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Methionine supplementation influences melanin-based plumage colouration in Eurasian kestrel, *Falco tinnunculus*, nestlings

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SUMMARY

The extent to which the expression of melanin-based plumage colouration in birds is genetically or environmentally determined is controversial. Here, we performed a between-nest design supplementation with either the sulphur amino acid DL-methionine or with water to investigate the importance of the non-genetic component of melanin-based plumage colouration in the Eurasian kestrel, *Falco tinnunculus*. Methionine affects growth and immunity, thus we aimed to modify nestling growth and immunity before feather development. Then, we measured the effect of the experiment on colouration of two melanin-based plumage patches of nestling kestrels. We found that methionine slowed down nestling growth through treatment administration and that nestlings compensated by speeding up their growth later. We did not find any effects of methionine on nestling immunity (i.e. lymphocyte counts, natural antibody levels or complement-mediated immunity). Effects on growth seemed to be mirrored by changes in nestling colouration in the two sexes: methionine-nestlings showed less intense brown plumage on their backs compared with control nestlings. These results provide support for a non-genetic determination of a melanin-based plumage patch in the two sexes of nestling kestrels.

Key words: colour, non-genetic determination, growth, immunity, melanin-based trait, nestling.

INTRODUCTION

Colours of structures such as skin, feathers and cuticles are among the most striking visible traits in animals. In birds, the expression of plumage colouration has received increasing attention within signalling theories (reviewed in Hill and McGraw, 2006). Three main mechanisms have been proposed to produce plumage colour: (1) feather microstructure, (2) carotenoid pigments and (3) melanin pigments. Melanin colouration is the result of the concentration of two different melanin pigments, eumelanin and phaeomelanin (Haase et al., 1992; Kerje et al., 2003), and is the most widespread mechanism explaining feather colour variation in birds (many black, grey and brown colours) (McGraw, 2006). Reddish-brown and brown colourations correspond to higher concentrations of phaeomelanin relative to eumelanin, while grey and black colourations result from higher concentrations of eumelanin relative to phaeomelanin (Haase et al., 1992). The environmental control of melanin-based colouration is controversial. A number of studies have shown significant environmental variation in melanin colouration in birds (Veiga and Puerta, 1996; Griffith et al., 1999; Fitze and Richner, 2002; Fargallo et al., 2007a), whereas others have considered melanin colouration to be under strong genetic control and independent of environmental conditions (Badyaev and Hill, 2000; Roulin and Dijkstra, 2003; Bize et al., 2006). The proposed low environmental component of melanin colouration is based on the fact that melanins are synthesized *de novo* by animals from the amino acids phenylalanine and tyrosine (Hearing, 1993), which do not seem to be involved in many physiological processes. Therefore, melanin seems less likely to reveal individual quality than other pigments, such as carotenoids, which must be obtained from the diet (Goodwin, 1984). A recent meta-analysis of studies analysing the origin of carotenoid- and melanin-based plumages, however, has shown no quantitative evidence of differences in the ability of these two types of plumage colouration to reliably indicate the quality of their bearers (Griffith et al., 2006). Hence, further studies reassessing the extent of environmental determination of melanin-based ornaments are needed (Griffith et al., 2006).

The Eurasian kestrel (hereafter referred to as 'kestrel') is a sexually dimorphic species, with females being 20% heavier than males (Village, 1990). Differences in size can be detected at early nestling stages (Fargallo et al., 2003), being obvious at the end of nestling development (Martínez-Padilla, 2006). Sexes also differ in plumage colouration at the adult stage. Females are predominantly brown, while males show blue-grey on the head, rump, upper tail-coverts and tail and pinkish-red on the back (Palokangas et al., 1994). Fledglings of the two sexes all look like females, and sexual differences are only obvious in the rump and tail. However, not all fledgling males show grey in the rump and tail zone (Fargallo et al., 2007a). Male kestrels that were greyer in the back, rump and tail were found to be more productive during the breeding period than browner males (Palokangas et al., 1994). Furthermore, captive females preferred greyer males (Palokangas et al., 1994). Our own data also reveal that rump plumage colour is related to immune capacity in adult males (Silva, 2008). In addition, Fargallo et al. (Fargallo et al. 2007a; Fargallo et al. 2007b) have shown experimentally that the size of the grey patch on the rump of male nestlings just before fledging increases in good environmental conditions (Fargallo et al., 2007a) and decreases with increased concentrations of plasma testosterone (Fargallo et al., 2007b). Also, male fledglings with a larger grey area on the rump have been shown to be more efficient hunters (Vergara and Fargallo, 2008), which

suggests that rump colouration could function as an intra-age class indicator of dominance (Senar, 2006). Altogether, these pieces of evidence would suggest that melanin plumage colouration of the rump and, to a lesser extent, the back may function as indicators of phenotypic quality in adult males and young kestrels. By contrast, the role of plumage colouration is not well-known for female kestrels. In our population, head and rump colour in adult females was positively related to individual immunity and to the onset of reproduction, which seems to support condition dependence of adult female rump plumage colouration (Silva, 2008).

Here, we performed a between-nest design supplementation with either the sulphur amino acid DL-methionine or water in Eurasian kestrel nestlings. Specifically, our aim was to test whether methionine supplementation could affect melanin-based colouration in the species. The role of amino acids on melanin-coloured plumage patches has recently been suggested as a new and profitable line of research (McGraw, 2008). DL-methionine has been used in poultry research to increase general performance and immunocompetence of chickens (Tsiagbe et al., 1987; Grimble and Grimble, 1998; Swain and Johri, 2000). Experimental work with wild birds shows that methionine supplementation induces the production of lymphocytes at the expense of growth (Soler et al., 2003; Brommer, 2004; Tschirren and Richner, 2006). Allocation trade-offs with immunity can enforce honesty of signals, indicating individual quality (Hamilton and Zuk, 1982; Andersson, 1994; Wedekind and Folstad, 1994; von Schantz et al., 1999; Peters et al., 2004). Therefore, by providing nestlings with methionine, we aimed to modify growth and immunity in the nesting period. In a first step, we tested the effect of our experiment (methionine supplemented and control groups) on nestling growth and immunity. Secondly, once nestlings were feathered, we measured colouration of two melanin-based plumage patches (i.e. the back and the rump plumage) likely to function as indicators of condition (see above), in both adults and nestlings, between control and experimental groups. We then compared colouration of nestlings between the two experimental groups.

