Proximate mechanisms of variation in the carotenoid-based plumage coloration of nestling great tits (*Parus major* L.)

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Abstract

Many vertebrates use carotenoid-based signals in social or sexual interactions. Honest signalling via carotenoids implies some limitation of carotenoid-based colour expression among phenotypes in the wild, and at least five limiting proximate mechanisms have been hypothesized. Limitation may arise by carotenoid-availability, genetic constraints, body condition, parasites, or detrimental effects of carotenoids. An understanding of the relative importance of the five mechanisms is relevant in the context of natural and sexual selection acting on signal evolution. In an experimental field study with carotenoid supplementation, simultaneous cross-fostering, manipulation of brood size and ectoparasite load, we investigated the relative importance of these mechanisms for the variation in carotenoid-based coloration of nestling great tits (*Parus major*). Carotenoid-based plumage coloration was significantly related to genetic origin of nestlings, and was enhanced both in carotenoid-supplemented nestlings, and nestlings raised in reduced broods. We found a tendency for ectoparasite-induced limitation of colour expression and no evidence for detrimental effects of carotenoids on growth pattern, mortality and recruitment of nestlings to the local breeding population. Thus, three of the five proposed mechanisms can generate individual variation in the expression of carotenoid-based plumage coloration in the wild and thus could maintain honesty in a trait potentially used for signalling of individual quality.

Introduction

Carotenoid-based coloration is widespread in nature and is used by different vertebrate species as signals in the context of natural and sexual selection (e.g. Baker & Parker, 1979; Kodric-Brown, 1989; Hill, 1990, 1991; Milinski & Bakker, 1990; Savalli, 1995; Sundberg, 1995). Signalling theory predicts that signals have to be costly to function as an honest indicator of individual quality (Zahavi, 1975; Grafen, 1990), and thus predicts variation in signalling traits among phenotypes.

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Despite the increasing number of studies indicating that carotenoid-based colours are important signalling traits, surprisingly little is known about the proximate mechanisms that lead to variation in carotenoid-based colours among phenotypes in the wild (e.g. Bortolotti *et al*., 2000). The understanding of the proximate mechanisms determining colour expression, however, is essential for the understanding of how honesty of carotenoid-based signals is maintained. Several not mutually exclusive hypotheses have been proposed to explain variation in carotenoid-based colour expression (reviewed in Olson & Owens, 1998) but experimental field studies investigating the importance of these hypotheses are still rare. Carotenoids cannot be synthesized by animals and thus have to be ingested with the food (Goodwin, 1984). The knowledge of whether carotenoid-availability is limited in nature or whether carotenoids are a widespread resource is therefore crucial

for the understanding of all carotenoid-based functions and structures. Several studies suggest that carotenoid-availability is limited in nature (carotenoid-availability hypothesis, e.g. Slagsvold & Lifjeld, 1985; Hill, 1992, 1994; Grether *et al.*, 1999; Craig & Foote, 2001). Signalling theory therefore predicts that access to large quantities of carotenoids and thus development of an intense coloration is restricted to high quality individuals (e.g. Kodric-Brown, 1989). However, the carotenoid-availability hypothesis is controversial as other studies assume that carotenoids are ubiquitous in the natural diet of animals and therefore not a limited resource (e.g. Hudon, 1994; Thompson *et al.*, 1997).

Limitation of the colour expression may also occur post-consumption as a result of the physiological ability of an individual to absorb or deposit ingested carotenoids. This ability may have a genetic, a condition-dependent, and/or a parasite-dependent determination. The genetic-limitation hypothesis suggests that colour differences between individuals may arise by genetic control of colour expression (Brush, 1990; Bortolotti *et al.*, 2000). Heritable variation is a basic assumption for the evolution of a signal. Despite the numerous studies suggesting a signalling function of carotenoid-based colours, only Hill (1991) found a correlation between father and son plumage colour and thus an indication for a genetic determination of the carotenoid-based plumage coloration in birds (see also Craig & Foote, 2001 for differences in carotenoid-use by sockeye salmon morphs). An experimental cross-fostering approach is required to investigate origin-related variation in plumage coloration of birds. However, both genetic and maternal effects may contribute to origin-related variation measured with this approach (see Discussion).

Besides genetic constraints, condition-dependent physiological mechanisms involved in carotenoid absorption, transport, metabolism, storage or deposition may restrict the colour intensity (condition-dependence hypothesis Brockmann & Völker, 1934; Brush, 1978; Frischknecht, 1993; Hill & Montgomerie, 1994; Hudon, 1994; Bortolotti *et al.*, 1996; Thompson *et al.*, 1997).

