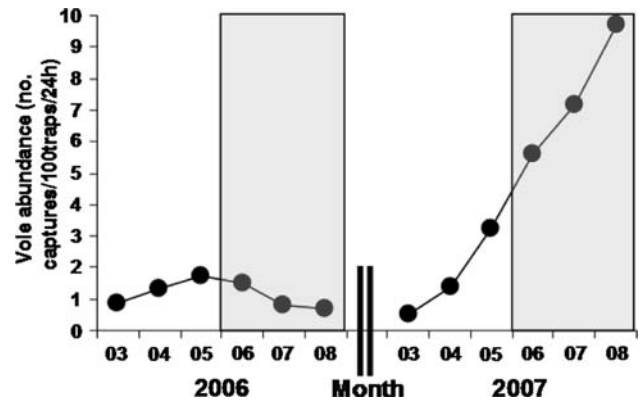
**Nestlings’ coloration**

When close to fledging, cere and tarsi color scores were strongly positively related (Mixed model with nest as a random effect:  $F_{1,97} = 1279.8$ ,  $P < 0.0001$ ,  $n = 166$ ; slope  $\pm$  SE =  $0.953 \pm 0.027$ ). There was no difference between sexes, year or origin (wild vs. reared nestlings) in the relationship between cere and tarsi color (all  $P > 0.11$ ), so we summed cere and tarsi color scores to get an overall score of carotenoid-based coloration, hereafter referred to as “color score” (range 0–11).

**Effect of vole abundance on nestlings’ coloration and body condition**

In 2006 (crash vole year), vole abundance averaged  $1.00 \pm 0.42$  capture/100 traps per 24 h ( $N = 160$  lines of 51 traps) between June and August. In 2007 (peak vole year), vole abundance averaged  $7.51 \pm 2.10$  captures/100 traps per 24 h between June and August. When harriers were at the nestling stage, vole abundance was thus more than 7 times higher in 2007 than in 2006 (Fig. 1).

In control wild nestlings, variation in carotenoid-based coloration was explained by age, year and their interaction (Table 1). Coloration of both sexes increased linearly with age, but the increase with age was greater in 2006 than in 2007. In addition, coloration was higher in 2006 than in 2007, in both sexes (Table 1; Fig. 2a). Variation in nestling body condition (i.e. body mass corrected for tarsus length

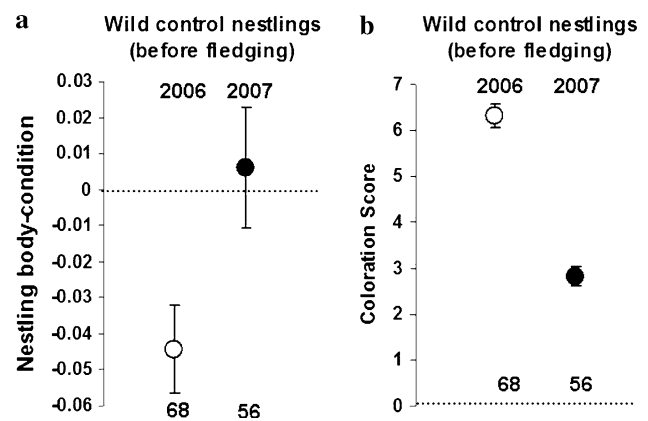


**Fig. 1** Vole abundance in the study site (no. captures/100 traps per 24 h) from March to August during the 2 years of the study. Vole abundance was higher in 2007 (peak vole year) contrasted to 2006 (crash vole year). Rearing period is shaded

**Table 1** Effects of age, sex, year, and their interactions on carotenoid-based coloration and body condition (body mass corrected for tarsus length and wing length) in control wild nestlings (2006 and 2007)

Source of variation	Coloration			Body condition		
	df	F	P	df	F	P
Age	1,148	127.29	<0.0001	1,162	15.17	0.0001
Sex	1,148	1.15	0.28	1,162	22.80	<0.0001
Year	1,148	79.15	<0.0001	1,162	7.90	0.005
Age $\times$ sex	1,148	1.75	0.19	1,162	5.67	0.018
Age $\times$ year	1,148	4.97	0.027	1,162	6.72	0.010