MATERIALS AND METHODS

Study population

The study was conducted during the 2007 breeding season in a Eurasian kestrel (*Falco tinnunculus* L.) population breeding in nest boxes in the Cáceres province in western Spain (39°27′N, 6°20′E). Nest boxes were placed on electric poles, and the study area is characterized by the predominance of dry pastures with a general lack of trees (for details, see Avilés et al., 1999).

The kestrel is a small raptor that inhabits a variable range of habitats and nest types across its distributional range (Village, 1990; Avilés et al., 2001). In the study area, clutch size ranged from 3 to 7 eggs (mean=4.98, N=64). Mainly, the female incubates the eggs and broods the young. Incubation takes 34 days and rearing takes 31 days (Cramp and Simmons, 1988).

Experimental protocol

We randomly assigned kestrel nests with similar laying dates into control and methionine-supplemented groups. In total, our sample included 40 control nestlings and 35 methionine-supplemented nestlings from nine control and nine methionine-supplemented nests that did not differ significantly in hatching dates (ANOVA model, $F_{1,16}$=2.72, P=0.12), clutch size (ANOVA model, $F_{1,16}$=0.36, P=0.55) or initial brood size (ANOVA model, $F_{1,16}$=0.0, P=0.99). In addition, body measurements of nestlings just before the treatment (LMMs: tarsus length, $F_{1,16}$=1.64, P=0.22; wing length, $F_{1,16}$=1.42, P=0.25; body mass, $F_{1,16}$=1.66, P=0.21) did not differ among nestlings from the two experimental groups. Finally, we measured colouration of the 18 females and 15 out of 18 males (seven and eight males from control and methionine-supplemented nests, respectively) breeding in the experimental nests to assess whether treatments were randomly allocated with respect to plumage colouration of parents. Plumage colouration of the back and rump of adults was measured as in nestlings (see below). Plumage colouration in the back and rump of the parents did not differ among nests of the two groups either in females (one-way ANOVAs on scores derived from principal component analyses on brightness of the brown and black, yellow-red chroma of the brown, UV chroma of the black and hue of the brown: back PC1, $F_{1,16}$=0.64, P=0.43; back PC2, $F_{1,16}$=2.08, P=0.17; rump PC1, $F_{1,16}$=1.38, P=0.26, rump PC2, $F_{1,16}$=0.06, P=0.82) or in males (back: one-way ANOVA on scores derived from principal component analyses on brightness of the brown and black, yellow-red chroma of the brown, UV chroma of the black and hue of the brown: back PC1, $F_{1,8}$=0.04, P=0.85; back PC2, $F_{1,8}$=1.39, P=0.27) (rump: one-way ANOVA on scores derived from a principal component analysis on brightness of the grey and UV chroma of the grey: rump PC1, $F_{1,13}$=1.98, P=0.18). Together, our analyses suggested a proper treatment randomization of the experiment across nests and/or parental individual qualities.

The treatment began when the youngest nestling of the brood was three days old and finished after 4 days. Nestlings in methionine nests were supplemented daily during the four consecutive days with a mass-dependent dose of DL-methionine (Sigma Chemicals, Berlin, Germany) suspended in tapwater. The same amount of tapwater was administered to nestlings in control nests during the same period. Therefore, experimental and control nests were disturbed equally. Methionine dosage was 0.02 ml of solution per 1 g of nestling body mass. The solution was made by suspending 0.1 g methionine in 1 ml tapwater for methionine-supplemented nests (Brommer, 2004). Nestlings were individually identified from the beginning of the treatment by colouring one of their tarsi with felt-tip waterproof markers and weighed daily before the administration of the dosage. The same dose significantly affected nestling mortality in blue tits (Brommer, 2004) [but see the following references (Soler et al., 2003; Tschirren and Richner, 2006) for no effects on other species]; consequently, we paid special attention to nestling mortality in methionine-supplemented nests to be able to stop the manipulation at early stages if needed. On the last day of supplementation, mortality rate at the nest (mean ± s.e.m.: control nests, 0.11±0.08; methionine nests, 0.04±0.08) did not differ between treatments (GLM, $F_{1,16}$=0.41, P=0.53). Later on, nestling survival was not affected by the treatment either (see Results section), confirming that the used dose was not harmful for nestling kestrels.

Data collection

Nest boxes were visited regularly to determine laying and hatching dates. Nests were visited daily from 2 days before the estimated hatching date (24 days after the laying of the last egg).

On each day of the treatment administration, nestlings were weighed before dosage administration. Nestling body mass, tarsus and wing length were measured on the first and last day of the treatment and 15 days after the end of the treatment. For this purpose, we used digital callipers and rulers to measure tarsus and wing length to the nearest 1 mm, and an electronic balance to measure body mass to the nearest 0.01 g.