Furthermore parasites may limit the expression of carotenoid-based colours by reducing pigment uptake or host-condition, or by provoking a trade-off between carotenoid demand for coloration vs. immune function (parasite hypothesis, e.g. Hamilton & Zuk, 1982; Milinski & Bakker, 1990; Zuk *et al.*, 1990; Houde & Torio, 1992; Lozano, 1994; Skarstein & Folstad, 1996; Hill & Brawner III, 1998). However, whether carotenoid-based colours signal parasite load or health status is controversial as positive relationships between coloration and parasite load have also been reported in the literature (Shykoff & Widmer, 1996; reviewed in Møller *et al.*, 2001).

In contrast to the idea that carotenoids act beneficially on the immune system of animals (e.g. free radical absorption or enhancement of immune response, reviewed in Bendich, 1989a and Møller *et al.*, 2001), detrimental effects of carotenoids have been suggested recently (Nowak, 1994; Olson & Owens, 1998). A trade-off between coloration and health status, due to carotenoid ingestion, may therefore arise. Thus only individuals of good quality would be able to carry the costs of developing an intense coloration.

To investigate the importance of the five proposed mechanisms that potentially limit carotenoid-based colour expression and lead to variation in carotenoid-based coloration among phenotypes, we performed a field experiment on nestling great tits (*Parus major*, Passeriformes). The great tit is one of the few bird species where a conspicuous carotenoid-based coloration is developed already during the nestling stage (Brush, 1990). It arises by unmodified deposition of the two carotenoids lutein and zeaxanthin in the developing feathers (Partali *et al.*, 1987).

By supplementing nestling great tits with carotenoids, we experimentally investigated whether access to dietary carotenoids limits the expression of the yellow plumage coloration. According to the carotenoid-availability hypothesis we predict a more intense coloration of carotenoid-supplemented nestlings. To investigate the role of both origin-related and condition-dependent effects, nestlings were cross-fostered and raised in naturally sized or reduced broods. According to the genetic-limitation and the condition-dependence hypotheses, we expect origin-related variation in the carotenoid-based coloration and predict a more intense coloration of nestlings raised in reduced broods. To test for the parasite hypothesis we infested half of the nests with the hen flea *Ceratophyllus gallinae,* a common haematophagous ectoparasite that affects reproduction and condition of great tits (e.g. Richner *et al.*, 1993; Heeb *et al.*, 1999). According to the parasite hypothesis we predict a reduced colour intensity of nestlings raised in infested nests. Finally, we tested for detrimental effects of carotenoids by comparing nestling body mass, mortality between hatching and fledging, and local recruitment of carotenoid-supplemented and control nestlings. The combination of the treatments applied simultaneously to the nests allowed us to investigate the importance of the proposed mechanisms within one single study design. Thus our experimental set-up allowed a comparative investigation of the relative importance of the different proposed mechanisms, as opposed to approaches where the effects are investigated separately and independently.

Materials and methods

General experimental procedure

The experiment was performed during the breeding season, 1999, in a great tit population breeding in nest boxes in the Forst, a forest near Bern, Switzerland (46°54′N 7°17′E/46°57′N 7°21′E). Nest boxes were regularly visited from the beginning of the breeding

season onwards to determine the start of egg laying and the hatching date. To eliminate initial differences in nest-based ectoparasite levels and thus additional variance in the measured traits, we heat-treated all nests in a microwave oven the day the birds laid their sixth egg (following Richner *et al.*, 1993). Nestling body mass was measured on day 2 (day 1 = day of hatching), day 8 and day 16, using an electronic balance with a precision of 0.01 g. Nestlings were marked individually by clipping down feathers on day 2 and were ringed with aluminium rings on day 8. In the following year, breeding great tits were captured in the nest boxes during the nestling period to assess local recruitment.

