doi: 10.1111/j.1420-9101.2006.01095.x

Strong environmental determination of a carotenoid-based plumage trait is not mediated by carotenoid availability

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Keywords:

carotenoids;
colour;
genetic variance–covariance matrix;
Parus caeruleus.

Abstract

Carotenoid-based colours are recognized as having an important signalling function, yet the nature of the mechanisms that maintain their honesty is not well understood. By combining a carotenoid-feeding experiment with a quantitative genetic experiment in a wild population of blue tits (*Parus caeruleus*), we were able to test predictions that differentiate between proposed mechanisms. If variation in carotenoid ingestion underlies variation in carotenoid-based colour expression, then carotenoid-supplemented birds should have reduced variance in colour. In this study, carotenoid supplementation produced a small but significant change in plumage colouration, but no significant change in variation. These results suggest that variation in carotenoid acquisition is not an important source of variation for this colour trait, and that variation in post-ingestion processes are likely to be more important. The low heritability of this colour trait suggests environmental factors are likely to underlie the majority of variation in these processes.

Introduction

Carotenoid pigments are responsible for many of the highly saturated yellows, oranges and reds expressed in the integuments of several animal taxa (Fox & Vevers, 1960; Goodwin, 1980). Both experimental (Endler, 1980; Kodric-Brown, 1983; Milinski & Bakker, 1990; Hill, 1991; Houde & Torio, 1992) and comparative (Gray, 1996; Badyaev & Hill, 2000; Badyaev *et al.*, 2002; but see Owens & Hartley, 1998) work have demonstrated that these colours play an important role in sexual selection. Recently, much work has focused on testing hypotheses put forward to explain the proximate mechanisms of variation in integumentary carotenoids. These hypotheses centre on the fact that carotenoid-based colour expression should be costly to ensure honesty of signal content. The three main hypotheses put forward contend that carotenoids are costly to acquire ('rare'), costly because they are required by other physiological processes ('required'), or costly because they are toxic ('risky'; Olson & Owens, 1998). The distinction between these hypotheses is that the intrinsic worth of carotenoids are zero, positive, or negative respectively. The overall aim of this study was to estimate the relative roles of genetic and environmental factors in determining the variation in the development of carotenoid-based colouration in the blue tit, and apply this to the question of whether such signals are based on variation in carotenoid acquisition.

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Rare

The earliest of these hypotheses assume that carotenoids have no intrinsic worth other than as integumentary pigments, and that their expression is limited simply because they are rare. Because carotenoids cannot be synthesized by animals, and must be acquired in the diet, this hypothesis initially rested on the scarcity of ingested carotenoids (Endler, 1980). More recently this hypothesis has come to include the potentially limiting processes of carotenoid absorption, transportation, metabolism and deposition (Hill, 2002). The trade-off necessary for honest signalling is that resources used for carotenoid assimilation cannot be used in other physiological processes.

The hypothesis that carotenoid scarcity is a key factor is supported by a positive association between colouration and carotenoid concentrations of gut contents in a wild population of birds (Hill *et al.*, 2002) and between

populations of fish (Grether *et al.*, 1999). In addition, experimental manipulation of carotenoid access consistently produces changes in colouration and/or circulating levels of carotenoid in the blood plasma (Hill, 1992, 1993; Grether, 2000; Blount *et al.*, 2002b, 2003; McGraw *et al.*, 2002, 2004; Bortolotti *et al.*, 2003; Fitze *et al.*, 2003b; McGraw & Ardia, 2003; Tschirren *et al.*, 2003; Alonso-Alvarez *et al.*, 2004). As yet, there is no direct evidence that carotenoid absorption is costly, but plasma carotenoid concentrations are known to change seasonally (Negro *et al.*, 1998), and differ between the sexes (Bortolotti *et al.*, 1996; Negro *et al.*, 1998, 2001; McGraw *et al.*, 2003) despite individuals being fed a standardized diet. Studies on the costs associated with the metabolism of dietary carotenoids to produce many of the red pigments seen in birds are few, but are consistent with carotenoid metabolism being costly (Hill, 1996; Inouye *et al.*, 2001; Olson & Owens, 2005).

Required

The second hypothesis assumes that carotenoids are actually beneficial in many ways, and that the primary cost of expressing carotenoids in a non-recoverable form is that they cannot be used for the other physiological processes for which they are required (Lozano, 1994). This hypothesis shifts the currency of the trade-off from the resources needed to assimilate carotenoids to the carotenoids themselves, often by invoking the immune system as a causal link (Olson & Owens, 2005).

