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## Carotenoid-based colour expression is determined early in nestling life

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**Abstract** Carotenoid-based colours are widespread in animals and are used as signals in intra- and interspecific communication. In nestling birds, the carotenoids used for feather pigmentation may derive via three pathways: (1) via maternal transfer to egg yolk; (2) via paternal feeds early after hatching when females are mainly brooding; or (3) via feeds from both parents later in nestling life. We analysed the relative importance of the proposed carotenoid sources in a field experiment on great tit nestlings (*Parus major*). In a within-brood design we supplemented nestlings with carotenoids shortly after hatching, later on in the nestling life, or with a placebo. We show that the carotenoid-based colour expression of nestlings is modified maximally during the first 6 days after hatching. It reveals that the observed variation in carotenoid-based coloration is based only on mechanisms acting during a short period of time in early nestling life. The experiment further suggests that paternally derived carotenoids are the most important determinants of nestling plumage colour.

**Keywords** Carotenoids · Honest signalling · Maternal effects · Paternal effects · Plumage coloration

### Introduction

Carotenoid-based coloration is widespread in animals and is used by several vertebrate species as a signal in inter- and intraspecies interactions (e.g. Hamilton and Zuk

1982; Kodric-Brown 1989; Milinski and Bakker 1990; Hill 1990, 1992, 1994; Bakker and Mundwiler 1994; Götmark and Hohlfalt 1995; Brawner et al. 2000; Senar et al. 2002). As animals are not able to synthesize carotenoids per se, they have to ingest them with the food (Partali et al. 1987). This led to the hypothesis that carotenoids may be a limiting resource for colour expression in nature, as evidenced in a range of species and taxa (Kodric-Brown 1989; Hill 1992; Grether et al. 1999; Tschirren et al. 2003). Experimental work on sticklebacks (Milinski and Bakker 1990; Frischknecht 1993; Bakker and Mundwiler 1994), house finches (Hill 1990, 1992), and great tits (Hörak et al. 2000, 2001; Fitze et al. 2003; Tschirren et al. 2003) demonstrated that carotenoid-based coloration reflects condition, parasite load or genetic variation in the ability to incorporate carotenoids, and supports the idea that carotenoid-based coloration honestly reveals an individual's quality (Olson and Owens 1998).

As nestlings depend entirely on the carotenoids provided by their parents, the carotenoids used for nestling plumage coloration may either be of maternal origin by transfer via the egg yolk (Blount et al. 2000; Nys 2000), or paternally derived via the food ingested shortly after hatching when males are the principal food providers (Kluyver 1950), or be provided by both parents for a longer period in nestling life when, commonly, both parents feed young. Understanding the evolution of carotenoid-based plumage colour and its signalling function thus requires knowledge of the timing of plumage colour determination.

To investigate the timing of carotenoid deposition in the plumage during nestling growth and development, we conducted an experimental field study on great tit nestlings (*Parus major*).

Great tits are one of the few bird species where a carotenoid-based plumage coloration is displayed in nestlings (Brush 1990). Their yellow breast plumage coloration results from the carotenoids lutein and zeaxanthin (Partali et al. 1987; Gosler 1993).

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Within each brood, nestlings were assigned to one of three treatments. They received additional carotenoids either 3 and 4 or 7 and 8 days after hatching, or were fed with a placebo on both occasions. Without a timing effect, we predict no difference between early and late supplemented nestlings, but significant differences between placebo- and carotenoid-treated nestlings within each brood. If the timing of carotenoid ingestion is important, we predict significant colour differences between early and late supplemented nestlings.

## Materials and methods

### General procedures

This experiment was performed in a great tit population in 1999 in the 'Forst', a deciduous forest near Bern, Switzerland (46°54'N 7°17'E / 46°57'N 7°21'E). The precise hatching date of the broods was determined by daily visits until hatching (hatching day = day 1). Nestlings were individually marked by clipping dorsal tufts on day 2, and were ringed with individual aluminium rings on day 8. Body mass was measured on days 2, 8 and 16 using an electronic scale (precision 0.01 g). On day 16, the length of the metatarsus was measured to the nearest 0.1 mm and the length of the third primary feather to the nearest 0.5 mm (Svensson 1992). A total of 15 second or replacement broods containing 94 nestlings were experimentally manipulated.