Brood size manipulation and cross-fostering

To investigate condition-dependent variation of the carotenoid-based coloration we experimentally manipulated the condition of nestlings by means of a brood size reduction. First, all eggs were weighed after clutch completion and the medium-sized egg of each nest was replaced by an artificial egg (Fig. 1a). Thus, all females incubated a clutch with the original clutch size but with one egg being replaced by an artificial egg. To create reduced and naturally sized broods, nestlings were exchanged among pairs of nests (hereafter referred to as nest pairs) with the same hatching date and a similar brood size (maximal difference of two nestlings, total 62 nests) 1 day post-hatching. One nest of each nest pair ended up with two nestlings less after the brood size manipulation compared with the brood size before egg replacement (hereafter referred to as reduced nest), whereas in the other nest the brood size before egg replacement was restored (hereafter referred to as control nest, Fig. 1a). Because of the egg replacement before incubation, no surplus nestlings remained after brood size reduction, which otherwise could be of ethical concern. The brood size of reduced and control nests was not significantly different before the brood size manipulation (mean brood size of reduced nest: 7.4 ± 0.2 nestlings, mean brood size of control nest: 7.4 ± 0.2 nestlings, paired *t*-test: $t_{30} = 0.0$, n.s.), whereas differences after brood size manipulation were highly significant (mean brood size of reduced nest: 6.4 ± 0.2 nestlings, mean brood size of control nest: 8.4 ± 0.2 nestlings, paired *t*-test: $t_{30} = -9.89$, $P = 0.0001$). Mean nestling body mass of reduced vs. control broods was not significantly different immediately after manipulation (mean nestling body mass in reduced nests: 2.48 ± 0.07 g, mean nestling body mass in control nests: 2.44 ± 0.08 g, paired *t*-test: $t_{30} = 0.595$, n.s.), indicating that there was no experimenter bias due to initial body mass differences between treatment groups.

Simultaneously with the brood size manipulation, nestlings were partially cross-fostered within nest pairs (total 460 nestlings of 62 nests, Fig. 1a) to investigate origin-related variation of the carotenoid-based coloration. Cross-fostering allowed a separation of effects due to either common environment or common origin. First, nestlings were ranked according to their body mass in the nest where they were born (hereafter referred to as origin). The heaviest nestling was randomly assigned to stay in the nest of origin or to be exchanged to the partner nest by throwing a coin. Cross-foster treatment (exchange / stay) was then alternated through the

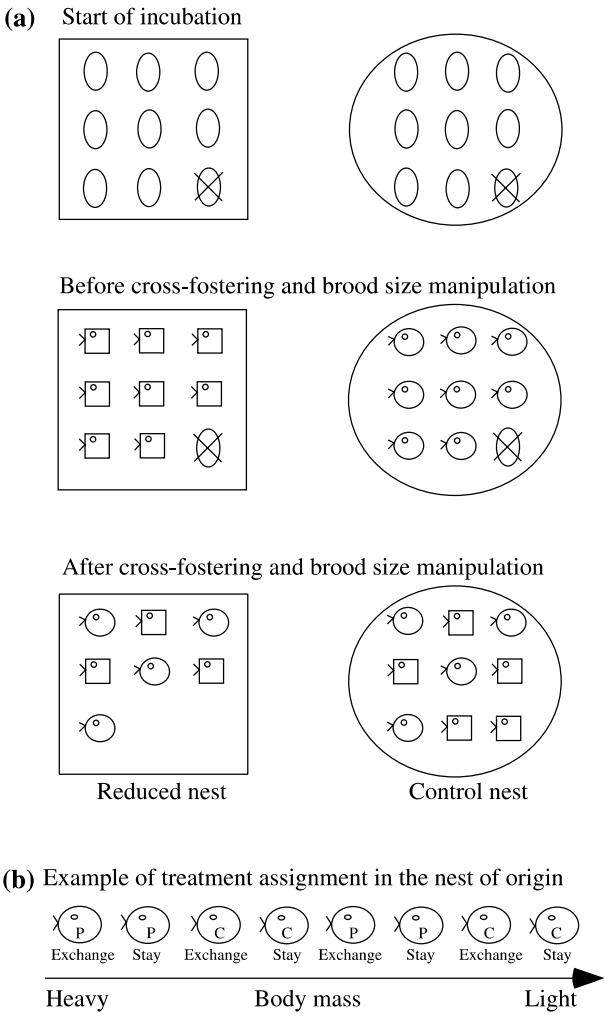

Fig. 1 Experimental design (a) In all clutches, the medium-sized egg was replaced by an artificial egg. Thus, compared with their originally planned family size, all broods were reduced by one nestling 1 day post-hatching. Among pairs of nests, nestlings were partially cross-fostered and the brood size of the nests was manipulated simultaneously. As a consequence of both treatments, one nest of each pair was reduced by two nestlings, whereas the other one held the originally planned number of offspring and both nests consisted of own and foreign nestlings. (b) Cross-foster treatment (exchange / stay) and feeding treatment (carotenoid C / placebo P) were randomly assigned to the heaviest nestling of each nest, and then alternated through the mass-based rank list of nestlings.

mass-based rank list (Fig. 1b) (see Hurlbert (1984) for the validity of the random-systematic treatment interspersion). In the two nests of a nest pair, cross-foster treatments were assigned randomly and independently of the treatment applied to the partner nest (Fig. 1b).