Many observational studies have shown a positive relationship between colour and immune response, or between colour and levels of parasitism (Dufva & Allander, 1995; Skarstein & Folstad, 1996; Thompson *et al.*, 1997; Figuerola *et al.*, 1999; Saino *et al.*, 1999; Bortolotti *et al.*, 2000; Saks *et al.*, 2003; Peters *et al.*, 2004; but see Fitze & Richner, 2002). However, although these results are suggestive, they fail to distinguish whether the change in carotenoid-based colouration is due to allocation of resources to the immune system, and away from carotenoid assimilation, or direct allocation of the carotenoids themselves to an immune function (Thompson *et al.*, 1997). Likewise, experimental studies in which parasite loads have been manipulated and also fail to make this distinction (e.g.Houde & Torio, 1992; Hill & Brawner, 1998; Saino *et al.*, 2002; Faivre *et al.*, 2003; Hill *et al.*, 2004).

Carotenoid supplementation experiments in which carotenoid-supplemented birds have been found to have stronger immune responses than non-carotenoid-fed birds are more conclusive (Blount *et al.*, 2002b, 2003; Grether *et al.*, 2004a). However, the two experiments that have used a carotenoid supplementation treatment together with an immune challenge have failed to find a significant interaction with respect to colouration or concentration of plasma carotenoids (Tschirren *et al.*, 2003; Alonso-Alvarez *et al.*, 2004).

Risky

The risky hypothesis (Zahavi & Zahavi, 1997; Hartley & Kennedy, 2004) has received little attention, and contends that the oxidation products of carotenoid pigments are toxic. Rather than carotenoids being required for their role as antioxidants (Blount *et al.*, 2000), as is consistent with the required hypothesis, the risky hypothesis suggests that carotenoid expression may be honest because only individuals with a wealth of antioxidant resources are able to limit the bleaching of pigmentary carotenoids. Only one direct test of this hypothesis has been made (Tschirren *et al.*, 2003) and carotenoid supplementation was not shown to affect the probability of survival. However, the experiments that have shown sexual and seasonal variation in carotenoid absorption (see required hypothesis) are also consistent with this hypothesis.

Carotenoid supplementation experiments are a key tool in assessing the relative importance of these hypotheses (Hill, 1992, 1993; Surai & Speake, 1998; Grether, 2000; McGraw & Hill, 2001; Blount *et al.*, 2002a,b, 2003, 2004; Bortolotti *et al.*, 2003; Fitze *et al.*, 2003b; McGraw & Ardia, 2003; Tschirren *et al.*, 2003; Alonso-Alvarez *et al.*, 2004; Grether *et al.*, 2004a; McGraw *et al.*, 2004), yet these experiments have only been used to investigate the role of carotenoids in two wild populations. Given that the environment is likely to play a key role in the amount and type of carotenoids available in the diet, such experiments in captive populations have been strongly criticized (Hudon, 1994).

The overall aim of this study was to test whether variation in carotenoid availability or variation in post-ingestion processes underlie variation in a carotenoid-based colour trait in a wild population of blue tits. By feeding birds super-abundant quantities of carotenoid we aimed to saturate their absorption mechanisms, and eliminate the effects of natural variation in carotenoid availability (Tschirren *et al.*, 2003). Incorporating this carotenoid supplementation experiment within a cross-fostering experiment allowed us to test two predictions. First, if natural variation in carotenoid availability is responsible for natural variation in colour, then the variance in colour of carotenoid-fed birds should be reduced. Secondly, if post-ingestion processes underlie variation in carotenoid-based colour expression, then birds of different genetic or environmental origin would be expected to respond differently to carotenoid supplementation.

Methods

Study site and species

Work was carried out in the spring of 2003, on a population of blue tits at Silwood Park, a 100 ha deciduous woodland site 40 km west of London, UK (grid reference SU 939693). Of the 200 nest boxes monitored for nest building, 177

contained pairs that had commenced egg laying. However, levels of nest desertion and chick mortality were high and only 60% of these nests had surviving chicks 15 days after hatching. This trend was nationwide (Clark *et al.*, 2004), and mortality was not significantly related to any of the experimental manipulations (J. D. Hadfield, unpublished data). Some analyses use data collected the previous spring (Hadfield, 2005).

The UV-yellow chest colouration of blue tits is characteristic of carotenoid-based plumage colouration (Shawkey & Hill, 2005), and previous biochemical work has identified the carotenoids lutein and zeaxanthin (Partali *et al.*, 1987) as the major pigmentary compounds. Previous work on the genetics of carotenoid-based plumage colour in the blue and the closely related great tit (*Parus major*) suggests that variation in colour is only weakly heritable and has a large environmental component (Slagsvold & Lifjeld, 1985; Horak *et al.*, 2000; Fitze *et al.*, 2003a; Hadfield, 2005).