A drop of blood was extracted from each nestling by brachial venipuncture and stored in ethanol for later molecular sexing (see Fridolfsson and Ellegren, 1999) the day that the youngest nestling

of each brood was 14 days old. A second drop was smeared on a slide, air-dried and fixed in ethanol until examination. Blood smears were then stained with azure-eosin and examined to estimate the number of lymphocytes per 10,000 erythrocytes. As methionine induces the production of immune cells (lymphocyte repertoire) (Soler et al., 2003), we measured the effect of methionine on lymphocyte counts. However, the supplementation of methionine is likely to enhance other components of the immune system, e.g. the humoral immune defence (Tsiagbe et al., 1987). Therefore, we additionally extracted 225 µl of blood from each nestling to assess innate humoral immunity (see Matson et al., 2005; Parejo and Silva, 2009). Collected blood was stored in a refrigerator until centrifugation and plasma removal later on the day of collection. Then, plasma was frozen at –20°C for storage until the performance of the innate immune assay. This assay allows us to obtain values of both circulating natural antibodies (NAb), by measuring red blood cell agglutination, and complement-mediated activity, by measuring lysis of exogenous erythrocytes, which is a function of the amount of lytic complement proteins present in the sampled blood. NAbs recognise and attach foreign agents and also initiate the complement cascade (Ochsenbein and Zinkernagel, 2000). The complement cascade recognises and lyses cells of invading organisms (Matson et al., 2005). Quantification was done by serial dilution of plasma samples (25 µl per bird in polystyrene 96-well assay plates) and assessment of the dilution step at which either the agglutination or lysis reaction against the same amount of rabbit blood cell suspension stopped. Plates were vortexed for 10 s at a low speed and set to incubate at 37°C for 90 min. After incubation, plates were tilted at a 45 deg. angle along their long axis for 20 min at room temperature and then scanned (Microtek Scanmaker 5900, Carson, CA, USA) using the positive transparency (top-lit) option and a full-size image (300 d.p.i.). We then quantified agglutination (to measure NAb). Subsequently, plates were kept at room temperature for an additional 70 min and scanned for a second time to record complement-mediated maximum lysis. The assessment of the dilution stage (on a scale from 1 to 12) at which agglutination and lysis stopped provided NAb and complement levels, respectively (for details, see Matson et al., 2005).

Colour measurements and variables

Twenty-one days after hatching, when eruption of feathers from their feather sheets was complete, we plucked three to five feathers from the back and rump of nestlings. Feathers from these body regions show a typical brown-black barred pattern. A small proportion of male nestlings showed some grey on the rump at the end of the nestling period (30 days after hatching). However, 21-day-old nestlings did not show grey in the rump in our population. Therefore, the range of colouration we measured in brown bars was from dark brown to light reddish-brown. As brown and black colours differ in the amount of phaeomelanin and eumelanin they contain (Fargallo et al., 2007a), they were analysed separately. For colour measurements, feathers were carefully placed on black paper in a fashion that mimicked the way the feathers naturally lay on the bird. Spectral data were always recorded by the same person (N.S.) in total darkness with an Ocean Optics DH 2000 spectroradiometer (Dunedin, FL, USA). Plumage reflectance was quantified in the range 300–700 nm with a deuterium and a halogen light source using a bifurcated micron fibre optic probe at a 45 deg. angle from the feather surface and illuminating an area of 1 mm^2. Using the spectra acquisition software package OOIBase (Ocean Optics), we sequentially recorded 10 spectra relative to a standard white reference (WS-2) and then averaged the spectra to reduce electrical noise from the collection array within the spectrometer. This process was repeated three times, with the probe lifted and replaced on the feather sample between each scan. We then averaged the three spectra for each body region and individual. This technique provided highly repeatable measures of plumage colour of nestlings for the first three PC scores of a PCA, summarizing 78.41% of whole variation in nestling colouration for the two plumage patches (brown part of the back, R=0.99–1, $F_{50,102}$=257.7–3208.5, P<0.001; black part of the back, R=0.98–0.99, $F_{50,102}$=150.9–647.5, P<0.001; brown part of the rump, R=0.99–1, $F_{50,102}$=502.8–5635.5, P<0.001; black part of the rump, R=0.99–1, $F_{50,102}$=270.4–1926.9, P<0.001).

Reflectance curves for each body region are shown in Fig. 1. Colour of the brown parts of back and rump was summarized using three standard descriptors of reflectance spectra: brightness, chroma and hue. Brightness is calculated as the summed reflectance from 300 to 700 nm. Chroma is the ratio of the total reflectance in the range of interest (550–700 nm), i.e. since curve changes (Fig. 1), and the total reflectance of the entire spectrum (300–700 nm). Hue refers to the mean wavelength between the minimum and maximum values of reflectance. Reflectance data from black parts of back and rump feathers are summarized using the brightness (McGraw et al., 2002).

As brightness and chroma are usually correlated descriptors of reflectance spectra, colour variables from each body part were entered into separate PCAs. By doing this we aimed to reduce the number of independent variables for analyses and the possibility of analysing the relationship of correlated variables, and thus redundant information, with other (non-colour) variables (Montgomerie, 2006). PC scores originated from the PCAs were then used to define inter-individual differences in nestling colouration. Back colouration was reduced to two PCs: (1) the first PC explained 52% of the variation of colour variables and received strong loadings for brightness of the brown (0.57) and the black (0.60) part of back feathers and (2) the second PC explained 35% of the variation and received strong loadings for yellow-red chroma (0.66) and hue (0.60). An individual with a high positive PC1 score for back colour therefore showed brighter feathers in both the brown and black. On the other hand, individuals with high positive PC2 scores for feathers of the back displayed more brownish back plumage. Finally, rump colouration was also reduced to two PCs: (1) the first PC explained 45% of the variation of colour variables and received strong positive loadings for yellow-red chroma (0.67) and hue (0.52) and (2) the second PC explained 39% of the variation and had strong loadings for brightness of the brown (0.50) and the black (0.68) part of feathers.