Thirty-eight eggs in 33 nests had not hatched at the time of cross-fostering. They were not exchanged but stayed in their nest of origin because of the typically very low survival chance of late hatched nestlings. Seventeen of the 38 unhatched eggs mentioned above were sterile. Only nine late hatched nestlings survived till day 16 and were included in the analysis. Unhatched eggs were distributed similarly over the treatment groups and the results of the statistical analyses did not change if the late hatched nestlings were excluded.

Carotenoid supplementation

The influence of access to dietary carotenoids on the expression of the plumage coloration was investigated by a carotenoid supplementation experiment. Before cross-fostering, the heaviest two nestlings of each nest were assigned to be either carotenoid-supplemented or to receive placebo beadlets by throwing a coin. Feeding treatment was thereafter alternated in pairs through the rank list (Fig. 1b). Thus, siblings with a similar body mass on day 2 growing up in a reduced and a control brood, respectively, were assigned to the same feeding treatment (Fig. 1b) [see Hurlbert (1984) for validity of the random-systematic treatment interspersion].

The nestlings were fed six times every second day starting 3 days post-hatching. Nestlings of the carotenoid-supplemented group were fed with 17 mg (±0.25 mg) carotenoid beadlets per feeding containing 5.58% lutein and 0.44% zeaxanthin, whereas nestlings of the control group were fed with 17 mg (±0.25 mg) placebo beadlets. The beadlets were inserted in the throat of the nestlings together with a small bee larvae (mean of 150 randomly chosen larvae: 134.7 ± 0.13 mg, all of the same larval stage) to ensure swallowing of the beadlets. The lutein/zeaxanthin ratio of the carotenoid-beadlets was similar to the ratio found in the natural diet of great tit nestlings (Partali *et al.*, 1987). Mean body mass per nest of carotenoid and placebo treated nestlings on day 2 was not significantly different (mean body mass of carotenoid-supplemented nestlings: 2.46 ± 0.05 g, mean body mass of placebo-fed nestlings: 2.43 ± 0.05 g, paired *t*-test: $t_{30} = -0.05$, n.s.). Thus, there was no experimenter bias due to initial body mass differences between treatment groups.

Flea infestation

To investigate the influence of flea infestation on the expression of the carotenoid-based coloration, nest pairs were deparasitized 3 days post-hatching as described above, and the nest height was standardized to 7 cm to avoid density-dependent effects on the flea population (Eeva *et al.*, 1994; Tripet & Richner, 1999).

Two days later, nest pairs were alternately assigned to be infested with 40 female and 20 male hen fleas obtained from naturally infested nests of the same forest, or to remain uninfested. Thus, both nests of a nest pair had the same flea treatment and treatments were equally distributed over the season.

Colour analysis

For the colour analysis a photograph of the breast plumage of the nestlings was taken 15 days post-hatching using a digital camera and two factory-calibrated flashes providing a standardized amount of light as described in Fitze & Richner (2002). Four standard white chips (Kodak Colour Separation Guide and Gray Scale, Q13/Q14, Red = 255, Green = 255, Blue = 255) were photographed together with the birds for calibration of the photographs. The photographs were taken inside an opaque box with a fixed distance between the plumage and the lens. This method led to highly repeatable measurements [all $r \geqslant 0.80$, see Fitze & Richner (2002) for details]. The pictures were analysed by measuring 10 a *priori* selected squares of 400 pixels each, and by subsequent calculation of a mean colour value (RGB) per bird. Photographing and colour analyses were performed blindly with respect to origin and treatment of the nestlings.

Hue (H), saturation (S) and brightness (B) of the birds' plumage coloration was calculated (Fitze & Richner, 2002). Variation in light exposure during photographing, assessed from the measurements of the white reference chips, was corrected by using the residuals of the correlations between the H–S–B values of the plumage coloration and those of the white reference chips. Residuals were used in the further analyses. Using Principal Component Analysis, the first principal component of the residual colour parameters HSB was derived and taken as an overall measure of the plumage coloration (hereafter referred to as Colour PC1). Colour PC1 explained 54.12% of the total variance (Eigenvectors: H = −0.634, S = 0.668, B = 0.390).