Cross-fostering protocol

Nest boxes were monitored every other day to ascertain the date on which the first egg was laid. Blue tits lay an egg each day, and nests with a single egg were assigned the day of checking as their lay date, and nests with two eggs, the previous day. The nests were rechecked 14 days after their lay date to count the clutch size and estimate a predicted date of hatching (lay date + clutch size + 13). Nests were checked daily from 2 days prior to the predicted hatch date until the first chick had hatched. Chicks within broods generally hatch synchronously and within 24 h, and nests were categorized on the proportion of chicks that had hatched on checking the nest ($<\frac{1}{3}$: just hatched; $\frac{1}{3}$–$\frac{2}{3}$: half hatched; $>\frac{2}{3}$: full hatched). Nests hatching on the same day were paired with respect to these categories where possible, and the following day an equal number of randomly allocated chicks were swapped between the two nests. Chicks were marked with a combination of indelible red and black markers to identify whether they had been cross-fostered, and to which feeding treatment (See Carotenoid feeding) they had been allocated. All chicks were ringed by day 8 (hatch day = day 1) with individually numbered BTO rings.

This manipulation results in roughly equal numbers of nestlings from two different families being raised together, forming a cross-classified design (Riska *et al.*, 1985). Under this design, chicks within the two nests that make up the cross-fostering unit (dyad) are distributed across four combinations of random effects: (1,1), (2,2), (1,2) and (2,1) with the first number designating the chick's original nest and the second number designating the chick's recipient nest. The first two combinations therefore belong to non-cross-fostered chicks, and the second two to cross-fostered chicks. Of the 142 nests that successfully hatched chicks, 130 (65 dyads) were cross-fostered.

Carotenoid feeding

Of the 130 cross-fostered nests, 74 were allocated to a carotenoid-feeding experiment. This was performed randomly, with the stipulation that each nest of the dyad had six or more nestlings. This stipulation allowed three or more chicks to be present in each of the four cross-fostering combinations. Within each combination, three treatments were applied to different chicks randomly: Carotenoid + Mealworm (CM), Mealworm (M) and No Treatment (NT). Because chicks within each combination received each treatment, the main effects of treatment, together with the variance components for genetic effects, brood effects, treatment × genetic effects and treatment × brood effects are all estimable. This is preferable over the design in which the two nests of a dyad are allocated different treatments, and this treatment then applied to all chicks in a given nest (Merila & Fry, 1998), because genetic × treatment interactions are not confounded with brood × genetic interactions.

Nestlings in the CM and M treatment group were fed twice daily from 3 days after hatching to 11 days after hatching. On days 3, 4 and 5 the M treatment group received 1 mL of pureed mealworms administered through a syringe. From days 6 to 11 the feed was increased to 2 mL. Birds in the CM treatment group received identical amounts of food, but each millilitre of puree contained 17.5 mg of lutein beadlets and 1.25 mg of zeaxanthin beadlets, each at a concentration of 5%. Birds in the NT treatment group received no food supplement.

Birds in the CM treatment group received 28.1 mg of carotenoid at a lutein : zeaxanthin concentration ratio of 14 : 1 over the nestling period. This compares directly with similar study on great tits (36.7 mg of carotenoid at a ratio of 12.6 : 1; Tschirren *et al.*, 2003) and work (Partali *et al.*, 1987) assaying carotenoid ratios in the feathers of nestling blue tits (7.8 : 1) and lepidopteran larvae (27 : 1), the main food item of nestling blue tits (Cramp & Perrins, 1993). However, the quantity of carotenoid the birds in the CM treatment group received, far exceeded natural levels by approximately two orders of magnitude [estimated from Partali *et al.* (1987), and a daily food intake estimate of 5 g].

Colour measurement and quantification

On day 15 after hatching, three colour measurements were taken from the upper chest of each nestling. A total of 546 chicks [170 (NT treatment), 77 (M treatment), 81 (CM treatment) and 218 other chicks from non-fed nests] were measured from 107 nests [55 (received experimental-feeding treatment), 42 (cross-fostered but not fed) and 10 (non-experimental)]. The colour measurements were made using a well-established protocol (see Andersson, 1996 for details). An Avantes AVSUSB2000 miniature fibre optic spectrometer coupled to a Xenon-pulsed light source (XE-2000, Avantes, Boulder, CO, USA) was used

for measuring all reflectance spectra. The optic fibre was held at 90° to the colour patch and a Teflon white reference tile (WS-2, Avantes) was used to standardize the reflectance of each measurement. Measurements taken from the same individual were averaged.