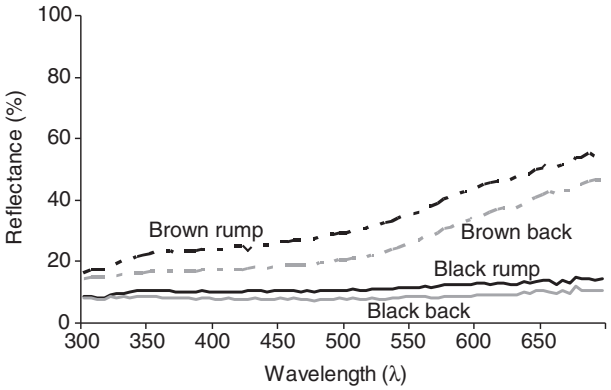

Fig. 1. Mean reflectance spectra for each body region of fledgling kestrels (N=57 individuals). As the two body regions show clearly separated and alternative brown and black bars, one curve for each colour is shown.

Statistical analyses

Analyses were performed using SAS v. 9.1 statistical software (SAS Institute, Cary, NC, USA).

ANOVA models (GLM SAS procedure) were used to test for differences between experimental groups in hatching date, clutch size, initial brood size, mortality rate measured as the proportion of hatching nestlings in a given nest that died before fledging, and female and male parental plumage colouration. Linear Mixed Models (LMM, MIXED SAS procedure) were performed to test for differences in tarsus length, wing length and body mass before the beginning of the treatment among nestlings from the experimental groups. As nestlings from the same nests are not independent, the nest nested within the treatment was introduced as a random factor in analyses.

LMMs (MIXED SAS procedure) where body measures at the different days are the repeats were performed to test for differences in growth during the treatment and afterwards among nestlings from the two experimental groups (methionine-supplemented and control nests). In these analyses, to analyse whether the treatment mediated nestling growth we tested the interaction time × treatment on body measures. The nest (nested within the treatment, see below) and the nestling were introduced as random factors in these analyses. LMMs were also performed to test for differences in lymphocyte counts, NAb levels, complement-mediated lysis and PC colour scores at the end of the nestling period among nestlings from the experimental groups. We performed Generalized Linear Mixed Models (GLMM, GLIMMIX SAS procedure) to test for the effect of the experiment on nestling mortality probability at the nests.

In all these analyses, nestlings were used as statistical units and then the nest was introduced as a random factor to account for the non-independence of nestlings from the same nests. The nest effect was nested within the treatment to show whether treatment affected residual variances. The treatment and the sex were introduced as fixed factors in analyses. Because we aimed to study whether sex mediated nestling allocation between plumage color and growth and immunology, two-way interactions of fixed terms were also entered in our models. Once we found a significant interaction including the treatment effect, we analyzed the effect of the treatment on the split dataset (Engqvist, 2005).

Non-significant covariate interaction terms were removed, starting with the highest-order interactions, down to the main effects following Engqvist (Engqvist, 2005).

RESULTS

Nestling growth

Nestling growth was significantly affected by the experimental manipulation (Table 1, Fig. 2). The growth of the three body measurements (tarsus length, wing length and body mass) was significantly affected by the treatment, as indicated by the statistically significant interactions between time and treatment (Table 1, Fig. 2). Nestling growth in methionine nests during the treatment either did not differ from nestling growth in control nests or was reduced, and it was accelerated afterwards for the three measures (Fig. 2).

Nestling condition and survival

There was no significant effect of the manipulation on lymphocyte counts of nestlings (LMM, $F_{1,12}$=0.63, P=0.44). Lymphocyte counts were unaffected by sex (LMM, $F_{1,39}$=0.76, P=0.39), the interaction term (sex × treatment: LMM, $F_{1,38}$=0.10, P=0.76) and the random effect of nest (Z=0.04, P=0.5). The experiment did not affect NAb levels (LMM, $F_{1,13}$=2.30, P=0.15) or complement-mediated lysis (LMM, $F_{1,13}$=0.76, P=0.40) of nestlings. These two immune indexes were also unaffected by sex (NAb level, LMM, $F_{1,42}$=2.29, P=0.14; complement-mediated lysis, LMM, $F_{1,42}$=1.27, P=0.27) and the interaction term (sex × treatment LMM, NAb level, $F_{1,41}$=0.07, P=0.79; complement-mediated lysis, $F_{1,41}$=0.26, P=0.61). NAb levels (Z=1.76, P=0.04) and complement-mediated lysis (Z=2.08, P=0.02) only varied among different nests.

Neither mortality rate at the nest (GLM, $F_{1,16}$=0.04, P=0.84) nor nestling mortality probability (GLMM, treatment effect, $F_{1,16}$=0.85, P=0.37; nest random effect, Z=1.14, P=0.13) were affected by the experiment.

Plumage colouration

Nestling back colouration was affected by the manipulation (Table 2). Nestlings raised in control nests had more yellow-red coloured and more long-wavelength biased backs (i.e. higher PC2 scores) than nestlings from methionine-supplemented nests (Fig. 3).

Rump colouration was not significantly affected by the experiment but varied with sex (Table 2).

DISCUSSION

We have found that methionine had an immediate effect on nestling growth by decreasing it through treatment administration. Nestlings from methionine-supplemented nests compensate for this deceleration in growth, speeding up later to finish the nesting period

Table 1. Effect of the supplementation treatment, sex and time on nestling body measurements on days 3, 6 and 21 of the youngest nestling of each nest

