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Carotenoid availability in diet and phenotype of blue and great tit nestlings

Clotilde Biard[1,*], Peter F. Surai[2] and Anders P. Møller[1]

[1]*Laboratoire de Parasitologie Evolutive, CNRS UMR 7103, Université Pierre et Marie Curie–Paris 6, Bât. A–Case 237, 7 quai Saint Bernard, F-75252 Paris Cedex 05, France and* [2]*Lipid and Antioxidant Group, Department of Biochemistry and Nutrition, Avian Science Research Centre, Scottish Agricultural College, Auchincruive, Ayr, KA6 5HW, UK*

*Author for correspondence at present address: Equipe Ecologie Evolutive, UMR CNRS 5561 Biogéosciences, Université de Bourgogne, 6 bd Gabriel, F-21000 Dijon, France (e-mail: Clotilde.Biard@u-bourgogne.fr)

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Summary

Carotenoids are biologically active pigments of crucial importance for the development of avian embryos and nestlings. Thus parental ability to provide nestlings with a carotenoid-rich diet may enhance offspring fitness. However, very little is known about the possible effects of carotenoid availability in the diet on growing nestlings in natural populations. We experimentally manipulated dietary intake of carotenoids by nestlings of two closely related passerine species, the great tit *Parus major* and the blue tit *Parus caeruleus*, and measured nestling antioxidants, body condition, immunity and plumage colour. There was no detectable increase in plasma carotenoids after treatment in carotenoid-fed nestlings of either species despite regular supply of dietary carotenoids. However, in carotenoid-fed blue tit nestlings, plasma vitamin E concentration increased with plasma carotenoid concentration, while that was not the case for control nestlings. In both species, there was no significant effect of carotenoid supply on immune function. Carotenoid supplementation enhanced yellow feather colour in great tit nestlings only. In both species a strong effect of carotenoid supply was found on body condition with an increase in body mass for small carotenoid-fed nestlings compared to similarly sized control nestlings. Dietary availability of carotenoids may thus have important fitness consequences for tits. We hypothesise that the difference in effect of dietary carotenoids on the two species is due to relatively larger clutch size and higher growth rates of blue tits compared to great tits, leading to blue tit nestlings being more in need of carotenoids for antioxidant function than great tit nestlings.

Key words: antioxidants, early development, feather colour, fledgling body mass, immune function.

Introduction

Carotenoids form a large family of biologically active pigments with antioxidant properties synthesised by plants, algae and fungi (Goodwin, 1984; Surai, 2002). In animals, they are acquired from food and used for prevention of oxidative stress, regulation of immune function and development of bright yellow to red coloration of feathers and skin (reviewed by Møller et al., 2000). Antioxidants and particularly carotenoids have been shown to be of crucial importance during embryo development, at hatching and during the nestling phase, as this period of intense growth is associated with increased oxidative stress (Surai et al., 1999). Efficient antioxidant protection is essential for normal development of embryos and chicks, and any impairment in antioxidant defences leads to profound, and in many cases, irreversible damage to physiological functions (for a review, see Surai, 2002). For example, chicks reared in carotenoid-poor environments have a compromised ability to assimilate carotenoids from the diet in later life (Blount et al., 2003a; Hõrak et al., 2000; Koutsos et al., 2003). Furthermore, in several passerine species carotenoids are involved in the development of coloured signals like gape colour (Hunt et al., 2003), which may mediate parent-offspring communication (Saino et al., 2000). Carotenoids may also be transferred into growing feathers, and determine yellow to red coloration, as well as green and blue coloration when associated with proteins (Ong and Tee, 1992). Carotenoid availability during early stages of development may thus modulate several functions of potential importance for nestling fitness.

Carotenoids may be limiting in the environment as a result of environmental scarcity or individual variation in foraging ability (Møller et al., 2000; Olson and Owens, 1998). Thus parents able to provide their nestlings with a carotenoid-rich diet should enhance their offspring's antioxidant protection, body condition and immunity, and modulate their phenotype. Parental provisioning with carotenoids takes place very early

in nestling life when females invest carotenoids in egg yolk (Blount et al., 2000), but also later when parents feed nestlings. However, very little is known in natural populations about the possible effects of carotenoid availability in the diet on growing nestlings. To our knowledge, the effect of dietary carotenoids has only been investigated in nestling great tits (*Parus major*) in a population breeding in deciduous woodland (Fitze et al., 2003; Tschirren et al., 2003). Nestlings in the study by Fitze et al. were provided with extra carotenoids on two feeding occasions, either shortly after hatching or later during the nestling period. An increase in carotenoid supply affected feather colour only if occurring before 6 days of age, and no effect of carotenoid supply was found on nestling growth independent of age at which carotenoids were provided (Fitze et al., 2003). A similar increase in yellow feather colour was observed in carotenoid-fed nestlings in the study by Tschirren et al. (Tschirren et al., 2003).

The aim of the present study was to investigate the potential effects of carotenoid availability in the diet for growing nestlings. We experimentally manipulated dietary intake by nestlings of two closely related species, the blue tit (*Parus caeruleus*) and the great tit during the nestling period, and measured several aspects of nestling condition and phenotype before fledging. Blue and great tits are small hole-nesting passerines of similar ecology but differing in life-history traits (Blondel et al., 1990; Newton, 1989). In both species, nestling diet is composed of various insects and arachnids (Minot, 1981). We hypothesised that increasing the amount of carotenoids in the diet could modulate one or several of the following four types of parameters. First, an increase in circulating carotenoids could be expected in nestling blood, but also an increase in the concentration of other lipid-soluble antioxidants, as interactions such as recycling and mutual protection are known to occur between different antioxidants (e.g. Surai and Speake, 1998; Surai et al., 2001b). We thus measured the concentration of carotenoids and the two other major antioxidants for birds, vitamin E and vitamin A, in nestling plasma following carotenoid supplementation.

Second, an increase in general body condition of nestlings could be expected because of the antioxidant properties of carotenoids. Indeed, an increase in dietary carotenoids has been found to lead to an increase in antioxidant protection (Blount et al., 2002; Surai, 2002), which could be beneficial to rapidly growing chicks under intense oxidative stress produced by metabolism. Thus an increase in dietary intake of carotenoids could facilitate nestling growth and lead to better condition of nestlings, and/or to a better utilisation of nutrients. Body mass at fledging is a good predictor of immediate post fledging and overwinter survival in tits (Naef-Daenzer et al., 2001; Tinbergen and Boerlijst, 1990), and body mass with tarsus length as a covariate was therefore used as a measure of nestling body condition.

Third, carotenoids play important roles in immunoregulation in vertebrates. For example, they are known to enhance T and B lymphocyte proliferation, enhance macrophage and cytotoxic T cell capacities, and stimulate the production of various cytokines and interleukins in humans and other animals (reviewed by Chew, 1993; Møller et al., 2000). An increase in carotenoid availability could be expected to help clear infections, reduce the intensity of current infections, and enhance the ability to respond to an antigen challenge. The state of development and activation of the immune system was measured using red blood cell sedimentation rate, amount of leukocytes circulating in blood and cell-mediated immune response. Blood sedimentation is useful for detecting elevated levels of immunoglobulins and fibrinogen, high sedimentation rate being indicative of acute infections and inflammatory diseases (e.g. Sturkie, 1986; Svensson and Merilä, 1998). The number of leukocytes increases in case of stress and infection (e.g. Ots et al., 1998; Sturkie, 1986), and in chicken *Gallus gallus* and quail *Coturnix* sp. offspring the number of leukocytes in blood increases to reach adult levels by three weeks of age (Sturkie, 1986). Nestlings were tested for their ability to raise a cell-mediated immune response to phytohemagglutinin (PHA). This test reflects the combined responses of T-cells, cytokines and inflammatory cells (Davison et al., 1996).