Using the SPEC package (Hadfield, 2005), and data on the spectral sensitivity of the blue tit visual system (Hart *et al.*, 2000), we reduced complicated spectral data into four quantal cone catches (Vorobyev *et al.*, 1998) that quantify the amount of light captured by each of the avian single cones. The cone catches were normalized using a von Kreis algorithm with standard daylight (D65) illumination and an achromatic adapting background (Neumeyer, 1998). This method has the useful property that a reflectance spectrum can be described by four parameters without loss of any biologically relevant information. Such data are particularly suited to multivariate statistical techniques (Grether *et al.*, 2004b), and a useful concept is that the data can be treated geometrically as an object within a four-dimensional 'receptor space', with each axis of the receptor space representing the stimulation of each of the four single cone types (Kelber *et al.*, 2003). These four types of cone are denoted UVS (very short – UV), SWS (short – blue), MWS (medium – green) and LWS (long – red) throughout this study, and refer to the range of wavelengths to which they are most sensitive (Hart, 2001).

In birds, variation in spectral composition (the chromatic signal) is perceived by comparing the relative outputs of the four single cones (Hart, 2001). Variation in overall light intensity (the achromatic signal), however, is believed to be perceived through a distinct pathway involving a fifth photoreceptor, the double cone (Campenhausen & Kirschfeld, 1998; Osorio *et al.*, 1999). Because of this we eliminate variation in overall intensity by standardizing each cone catch by the sum of the four cone catches:

$$R_i = Q_i / \sum_{k=1}^{4} Q_k,$$

where Q_i is the cone catch for the ith cone type. The four standardized variables (denoted with cone type subscripts: R_{UVS}, R_{SWS}, R_{MWS} and R_{LWS}) are unsuitable for multivariate analyses because they only contain three independent axes of variation, resulting in a singular sample covariance matrix (Aitchison, 1986). To break the unit-sum ($\sum_{i=1}^{4} R_i = 1$) constraint in the data we divide three of the standardized cone catches by a fourth, in this case R_{LWS}, and take the natural logarithm to give three contrasts (UVS log-contrast, SWS log-contrast and MWS log-contrast). These log-contrasts now define the axes of a three-dimensional chromatic receptor space, and the results are independent of the denominator ratio. Although developed for geological survey work it should be noted that treating these data as a multivariate response are equivalent to specifying an opponency model of colour vision where the exact opponency mechanisms are unspecified (Vorobyev & Osorio, 1998; Endler & Mielke, 2005). In other words, chromaticity is believed to be coded by a physiological mechanism that contrasts the relative outputs of the different cone types. The exact way in which the outputs of different cone types are compared is unknown, except in humans, and so the model leaves the details of the opponency mechanism undefined. Although log-contrasts are commonly used for compositional data [data that satisfies the unit-sum constraint see Blows & Allan (1998), for example], colour data transformed in such a way often contravenes the assumption of multivariate normality common to most multivariate techniques (Endler & Mielke, 2005). However, the level of heteroscedasticity in the presented data (see Fig. 1) is minimal, so the conclusions drawn remain valid.

Molecular sex determination

At the time of measuring, blood samples were also taken from the brachial veins of the majority of chicks for the purpose of sexing. Blood samples were stored in 95% ethanol, and DNA extracted using a Chelex®-based method (Walsh *et al.*, 1991). The P2 and P8 primers were used to amplify the CHD-W and CHD-Z genes located on the avian sex chromosomes following Griffiths *et al.* (1998). The polymerase chain reaction (PCR) products were separated by electrophoresis on a 10% polyacrylamide gel and visualized by ethidium bromide staining.

Quantitative genetic analysis and the mean response of carotenoid supplementation

The three log-contrast colour variables were projected onto orthogonal axes and then treated as a multivariate response in a multivariate 'animal' model (Kruuk, 2004). Strong colinearity was present between the log-contrasts on their original axes and projection onto orthogonal axes improved the convergence properties of the model. The eigenvectors of the sample covariance matrix estimated from the raw data defined the projection matrix (see Table 1). Data from 2002 (Hadfield, 2005), and nests that were not included in the feeding experiment were also included in the analysis so that quantitative genetic parameters could be estimated more accurately. Animal (genetic) and brood effects were fitted as random main terms allowing us to partition phenotypic colour variation into additive genetic variance, brood variance and a residual variance term that includes environmental sources of variation peculiar to individuals and measurement error. Year, sex, hatch date, year × hatch date and carotenoid treatment were fitted as fixed effects. Chicks from non-experimental nests were assigned a dummy variable as their treatment level. Estimates associated with this level of the treatment can be thought of as accounting for systematic differences between fed and non-fed broods through differences in levels of nest

disturbance and/or differences due to the fact that nests were not allocated randomly with respect to clutch size.

Treatment × brood and treatment × genetic interactions

Chick mortality was exceptionally high with approximately 25% of experimental nests being abandoned before measurements could be made on the nestlings. In addition, within brood mortality was also high, resulting in an unbalanced data set for which a high degree of aliasing between random effects is to be expected. When testing the treatment × brood and treatment × genetic interactions we therefore merged NT and M treatments into a single level to make the tests more robust. The plumage colour of NT and M birds did not differ along any axis in chromatic space (see Table 5 and Fig. 1). The interactions were only fitted for experimental nests, and for the first principal component. The robustness and power of the tests were checked by simulating data according to the linear model outlined above for a range of parameter values (See Electronic Appendix).