The visual system of birds is rather different from the human vision. Birds have at least four types of cones (compared with three as in humans), they see in the UV and possess a system of oil droplets filtering the light (reviewed in Bennett & Cuthill, 1994). As we used a digital camera insensitive to UV, the colour measures used for the analysis may not exactly correspond to the colours perceived by the birds. However, 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'. We manipulated the plumage coloration by controlled supplementation of the pigments responsible for the coloration. Furthermore, colour measurements were performed blindly with respect to the treatment applied to the nestlings, and

the method used in this study was highly repeatable as mentioned above. Therefore, we assume that differences perceived by the digital camera correlate with differences visible to birds.

Statistical analysis

Coloration

The influence of the flea treatment, the nest pair and the origin on the expression of the carotenoid-based coloration was assessed by a hierarchical mixed-model nested ANOVA (Type 1 SS) (see, e.g. Merilä, 1997). Flea treatment was the main nesting factor (fixed effect) with the nest pair (random effect) nested as a factor within the flea treatment, and the origin (random effect) nested as a factor within the nest pair and the flea treatment. Prior to analysis, Colour PC1 values were statistically corrected for the brood size manipulation and the carotenoid supplementation (factors that were manipulated, but not analysed in this model) using a two-way ANOVA. Residuals were then used in the model mentioned above. The origin accounts for origin-related factors like common genes, maternal effects or a shared environment before cross-fostering. Nest pair controls for seasonal variation in coloration, e.g. seasonal variation in the availability of carotenoids. It further accounts for the correlation between season and both genetic and environmental sources of variance, e.g. for a correlation between season and parental or territorial quality.

To analyse the effect of the brood size manipulation and the carotenoid supplementation on the colour expression, we used a repeated-measures ANOVA with mean Colour PC1 per nest of the carotenoid-supplemented and placebo-fed nestlings as repeated measurements, and brood size manipulation as a factor. Prior to analysis, Residual Colour PC1 values were calculated in a nested model, including flea treatment, nest pair and origin. Residuals were used in the statistical analysis mentioned above.

The experimental effects on nestling coloration were analysed in two separate models to estimate the denominator d.f. of the different manipulated factors correctly in our experimental design with cross-fostering between two nests only.

Body mass

The effect of the brood size manipulation on the body mass of the nestlings was analysed by a repeated measures ANOVA with mean body mass per nest on days 2, 8 and 16 as repeats. Body mass was statistically corrected for the time (hour) of measurement to account for diurnal body mass changes and for the treatments not analysed in this model. Residuals were then used in the analysis.

Detrimental effects of carotenoids

To analyse detrimental effects of carotenoids on growth, the mean body mass of carotenoid- and placebo-fed nestlings per nest on days 2, 8 and 16 was analysed by a repeated General Linear Model with two trial factors. Prior to analysis, residual body mass was calculated as mentioned above. Furthermore, mortality from hatching to fledging of carotenoid-supplemented and control nestlings, and recruitment of the fledglings to the local breeding population of the following year, was analysed by logistic regression.

General comments

Lower sample size, as mentioned in the Method section *Brood size manipulation and cross-fostering*, is due to nestling mortality or rare technical problems with the digital camera (one nest pair with 17 nestlings). Complete brood mortality occurred in 10 nests (72 nestlings). Additionally 10 nestlings from nine different nests died between day 2 and day 16. Nestling mortality was not significantly different between reduced and control nests (logistic regression: $D = 13.77$, Scale $= 6.217$, $F_{1,60} = 2.21$, $P = 0.142$), between flea infested and uninfested nests (logistic regression: $D = 14.06$, Scale $= 6.213$, $F_{1,60} = 2.26$, $P = 0.138$), and between carotenoid-supplemented and placebo-fed nestlings ($D < 0.001$, Scale = fixed at 1, $\chi^2_{61} < 0.001$, n.s.), indicating that our results were not significantly biased by differential mortality.

Reduced broods can be manipulated faster than control broods because of the lower number of nestlings. We corrected for this difference by spending approximately the same amount of time at the two nests of a nest pair during manipulations (summed time spent at the nests (min): brood size manipulation, paired *t*-test: $t_{30} = -0.424$, n.s.; flea treatment, one-way ANOVA: $F_{1,60} = 1.626$, $P = 0.207$).