### Experimental carotenoid supplementation

In a within-brood design, nestlings were randomly assigned to one of three different feeding treatments: early fed nestlings received carotenoids shortly after hatching (days 4 and 5) and placebos later on (days 8 and 9); in late fed nestlings the feeding order was inverted (placebo early, carotenoids late); and placebo fed nestlings received placebos early and late.

Early fed nestlings were supplemented on both days 4 and 5 with 17 mg ( $\pm 0.25$  mg) carotenoid beadlets per feed containing 5.58% lutein and 0.44% zeaxanthin (Roche, Basel), and on both days 8 and 9 with 17 mg ( $\pm 0.25$  mg) placebo beadlets (Roche, Basel) per feed. Late fed nestlings were supplemented with 17 mg ( $\pm 0.25$  mg) placebo beadlets on days 4 and 5, and with 17 mg ( $\pm 0.25$  mg) carotenoid beadlets on days 8 and 9. Control nestlings were supplemented with 17 mg ( $\pm 0.25$  mg) placebo beadlets on all four days.

Nestlings were experimentally fed with the above-mentioned quantities in a single feed on each of the four days. The supplemented dose of lutein (0.95 mg/day) per nestling is approximately 5–15 times higher than the natural daily lutein intake (Royama 1966; Partali et al. 1987; Fitze, personal observation). The yellow plumage coloration of the carotenoid supplemented nestlings was in the natural range of the nestling plumage coloration [range of nestling plumage coloration as measured in an earlier study in the same study area (Fitze et al. 2003): H=41–47; S=0.40–0.61; B=0.64–0.92 and range of the carotenoid fed nestlings of this study: H=40–44; S=0.52–0.59; B=0.70–0.85]. The carotenoid supplementation took place before the first breast feathers appeared (Winkel 1970; Fitze, personal observation) and the lutein/zeaxanthin ratio of the supplemented carotenoid beadlets was similar to the ratio found in the natural diet of great tit nestlings (Partali et al. 1987). The beadlets were inserted into the throat of the nestlings, together with a small bee larva, to ensure swallowing of the beadlets [for further details see (Tschirren et al. 2003)].

### Plumage colour quantification

Fifteen days post-hatching, nestling great tits were photographed under standard light conditions using a digital camera and two flashes as described in Fitze and Richner (2002), Fitze et al. (2003) and Tschirren et al. (2003). Standard white chips (Kodak Colour Control Patches, Kodak, New York) were used for the calibration of the photos (see Statistical analyses section). Mean Hue-Saturation-Brightness (HSB) values of each nestling's breast plumage colour were calculated (Fitze and Richner 2002). Both the photographing and the analyses were done blindly with respect to treatment and condition of the birds.

The colour measurements do not exactly correspond to the colours perceived by birds. Also, birds possess biologically functional receptors for UV light (e.g. Cuthill et al. 2000) to which our equipment was insensitive. As remarked by Bennett et al. (1994), "for heuristic purposes, it may be useful to express colour patterns in subjective terms that humans can readily understand". Most importantly, the supplemented carotenoids have an absorption maximum within the human visible spectrum, the differences between carotenoid supplemented and placebo fed nestlings can be reliably measured by the camera (Tschirren et al. 2003), the method used in this study is highly repeatable (see Fitze and Richner 2002) and the measured colour differences were experimentally induced. Therefore, we assume that differences perceived by the digital camera correlate with differences visible to birds.