	Tarsus length		Wing length		Body weight	
Variable	Test statistic	P	Test statistic	P	Test statistic	P
Sex	$F_{1,111}$=0.67	0.41	$F_{1,111}$=0.02	0.88	**$F_{1,109}$=4.90**	**0.03**
Treatment	**$F_{1,13}$=6.03**	**0.029**	**$F_{1,13}$=3.48**	**0.08**	**$F_{1,13}$=8.66**	**0.01**
Time	**$F_{2,111}$=1172.03**	**<0.0001**	**$F_{2,111}$=6172.47**	**<0.0001**	**$F_{2,109}$=2180.20**	**<0.0001**
Sex × Treatment	$F_{1,109}$=0.01	0.90	$F_{1,109}$=0.12	0.73	$F_{1,109}$=0.10	0.75
Sex × Time	$F_{2,109}$=2.81	0.06	$F_{2,109}$=0.32	0.72	**$F_{2,109}$=10.45**	**<0.0001**
Treatment × Time	**$F_{2,111}$=16.84**	**<0.0001**	**$F_{2,111}$=5.80**	**0.004**	**$F_{2,109}$=15.58**	**<0.0001**
Sex × Treatment × Time	$F_{2,107}$=0.40	0.67	$F_{2,107}$=0.09	0.91	$F_{2,107}$=0.05	0.95
Nest	**Z=1.86**	**0.03**	**Z=2.14**	**0.02**	**Z=1.88**	**0.03**
Nestling code	**Z=2.88**	**0.02**	**Z=7.43**	**<0.0001**	**Z=2.49**	**0.006**

We performed Linear Mixed Models in which body measures on the different days were the repeats and the nest (nested within the treatment) and nestling identification were introduced as random factors. Interactions among treatment, sex and time were included in the models. Dependent variables were tarsus length, wing length and body weight. Retained effects are in bold.

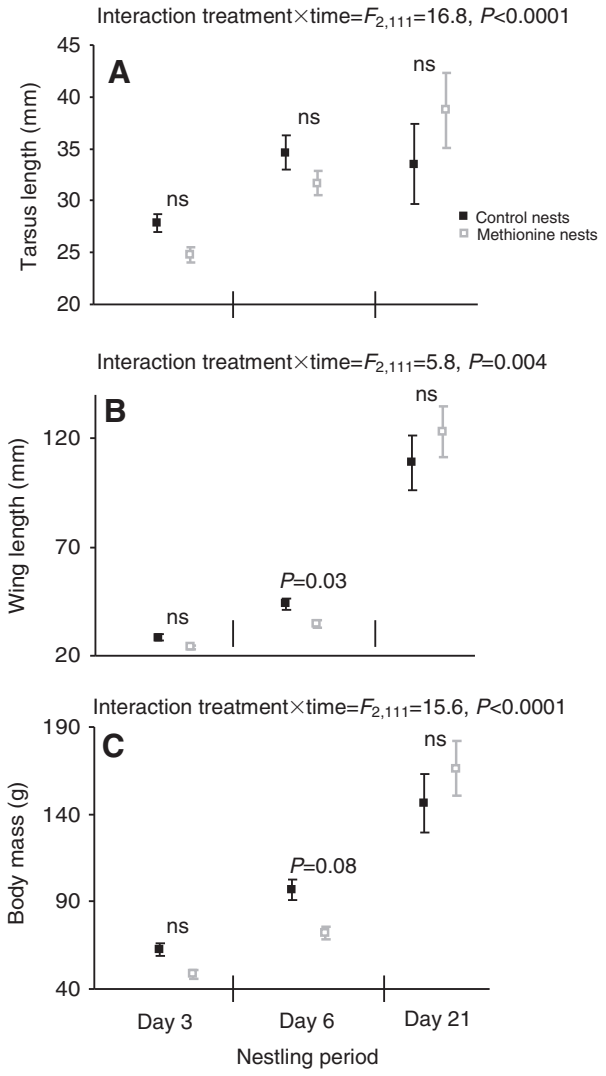

Fig. 2. Mean (± s.e.m.) (A) tarsus length (mm), (B) wing length (mm) and (C) body mass (g) of nestling kestrels being raised in control (filled symbols) and methionine-supplemented (open symbols) nests at days 3, 6 and 21 of the youngest nestling of each nest. *P* values of *post-hoc* tests comparing size between treatments within day 6 (Linear Mixed Models with the nest as a random effect) are shown. *Post-hoc* tests comparing treatments within day 3 and 21 were all non significant (ns; P>0.21).

Table 2. Effects of supplementation treatments and sex on plumage colour measurements

Variable	Test statistic	*P*
Back PC1		
Sex	$F_{1,37}$=0.21	0.65
Treatment	$F_{1,13}$=0.07	0.80
Sex × Treatment	$F_{1,36}$=0.14	0.71
Nest	Z=0.01	0.50
Back PC2		
Sex	$F_{1,37}$=0.03	0.86
Treatment	**$F_{1,13}$ 11.95**	**0.004**
Sex × Treatment	$F_{1,36}$=0.01	0.93
Nest	Z=0.23	0.41
Rump PC1		
Sex	**$F_{1,36}$ 4.36**	**0.04**
Treatment	$F_{1,13}$=0.25	0.63
Sex × Treatment	$F_{1,35}$=1.13	0.29
Nest	Z=0.77	0.22
Rump PC2		
Sex	$F_{1,36}$=1.63	0.21
Treatment	$F_{1,13}$=1.90	0.19
Sex × Treatment	$F_{1,35}$=0.84	0.36
Nest	Z=0.26	0.40

Interactions among these variables were included in the models. Dependent variables were back PC1, back PC2, rump PC1 and rump PC2. The nest nested within the treatment was introduced as a random effect in all statistical models. Retained effects are in bold.

as big as nestlings from control nests. Immunity (i.e. nestling lymphocyte circulation, NAb levels and complement-mediated lysis), however, was not improved in methionine-supplemented nests as compared with control nests. The effects of our experiment on nestling growth seemed to be mirrored by changes in nestling colouration since methionine-supplemented nestlings showed less intense brown plumage in the back than nestlings from control nests.

