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RESEARCH ARTICLE

Differential effects of vitamins E and C and carotenoids on growth, resistance to oxidative stress, fledging success and plumage colouration in wild great tits

Viviana Marri* and Heinz Richner

ABSTRACT

Oxidative stress is the imbalance between the production of reactive species and antioxidants, which causes damage to lipids, proteins and DNA. Antioxidants, like vitamins and carotenoids, can limit oxidative damage and can therefore regulate the trade-off between growth, which is a period of high reactive species production, and self-maintenance. However, the role of carotenoids as antioxidants *in vivo* has been debated, and it has been suggested that carotenoid-based signals indicate the availability of non-pigmentary antioxidants (e.g. vitamins) that protect carotenoids from oxidation, known as the 'protection hypothesis'. To evaluate the importance of vitamins versus carotenoids as antioxidants during growth and to test the protection hypothesis, we supplemented nestling great tits, *Parus major*, 3, 5 and 7 days after hatching with a single dose of carotenoids and/or vitamins in a 2×2 full-factorial design. We subsequently measured body condition, antioxidant capacity, oxidative damage, fledging success and plumage reflectance. Vitamins enhanced antioxidant capacity, but did not affect oxidative damage. Vitamin-treated nestlings had higher growth rates and higher probability of fledging. In contrast, carotenoids did not affect any of these traits. Furthermore, carotenoid-based colouration increased over the breeding season in nestlings that received vitamins only. This study shows that vitamins are limiting for growth rate and fledging success, and suggests that vitamins could regulate the trade-off between growth and self-maintenance in favour of the former. Moreover, our results are consistent with the idea that carotenoids are minor antioxidants in birds, but they do not support the protection hypothesis.

KEY WORDS: Carotenoids, Free-radicals, Growth, Oxidative damage, *Parus major*, Protection hypothesis, Trade-off, Vitamins

INTRODUCTION

Oxidative stress is defined as an imbalance between reactive species and antioxidants, in favour of the former (Sies, 1991). Reactive species are by-products of the metabolic activity that cause damage to lipids, proteins and DNA (Finkel and Holbrook, 2000). Because oxidative stress is ubiquitous, and occurs throughout an individual's life, it is thought to influence different life-history traits and to be a constraint in many biological processes (Monaghan et al., 2009). To limit the toxic effects of reactive species, animals use different antioxidants, including vitamins, enzymes and minerals (Surai, 2002). The antioxidant system comprises dietary antioxidants, which are compounds that cannot be synthesized by animals and thus must be acquired with the diet. Among these, carotenoids and vitamin E have been the target of many studies. Vitamin E is a reactive species scavenger that protects lipids from peroxidation (Burton et al., 1983). Importantly, because oxidized vitamin E can be transformed back to the active form by vitamin C (Chan, 1993), a combination of these two vitamins has a better antioxidant effect than either of the two alone (Rinne et al., 2000). Carotenoids are also considered as dietary antioxidants, occur as natural pigments in fish and birds, and are important for processes of sexual selection (Hill, 1991) and parent–offspring interactions (Saino et al., 2000). Recently, the importance of carotenoids as antioxidants *in vivo* has been debated because an increasing number of field experiments could not prove a direct effect of carotenoids on oxidative stress (Hõrak et al., 2006; Costantini et al., 2007; Isaksson et al., 2007; Isaksson and Andersson, 2008; Larcombe et al., 2010). Additionally, Costantini and Møller (Costantini and Møller, 2008) showed in a meta-analysis that carotenoids are only minor antioxidants for birds. The finding that carotenoids are not important antioxidants *in vivo* is in line with the 'protection hypothesis' of Hartley and Kennedy (Hartley and Kennedy, 2004). Given that oxidative stress bleaches carotenoids (Woodall et al., 1997), the hypothesis holds that carotenoid-based signals may reflect the amount of other non-pigmentary antioxidants that prevent the oxidation of carotenoids and make these available for signals.