Fourth, feather colour may mirror the amount and type of carotenoids available for an individual at the time of feather growth, and individual nestlings must trade allocation of pigments deposited in feathers against their use for other physiological functions. Both species show a carotenoid based yellow plumage on the breast (Partali et al., 1987), and we therefore investigated the effect of dietary carotenoids on development of juvenile plumage coloration.

Materials and methods

Data collection and experimental protocol

Data were collected in 2001 in a population of blue and great tits breeding in eastern France (48°17′N, 4°18′E). The blue tit *Parus caeruleus* (Linnaeus 1758) and great tit *Parus major* (Linnaeus 1758) are small (i.e. adult body mass of 11 g and 19 g, respectively) hole-nesting passerines often living in the same wooded areas and that are in competition for access to breeding sites and for food (Gosler, 1993). Both species lay large clutches (i.e. generally more than seven eggs), although blue tit females lay on average more eggs than great tit females (Blondel et al., 1990; Newton, 1989). Nestlings of both species have a similar diet of arthropods, mainly lepidopteran larvae and spiders (Minot, 1981). The study area (about 250 ha) contained 400 nestboxes evenly distributed among a homogenous deciduous old woodland composed mainly of oak (*Quercus* spp.), hornbeam (*Carpinus betulus*) and beech (*Fagus sylvatica*). Nests were regularly inspected to determine laying date, clutch size, start date of incubation, hatching date and number of hatchlings and fledglings.

Carotenoids were obtained from Kemin Foods (Nantes, France; OroGlo Layer Dry 20) in the form of a dietary supplement made of crystalline lutein derived from marigold flowers. Xanthophyll concentration in the product was 1.8% lutein and 0.2% zeaxanthin, confirmed with HPLC (high-

performance liquid chromatography) analysis, and both carotenoids were in free alcohol forms readily available for absorption. It is generally accepted that efficiency of carotenoid absorption in birds is about 20% of the used dose as determined from supplementation experiments (Surai, 2002; Surai et al., 2001a). The quantity of carotenoids to be given with each supplementation was therefore fixed to be five times the total quantity of carotenoids circulating in nestling plasma, which was estimated as follows, based on data collected in 2000 in the same population. Mean fledgling body mass was 10.5 g and 16.5 g and mean carotenoid concentration in plasma was 37 μg ml^{-1} and 56 μg ml^{-1} for blue and great tit nestlings, respectively. In nestling birds, blood volume represents about 10% of body mass (Sturkie, 1986), thus total quantity of circulating carotenoids can be estimated as 38 μg and 92 μg, respectively. Therefore, carotenoid treatment consisted of 200 μg and 500 μg of carotenoids diluted in 0.05 ml sunflower seed oil per feeding, for blue and great tit nestlings, respectively. Control treatment consisted of 0.05 ml pure sunflower seed oil per feeding. Sunflower seed oil was chosen because it is rich in mono- (24%) and polyunsaturated (65%) fatty acids, and contains natural vitamin E. Vitamin E will ensure protection of carotenoid pigment against oxidation before ingestion, and unsaturated fatty acids will enhance their absorption from the food matrix (Surai et al., 2001a). Supplemental food was freshly prepared each evening and stored at 4°C and in darkness until use the following day. We started feeding nestlings when they were sufficiently large to be identified with a numbered aluminium ring (mean nestling age ± s.e.m., blue tit: 6.64±0.25 days, *N*=14 nests and 141 nestlings, great tit: 5.89±0.18 days, *N*=19 nests and 182 nestlings). All broods used in this experiment were first broods. In all nests, half the brood was randomly attributed to each treatment, and nestlings were fed every 2 days until just before fledging (mean number of feedings ± s.e.m., blue tit: 4.82±0.04, great tit: 5.47±0.05). Supplementary food was delivered into the nestlings' throat with a graduated 1-ml syringe, and complete swallowing was checked.

Nestlings were measured, and feather and blood sampled on the day after the last supplemental feeding (mean nestling age ± s.e.m., blue tit: 15.35±0.1 days, *N*=108 nestlings from 12 nests, great tit: 15.58±0.16 days, *N*=82 nestlings from 10 nests). Sample size was reduced as a result of early fledging or predation. We measured tarsus length to the nearest 0.1 mm with a calliper and weighed nestlings to the nearest 0.25 g with a Pesola spring balance. A sample of five to eight yellow feathers was plucked from the centre of the yellow breast from each bird, and stored in individual plastic bags in the dark until later colour analysis. A blood sample (50–100 μl) was taken from the brachial vein in heparinized micro-haematocrit tubes. Blood samples were stored in a cooling bag in the field, and when back in the lab, stored at 4°C and upright to measure sedimentation after 8 h [for a randomly chosen sub-sample of nests: seven nests (*N*=59) for blue tit, eight nests (*N*=64) for great tit]. All blood samples were then centrifuged for 5 min at 2800 ***g***. Length of the red blood cell layer was measured to the nearest 0.5 mm and length of the 'buffy coat' layer was measured to the nearest 0.01 mm with a graduated magnifying ocular. Plasma was then separated from blood cells and stored at –20°C. Sedimentation and haematocrit were measured as the amount of red blood cells divided by the total length of the tube filled with blood. In the same way, the relative amount of leukocytes in total blood volume was measured as the ratio of the 'buffy coat' layer to total length. Sedimentation rate and relative proportion of leukocytes are routinely measured with this method to describe different aspects of health both in physiological ecology (e.g. Hõrak et al., 1998; Ots et al., 1998; Svensson and Merilä, 1998) and veterinary studies (Coles, 1997).

After capture and measurements, nestlings were injected subcutaneously with 0.2 mg phytohemagglutinin (PHA; Sigma L-8754, Saint-Quentin Fallavier, France) in 0.04 ml sterile PBS within the patagium first plucked of feathers and marked for injection with permanent ink. Following recommendations by (Smits et al., 1999), no control injection of PBS was made on the other wing web. PHA first induces an acute response 4 h after injection, primarily characterised by oedema. Then it induces a delayed-type hypersensitivity response through stimulating heterophils, basophils, granulocytic and mononuclear cell infiltration in dermis and dense perivascular infiltration of T lymphocytes at the site of injection (Parmentier et al., 1998; Sharma, 1990). This late response generally peaks 18 h after injection, and may last up to 36 h. Wing web thickness was measured with a spessimeter (Alpa S.p.A., Milan, Italy; the spring was removed from the spessimeter and replaced with a fixed weight of 15 g) with an accuracy of 0.01 mm just before injection and at least 19 h after injection to assess the intensity of the immune response (mean time ± s.e.m., blue tit: 26.78±0.41 h, *N*=48, great tit: 21.24±0.37 h, *N*=27). The immune response index was calculated as the difference between thickness after and before injection.