Mixed model included individual nested within nest of origin as random effects



**Fig. 2** Mean  $\pm$  SE. **a** Coloration score, and **b** body condition of wild control nestlings at fledging age in years of contrasting vole abundance (low abundance: 2006, high abundance: 2007). Years and sample sizes are indicated above and below the histograms, respectively. Body condition was calculated as the residuals from a GLM of body mass on tarsus length and wing length, calculated for each sex separately

and wing length) was explained by age, year and their interaction, in addition to sex and its interaction with age (Table 1). This sex  $\times$  age interaction on body condition emphasized different growth strategies in males and females. Thus, in order to control for the sex growth strategies effect, we re-analyzed variation in body condition with only one measure per nestling, at the oldest age prior to fledging. Upon fledging age (last sampling at 20–30 days of age), nestlings were in poorer body condition (lighter relative to their size) in 2006 than in 2007, irrespective of sex (Fig. 2b; dependent: body condition; year:  $F_{1,108} = 5.55$ ,  $P = 0.020$ ; sex:  $F_{1,108} = 0.39$ ,  $P = 0.534$ ; sex  $\times$  year:  $F_{1,108} = 1.37$ ,  $P = 0.244$ ). Furthermore, we found a negative body condition dependence of wild nestling coloration (Mixed model included individual nested within nest of origin as random effects:  $F_{1,143} = 4.99$ ,  $P = 0.027$ ,  $n = 252$ ; slope  $\pm$  SE =  $-0.007 \pm 0.003$ ).

#### Effects of carotenoid supplementation on wild nestlings

We first tested for an effect of supplementation on circulating carotenoids, comparing supplemented and non-supplemented nestlings, irrespective of the amount received. Overall, control chicks circulated on average  $9.68 \pm 7.90$  and  $6.53 \pm 5.53$   $\mu\text{g/ml}$  of carotenoids (for male and

female, respectively), whereas supplemented nestlings circulated in average  $7.48 \pm 8.52$  and  $10.33 \pm 8.24$   $\mu\text{g/ml}$  (for male and female, respectively). Circulating carotenoids increased with nestling age, but this increase was steeper in supplemented nestlings (Table 2). Treatment effects on circulating carotenoids did not vary with the dose supplemented but differed between sexes (significant age  $\times$  sex  $\times$  treatment interaction; Table 2). In female nestlings, circulating carotenoids were higher in supplemented than control chicks ( $F_{1,142} = 4.22$ ,  $P = 0.042$ ), increased with age ( $F_{1,142} = 4.94$ ;  $P = 0.023$ ), but increased similarly in supplemented and control nestlings (same slope: age  $\times$  treatment:  $F_{1,142} = 0.60$ ,  $P = 0.439$ ). In male nestlings, circulating carotenoids were lower in supplemented chicks ( $F_{1,72} = 5.27$ ,  $P = 0.025$ ), but increased more with age in supplemented than in control nestlings (age:  $F_{1,72} = 0.22$ ,  $P = 0.644$ ; age  $\times$  treatment:  $F_{1,72} = 7.06$ ,  $P = 0.01$ ). Therefore, with regard to circulating carotenoids, male nestlings responded more to the treatment than female nestlings (Table 2). Variation in color score was explained by age, treatment and by age  $\times$  treatment interaction (Table 2). Supplemented nestlings increased more coloration with age than control ones. This effect was independent of the dose supplemented and similar in male and female chicks (Table 2). Therefore, in

**Table 2** Effects of age, sex, treatment (carotenoid supplemented or not), dose (amount of carotenoids supplemented) and their interactions on circulating carotenoids levels and carotenoid-based coloration in wild (2006) and captive (2006–2007) nestlings

Source of variation	Circulating carotenoids			Coloration		
	<i>df</i>	<i>F</i>	<i>P</i>	<i>df</i>	<i>F</i>	<i>P</i>
Wild nestlings						
Age	1,214	1.19	0.276	1,273	1,053.69	<0.0001
Sex	1,214	0.42	0.518	1,273	1.13	0.289
Treatment	1,214	8.51	0.004	1,273	59.60	<0.0001
Dose	1,214	2.00	0.518	1,273	0.12	0.725
Age $\times$ sex	1,214	0.57	0.451	1,273	1.18	0.279
Treatment $\times$ sex	1,214	9.31	0.003	1,273	2.14	0.145
Treatment $\times$ age	1,214	6.53	0.011	1,273	41.74	<0.0001
Treatment $\times$ age $\times$ sex	1,214	4.68	0.032	1,273	2.55	0.112
Captive nestlings						
Age	1,109	1.24	0.268	1,218	1.11	0.294
Sex	1,109	2.56	0.112	1,218	6.18	0.014
Treatment	1,109	3.35	0.070	1,218	17.92	<0.0001
Dose	1,109	2.98	0.087	1,218	5.14	0.024
Age $\times$ sex	1,109	0.61	0.437	1,218	10.50	0.001
Treatment $\times$ sex	1,109	0.38	0.537	1,218	7.44	0.007
Treatment $\times$ age	1,109	4.11	0.045	1,218	47.08	<0.0001
Treatment $\times$ age $\times$ sex	1,109	0.17	0.683	1,218	13.43	0.001

Dependent variables were log-transformed. Mixed model included individual nested within nest of origin as random effects. All analyses are Type III (SAS 2001)

contrast to circulating carotenoids, nestlings of both sexes responded equally to treatment with regard to coloration.