We expected that the methionine supplementation affected nestling growth and immunity, thus producing two levels of effects that could trade with plumage colouration. Nestling growth was effectively affected by methionine. This result is in agreement with the reduced growth found during methionine administration in methionine-supplemented nestlings of magpies (*Pica pica*) (Soler et al., 2003) and blue tits (*Cyanistes caeruleus*) (Brommer, 2004). However, we have failed to find an effect of methionine on lymphocyte counts or innate immunity (NAb levels and complement-mediated immunity) while those authors found an increase in lymphocytye-mediated immunity in methionine-supplemented nestlings compared with controls. The lack of effect of our experiment on immunity could reside in the fact that lymphocyte counts in blood smears measure the basal number of circulating lymphocytes rather than the capacity of lymphocyte production whenever they are needed, which is the more likely effect of methionine (Tsiagbe et al., 1987). Indeed, Soler et al. (Soler et al., 2003) and Brommer (Brommer, 2004) measured response to a novel antigen, the phytohaemagglutinin PHA, that assesses the lymphocyte production in response to a novel antigen, i.e. the ability of mounting an immune response based on lymphocytes. Also, it cannot be ruled out that methionine did not work properly and reduced short-term growth, as any other substance producing harmful short-term effects. In this last case, one would also observe later compensatory growth. Nevertheless, our study would still show that melanin-based plumage colouration in nestling kestrels is plastic.

Back colouration was affected by methionine supplementation in both male and female nestling kestrels. The amount of grey *versus*

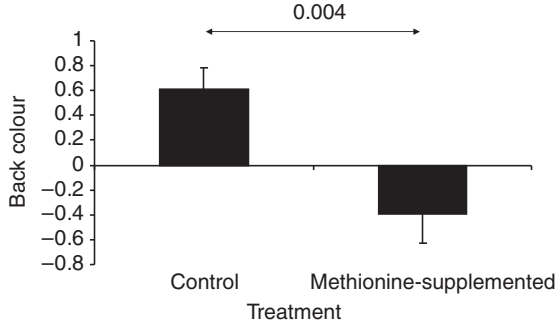

Fig. 3. Variation of the back PC2 (mean ± s.e.m.) in control and methionine-supplemented nests. *P* values of differences are shown over the arrow.

brown in the rump of male nestlings has been previously shown to be affected by the rearing environment (Fargallo et al., 2007a), but nothing was known for female nestlings and for the back plumage patch. We have shown that nestlings raised in methionine-supplemented nests have reduced colour on the back compared with nestlings raised in control nests. Methionine thus modifies a trait of nestling plumage of relatively unknown function compared with rump colouration. The fact that back plumage colouration has a relatively low signalling importance may be the key factor in the trade-offs found. Our results suggest that back colouration was not compromised by at least some components of immunity (i.e. lymphocyte counts, NAb levels and complement-mediated immunity). However, it cannot be discounted that other components of immunity not considered in this study (see above) were affecting methionine-supplemented nestlings. On the other hand, methionine nestlings developed faster at the end of the nestling period than control nestlings, which could have caused their reduced back colouration. There is increasing evidence for compensatory growth from a wide range of taxa (e.g. Wilson and Osbourn, 1960; Nicieza and Metcalfe, 1997; Jespersen and Toft, 2003), despite its potential costs (Lindström et al., 2005). Patterns of growth after a bad start in life depend on the expected fitness returns (Lindström et al., 2005). Thus, it follows that for nestling kestrels allocating resources to somatic growth could be more advantageous than allocation to the development of a plumage trait with a minor signalling importance (see above). Indeed, nestling kestrels might attain more benefits through larger body fledging sizes than by bearing highly coloured plumage at the back. However, Vergara et al. have shown that, during the post-fledging dependence period, males showing a higher proportion of grey in the rump captured larger prey than browner males (Vergara and Fargallo, 2008), which could indicate that rump colouration in male kestrel nestlings could act as an intra-age class indicator of dominance and, hence, that at least plumage colouration of some body parts may also confer benefits to nestlings.

Methionine is involved in the regulation of glutathione (Soler et al., 2003), a substance involved in the melanogenesis pathway (Galván and Alonso-Álvarez, 2008). Therefore, it is still possible that the decrease in phaeomelanin deposition was obtained by means of a methionine-induced change in glutathione regulation and the effects of methionine on nestling growth *via* another completely independent pathway. Indeed, methionine protects glutathione and prevents depletion; therefore, methionine supplementation is expected to result in increased levels of glutathione. There are two proposed alternatives for how glutathione functions. (1) Glutathione inhibits melanogenesis. This mechanism could explain why methionine-supplemented nestlings show less intense and pure brown feathers at the back. (2) Glutathione joins to other substances to form precursors of phaeomelanin, leading to an apparent inhibition of pigmentation due to the lighter colour of phaeomelanin (del Mármol et al., 1993; Solano et al., 2006). Our results show that methionine-supplemented nestlings have less phaeomelanin in the back feathers, which thus reduces the plausibility of this last possibility.

Finally, methionine might behave as a stressful factor, because it makes nestlings grow faster and then induces a rise in circulating corticosterone, which has been proposed to inhibit melanin production (Roulin et al., 2008). This would mean that there is a limitation to produce melanin under stressful factors and it would explain the mechanism by which back colouration would compromise growth.

The manipulation did not affect male and female nestlings differently in spite of the fact that the kestrel is a sexually dimorphic species in size. Sexual size dimorphism, by generating differences in energy demands and/or competitive abilities, has been found to play an important role in sex-specific sensitivity to environmental conditions in early life (e.g. Velando, 2002; Fargallo et al., 2006). Additionally, sex-specific hormones may also cause sex-specific vulnerability (Grossman, 1985; Owens and Short, 1995). However, as kestrels are reverse sexually size-dimorphic, the smaller size of males compared with females might compensate the negative effects of testosterone. In other words, males' sensitivity to the environment may be reduced as a consequence of their smaller growth but increased by their higher levels of testosterone. However, Fargallo et al. found no sex differences in circulating levels of testosterone in nestling kestrels (Fargallo et al., 2007b), which suggests that at least this factor does not affect postnatal vulnerability in the species.