Plasma antioxidant analysis

Antioxidants were extracted from plasma as follows. 20 μl of plasma was mixed with 40 μl ethanol, then extracted twice with 500 μl hexane. Hexane extracts were pooled and evaporated at 60–65°C under nitrogen flow and the residue was dissolved in 0.1 ml dichloromethane and 0.1 ml methanol. Carotenoid composition and concentration, and vitamin A and E concentration were determined using reverse phase HPLC following previously published procedures (Surai, 2000; Surai et al., 2001c) (for details see Biard et al., 2005). All nestling plasma samples of at least 20 μl (blue tit *N*=99, great tit *N*=82) were analysed for total carotenoid concentration, and a random sub-sample was used for carotenoid composition and vitamin analysis (one nestling of each treatment for blue tit *N*=10 nests, great tit *N*=7 nests). Concentrations are given in μg ml^{-1}.

Feather colour analysis

Nestling breast feather colour was analysed blindly with respect to treatment, in the laboratory, using a spectrometer

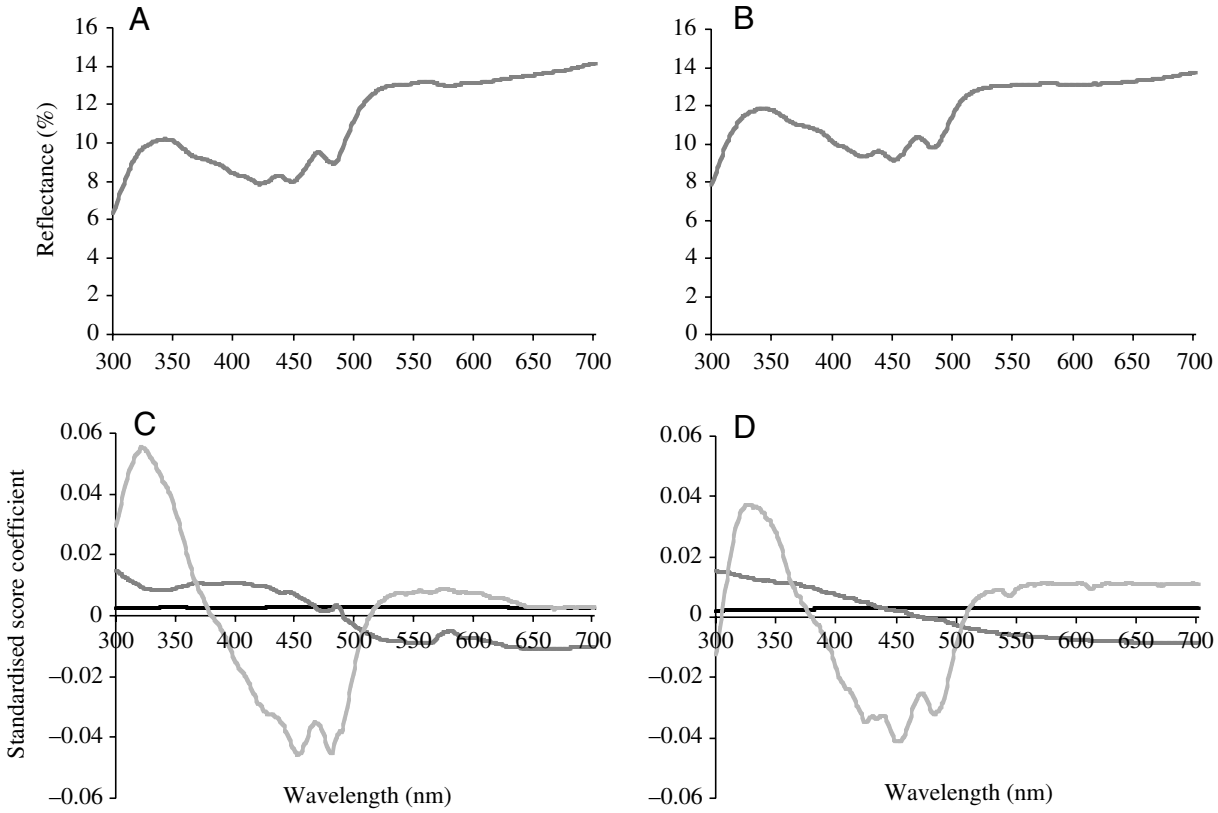

Fig. 1. Breast feather colour. (A,B) Average reflectance spectra of nestling breast feathers for (A) great tits (N=656 spectra) and (B) blue tits (N=864). (B,C) Association between principal component standardised score coefficients for PC1 (black line) PC2 (dark grey line) and PC3 (light grey line) and wavelength for (C) great tits and (D) blue tits.

(Ocean Optics, Duiven, The Netherlands) following methods described previously (Hõrak et al., 2000; Saino et al., 1999). Feathers were illuminated at an angle of 90° with a deuterium-halogen lamp, and reflected light was measured at an angle of 45°. Percentage of reflectance at each 1 nm interval was calculated between 300 and 700 nm, with respect to white and dark references, as $R_{(\lambda)}$=100×[(sample–white)/(white–dark)]. The reflectance spectra of breast feathers were similar for both species (Fig. 1A,B) and typically show two peaks, in the ultraviolet and the yellow parts of the spectrum [for blue tit nestlings (see Johnsen et al., 2003)]. Variation in reflectance spectra was summarised performing a principal component analysis (PCA) on raw spectra (Cuthill et al., 1999; Endler, 1990; Hill et al., 2005) separately for each species. Based on the scree plot of eigenvalues, the three first principal components that together explained more than 99% of the variation in reflectance were retained. PC1 explained 87% and 89.7%, PC2 10.6% and 8.8% and PC3 1.6% and 1% of the variance in reflectance spectra in blue and great tit, respectively. Factor loading for principal components was qualitatively similar for both species (Fig. 1C,D). PC1 described mean reflectance, as it is generally the case in a PCA on raw spectra: brightness is the main source of variation between spectra, and subsequent principal components describe variation in spectral shape (Cuthill et al., 1999). Factor loading for PC2 was positive below and negative above 475 nm, suggesting that it represents the relative importance of ultraviolet and yellow peaks. Factor loading for PC3 coefficient was positive in both short and long wavelengths and negative in medium wavelengths. Feathers with high values of PC3 coefficients will show greater differences among parts of the spectrum and will therefore appear more chromatic (Endler, 1990). Two feathers were analysed for each individual, with four measures per feather. Repeatability of measurements calculated as the intra-class correlation coefficient (Lessells and Boag, 1987) was always highly significant (all P<0.0001) with the following values (blue tit; great tit): (a) repeatability within feathers, PC1: 0.57; 0.69, PC2: 0.66; 0.73, PC3: 0.61; 0.61; and (b) repeatability within individuals, PC1: 0.42; 0.48, PC2: 0.55; 0.62, PC3: 0.53; 0.40. Average values for the eight measures per individual were used in subsequent statistical analyses.

Statistical analyses

All statistical analyses were made using SAS v8.2 (SAS Institute Inc. 1999–2001, Cary, NC, USA). Tests of the residuals for normality and homoscedasticity were used to check the validity of the model. In order to control for the effect of the proportion of red blood cells on sedimentation,

size on body mass, and time between measures of initial and post-reaction wing web thickness on immune response, haematocrit, tarsus length and time were entered as covariates in all models with sedimentation rate, body mass, and cell-mediated immune response as dependent variables, respectively (Freckelton, 2002). As the effect of supplementation may differ according to body size, tarsus length and its interaction with treatment were initially included in all models. Hatching date, brood size and nestling age were also first included in all models to account for seasonal variation, sibling competition and nestling growth, respectively. Retinol and vitamin E concentrations in nestling plasma were investigated including carotenoid concentration and its interaction with treatment in the models. Indeed, it is known that physiological interactions occur between these different antioxidants, and we may therefore expect plasma levels of vitamin A and E to be related to plasma levels of carotenoids, and these relationships to be modulated by experimental treatment. Interactions and main effects were dropped from the models when not significant. The age at first experimental feeding and the total number of feedings received were both initially included as covariates in all models, but they never explained a significant amount of variance, and thus were not retained in the models.