### Statistical analyses

Prior to the colour analysis, the hue-saturation-brightness values of the breast colour were corrected for variation in light exposure during photographing, as assessed from the measurements of the white reference chips. Residuals of the correlations between the HSB values of the plumage coloration and those of the white reference chips were used in the subsequent analyses. As H, S and B values were intercorrelated in earlier studies, we used principal component analysis as an overall measure of the plumage coloration [hereafter referred to as Colour PC1; see Fitze et al. (2003) and Tschirren et al. (2003)]. The first principal component of the residual colour parameters HSB explained 52.42% of the total variance (factor loadings: H=-0.702, S=0.711, B=0.048).

We included the treatment and the brood where the nestlings grew up as factors, the individual body condition, defined as the residuals of the regression of body mass on tarsus, as a covariate, and the interaction between brood and treatment into an ANCOVA model for the analysis of Colour PC1. The factor brood accounts for differences between broods due to common genetic and maternal effects, and differences due to a common environment, including food abundance, territory quality and parental behaviour. Non-significant interactions were eliminated backwards.

The effect of the feeding treatment on the body mass development of the nestlings was analysed by a repeated-measures ANOVA with body mass on days 2, 8 and 16 as repeats. Because all the nestlings died in 5 of the 15 broods ( $n=21$ ) before 15 days post-hatching, and as 27 nestlings died in the other ten broods, all analysis was conducted on the basis of the 46 surviving nestlings. Mortality was analysed using logistic regression (Beath 2000) including both feeding treatment and brood as a factor into the model.

Prior to analysis, dependent variables were checked for unequal variances, using Bartlett tests, and residuals of the model were tested for normality (Sokal and Rohlf 1981). Two-tailed tests were applied throughout, with the significance level set at  $P \leq 0.05$ , means  $\pm$  SE are given throughout. Data were analysed using the JMP IN 4.0 statistical package (Sall and Lehman 1996) except for the mortality analysis where we used GLM Stat Version 5.0.4. (Beath 2000).

## Results

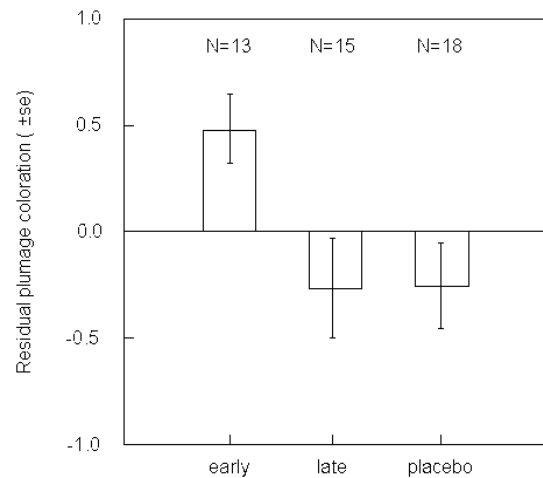
### Effects of timing of carotenoid supplementation on plumage coloration

Nestling plumage coloration was significantly influenced by the feeding treatment (see Table 1; Fig. 1) and the brood. The feeding treatment explained 12.19% of the total variation, the brood 37.81%. Body condition did not affect plumage coloration significantly, and the interaction between feeding treatment and brood was not significant ( $F_{18,15}=1.676$ ,  $P=0.159$ ). As the feeding treatment consisted of three different levels, we applied individual contrasts, based on least square means (Sokal and Rohlf 1981; Sall and Lehman 1996) to locate significant differences between the treatment groups. There was a significant difference between early and late fed nestlings ( $F_{1,33}=6.598$ ,  $P=0.015$ ), and between early and placebo fed ones ( $F_{1,33}=7.221$ ,  $P=0.011$ ; see Fig. 1), but no significant difference between placebo and late fed nestlings ( $F_{1,33}=0.009$ ,  $P=0.923$ ), as predicted if early carotenoid intake is important for plumage colour determination.