Normality of the data was tested prior to analysis. All tests are two-tailed and the significance level is set at $P = 0.05$. Mean ± SE are given. Most statistical analyses were performed using the JMP IN 3.2.1. statistical package (Sall & Lehmann, 1996). SYSTAT 5.2.1. statistical package (Wilkinson, 1989) was used for the General Linear Model with trial factors. Logistic regression analysis with binomial error and a logit link was performed using GLM Stat 5.3.1 (Beath, 2000). Chi-squared tests were applied if the estimated scale was $\leqslant 1$ and *F*-tests were applied if the scale was >1.

Results

Carotenoid-based coloration

The expression of the carotenoid-based coloration in nestling great tits was significantly influenced by the origin and the nest pair, whereas there was a tendency only of the flea treatment to affect nestling plumage coloration (Table 1).

Furthermore, colour expression was significantly influenced by the brood size manipulation and the

Table 1 Effects of the flea treatment, the nest pair (nested within the flea treatment) and the origin (nested within the nest pair and the flea treatment) on the expression of the carotenoid-based plumage coloration (Colour PC 1) of great tit nestlings. Hierarchical mixed-model nested ANOVA with sequential sum of squares, see Materials and methods for statistical details.

	SS	F	d.f.	*P*-value
Flea treatment	2.85	3.16	1, 19.33	0.076
Nest pair	127.75	4.88	29, 25.86	<0.0001
Nest of origin	61.46	2.20	31, 297	0.0004
Error	267.87		297	

Table 2 Influence of the brood size manipulation and the carotenoid supplementation on the expression of the carotenoid-based plumage coloration (Colour PC 1) of great tit nestlings. Repeated-measures ANOVA with mean Colour PC1 per nest of carotenoid- and placebo-fed nestlings as repeated measurements and brood size manipulation as a factor, see Materials and methods for statistical details.

	SS	F	d.f.	*P*-value
Between nests				
Brood size manipulation	2.61	6.81	1	0.012
Error	18.42		48	
Within nests				
Carotenoid supplementation	29.38	106.34	1	<0.0001
Brood size manipulation × Carotenoid supplementation	0.14	0.50	1	0.482
Error	13.26		48	

carotenoid supplementation (Table 2). Carotenoid-supplemented nestlings and nestlings raised in reduced nests had higher Colour PC1 values than placebo-fed nestlings and nestlings raised in control nests (Figs 2 and 3). The interaction between the brood size manipulation and the carotenoid supplementation was not significant, indicating that the brood size manipulation did not influence the colour expression of carotenoid-supplemented and control nestlings differently. Variation of the carotenoid-based coloration was not different between nestlings raised in reduced and control nests nor between carotenoid-supplemented and placebo-fed nestlings (repeated measures ANOVA with Colour PC1 Coefficient of Variation of carotenoid- and placebo-fed nestlings as repeats and brood size manipulation as a factor; between subjects: brood size manipulation $F_{1,48} = 1.786$, n.s., within subjects: carotenoid supplementation $F_{1,48} = 1.406$, n.s., brood size manipulation × carotenoid supplementation $F_{1,48} = 1.493$, n.s.).

Nestling body mass

Mean body mass of nestlings (per nest) tended to be higher in reduced than in control brood (repeated measures ANOVA with body mass on days 2, 8 and 16 as repeated measures; between subjects: brood size manipulation $F_{1,48} = 3.61$, $P = 0.063$). Nestling growth, measured as the increase of body mass between day 2 and day 16, was significantly different between nestlings raised in reduced and control broods (repeated measures ANOVA, within subjects: brood size manipulation × day of measurement: $F_{2,47} = 4.23$, $P = 0.021$), showing that the brood size manipulation influenced the nestlings' rate of growth.

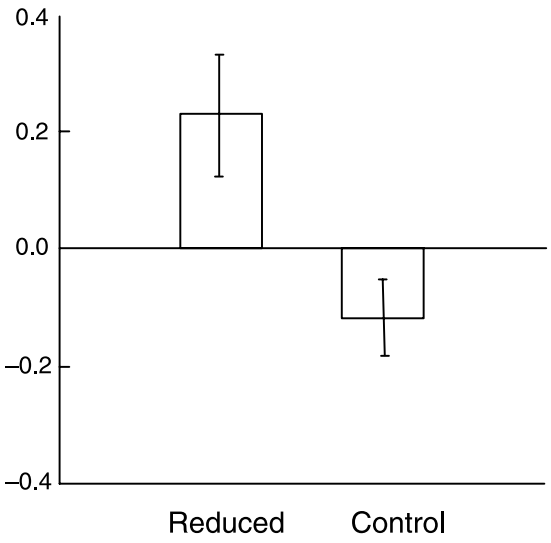

Fig. 2 Residual plumage coloration of nestlings raised in reduced and control broods.