Effect of treatment on plasma carotenoid composition was investigated using a multivariate analysis of variance (MANOVA) model with the procedure GLM, on log-ratio-transformed proportions (Reyment, 1989). Effect of treatment on nestling characteristics was tested using the MIXED procedure of mixed linear models (Goldstein, 2003), with treatment as fixed effect and nest as random effect to account for non-independence of nestlings belonging to the same nest. The null model likelihood ratio χ^2 test was used to assess whether the model with random effect provided a significantly better fit than the same model without random effect. If that was not the case, the model was constructed without random effect. Models were compared by Akaike's Information Criterion (AIC), and the most parsimonious was retained [lowest AIC (Burnham and Anderson, 1998)]. Effect of treatment on nestling retinol and vitamin E was tested using the GLM procedure of generalised linear models.

Significant models were followed by comparisons of means or least square means between treatments with adjusted values of P using the Tukey-Kramer method. Values are reported as mean ± 1 s.e.m.

Results

Blue tit nestlings

Carotenoid profile and antioxidant concentration in plasma

Feeding treatment did not change relative concentrations of individual carotenoids in nestling plasma (Wilks' λ=0.55, $F_{6,12}$=1.62, P=0.22) (Table 1). Total carotenoid concentration in plasma was not significantly affected by feeding treatment ($F_{1,86}$=1.00, P=0.32; carotenoid supplemented: 35.8±4.4 μg ml^{-1}, control: 42.8±4.9 μg ml^{-1}) (Fig. 2). Retinol was not significantly affected by feeding treatment ($F_{1,17}$=0.45, P=0.51; carotenoid supplemented: 1.0±0.1 μg ml^{-1}, control: 1.3±0.1 μg ml^{-1}) (Fig. 2), but was marginally and positively related to carotenoid concentration ($F_{1,17}$=2.18, P=0.16; when removing feeding treatment from the model: $F_{1,18}$=4.36, P=0.05, R^2=0.19, slope estimate ± s.e.m. = 0.0064±0.0030). Total vitamin E (summed δ-, γ-, α-tocopherol) concentration was affected by the interaction between feeding treatment and total carotenoid concentration (carotenoid main effect: $F_{1,16}$=0.01, P=0.94, feeding treatment main effect: $F_{1,16}$=10.94, P=0.004, interaction: $F_{1,16}$=9.99, P=0.006). There was a positive relationship between vitamin E and carotenoids in carotenoid-fed nestlings, but that was not the case for control-fed nestlings (Fig. 3). However, there was no significant difference in plasma vitamin E concentration between treatments (carotenoid supplemented: 3.6± 1.3 μg ml^{-1}, control: 6.9±1.3, difference of least squares means: t=2.04, P=0.06) (Fig. 2).

Body condition

Nestling body mass was significantly affected by feeding treatment and its interaction with tarsus length (Table 2). Overall, nestlings from the carotenoid-supplemented group were slightly heavier than nestlings from the control group (least square mean ± s.e.m., carotenoid supplemented: 10.8±0.1 g, control: 10.6±0.1 g, t=2.04, P=0.04) (Fig. 4). Feeding treatment modified the relationship between body mass and size (Fig. 5A): small (i.e. tarsus length below mean tarsus length) carotenoid-fed nestlings were heavier than small control-fed nestlings, this difference increased with decreasing tarsus length. The smallest carotenoid-fed nestlings were between 0.50 and 0.75 g heavier than similarly sized control-fed nestlings (Fig. 5A).

Immune function

Nestling blood sedimentation rate increased with haematocrit ($F_{1,53}$=6.78, P=0.01), hatching date ($F_{1,53}$=11.72,

Table 1. *Plasma carotenoid composition in percentage of total carotenoids in blue and great tit nestlings*

Species	Treatment	*N*	Lutein	Zeaxanthin	*cis*-lutein	*cis*-Zeaxanthin	β-Cryptoxanthin	β-Carotene	Unidentified
Blue tit	Carotenoid	10	66.8±2.4	10.9±1.0	5.0±0.6	1.0±0.3	1.1±0.4	0.5±0.1	14.7±2.7
	Control	9	73.7±0.8	11.8±0.8	6.0±0.3	0.5±0.1	0.30±0.03	0.26±0.04	7.5±0.7
Great tit	Carotenoid	7	73.1±3.6	10.1±0.4	6.9±0.6	0.5±0.1	0.8±0.6	0.4±0.2	8.0±3.6
	Control	7	75.5±0.4	10.8±0.4	6.8±0.3	0.8±0.1	0.28±0.05	0.36±0.06	5.4±0.4

Values are means ± s.e.m.

P=0.001) and nestling age ($F_{1,53}$=12.79, P=0.0008), but did not depend on feeding treatment ($F_{1,53}$=0.08, P=0.77; carotenoid supplemented: 72.9±0.9%, control: 72.4±0.9%) (Fig. 4). The relative amount of leukocytes in nestling blood increased with brood size ($F_{1,101}$=3.83, P=0.05), but was not significantly affected by feeding treatment ($F_{1,101}$=2.31, P=0.13; carotenoid supplemented: 1.45±0.05%, control: 1.56±0.06%) (Fig. 4). Cell-mediated immune response decreased with nestling age ($F_{1,44}$=22.87, P<0.0001) and hatching date ($F_{1,44}$=24.56, P<0.0001), without any significant effect of feeding treatment ($F_{1,44}$= 0.00, P=0.95; carotenoid supplemented: 0.25±0.04 mm, control: 0.25±0.03 mm) (Fig. 4).

Breast feather colour

Juvenile plumage colour scores for PC1, PC2 and PC3 did not differ significantly with feeding treatment (Table 3; Fig. 6).

Great tit nestlings

Carotenoid profile and antioxidant concentration in plasma

Feeding treatment did not significantly modify relative concentrations of individual carotenoids in nestling plasma (Wilks' λ=0.30, $F_{6,7}$=1.62, P=0.11) (Table 1). Total carotenoid concentration in plasma was not significantly affected by feeding treatment ($F_{1,80}$=0.00, P=0.97; carotenoid supplemented: 45.5± 4.7 μg ml^{-1}, control: 45.2±3.4 μg ml^{-1}) (Fig. 2). Retinol concentration in plasma was found to decrease with increasing hatching date ($F_{1,9}$=13.14, P=0.005), and to increase with brood size ($F_{1,9}$=17.00, P=0.002), with no significant effect of treatment ($F_{1,9}$=0.55, P=0.48; carotenoid supplemented: 0.23±0.11 μg ml^{-1}, control: 0.39± 0.25 μg ml^{-1}) (Fig. 2). Total vitamin E concentration in plasma decreased with nestling age ($F_{1,11}$=6.38, P=0.03), but was not significantly affected by treatment ($F_{1,11}$=0.08, P=0.79; carotenoid supplemented: 11.8±2.9 μg ml^{-1}, control: 12.5±1.3 μg ml^{-1}) (Fig. 2).