### Effects of the carotenoid supplementation on increase in body mass, body size and mortality

The increase in nestling body mass between days 2 and 16 was not significantly affected by the feeding treatment [repeated measures ANOVA with body mass on day 2 (early:  $2.27\pm 0.14$  g, late:  $2.21\pm 0.13$  g, placebo:  $2.19\pm 0.12$  g), 8 (early:  $10.78\pm 0.54$  g, late:  $10.58\pm 0.50$  g, placebo:  $10.27\pm 0.45$  g), and 16 (early:  $15.36\pm 0.50$  g, late:  $14.98\pm 0.47$  g, placebo:  $14.41\pm 0.42$  g) as repeats:  $F_{4,68}=0.78$ ,  $P=0.542$ ] but was significantly different between broods ( $F_{18,68}=5.511$ ,  $P<0.0001$ ). Neither metatarsus length (early:  $19.04\pm 0.20$  mm, late:  $18.91\pm 0.19$  mm, placebo:  $18.85\pm 0.17$  mm;  $F_{2,34}=0.894$ ,  $P=0.418$ ) nor primary length (early:  $33.73\pm 1.11$  mm, late:  $33.67\pm 1.03$  mm, placebo:  $32.50\pm 0.94$  mm;  $F_{2,34}=0.332$ ,  $P=0.720$ ) were influenced by the treatment. However, differences between broods were significant (metatarsus length:  $F_{9,34}=6.487$ ,  $P<0.001$ ; primary length:  $F_{9,34}=3.675$ ,  $P=0.003$ ). There were no significant interactions between brood and treatment (all  $P>0.4$ ).

Nestling mortality was not significantly different between treatment groups (early fed group: 57% mortality, late fed group: 54%, and placebo fed group: 44% mortality; logistic regression:  $\Delta D=2.131$ ,  $F_{2,77}=0.968$ ,  $P=0.383$ , scale=1.100). It was, however, significantly



**Fig. 1** Effect of the feeding treatment on plumage coloration. The residual plumage coloration derived from an ANOVA including the feeding treatment and the nest effect (see Materials and methods) is shown

different between broods ( $\Delta D=44.42$ ,  $F_{14,77}=2.884$ ,  $P=0.002$ ). As 5 (consisting of 21 nestlings) of the original 15 broods died before day 16, we restricted the mortality analysis in a second step to the ten broods where at least one nestling was alive on day 16. A total of 27 nestlings died in these broods. Again, mortality was not significantly different between treatment groups (early fed group: 43% mortality, late fed group: 42%, and placebo fed group: 25% mortality;  $\Delta D=2.131$ ,  $F_{2,61}=0.767$ ,  $P=0.489$ , scale=1.389) and it was not significantly different between broods ( $\Delta D=9.187$ ,  $F_{9,61}=0.735$ ,  $P=0.675$ ).

## Discussion

This study shows that nestlings supplemented with carotenoids shortly after hatching developed intense yellow plumage coloration, whereas nestlings supplemented later on, but before feathers were visible, did not differ in colour expression from control nestlings supplemented with a placebo. Therefore, only carotenoids ingested during the first 6 days of life and carotenoids derived from the egg yolk significantly contribute to plumage coloration.

Nestlings completely depend on the carotenoids provided by their parents, thus the carotenoids used for nestling plumage coloration may either be paternally derived, of maternal origin, or provided by both parents. Because, during the first days of life, nestling passerines are unable to properly thermoregulate (e.g. Mertens

**Table 1** Effects of the feeding treatment on the coloration of the nestling plumage. The results of an ANCOVA with feeding treatment and brood as factors and body condition as a covariate are presented

Variable	Sum of squares	df	F	P	Variance explained
Feeding treatment	8.646	2, 33	4.497	0.019	12.19%
Brood	26.822	9, 33	3.099	0.008	37.81%
Condition	2.177	1, 33	2.264	0.142	3.07%

1977), they rely on the heat provided by their mother. Consequently, mothers brood regularly during the first 5–7 days after hatching, and males are the main food providers (Kluyver 1950; Betts 1955; Fitze, personal observation). Here we show that only early, but not late, ingested carotenoids contribute to plumage colour expression, suggesting that mainly paternally derived carotenoids determine plumage coloration. The importance of paternally derived carotenoids has further been demonstrated in an earlier cross-foster study, where a significant positive correlation between nestling and foster father plumage colour, but none between the nestlings and their foster mothers, has been shown (Fitze et al. 2003). Both studies together suggest that nestling plumage colour reflects the male's food provisioning abilities or territory quality and, further, that male food provisioning during early nestling life correlates with male plumage colour, as found in house finches *Carpodacus mexicanus* (Hill 1991) and northern cardinals *Cardinalis cardinalis* (Linville et al. 1998).