Effects of carotenoid supplementation on captive nestlings and comparison with wild nestlings

Both male and female captive chicks fed only with mice were pale and their circulating carotenoids low, although not null (Fig. 3d and b, respectively). Variation in circulating carotenoids was not explained by sex or dose, but increased with age, this increase being more pronounced in supplemented than control nestlings (Table 2; Fig. 3a, b). Variation in color score was explained by age and by its interaction with treatment (Table 2). Supplemented nestlings increased more coloration with age than control ones (Fig. 3c, d).

Treatment effects on coloration also depended on the dose supplemented (positive effect) and differed between sexes (significant age × sex × treatment interaction; Table 2), the increase in coloration with age being steeper for supplemented female nestlings (slope ± SE: 0.045 ± 0.006) than for supplemented male nestlings (slope: 0.031 ± 0.007). When considering only control captive nestlings, age effects on both circulating carotenoid ( $F_{1,43} = 0.20, P = 0.657$ ) and coloration ( $F_{1,43} = 0.22, P = 0.638$ ).

Carotenoid-supplemented captive nestlings were less colored (“type of rearing” i.e. captive vs. wild:  $F_{1,59} = 12.27, P = 0.001$ ) but circulated as much carotenoids (“type of

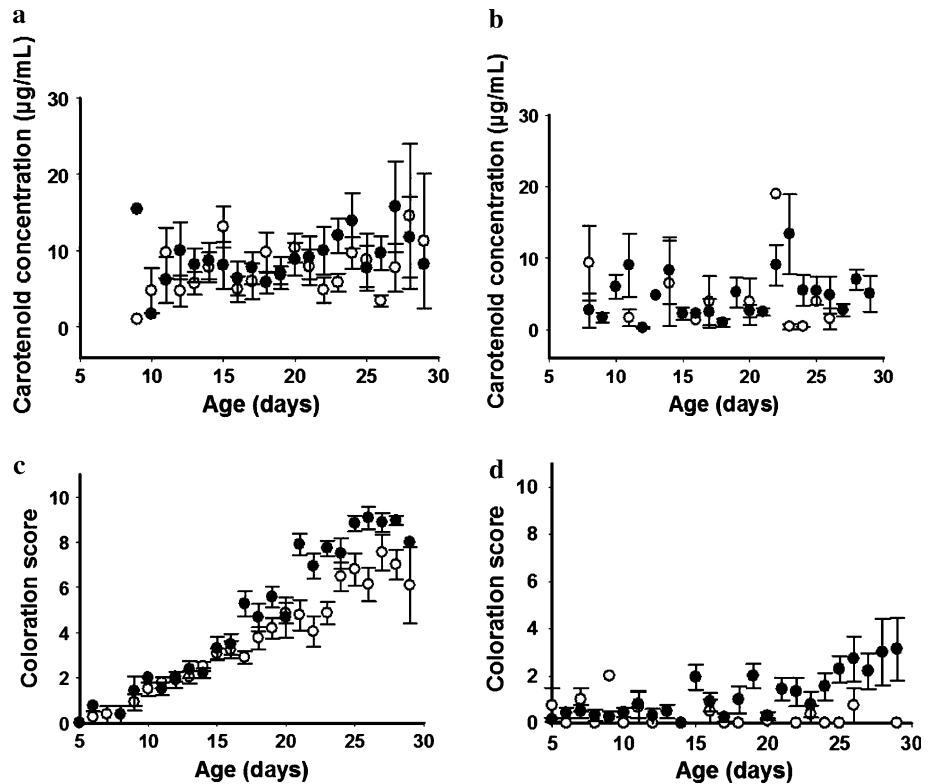
rearing”:  $F_{1,20} = 0.01, P = 0.941$ ) as wild control nestlings. Furthermore, nestling body condition was affected by “type of rearing” ( $F_{1,123} = 7.15, P = 0.008, N = 195$ ): captive nestlings were in better body condition than wild ones.