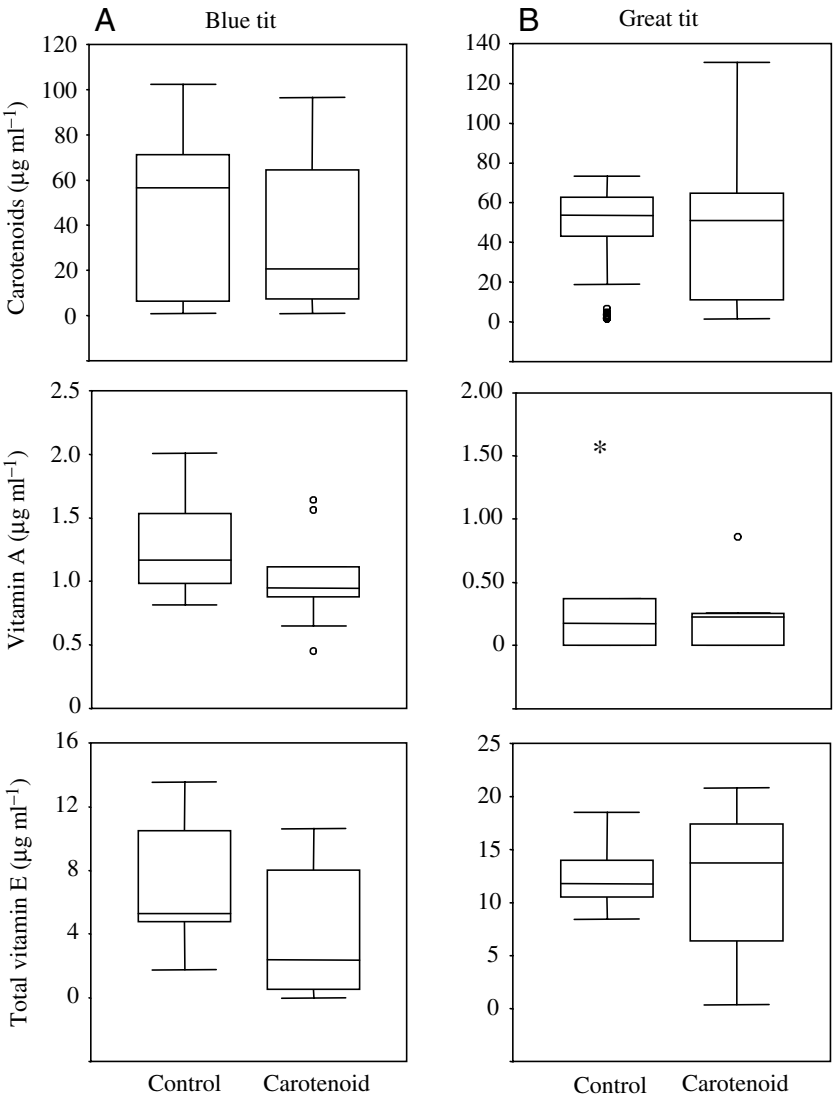

Fig. 2. Box plots describing variation in plasma antioxidants in blue tit (A) and great tit (B) nestlings as a function of feeding treatment. Box plots show median, quartiles (boxes), tenth quintiles (bars), distant (circles) and extreme observations (asterisk). Means and associated standard errors are given in the text, together with corresponding statistical tests.

Body condition

Body mass was significantly affected by feeding treatment in interaction with tarsus length, in a model controlling for nestling age (Table 2), although there was no difference in mean body mass between feeding groups (least square mean ± s.e.m., carotenoid supplemented: 16.96±0.16 g, control: 16.91±0.16 g, t=0.33, P=0.74) (Fig. 4). Smaller than average carotenoid-fed nestlings were heavier than similarly sized control-fed nestlings and the reverse was true for nestlings larger than the average (Fig. 5B). The smallest carotenoid-fed nestlings were about 1 g heavier than similarly sized control-fed nestlings, while the largest carotenoid-fed nestlings were about 1 g lighter than similarly sized control-fed nestlings (Fig. 5B).

Immune function

Nestling blood sedimentation rate increased with haematocrit ($F_{1,60}$=14.17, P=0.0004) and brood size ($F_{1,60}$=4.31, P=0.04), but was not significantly affected by feeding treatment ($F_{1,60}$=0.63, P=0.43; carotenoid supplemented: 68.9±0.7%, control: 70.3±1.2%) (Fig. 4). There was no significant effect of feeding treatment on the relative amount of leukocytes in nestling blood ($F_{1,80}$=0.35, P=0.55; carotenoid supplemented: 1.29±0.09%, control: 1.35±0.07%) (Fig. 4). Cell-mediated immune response was not significantly affected by feeding treatment ($F_{1,25}$=0.09, P=0.77; carotenoid

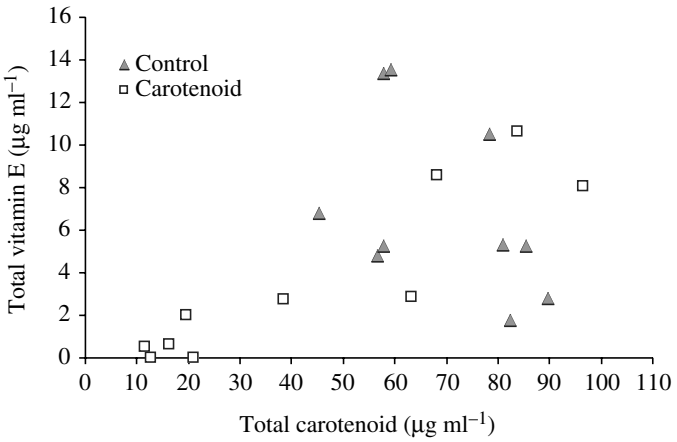

Fig. 3. Total vitamin E (summed δ-, γ-, α-tocopherol) concentration (μg ml^{-1}) in plasma as a function of carotenoid concentration (μg ml^{-1}) in nestling blue tits from control ($F_{1,8}$=1.94, P=0.20, R^2=0.19, slope estimate ± s.e.m. = –0.12±0.08) and carotenoid-fed groups ($F_{1,8}$=37.59, P=0.0003, R^2=0.82, slope estimate ± s.e.m. = 0.11±0.02).

supplemented: 0.16±0.03 mm, control: 0.17±0.03 mm) (Fig. 4).

Breast feather colour

Feeding treatment influenced all colour parameters (Table 3; Fig. 6). PC1 was significantly greater, i.e. feathers were brighter in carotenoid-fed than control-fed nestlings. PC2 was smaller for carotenoid-fed nestlings than for control-fed nestlings: feathers from carotenoid-fed nestlings showed a proportionally higher peak in the yellow than in the ultraviolet part of the spectrum, as compared to control-fed nestlings. Finally, PC3 was marginally greater in carotenoid-fed than control nestlings: feathers from carotenoid-fed nestlings appeared slightly more chromatic than feathers from control fed nestlings.