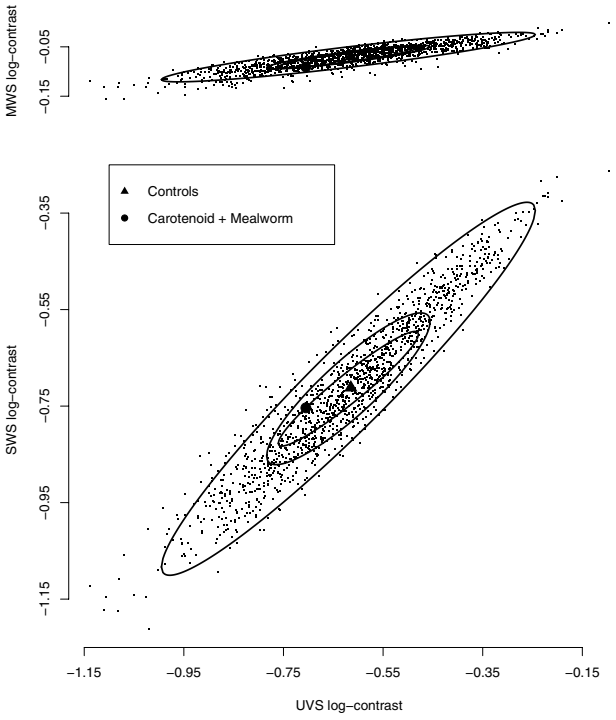

Fig. 1 Graphical representation of the subspace described by chest colouration within the blue tit's chromatic colour space. The three axes are the UVS, SWS and MWS log-contrasts with the L cone catch as the denominator. Lower and upper plots can be thought of as looking at the same solid from above and from the side. The outer ellipse describes the 95% interquantile range for phenotypes based on the phenotypic covariance matrix estimated from an animal model (Table 2). The inner ellipse is the equivalent for breeding values, and can be thought of as the dispersion around the population mean attributable to genetic effects (Table 3). The middle ellipse describes dispersion attributable to environmental brood effects (Table 4). ● and ▲ are the mean values of CM and remaining chicks respectively.

Hypothesis testing

The significance of the random effect (co)variances was assessed by fixing the parameter of interest at zero and using a log-likelihood ratio test with 1 d.f. (Pinheiro & Bates, 2000). The significance of the fixed effects was assessed using the incremental Wald statistic, and the number of families included in the analysis (205) was used as a conservative estimate of the denominator degrees of freedom. The significance of differences between feeding treatment levels was assessed using a two-tailed *t*-test (the *t* statistic was calculated as the ratio of the estimate to its standard error) with degrees of freedom equal to the number of families included in the carotenoid supplementation experiment for which chicks were measured (55 families). Because many analyses use data projected onto orthogonal axes we also derive estimates and approximate standard errors on the original log-contrast axes by developing the methods of Fischer *et al.* (2004; see Electronic Appendix).

Although cross-fostering experiments allow genetic and brood effects to be disentangled, it has long been recognized that estimates of genetic variation are confounded with precross-fostering brood effects in such designs (Lynch & Walsh, 1998). Carotenoid-supplemented female birds have been shown to allocate some of these carotenoids into the egg (Blount *et al.*, 2000; Bortolotti *et al.*, 2003) and heritability estimates may therefore be upwardly biased by precross-fostering effects. However, multigenerational animal models will tend to alleviate these biases to some extent (Hadfield, 2005) and extra-pair paternity rates of 11–14% (Kempenaers *et al.*, 1997) will downwardly bias heritability estimates by about 6% in this system (J. D. Hadfield, unpublished data).

Variance response of carotenoid supplementation

Differences in the amount of phenotypic variation observed between birds in the CM treatment group and the M and NT treatment groups were tested using an *F*-test, with degrees of freedom of 80 (CM treatment), and 246 (M and NT treatment). Overall variation in colour was taken as the sum of the eigenvalues of the log-contrast covariance matrices estimated for each group. Because a large amount of the total colour variation present may not be associated with changes in carotenoid, we repeated the tests with variances estimated for the data projected onto the vector that described the change induced by carotenoid supplementation. This vector can be visualized as a line that passes through the mean colour of unsupplemented birds and the mean

Table 1 Principal components (eigenvectors) and associated eigenvalues of the log-contrast sample covariance matrix.