Discussion

Effects of carotenoid supplementation on Montagu’s harrier nestlings

In the wild, carotenoid-supplemented nestlings developed more colored integuments and, but to a lesser extent, circulated higher carotenoid concentrations than control ones. Even when voles were scarce and nestlings had a more diversified diet potentially richer in carotenoid pigments, supplementation increased both skin coloration and circulating carotenoids. This result indicated that carotenoid-based coloration of wild chicks is under limited expression, consistent with the carotenoid-limitation hypothesis (Blount et al. 2004; Endler 1983; Hill 1992). It also suggested, as previously proposed by Casagrande et al. (2007), that carotenoids might be limited by physiological thresholds (Alonso-Alvarez et al. 2004), such as the ability to absorb carotenoids in the gut, explaining therefore the differential increase between circulating carotenoids and coloration in wild nestlings. In contrast, captive nestlings (carotenoid deprived) did not develop colored integuments

**Fig. 3** Variation (mean ± SE) in (a, b) circulating carotenoids (µg/ml) and (c, d) score coloration in relation to age (days), according to treatment (control: open dots, supplemented: solid dots) in (a, c) wild (2006 only) and (b, d) captive nestlings (2006–2007 data combined). Data from both sexes are combined on the figure



like wild ones. As for captive kestrels maintained on a diet of laboratory mice (J. J. Negro, unpublished data), captive harrier chicks almost completely lacked (yellow) coloration. However, although low, circulating carotenoids of these deprived nestlings were not null. This could be because chicks store residual pigments, such as those obtained from eggs yolk (Laaksonen et al. 2008; Royle et al. 1999). Alternatively, as we provided 1-day-old chicks to nestlings before the start of the experiment, they may have used these pigments subsequently during growth either for coloration or more probably for health-related functions.

Skin coloration increased much more strongly with age than circulating carotenoids in wild control harrier nestlings. A similar result was found in kestrel nestlings by Casagrande et al. (2007), who suggested that increasing coloration with age can be a consequence of maturation of absorption and deposition processes, or a consequence of longer times of accumulation. In contrast to control wild nestlings, which may receive additional carotenoid pigments through parental food provisioning, control captive nestlings (fed only mice) remained pale but showed increasing levels of circulating carotenoids with age. This suggests differences in carotenoid allocation (skin coloration vs. blood circulation) between wild and captive control nestlings. Such differences could be due to differences in sibling competition. Nestling rearing condition should indeed greatly affect sibling competition, the latter being weak in captivity due to the ad libitum diet given to chicks, in contrast to natural rearing conditions. A possible explanation could be that under high competition, nestlings allocated preferentially carotenoid pigments to skin coloration because the latter may act as a signal toward parents or siblings, as has been proposed in other bird species (Bize et al. 2006; Kilner 2006). However, further investigations will be needed to infirm or confirm this hypothesis. In addition, due to a qualitative difference in how carotenoids are metabolized, we may not be able to infer as much as we would hope from the captive study. We further found that circulating carotenoids of wild male nestlings increased more after supplementation than those of females, whereas both responded similarly with regard to coloration. In this sexually dimorphic (size) species, a difference in growth strategy between males and females appears from nestling stage, females being larger and growing faster than males. Females might have used additional available carotenoids for their higher need in physiological detoxification during growth, or due to their higher body mass, or may have stored more circulating carotenoids than males in fat and other storage organs. In addition, intrinsic difference in carotenoid storage may exist between the two sexes, even at nestling stage, in relation to their future role in reproduction.

Skin coloration values recorded in captive supplemented nestlings never reached those of wild control individuals,

suggesting that dietary effects can influence coloration independently of carotenoid content. Indeed, during the experiment, captive nestlings have been fed with uniform diet of laboratory mice, containing low carotenoid and fat content. Lipoproteins are known to serve as carriers for carotenoids (McGraw 2006) and a low availability of such molecules may have constrained carotenoid transport and mobilization from integument coloration, thereby limiting the expression of captive nestling coloration. Alternatively, differences in environmental factors between captive and wild nestlings, such as light condition, general activity level, or stress levels induced by captivity could also have engendered such differences. In conclusion, supplementation of carotenoids led to faster increase in both circulating levels and coloration, suggesting a limitation of these pigments for wild Montagu's harrier nestlings through diet.

#### Natural variation in nestling coloration and body condition in years of contrasted vole abundance

We studied natural variations and relationship between carotenoid-based coloration and body condition of Montagu's harrier nestlings, in years of contrasted vole abundance. Voles are a preferred prey species, but are of poor quality, in terms of carotenoid content. We found that nestling's body condition and coloration changed markedly, but inversely, between high- and low-vole abundance years, suggesting a trade-off between energetic and carotenoid intakes.