Discussion

Experimental supplementation with carotenoids allowed us to investigate the consequences of dietary availability of carotenoids for growing nestlings, and to investigate possible differences between two closely related passerine species differing in life history. Information on this topic is currently very limited for nestling birds in the wild (Fitze et al., 2003; Negro et al., 2000; Slagsvold and Lifjeld, 1985; Tschirren et al., 2003). In both species, increasing carotenoid intake had a positive effect on body condition (body mass relative to body size), but this was not paralleled by an effect on immune function. Although carotenoid supplementation did not result in an increase in plasma carotenoids in either species, in blue tits a positive relationship between plasma carotenoids and vitamin E was observed in carotenoid-fed nestlings, while no such relationship was present in control nestlings. Increasing carotenoid intake enhanced plumage colour in great tit, but not in blue tit nestlings.

Carotenoids are hypothesised to be limiting in the environment (Olson and Owens, 1998), but studies investigating availability of these pigments in the natural food in different populations and species are very scarce (Partali et al., 1987). Plasma carotenoid levels in wild birds are documented mainly for adults (e.g. Biard et al., 2005; Blount et al., 2002; Bortolotti et al., 2000; Hõrak et al., 2004; Ninni et al., 2004; Tella et al., 2004; Wallace et al., 1996). In our population, plasma carotenoid concentration in breeding great tit females was similar to that found in females of a rural population studied by Hõrak et al. (Hõrak et al., 2004) (also our unpublished data). However, both physiology and diet may differ between nestlings and adults, and it would be hazardous to extrapolate plasma carotenoid levels of nestlings from plasma carotenoid concentrations of adults. There is very little information available on plasma carotenoid levels in nestlings (Biard et al., 2005; Negro et al., 2000). Therefore, it is difficult to determine whether and how much carotenoids may be limiting for nestlings in our population.

Carotenoid composition of supplementary food was 90% lutein and 2% zeaxanthin. However, natural food during nestling growth for blue and great tit is composed of various insects and arachnids rich in lutein and zeaxanthin, but also in β-carotene [example of composition of lepidopteran larvae: lutein: 80%, zeaxanthin: 3%, β-carotene: 17% (Partali et al., 1987)]. There was no difference in plasma carotenoid profile in carotenoid-fed nestlings compared to control-fed nestlings, although our carotenoid supply was deficient in β-carotene. Therefore, it is unlikely that the experimental protocol induced any modification in carotenoid absorption or metabolism that we would expect to be mirrored by changes in plasma carotenoid profile in carotenoid-fed nestlings. There was no detectable increase in plasma carotenoids after treatment in

Table 2. *Mixed linear models testing for effects of feeding treatment on body mass in blue and great tit nestlings*

Species	Nest	Effect	$F_{\text{d.f.}}$	P
Blue tit	Z=1.93, P=0.03	Tarsus length	$24.17_{1,104}$	<0.0001
		Feeding treatment	$10.16_{1,104}$	0.002
		Tarsus length × feeding treatment	$9.77_{1,104}$	0.002
Great tit	Z=1.39, P=0.08	Nestling age	$6.32_{1,77}$	0.01
		Tarsus length	$43.94_{1,77}$	<0.0001
		Feeding treatment	$7.11_{1,77}$	0.009
		Tarsus length × feeding treatment	$7.06_{1,77}$	0.009

carotenoid-fed nestlings of either species despite regular supply of dietary carotenoids. In a study on white storks, *Ciconia ciconia*, plasma carotenoid concentration was found to be five times higher in nestlings from a population feeding mainly on a carotenoid-rich diet (crayfish *Procambarus clarkii*) as compared to nestlings from a population having access to low carotenoid food only (Negro et al., 2000). Possibly, carotenoid concentration in plasma is maintained at a stable level preventing increase above a certain optimum, which may already be attained naturally in our population that was breeding in a rich habitat. Excess dietary carotenoids may be stored, with the most important storage organ being the liver, and then released in circulation when needed (Surai, 2002; Surai and Speake, 1998). Although plasma vitamin E concentrations were not significantly different in carotenoid-supplemented as compared to control nestlings, there was a tendency for control nestlings to show higher plasma vitamin E levels. The mechanism of carotenoid absorption in the intestine is similar to that of tocopherols (Surai, 2002; Woodall et al., 1996). Therefore, it could be speculated that increased carotenoid supplementation would have induced competitive interactions between carotenoids and tocopherol during absorption, and this may explain why some carotenoid-fed nestlings had low carotenoid and vitamin E levels. This particular question requires further study. Indeed, studies investigating the possible negative interactions between vitamin E and carotenoids in dietary supplementation experiments have yielded contradictory results (Furr and Clark, 1997). However, in carotenoid-fed blue tit nestlings vitamin E concentration increased with carotenoid concentration in plasma, while that was not the case for control nestlings. Dietary supplementation with carotenoids thus had an effect on plasma vitamin E and carotenoids, probably through interactions between them. This may have arisen because carotenoids were provided in oil containing vitamin E. However, we find this unlikely, because both groups received the same quantity of vitamin E in oil, and carotenoids are present in the nestlings natural food. In addition, when carotenoids were provided to female blue tits in low vitamin E vegetable oil, a similar enhancement of vitamin E availability without increase in plasma carotenoid level was found (Biard et al., 2005). Therefore, an increase in dietary availability of carotenoids may cause synergistic interactions between vitamin E and carotenoids even in the absence of a detectable increase in plasma carotenoid concentration. Such synergistic effects among antioxidants are described in the poultry literature (e.g. Surai and Speake, 1998; Surai et al., 2001b), but have generally been neglected in studies of wild birds. However, there was no such effect on plasma antioxidants in great tit nestlings. This may be linked to the increase in pigment deposition into growing