In conclusion, our study supports that melanin-based plumage patch colours in female and male nestling kestrels are phenotypically plastic, which adds to previous findings suggesting an environmental effect on colouration of nestling males in this species (Fargallo et al., 2007a). Future studies should assess how limiting methionine is in the diet of wild birds and how this may be traded off with physiological functions in order to determine whether plumage colouration reliably reflects quality in both male and female nestling kestrels.

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REFERENCES

Andersson, M. (1994). *Sexual Selection*. Princeton: Princeton University Press.
Avilés, J. M., Sánchez, J. M., Sánchez, A. and Parejo, D. (1999). Breeding biology of the roller *Coracias garrulus* in farming areas of the southwest Iberian Peninsula. *Bird Study* **46**, 217-223.
Avilés, J. M., Sánchez, J. M. and Parejo, D. (2001). Breeding rates of Eurasian kestrels (*Falco tinnunculus*) in relation to surrounding habitat in southwest Spain. *J. Raptor Res.* **35**, 31-34.
Badyaev, A. V. and Hill, G. E. (2000). Evolution of sexual dichromatism: contribution of carotenoid- versus melanin-based coloration. *Biol. J. Linn. Soc.* **69**, 153-172.
Bize, P., Gasparini, J., Klopfenstein, A., Altwegg, R. and Roulin, A. (2006). Melanin-based coloration is a nondirectionally selected sex-specific signal of offspring development in the alpine swift. *Evolution* **60**, 2370-2380.
Brommer, J. E. (2004). Immunocompetence and its costs during development: an experimental study in blue tit nestlings. *Biol. Lett.* **271**, S110-S113.
Cramp, S. and Simmons, K. E. L. (1988). *The Birds of the Western Palearctic*. Oxford: Oxford University Press.
del Mármol, V., Solano, F., Sels, A., Huez, G., Libert, A., Lejeune, F. and Ghanem, G. (1993). Glutathione depletion increases tyrosinase activity in human melanoma cells. *J. Invest. Derm.* **101**, 871-874.
Engqvist, L. (2005). The mistreatment of covariate interaction terms in linear model analyses of behavioural and evolutionary ecology studies. *Anim. Behav.* **70**, 967-971.
Fargallo, J. A., Laaksonen, T., Korpimäki, E., Poyri, V., Griffith, S. C. and Valkama, J. (2003). Size-mediated dominance and begging behaviour in Eurasian kestrel broods. *Evol. Ecol. Res.* **5**, 549-558.
Fargallo, J. A., Polo, V., de Neve, L., Martín, J., Dávila, J. A. and Soler, M. (2006). Hatching order and size-dependent mortality in relation to brood sex ratio composition in chinstrap penguins. *Behav. Ecol.* **17**, 772-778.
Fargallo, J. A., Laaksonen, T., Korpimäki, E. and Wakamatsu, K. (2007a). A melanin-based trait reflects environmental growth conditions of nestling male Eurasian kestrels. *Evol. Ecol.* **21**, 157-171.
Fargallo, J. A., Martínez-Padilla, J., Toledano-Díaz, A., Santiago-Moreno, J. and Dávila, J. A. (2007b). Sex and testosterone effects on growth, immunity and melanin coloration of nestling Eurasian kestrels. *J. Anim. Ecol.* **76**, 201-209.
Fitze, P. S. and Richner, H. (2002). Differential effects of a parasite on ornamental structures based on melanins and carotenoids. *Behav. Ecol.* **13**, 401-407.
Fridolfsson, A. K. and Ellegren, H. (1999). A simple and universal method for molecular sexing of non-ratite birds. *J. Avian Biol.* **30**, 116-121.
Galván, I and Alonso-Álvarez, C. (2008). An intracellular antioxidant determines the expression of a melanin-based signal in a bird. *PLoS ONE* **3**, e3335.