PC	1	2	3
Colour	UV/blue vs. green/red	UV vs. blue	Green vs. red
Eigenvalues	4.96	0.13	0.01
UVS log-contrast	0.702	0.687	−0.187
SWS log-contrast	0.707	−0.705	0.064
MWS log-contrast	0.087	0.177	0.980

An approximate biological interpretation of the principal components is given in terms of colour contrasts.

colour of supplemented birds in the three-dimensional chromatic receptor space of the blue tit (i.e. a line that passes through ● and ▲ in Fig. 1). The data are then collapsed onto this line, and colour variation of non-supplemented birds and supplemented birds estimated.

The importance of changes in variation between supplemented and non-supplemented birds has been noted elsewhere (McGraw & Hill, 2001) and can be framed in trade-off theory (Roff, 2002). Natural colour variation as represented by chromatic variance in non-treated birds (σ^2_{NT}) can be seen as a product of the effects of variance in ingested carotenoids (σ^2_I), variation attributable to other factors such as absorption or carotenoid allocation to an immune response (σ^2_R) and any covariation that may exist between these two processes ($\sigma_{(I,R)}$):

$$\sigma^2_{NT} = \sigma^2_I + \sigma^2_R + 2\sigma_{(I,R)}$$

Carotenoid absorption is known to be asymptotic with respect to carotenoid concentration (Alonso-Alvarez *et al.*, 2004), and if we assume that a >100-fold increase in carotenoid ingestion (as is the case with birds in the CM treatment group) saturates the absorption process, then:

$$\sigma^2_{CM} = \sigma^2_R$$

as variation in ingested carotenoids is not thought to contribute to chromatic variation in supplemented birds (σ^2_{CM}; Tschirren *et al.*, 2003). The difference in chromatic variance between supplemented and non-supplemented birds therefore gives us an estimate of the amount of chromatic variation attributable to natural variation in levels of carotenoid ingestion:

$$\sigma^2_{NT} - \sigma^2_{CM} = \sigma^2_I + 2\sigma_{I,R}$$

and $1-\sigma^2_{CM}/\sigma^2_{NT}$ gives us the proportion of variation in colour caused by variation in carotenoid ingestion in non-supplemented birds, in the absence of covariation between the ability to solicit carotenoids from the parents, and the ability to assimilate these carotenoids into the growing feather.

ASReml (Gilmour *et al.*, 2002) was used to fit the multivariate mixed models; data simulation and all other statistical models were fitted using R (R Development Core Team, 2004).

Results

Quantitative genetics

The variances of the three log-contrasts tend to be small (<0.03) so all variances have been multiplied by 100, and means by 10 for clarity. Eigen decomposition of the phenotypic covariance matrix (Tables 1 and 2) reveals high redundancy between the log-contrasts, with 97.3% of the phenotypic variance associated with the first eigenvector, and only 2.5% and 0.2% with the minor vectors. The major axis is largely determined by the VS and S cone catches vs. M and L, and the two minor axes VS vs. S, and M vs. L respectively (Fig. 1). These vectors closely correspond to possible opponency channels in chickens (see Osorio *et al.*, 1999).

Genetic sources of variation account for 12% of the total phenotypic variation (Table 3), and brood effects account for 18% (Table 4), calculated as the sum of the eigenvalues of the respective covariance matrices divided by the sum of the eigenvalues of the phenotypic covariance matrix. Phenotypic, genetic and environmental patterns of variation are similar, as is expected when the data are so redundant, with small angles between all corresponding major vectors of P, G and N (<7.5°; Fig. 1).

Carotenoid supplementation

Chicks fed nothing (NT treatment), or mealworm only (M treatment) were not significantly different along any

Table 2 Phenotypic parameters and their standard errors.

	Principal components				Log-contrast axes		
	PC1	PC2	PC3		UVS	SWS	MWS
PC1	4.77 ± 0.22**	−0.06 ± 0.03	−0.01 ± 0.01	UVS	2.35 ± 0.11**	2.31 ± 0.11**	0.29 ± 0.02**
PC2	−0.08	0.11 ± 0.01**	−0.00 ± 0.00	SWS	0.96	2.49 ± 0.11**	0.27 ± 0.01**
PC3	−0.05	−0.15	0.01 ± 0.00**	SWS	0.91	0.85	0.04 ± 0.00**

Variances are along the diagonal, covariances in the upper triangle, and correlations in the lower triangle. Parameters on both the principal component axes and the original axes are given.
*$P < 0.05$, **$P < 0.01$.

PC axes (Table 5 and Fig. 1). Carotenoid-fed chicks, however, were significantly different from all other chicks along the first two PC axes (see Table 5 and Fig. 1). However, despite having a highly significant effect, carotenoid supplementation failed to produce colouration that was as extreme as that found naturally in the population, even after accounting for sampling variance. There were no significant differences between the total colour variance of carotenoid-fed birds and non-carotenoid-fed birds in treated broods ($F_{[80,246]} = 1.07$, $P = 0.48$), or between the variances along the axis associated with carotenoid supplementation ($F_{[80,246]} = 1.10$, $P = 0.38$). The F-ratio in this case is equivalent to $\sigma^2_{CM}/\sigma^2_{NT}$, and $1 - F$ therefore provides an estimate of the proportion of chromatic variation attributable to variation in carotenoid ingestion. These estimates are not significantly different from zero and are −0.07 and −0.1 for the two measures of variance.