Maternally derived carotenoids may further contribute to nestling plumage colour expression, since females transfer a significant amount of carotenoids into the eggs (e.g. Blount et al. 2000), and these maternal carotenoids are transferred from yolk to embryo (Surai et al. 1999). As we further show that plumage coloration is determined early in life, the proportion of maternal carotenoids compared to the amount of carotenoids ingested during the first 6 days is higher than in a situation of late plumage determination where the carotenoids used for plumage coloration are ingested over a longer period. In addition, we showed in an earlier cross-foster study (Fitze et al. 2003) that the origin of the nestlings explained 11.73% of the variance in plumage coloration, supporting the possible relevance of maternally derived carotenoids for plumage coloration. Thus, plumage coloration may reflect—beside the paternally derived carotenoids—maternal investment and/or embryonic development. However, no significant positive correlation between genetic mother and offspring plumage colour could be demonstrated in the earlier cross-foster study (Fitze et al. 2003). The importance of maternal effects thus remains unclear.

Nestling plumage coloration is a signal of condition, as effects have been found of brood size manipulation on colour expression (Hörak et al. 2000; Tschirren et al. 2003). Due to the early determination of plumage coloration, we show that condition mainly affects colour expression during the first few days of the nestling period. Other environmental factors, such as parasites, have also been suggested for driving carotenoid-based coloration (Dufva and Allander 1995; Thompson et al. 1997; Hill and Brawnner III 1998; Hörak et al. 2001). Evidence for parasite-dependent colour expression was found in adult birds (Hill and Brawnner III 1998; Hörak et al. 2001; but see Fitze and Richner 2002) but could not be confirmed for nestlings (Tschirren et al. 2003). The lack of evidence in nestling plumage coloration, however, could be due to parasite manipulation after plumage colour determination

since nests were infested with ectoparasitic hen fleas, *Ceratophyllus gallinae*, not before 7 days post-hatching.

Olson and Owens (1998) hypothesised that carotenoids may be deleterious. However, we found no indication that carotenoids might have detrimental effects on body mass development, tarsus length, feather length or nestling mortality under natural conditions, neither in this nor in an earlier study which included 450 experimentally supplied nestlings (Tschirren et al. 2003), rendering detrimental effects of carotenoids at least under natural conditions unlikely. On the other hand, Olson and Owens (1998) proposed that carotenoids may have beneficial effects on an animal's physiology as they have important properties, like free radical scavenging and promotion of the immune system (e.g. Burton 1989; Bendich 1989, 1991; von Schantz et al. 1998; Blount et al. 2000). According to their hypothesis, animals supplemented with additional carotenoids should be able to manage oxidative stress and disease better. Consequently, carotenoid-supplemented individuals should grow faster, be in better condition and survive better than unsupplemented animals. In this and a previous study, we did not find significantly positive effects of carotenoid-supplementation on growth, condition or survival. The absence of positive effects may have different reasons. Firstly, the positive effects of carotenoids might be significant but too small to be demonstrated in the field due to large environmental variance. Secondly, carotenoids might stimulate the immune system only, and thus affect only immune responses rather than growth and condition.

This study shows that nestling plumage coloration depends to a large extent on paternal food provisioning, as only carotenoids ingested during the first 6 days, and probably those derived from the egg yolk, contribute to colour expression. The known environmental determinants of nestling plumage colour, including brood size and carotenoid availability, must therefore principally act shortly after hatching. Thus, if nestling plumage colour signals offspring quality, it is necessarily a property acquired early in life.

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