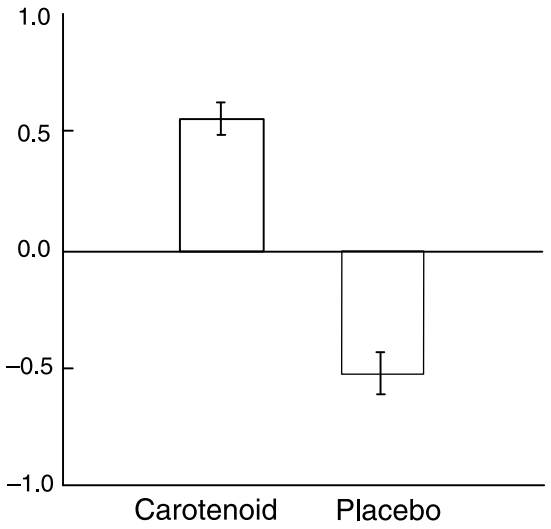

Fig. 3 Residual plumage coloration of carotenoid- and placebo-fed nestlings.

Effects of carotenoid supplementation on nestling body mass, mortality and local recruitment

Both mean nestling body mass and the increase of body mass from day 2 to day 16 were not significantly different between carotenoid- and placebo-treated nestlings (repeated-measures ANOVA with two trial factors, carotenoid supplementation: $F_{1,49} = 1.224$, n.s., carotenoid, supplementation × day of measurement: $F_{2,48} = 0.145$, n.s.). Neither nestling mortality (logistic regression: $D < 0.001$, Scale = fixed at 1, $\chi^2_{61} < 0.001$, n.s.) nor recruitment into the local breeding population (logistic regression: $D = 0.013$, Scale = fixed at 1, $\chi^2_{50} = 0.013$, n.s.) differed significantly between carotenoid-supplemented and placebo-treated nestlings.

Discussion

A growing number of studies reveal the importance of carotenoid-based colours in social and sexual signalling. However the mechanisms that limit the expression of carotenoid-based colours in the wild, which lead to variation in coloration among phenotypes, are controversial so far (reviewed in Olson & Owens, 1998). In this experimental field study we investigated the relative importance of five proposed mechanisms potentially responsible for differential colour expression.

As expected by the carotenoid-availability hypothesis (e.g. Hill, 1992, 1994; Grether *et al*., 1999), nestlings of the carotenoid-supplemented group developed a more intense yellow plumage coloration than placebo-fed nestlings. It shows that the carotenoid-based plumage coloration of great tit nestlings is limited by the amount of carotenoids ingested with the food and thus by the amount of carotenoids provided by the parents.

Olson & Owens (1998) suggested detrimental (toxic) effects of carotenoids on the physiology of animals. Thus, not the limited access to carotenoids but (active) avoidance of dietary carotenoids could explain a part of the observed variation in plumage coloration. Our results provide no indication for detrimental effects of the carotenoids lutein and zeaxanthin, as neither nestling body mass, growth rate, nestling mortality nor local recruitment were significantly different between carotenoid-supplemented and control nestlings. We thus suggest that active carotenoid avoidance due to detrimental effects may not be a crucial factor for colour expression in nature.

The origin of nestlings explained a significant amount of variation in the carotenoid-based plumage coloration. This effect may be explained both by an origin-related difference in the ability of nestlings to absorb or deposit ingested carotenoids (Brush, 1990), and/or by maternal effects. It is likely that maternal effects contribute at least partly to the observed variation because considerable amounts of carotenoids are deposited in the egg yolk by the mother (Goodwin, 1984; Partali *et al*., 1987; Blount *et al*., 2000). Thus origin-related variation in plumage coloration can at best be an indication of genetic limitation of colour expression.

Nest pair significantly influenced the colour expression. Nest pair accounts for seasonal variation in colour expression due to environmental factors and for correlation of seasonal effects with phenotypic or environmental sources of variance, e.g. for correlation of season and parental or territorial quality. The effect of the nest pair on colour expression corresponds to the results of Bortolotti *et al*. (2000) and Hõrak *et al*. (2000), who both found strong environmental determination of carotenoid-based plumage colours.