Carotenoid supplementation and quantitative genetics

The variance components for the genetic by treatment, and brood by treatment interactions were constrained on the edge of parameter space (0) during convergence. Simulated test data sets showed that the data structure allowed all fixed and random effect parameters to be estimated without bias (unpublished data). However, the power of these tests to detect variance associated with the random interaction terms was low (see Electronic Appendix).

Discussion

Carotenoid supplementation experiments have been a major tool in expanding our knowledge about the proximate mechanisms that underlie variation in

Table 3 Genetic parameters and their standard errors.

	Principal component axes				Log-contrast axes		
	PC1	PC2	PC3		UVS	SWS	MWS
PC1	0.12 ± 0.05*	0.05 ± 0.03*	−0.00 ± 0.00	UVS	0.14 ± 0.06**	0.27 ± 0.13*	0.05 ± 0.02*
PC2	0.49	0.17 ± 0.05**	0.01 ± 0.01	SWS	0.95	0.09 ± 0.05*	0.03 ± 0.02
PC3	−0.03	0.19	0.16 ± 0.05**	MWS	0.90	0.80	0.18 ± 0.06**

Heritabilities are along the diagonal, covariances in the upper triangle, and correlations in the lower triangle. Parameters on both the principal component axes and the original axes are given.
*$P < 0.05$, **$P < 0.01$.

Table 4 Brood parameters and their standard errors.

	Principal components				Log-contrast axes		
	PC1	PC2	PC3		UVS	SWS	MWS
PC1	0.17 ± 0.03**	0.02 ± 0.02	0.00 ± 0.01	UVS	0.19 ± 0.03**	0.38 ± 0.09**	0.06 ± 0.0
PC2	0.11	0.45 ± 0.04**	−0.01 ± 0.00**	SWS	0.89	0.17 ± 0.03**	0.05 ± 0.0
PC3	0.036	−0.42	0.34 ± 0.04**	MWS	0.89	0.80	0.23 ± 0.0

Ratios of brood to phenotypic variances are along the diagonal, covariances in the upper triangle and correlations in the lower triangle. Parameters on both the principal component axes and the original axes are given.
*$P < 0.05$, **$P < 0.01$.

Table 5 Summary statistics for the feeding experiments.

		PC1				PC2				PC3			
Treatment	d.f.	Coefficient	SE	*t*-value	*P*-value	Coefficient	SE	*t*-value	*P*-value	Coefficient	SE	*t*-value	*P*-value
M	55	−0.09	0.30	−0.31	0.76	−0.04	0.04	−1.25	0.22	−0.007	0.01	−0.67	0.51
CM	55	−1.10	0.36	−3.05	0.003**	−0.27	0.04	−6.24	<0.001**	0.009	0.012	0.75	0.46

Coefficients refer to the estimated difference between control (NT) and experimental birds (M and CM) along the three principal component axes.

carotenoid-based colour traits. However, most of these experiments have been carried out in captivity, where variation in carotenoid availability is unlikely to be representative of that found in wild populations (Hudon, 1994). Here, we show that in a wild population of blue tits, carotenoid supplementation can induce significant changes in the colour of carotenoid-based plumage traits. However, carotenoid supplementation did not reduce variation in plumage colouration or produce extreme phenotypes, suggesting that natural variation in carotenoid availability may play a minor role in maintaining colour variation in this population.

By including a carotenoid supplementation experiment within a reciprocal cross-fostering experiment we were also able to estimate genetic and environmental contributions to variation in plumage colouration. In accordance with other studies on quantitative carotenoid traits in birds (Slagsvold & Lifjeld, 1985; Bortolotti *et al.*, 2000; Horak *et al.*, 2000; Fitze *et al.*, 2003a) and fish (Brooks & Endler, 2001) we show that carotenoid-based traits are primarily determined by environmental factors such as brood effects, with genetic effects explaining only 12% of the phenotypic variance. Variance in the ability to assimilate dietary carotenoids into the growing feather was not detected, either at the genetic or the environmental brood level, but patterns of mortality reduced the power of this test, and no firm conclusions can be drawn.

Taken together these results suggest that although carotenoid availability may be limiting, it does not necessarily contribute to variation in the expression of integumentary carotenoids (see also Tschirren *et al.*, 2003). Nevertheless, the evidence does suggest that environmental factors are an important factor underlying carotenoid expression, and given the high residual variance of the models presented, these environmental factors are likely to be experienced at the individual level. Whether individuals that are able to invest more in carotenoid-based plumage do so because they are able to divert more resources to carotenoid assimilation or because they are under less pressure to divert assimilated carotenoids to other functions requires further work.