Growth is a period of high oxygen consumption (Stoks et al., 2006) that is related to high reactive species production (Loft et al., 1994). Thus oxidative damage caused by the increased metabolism could limit mass gain before fledging, which is correlated with fledging success (Losdat et al., 2013). Antioxidants could therefore play an important role during the nestling phase. In the present study, we evaluate the role of antioxidants in the regulation of the trade-off between growth and self-maintenance. Moreover, we compare the antioxidant role of vitamins and carotenoids, and test the protection hypothesis.

Using a 2×2 full-factorial design at the nest level, we supplemented great tit nestlings with carotenoids (a mixture of lutein and zeaxanthin) and/or vitamins (a mixture of α-tocopherol and ascorbic acid). We measured the effect of the experimental treatments on growth, oxidative damage, antioxidant capacity, fledging success and plumage colouration. The great tit, *Parus major* Linnaeus 1758, is a small hole-nesting passerine that exhibits a sexually dichromatic yellow breast plumage (Slagsvold and Lifjeld, 1985) due to the deposition of lutein and zeaxanthin in the feathers (Partali et al., 1987). The yellow breast plumage is already present in great tit nestlings, one of the few bird species where this colouration is expressed at nestling stage. Knowledge on sexual dichromatism at the nestling stage is scarce and contradictory (e.g. Slagsvold and Lifjeld, 1985; Isaksson et al., 2008), and yellow plumage colour of an individual from nestling to adult stage is

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List of abbreviations

DFI	daily food intake
KRL	Kit Radicaux Libres
MDA	malondialdehyde
SWS	short-wavelength sensitive
TBA	thiobarbituric acid

uncorrelated (Fitze et al., 2003a). Hence sexual selection is an unlikely cause for the evolution of the yellow plumage colour in nestlings and thus it has been speculated that it evolved in the context of post-fledging parent–offspring communication (Tschirren et al., 2005).

As there is evidence that carotenoids are minor antioxidants in birds, we predict that antioxidant capacity is enhanced mainly by available vitamins. Thus, we expect vitamin-treated nestlings to be able to invest more in growth, without a rise in oxidative damage. Moreover, if vitamins are protecting carotenoids from bleaching, as predicted by the protection hypothesis, we expect a positive effect of vitamins as well as carotenoids on colouration, and a synergistic effect when they are provided in combination.

RESULTS

Antioxidant capacity

Antioxidant capacity was influenced by vitamin treatment, independently of brood size and sex and after accounting for the effects of haematocrit and hatching date. In vitamin-treated nests, antioxidant capacity was significantly higher than in control nests (vitamin group: estimate ± s.e.=2.24±0.048 min, control group: estimate ± s.e.=2.08±0.046 min, $F_{1,91}$=6.62, P=0.01; Fig. 1). There was no significant effect of carotenoids ($F_{1,79}$=1.29, P=0.26), or the interaction between the two treatments ($F_{1,87}$=0.13, P=0.717). Moreover, antioxidant capacity was positively correlated with haematocrit levels ($F_{1,375}$=97.6, P<0.0001, estimate ± s.e.=0.41±0.042) and hatching date ($F_{1,91}$=15.3, P=0.0002, estimate ± s.e.=0.035±0.0009) in all experimental groups.

Oxidative damage

Plasma malondialdehyde (MDA) was significantly influenced by the interaction between vitamin treatment, carotenoid treatment and sex (Table 1): when only vitamins were supplemented, oxidative damage was lower in females than in males ($F_{1,39}$=4.6, P=0.038), while when both vitamins and carotenoids were supplemented there was no difference between the sexes ($F_{1,58}$=2.1, P=0.15). Plasma MDA was negatively correlated with hatching date (Table 1), showing a slight decrease in nestlings' oxidative damage over the breeding season.