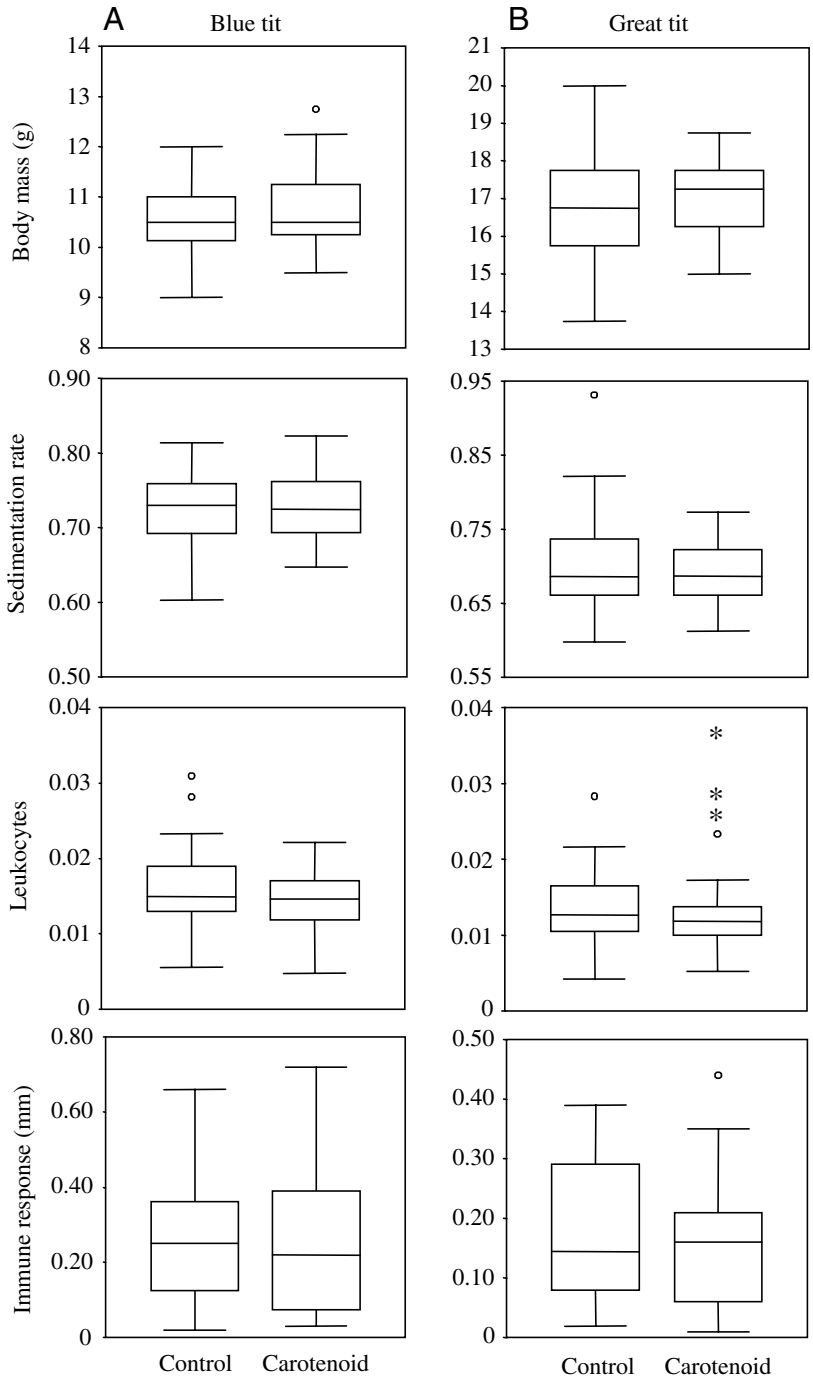

Fig. 4. Box plots describing variation in body condition in blue and great tit nestlings as a function of feeding treatment. Box plots show median, quartiles (boxes), tenth quintiles (bars), distant (circles) and extreme observations (asterisks) for body mass (g), blood sedimentation rate, proportion of leukocytes in blood and cell-mediated immune response (mm).

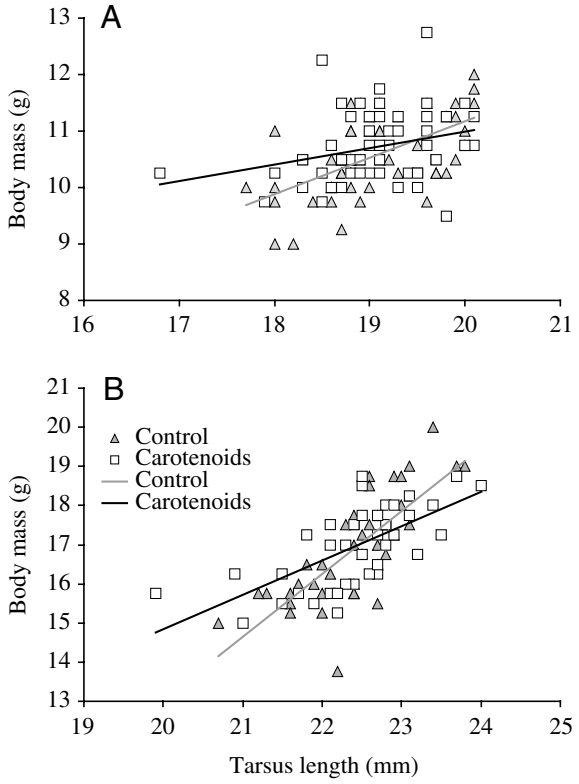

Fig. 5. Relationship between body mass (g) and tarsus length (mm) in blue tit (A) and great tit (B) nestlings depending on feeding treatment. Blue tit (mean tarsus length ± s.e.m.: 19.4±0.06 mm, N=108): control group: $F_{1,53}$=24.75, P<0.0001, slope estimate ± s.e.m. = 0.68±0.14 (nest: Z=1.51, P=0.06); carotenoid-fed group: $F_{1,51}$=3.23, P=0.08, slope estimate ± s.e.m. = 0.27±0.15 (nest: Z=1.17, P=0.12). Great tit (mean tarsus length ± s.e.m.: 22.43±0.08 mm, N=82): control group: $F_{1,39}$=28.15, P<0.0001, slope estimate ± s.e.m. = 1.26±0.24, (nest: Z=1.33, P=0.09); carotenoid-fed group: $F_{1,39}$=21.94, P<0.0001, slope estimate ± s.e.m. = 0.78±0.17 (nest: Z=1.24, P=0.11).

feathers in carotenoid-fed nestlings. Our data indicate that mean (±s.e.m.) carotenoid concentration in blue tit nestling plumage is 640±37 μg g^{-1} (N=4). For comparison, mean carotenoid concentration in the liver of the same nestlings was 145±62 μg g^{-1} (our unpublished data). Therefore, the amount of pigments deposited into plumage may not be negligible compared to circulating or stored carotenoids for a nestling. In conclusion, we suggest that assessment of treatment effects can only be made when carefully considering other antioxidants, different sites of storage, and the use of antioxidants in production of signals that will not allow subsequent use for physiological functions.

In both species a strong effect of carotenoid supplementation was found on body condition through a modification of the relationship between body mass and size, with an increase in body mass for small carotenoid-fed nestlings compared to similarly sized control nestlings. In great tit nestlings only, a decrease in body mass was also observed for large carotenoid-fed nestlings. Body mass at fledging is a good predictor of immediate post fledging and overwinter survival in tits (Naef-Daenzer et al., 2001; Tinbergen and Boerlijst, 1990). Thus parents able to provision their brood with a carotenoid-rich diet would increase the probability of survival of both smaller and larger nestlings. An increase in the availability of carotenoid pigments may enhance mass gain in nestlings by regulating oxidative stress resulting from rapid growth. The mechanism through which an increase in carotenoid availability could reduce mass gain in large great tit nestlings could be an increase in intensity of sib competition that may disproportionately affect large siblings.

In both great and blue tits, there was no effect of carotenoid supplementation on any of the variables used to describe activation of the immune system (sedimentation rate, relative amount of circulating leukocytes) or ability to raise a cell-mediated immune response. These were mostly influenced by hatching date, nestling age and brood size. A positive effect of an increase in dietary carotenoids was indeed expected because these pigments are involved in activation and regulation of immune function (reviewed by Chew, 1993; Møller et al., 2000). Cell-mediated immune response to PHA is localised at the site of injection and thus any effect of carotenoids on this immune response should be mediated through carotenoid concentration in plasma. However, carotenoid plasma levels were not enhanced by dietary supplementation, which could explain why there was no difference in cell-mediated immune response between treatments. In adult zebra finches *Taeniopygia guttata*, birds fed with a carotenoid-enriched diet showed an increase in plasma concentrations of carotenoids and an increase in both cell-mediated and humoral immune

Table 3. *Mixed linear models testing for effects of feeding treatment on feather colour parameters in blue and great tits nestlings*