This work builds on a sustained research programme that seeks to explain why carotenoid pigments play such an important role in the secondary sexual signals of several animal taxa. Adherents of the 'rare' hypothesis maintain that carotenoid assimilation is costly because the resources needed are diverted away from other physiological functions (Hill, 2002). On the other hand, followers of the 'required' hypothesis maintain that the carotenoids themselves are the basis of this trade-off, and that the cost of expressing them in a sexual signal is that they can no longer be used in the physiological processes for which they are required (Lozano, 1994). The body of work that is accumulating suggests that both mechanisms embodied by these hypotheses are likely to generate variation in integumentary carotenoids (Olson & Owens, 2005). Given this, carotenoid expression is likely to be governed by an allocation process, whereby resources are allocated to carotenoid acquisition, and the acquired carotenoids are then allocated either to a signalling function or to other physiological processes (Hill, 2002).

Theoretical work on sequential allocation processes (de Jong, 1993; Worley *et al.*, 2003) show that variation in resource acquisition and allocation downstream of a given trade-off can have a large affect on the sign and magnitude of the covariance between the traits involved. To take an example of current interest: if carotenoids play a role in immune function, then different levels of variation in the amount of resource allocated to the processes of carotenoid ingestion, absorption and transportation may lead to negative or positive covariation between a signal and parasite load at the population level, despite a trade-off existing between signalling and immunity within individuals. Moreover, recent clarifications of sexual selection theory show that the preconceived notion that the covariance between a signal and parasite load must be negative is naive (Getty, 1998, 2002; Kokko *et al.*, 2002).

We believe that explicit mathematical models of carotenoid/resource allocation processes, such as those put forward verbally by Shykoff & Widmer (1996) provide a framework within which carotenoid-based traits can be evaluated. Parameterization of these models from data collected in wild populations offer a unique chance for carotenoid-based traits to contribute to the empirical study of complex trade-offs and sexual selection. This study provides a quantitative estimate of a key parameter in these models (σ_I^2) and provides a coherent statistical methodology for estimating these parameters from spectral data.

This estimate of colour variation induced by variation in dietary carotenoids (σ_I^2) relies on two assumptions: (i) the absorption mechanisms of all treated birds are saturated and (ii) that there is no trade-off between the ability to solicit carotenoids from the parents, and the ability to assimilate these carotenoids into the growing feather. The first of these assumptions is supported by experimental work (Alonso-Alvarez *et al.*, 2004) and the fact that carotenoid-supplemented birds received levels of carotenoid far in excess of what they would encounter in the wild (Tschirren *et al.*, 2003). However, the remaining assumptions require further testing. In the light of allocation-acquisition theory, and the fact that carotenoid-supplemented birds have been shown to express greater colour variation in captivity (McGraw & Hill, 2001?, but see Hill, 1992; Tschirren *et al.*, 2003), the possibility of a trade-off between carotenoid ingestion and carotenoid expression warrants further study.

By applying statistical methods derived from mathematical models of colour vision (Vorobyev & Osorio, 1998; Endler & Mielke, 2005) we have ensured that these conclusions are relevant in the context of intraspecific signalling. These models offer a powerful tool to

behavioural ecologists and provide a coherent framework within which to study visual signals. This study highlights the advantages of such an approach and we recommend that future work recognize the fact that human or 'objective' measures of colour may be inappropriate. The fact that 98% of perceptible chromatic variation can be encoded by a single opponency mechanism is of particular importance. First, it suggests that the chromatic signal of UV-yellow colour patches is unlikely to act as multiple signals (Mays *et al.*, 2004). Secondly, it suggests that the multitude of objective colour measures currently used, such as hue, chroma and λ_{max}, may be redundant with respect to each other and/or associated with small amounts of the total chromatic variation.

Acknowledgments

We thank Hoffmann – La Roche for kindly providing the carotenoid beadlets. We also thank A. Phillimore, A. Lord, M. Burgess and S. Clegg for help during data collection, N. Hart for kindly providing data on the blue tit visual sytem, and S. Griffth for help with the molecular sexing. This manuscript benefited from insightful comments from D. Osorio, R. Baddeley, B. Sheldon and T. Coulson. This study was funded by the National Environment Research Council.

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Supplementary Material

The following supplementary material is available for this article online:

Appendix S1a. Estimating parameters and their standard errors from data projected onto eigenvectors.

Appendix S1b. Power of tests to detect a genetic or brood interaction with carotenoid supplementation

This material is available as part of the online article from http://www.blackwell-synergy.com

Received 31 October 2005; revised 3 January 2006; accepted 12 January 2006