Condition-dependent physiological constraints may cause differences in plumage coloration by affecting the physiological pathway of carotenoids, e.g. their deposition into the follicular cells of developing feathers (Brockmann & Völker, 1934; Hudon, 1994; Thompson *et al*., 1997). As predicted by the condition-dependence hypothesis, nestlings from reduced nests developed a more intense yellow plumage coloration than nestlings from naturally sized broods. This result is in accordance with the findings of De Kogel & Prijs (1996) and Hõrak *et al*. (2000) who also found effects of brood size manipulation on the expression of carotenoid-based traits in an experimental design without carotenoid supplementation. However, without manipulation of carotenoids it is difficult to argue whether the observed effect of the brood size manipulation is more likely explained by the carotenoid-availability or the condition-dependence hypothesis. Differences in nestling plumage coloration of reduced and control broods may simply arise by parents of reduced nests providing nestlings with more carotenoids, rather than by a condition-dependent effect *per se*. Due to the carotenoid-supplementation, our experimental set-up allowed to distinguish between these two effects. The carotenoid-fed nestlings were supplemented with relatively large amounts of carotenoids compared with the normal carotenoid content of their diet (Partali *et al*., 1987). We therefore assume that differences in the carotenoid content of the food provisioned by parents of reduced and naturally sized broods are of minor importance in carotenoid-supplemented nestlings. Thus if carotenoid-availability alone would explain the colour differences between nestlings from reduced and control nests, we would expect no significant effect of the brood size manipulation on the colour expression of carotenoid-supplemented nestlings. However, even within the carotenoid-supplemented nestlings, the nestlings of the reduced broods showed enhanced colour expression (ANOVA: $F_{1,48} = 8.445$, $P = 0.006$). It shows that the ability to incorporate carotenoids into the feathers depends, besides the access to carotenoids and origin-related constraints, on a bird's condition. This result

seems surprising given that carotenoids do not undergo costly metabolic transformation in great tits (Partali *et al.*, 1987; Hill, 1996). It suggests that the incorporation of carotenoids itself is costly.

It is known that endoparasites interfere with physiological mechanisms involved in carotenoid absorption, transport, or deposition (e.g. Ruff *et al.*, 1974; Milinski & Bakker, 1990; Houde & Torio, 1992; Hill & Brawner III, 1998). Moreover in a study on house finches (*Carpodacus mexicanus*) (Thompson *et al.*, 1997) plumage coloration was negatively associated with feather mite load *Proctophyllodes* sp. indicating that ectoparasite infestation as well can influence plumage coloration. Parasites may interfere with the plumage pigmentation by reducing the carotenoid uptake or deposition, by reducing the nutritional condition, or by activating the immune system of the host (Allen & Nelson, 1982). Parasitic infestations may thereby rise the demands of the immune system for carotenoids used for free radical absorption (Bendich & Olson, 1989; Bendich, 1989b; Allen, 1997). Therefore an allocation trade-off between carotenoids used for free radical scavenging and feather pigmentation, respectively, has been postulated (e.g. Saino *et al.*, 1999; von Schantz *et al.*, 1999; Saino *et al.*, 2000; but see Hill, 1999). As a consequence of parasitism, infested nestlings should develop duller plumage coloration. The flea infestation had no significant influence on the colour expression of the nestlings (Table 1), however, there was a tendency for nestlings raised in infested nests to have reduced colour intensity. Harmfulness of fleas is influenced by environmental factors such as weather or food availability (Merino & Potti, 1996; Allander, 1998), and overall benign conditions, or the comparatively late infestation in the reproductive cycle of the host, may explain the relatively weak effect of fleas on colour expression. Alternatively, a flea infestation may not interfere with the carotenoid metabolism or may not rise the demands of the immune system for carotenoids (e.g. for free radical absorption). The weak effect of the fleas on coloration corresponds to the findings of an earlier study where melanin- but not carotenoid-based colours were influenced by ectoparasites in adult great tits (Fitze & Richner, 2002).

This study investigates the proximate mechanisms that lead to variation in the carotenoid-based plumage coloration of free-living birds. We demonstrate that access to dietary carotenoids is important for the expression of the carotenoid-based coloration. Furthermore we show that carotenoid-based colour expression is limited by both origin-related and condition-dependent factors, leading to variation in coloration among individuals. Carotenoid-based colours are used by different vertebrate species for signalling of individual quality, and our study elucidates the relevance of some mechanisms potentially implicated in the maintenance of honesty of these traits in the wild.

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