		Blue tit			Great tit		
Colour parameter	Effect	$F_{d.f.}$	P	Nest	$F_{d.f.}$	P	Nest
PC1	Tarsus length	–	–	Z=1.76, P=0.04	$5.96_{1,79}$	0.02	Z=1.85, P=0.03
	Feeding treatment	$0.02_{1,106}$	0.88		$6.40_{1,79}$	0.01	
PC2	Feeding treatment	$0.00_{1,106}$	0.99	Z=2.11, P=0.02	$5.65_{1,80}$	0.02	Z=2.03, P=0.02
PC3	Hatching date	$8.99_{1,103}$	0.003	Z=1.76, P=0.04	–	–	Z=1.41, P=0.08
	Nestling age	$7.12_{1,103}$	0.01		–	–	
	Tarsus length	$4.16_{1,103}$	0.04		–	–	
	Feeding treatment	$0.44_{1,103}$	0.51		$3.70_{1,80}$	0.06	

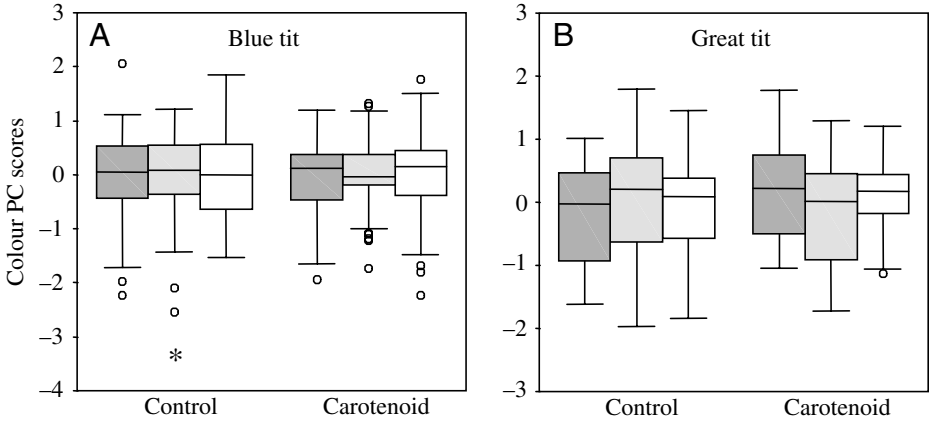

Fig. 6. Box plots describing variation in plumage colour scores for PC1 (black box) PC2 (grey box) and PC3 (white box) in blue tit (A) and great tit (B) nestlings as a function of feeding treatment. Box plots show median, quartiles (boxes), tenth quintiles (bars), distant (circles) and extreme observations (asterisks).

responses (Blount et al., 2003b; McGraw and Ardia, 2003). In nestlings, carotenoids may influence the development and maturation of the immune system and/or the immune response, depending on when they are available. In barn swallows *Hirundo rustica*, nestlings originating from experimentally carotenoid-enriched eggs had a stronger cell-mediated immune response than controls, and this response predicted survival to fledging (Saino et al., 2003). This suggests that an increase in carotenoid availability, if occurring during early embryo and hatchling development (i.e. maternally derived yolk carotenoids) may improve the development of an efficient immune system, whereas this may not be the case if carotenoids are provided later during nestling growth, as in our experiment. Alternatively, our population may not suffer from carotenoid scarcity in the environment, with the natural diet providing sufficient amounts of carotenoids to allow nestlings to efficiently mount an immune response. If that was the case, further increasing dietary availability of carotenoids for nestlings would not be expected to have an effect on the magnitude of immune responses.

In the great tit, carotenoid-supplemented nestlings grew brighter yellow feathers than control nestlings. In addition, in carotenoid-fed nestlings feathers showed a proportionally higher peak in the yellow than in the ultraviolet, and a tendency to be more chromatic than feathers from control-fed nestlings. These results show that dietary pigments were deposited into growing feathers, and that the development of juvenile plumage colour depends on nutritional conditions. This confirms the findings of previous studies showing that nestling great tits from carotenoid-poor habitats grew paler yellow plumage than nestlings from carotenoid-rich habitats (Slagsvold and Lifjeld, 1985). An enhancement of yellow plumage colour was also obtained in previous supplementary feeding experiments (Fitze et al., 2003; Tschirren et al., 2003). These studies did not investigate plasma carotenoid levels following supplementation. In our study, enhancement of plumage colour occurred in the absence of a detectable increase in plasma carotenoids. We may hypothesise that transitory increases in plasma carotenoid occurring after ingestion and absorption of supplementary carotenoids have stimulated follicles and increased the rate of pigment uptake and deposition into growing feathers. All great tit nestlings in this study were fed when older than 5 days, suggesting that in great tit nestlings, feather colour may be determined after an age of 6 days (contrary to what was stated by Fitze et al., 2003). In great tit fledglings, plumage colour has been shown to reflect rearing conditions in terms of habitat quality, year effects, and experimental reduction in brood size (Hõrak et al., 2000; Tschirren et al., 2003). However, at present nothing is known about the possible function of juvenile plumage colour in parent-offspring and/or offspring/offspring communication after fledging. Although there was an effect of dietary supplementation on feather colour in great tit nestlings, no such effect was found in blue tit nestlings. A different timing of feather development could explain this interspecific difference in response to carotenoid supplementation. However, blue and great tit nestlings show a very similar pattern of feather growth (Schoppe, 1977). Blue tits lay larger clutches, and thus invest relatively more in reproduction than great tits in terms of total clutch mass relative to female body mass (Blondel et al., 1990; Gosler, 1993; Newton, 1989). Beyond the energetic costs of producing larger clutches, female blue tits also need far more carotenoids for investment in their eggs. Carotenoid availability in the environment may indeed be lower at the time of egg production than at the time of rearing nestlings, when caterpillars reach peak abundance. This may cause egg yolk carotenoid concentration to be lower, and consequently embryos and hatchlings to develop in less favourable conditions in blue tits than in great tits. Indeed, data collected in 2001 and 2002 in this population indicate that mean (±s.e.m.) yolk carotenoid concentration for blue tits was 18.03±0.7 $\mu g\ g^{-1}$ (N=169) while it was 26.21±1.6 $\mu g\ g^{-1}$ (N=98) for great tit; a mean difference of 45% (our unpublished data). In addition, growth rate is reported to be higher in blue tit nestlings than in great tit nestlings [mean growth rate for blue tit=0.41 for an adult body mass of 11 g, mean growth rate for great tit=0.36 for an adult body mass of 19 g; a mean difference of 14% (Starck and Ricklefs, 1998)]. Blue tit nestlings may thus be subject to more intense oxidative stress than great tit nestlings. Therefore, blue tit nestlings are probably more in need of carotenoids for physiological functions than are great tit nestlings, and if provided with extra

carotenoids blue tit nestlings may primarily invest them in antioxidant function rather than plumage colour.

To our knowledge, this is the first study showing that an increase in dietary availability of carotenoids influences fledgling body mass independently of immune function or plasma carotenoid concentration. As body mass at fledging is a key parameter for offspring survival in tits, parental availability of dietary carotenoids may have important fitness consequences for their offspring. Furthermore, plasma carotenoids correlated positively with vitamin E levels in carotenoid-supplemented but not in control blue tit nestlings, while dietary supplementation with carotenoids enhanced plumage colour in great tit nestlings. We hypothesise that the differences in effect of experimental carotenoid supplementation on the two species may be due to a relatively larger clutch size and a higher growth rate in blue tits compared to great tits. This hypothesis of a differential need for antioxidants depending on early developmental conditions (i.e. maternal investment of carotenoids to eggs) and life history traits would require extensive comparative analyses taking phylogenetic relationships into account.

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