Goodwin, T. W. (1984). *The Biochemistry of the Carotenoides. Animals*. London and New York: Chapman and Hall.
Griffith, S. C., Owens, I. P. F. and Burke, T. (1999). Environmental determination of a sexually selected trait. *Nature* **400**, 358-360.
Griffith, S. C., Parker, T. H. and Olson, V. A. (2006). Melanin- versus carotenoid-based sexual signals: is the difference really so black and red? *Anim. Behav.* **71**, 749-763.
Grimble, R. F. and Grimble, G. K. (1998). Immunonutrition: role of sulfur amino acids, related amino acids, and polyamines. *Nutrition* **14**, 605-610.
Grossman, C. J. (1985). Interactions between the gonadal-steroids and the immune system. *Science* **227**, 257-261.
Haase, E., Ito, S., Sell, A. and Wakamatsu, K. (1992). Melanin concentrations in feathers from wild and domestic pigeons. *J. Hered.* **83**, 64-67.
Hamilton, W. and Zuk, M. (1982). Heritable true fitness and bright birds: a role for parasites? *Science* **218**, 384-387.
Hearing, V. J. (1993). Unraveling the melanocyte. *Am. J. Hum. Gen.* **52**, 1-7.
Hill, G. E. and McGraw, K. J. (2006). *Bird Coloration*. Harvard: Harvard University Press.
Jespersen, L. B. and Toft, S. (2003). Compensatory growth following early nutritional stress in the wolf spider *Pardosa prativaga*. *Funct. Ecol.* **17**, 737-746.
Kerje, S., Lind, J., Schutz, K., Jensen, P. and Andersson, L. (2003). Melanocortin 1-receptor (MC1R) mutations are associated with plumage colour in chicken. *Anim. Gen.* **34**, 241-248.
Lindström, J., Metcalfe, N. B. and Royle, N. J. (2005). How are animals with ornaments predicted to compensate for a bad start in life? A dynamic optimization model approach. *Funct. Ecol.* **19**, 421-428.
Martínez-Padilla, J. (2006). Cernícalo vulgar – *Falco tinnunculus*. In *Enciclopedia Virtual de los Vertebrados Españoles* (ed. L. M. Carrascal and A. Salvador). Madrid: Museo Nacional de Ciencias Naturales. http://www.vertebradosibericos.org/
Matson, K. D., Ricklefs, R. E. and Klasing, K. C. (2005). A hemolysis–hemagglutination assay for characterizing constitutive innate humoral immunity in wild and domestic birds. *Dev. Comp. Immunol.* **29**, 275-286.
McGraw, K. J. (2006). Mechanics of melanin-based coloration. In *Bird Coloration, Vol. I: Mechanisms and Measurements* (ed. G. E. Hill and K. J. McGraw), pp. 243-294. Cambridge, USA: Harvard University Press.
McGraw, K. J. (2008). An update on the honesty of melanin-based color signals in birds. *Pigm. Cell Melan. Res.* **21**, 133-138.
McGraw, K. J., Mackillop, E. A., Dale, J. and Hauber, M. E. (2002). Different colors reveal different information: how nutritional stress affects the expression of melanin- and structurally based ornamental plumage. *J. Exp. Biol.* **205**, 3747-3755.
Montgomerie, R. (2006). Analyzing colors. In *Bird Coloration, Vol. I: Mechanisms and Measurements*. (ed. G. E. Hill and K. J. McGraw), pp. 90-147. Cambridge, USA: Harvard University Press.
Nicieza, A. G. and Metcalfe, N. B. (1997). Growth compensation in juvenile Atlantic salmon: responses to depressed temperature and food availability. *Ecology* **78**, 2385-2400.
Ochsenbein, A. F. and Zinkernagel, R. M. (2000). Natural antibodies and complement link innate and acquired immunity. *Immunol. Today* **21**, 624-630.
Owens, I. P. F. and Short, R. V. (1995). Hormones, handicaps and bright birds – Reply. *Trends Ecol. Evol.* **10**, 121-122.
Palokangas, P., Korpimäki, E., Hakkarainen, H., Huhta, E., Tolonen, P. and Alatalo, R. V. (1994). Female kestrels gain reproductive success by choosing brightly ornamented males. *Anim. Behav.* **47**, 443-448.
Parejo, D. and Silva, N. (2009). Immunity and fitness in a wild population of Eurasian kestrels *Falco tinnunculus*. *Naturwissenschaften* **96**, 1193-1202.
Peters, A., Delhey, K., Denk, A. G. and Kempenaers, B. (2004). Trade-offs between immune investment and sexual signalling in male mallards. *Am. Nat.* **164**, 51-59.
Roulin, A. and Dijkstra, C. (2003). Genetic and environmental components of variation in eumelanin and phaeomelanin sex-traits in the barn owl. *Heredity* **90**, 359-364.
Roulin, A., Almasi, B., Rossi-Pedruzzi, A., Ducrest, A. L., Wakamatsu, K., Miksik, I., Blount, J. D., Jenni-Eiermann, S. and Jenni, L. (2008). Corticosterone mediates the condition-dependent component of melanin-based coloration. *Anim. Behav.* **75**, 1351-1358.
Senar, J. C. (2006). Color displays as intrasexual signals of aggression and dominance. In *Bird Coloration, Vol. 2: Function and evolution* (ed. G. E. Hill and K. J. McGraw), pp. 87-136. Cambridge, USA: Harvard University Press.
Silva, N. (2008). Rôle de l'information sociale dans les décisions de reproduction chez deux espèces d'oiseaux, le faucon crécerelle (*Falco tinnunculus*) et le rollier d'Europe (*Coracias garrulus*). Ph.D. Thesis. Université Paul Sabatier, Toulouse France.
Solano, F., Briganti, S., Picardo, M. and Ghanem, G. (2006). Hypopigmenting agents: an updated review on biological, chemical and clinical aspects. *Pigm. Cell Res.* **19**, 550-571.
Soler, J. J., de Neve, L., Pérez-Contreras, T., Soler, M. and Sorci, G. (2003). Trade-off between immunocompetence and growth in magpies: an experimental study. *Proc. R. Soc. Lond. B* **270**, 241-248.
Swain, B. K. and Johri, T. S. (2000). Effects of supplemental methionine, choline and their combinations on the performance and immune response of broilers. *Br. Poultry Sci.* **41**, 83-88.
Tschirren, B. and Richner, H. (2006). Parasites shape the optimal investment in immunity. *Proc. R. Soc. Lond. B* **273**, 1773-1777.
Tsiagbe, V. K., Cook, M. E., Harper, A. E. and Sunde, M. L. (1987). Enhanced immune-responses in broiler chicks fed methionine-supplemented diets. *Poultry Sci.* **66**, 1147-1154.
Veiga, J. P. and Puerta, M. (1996). Nutritional constraints determine the expression of a sexual trait in the house sparrow, *Passer domesticus*. *Proc. R. Soc. Lond. B* **263**, 229-234.
Velando, A. (2002). Experimental manipulation of maternal effort produces differential effects in sons and daughters: implications for adaptive sex ratios in the blue-footed booby. *Behav. Ecol.* **13**, 443-449.
Vergara, P. and Fargallo, J. A. (2008). Sex, melanic coloration, and sibling competition during the postfledging dependence period. *Behav. Ecol.* **19**, 847-853.
Village, A. (1990). *The Kestrel*. London: T. and A. D. Poyser.
von Schantz, T., Bensch, S., Grahn, M., Hasselquist, D. and Witzell, H. (1999). Good genes, oxidative stress and condition-dependent sexual signals. *Proc. R. Soc. Lond. B* **266**, 1-12.
Wedekind, C. and Folstad, I. (1994). Adaptive or non adaptive immunosuppresion by sex hormones? *Am. Nat.* **143**, 936-938.
Wilson, P. N. and Osbourn, D. F. (1960). Compensatory growth after malnutrition in mammals and birds. *Biol. Rev.* **35**, 324-363.