Carotenoid-based coloration differed between years, nestlings being more colored in crash vole year (2006) as compared with a peak vole year (2007). This variation suggested that carotenoid acquisition depends primarily on diet and food abundance (Casagrande et al. 2007). These results are consistent with the findings of Hill et al. (2002) and Negro et al. (2000) in showing that the expression of carotenoid-based coloration in wild vertebrates varies with the amount and types of carotenoid pigments that are acquired through diet. In wild raptor species, previous studies investigating variation of carotenoid-based skin coloration (Bortolotti et al. 2000; Casagrande et al. 2006; Negro et al. 2002) have also demonstrated such a relationship, in particular for specialized raptor species feeding on cyclic, carotenoid-poor small mammals, because carotenoid availability will depend almost entirely on cyclic abundance of these preys (Dawson and Bortolotti 2002). In peak vole years, our study population preys nearly exclusively on voles (>90% of the total prey biomass: Salamolard et al. 2000), thus providing plenty of energetic food but limited amounts of carotenoids to nestlings (Bortolotti et al. 2000; Olson and Owens 1998). In contrast, when voles are scarce, nestlings have a potentially lower access to energetic food but a higher carotenoids



intake, through the parental targeting of more diversified preys (e.g. passerine birds and insects: Salamolard et al. 2000) that are richer in carotenoid content than voles (Goodwin 1984). Variation in coloration between successive years may therefore be explained by differences in diet consumed by nestlings, though an alternative explanation could be that variation in chick coloration varied with parental foraging efficiency. Indeed, it has been suggested that at low-vole density only higher quality individuals may reproduce (Millon and Bretagnolle 2005). This might imply either merely better parental foraging efficiency (probably for males) or better ability for parents to select higher carotenoid content preys, allowing nestlings to be more colored. However, even in this case variation of nestling coloration lies in the prey consumed by chicks.

The negative body condition dependence of carotenoid-based traits we found contrasts sharply with results found in other studies investigating sexual selection in adult birds (Hill 2006), and perhaps more strikingly with studies that have investigated this issue in nestlings (Biard et al. 2006; Tschirren et al. 2003). However, most of these studies were performed on passerine species, especially frugivores and/or granivores species, which have a carotenoid-rich diet. In this case, food and carotenoid availability are confounded, conversely to species that feed on carotenoid-poor food. Consequently, the body condition index dependence of carotenoid-based coloration in wild species may depend on pigment availability in the food consumed: this relationship is usually positive with carotenoid-rich food but could be negative with carotenoid-poor food. As previously proposed by Bortolotti et al. (2000), natural variation in nestling coloration in years of contrasted vole abundance suggests a potential trade-off between diet quality and quantity. While the role of food abundance in limiting reproduction of raptors has been well explored (Dawson and Bortolotti 2002; Newton 1979), the potential for impacts of dietary micro-nutrients remains unknown. The negative association between food quantity (abundance) and quality (carotenoids content) may have important implications for the immune status and body condition of fledglings (Biard et al. 2006). For instance, our nestlings may be in good body condition but in weaker immune-condition (due to low carotenoid availability) in high-vole abundance years than in low vole abundance years. Further work is needed to properly evaluate how vole abundance and diet simultaneously influence body and immune conditions of nestlings, and examine ultimately the effects on fledging fitness.

#### Mechanisms and function of carotenoid-based coloration in nestlings

Nestlings responded to carotenoid supplementation by increasing both their coloration and their circulating

carotenoids, indicating that skin coloration is a plastic trait influenced by the environment. Recent theories suggest that hormonal activity (e.g., testosterone) is also used to regulate actively carotenoid levels (Blas et al. 2006b). Regardless of whether carotenoid-based coloration in nestlings play a role in intra-specific communication, variation in chick coloration appears to be passive plasticity (Senar and Quesada 2006), in which variation in trait expression is the result of a response to both environmental and physiological variations (Scheiner 1999). In altricial species such as Montagu's harrier, newborns' access to carotenoids relies exclusively on parental provisioning. Therefore, besides heritable variation in carotenoid assimilation (Laaksonen et al. 2008; Tschirren et al. 2003), foraging skills in interaction with the nutritional environment experienced by adults (Endler 1983; Hill 1992) may be of particular importance in governing the carotenoid status of their progeny and ultimately their coloration. In adults Montagu's harrier, carotenoid-based skin coloration is more pronounced in males than females, correlates positively with body condition in males, and may be used as a sexual signal during mate choice (Mougeot and Arroyo 2006). However, the potential function of carotenoid-based coloration in nestlings remains unknown and further investigations are required to clarify this particular issue.

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