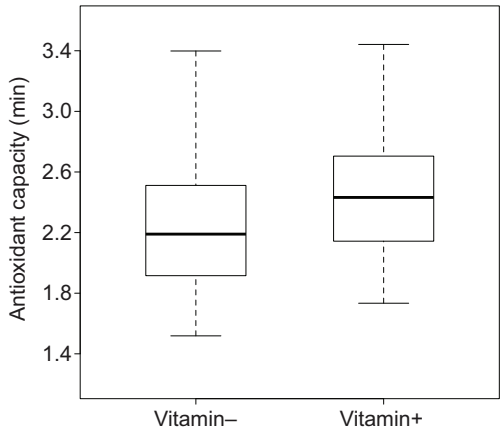

Fig. 1. Predicted square-root-transformed antioxidant capacity in relation to vitamin treatment. Antioxidant capacity was measured as the time (min) needed to hemolyse 50% of the red blood cells when exposed to a controlled free-radical attack. Horizontal lines are 25th, 50th (bold) and 75th percentiles; whiskers show the maximum and minimum values.

Change in body mass

Change in body mass during the supplementation (from day 3 to day 8 post-hatch) was affected by an interaction between both treatments and sex (Table 2): males gained more mass than females in the group of nestlings treated with vitamins and carotenoids ($F_{1,165}$=39.05, P<0.0001), and there was a similar tendency in nestlings treated with vitamins only ($F_{1,143}$=3.37, P=0.068).

Vitamin-treated nestlings showed a significantly higher increase in body mass (Fig. 2) from day 8 to day 14 post-hatch (Table 2). In addition, males gained significantly more mass than females over this time period (Table 2).

Fledging success

Carotenoid treatment did not have any significant effect on fledging success (χ^2=0.30, P=0.58), but nestlings supplemented with vitamins were significantly more likely to fledge (χ^2=7.48, P=0.006). Moreover, fledging success was negatively correlated with hatching date (χ^2=14.7, P=0.0001) and brood size (χ^2=11.3, P=0.0007).

Breast plumage colour

Breast plumage colour was significantly influenced by the interaction between vitamin treatment, carotenoid treatment and hatching date (Table 3). Analyses of the effect of vitamin treatment when carotenoid was not supplemented showed a significant interaction between vitamin treatment and hatching date ($F_{1,38}$=6.52, P=0.015), with a tendency of colouration to increase with hatching date in vitamin-treated nestlings ($F_{1,22}$=3.41, P=0.078; Fig. 3), and a tendency to decrease in control nestlings ($F_{1,16}$=4.0, P=0.063; Fig. 3). Analyses of the effect of vitamin treatment when carotenoid was supplemented did not show a significant interaction between treatment and hatching date ($F_{1,41}$=0.88, P=0.35). Males showed a significantly higher index of chromatic reflectance than females (Table 3).

DISCUSSION

We found that an increased availability of vitamins enhanced growth, antioxidant capacity and strongly improved fledging success, but did not have a main effect on oxidative damage. In contrast, carotenoid supplementation did not affect any of these fitness-related traits and, when supplemented together with vitamins, did not show any synergistic effects on the expression of a carotenoid-based signal. Vitamins, but not carotenoids, increased carotenoid-based colouration over the breeding season. The results thus show that vitamins have major effects on several fitness-related traits and suggest that vitamins may play a central role in the trade-off between growth and self-maintenance. Moreover, our results are consistent with the idea that carotenoids are minor antioxidants in birds (Costantini and Møller, 2008), but do not support the 'protection hypothesis' of Hartley and Kennedy (Hartley and Kennedy, 2004).

As expected, vitamins increased antioxidant capacity, confirming their role as powerful antioxidants, while carotenoids did not have any effect, supporting the idea that carotenoids are only minor antioxidants in birds (Costantini and Møller, 2008). Vitamins also positively influenced growth rate. Recent studies reported similar results in response to antioxidant supplementation in nestlings of

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Table 1. Linear mixed-effect model testing the effect of the vitamin and carotenoid treatments on oxidative damage (MDA), 8 days after hatching

Effect	Estimate ± s.e.	*F*	d.f.	*P*
Intercept	0.94±0.017	–	–	–
Brood size	−0.004±0.003	1.28	1, 82	0.26
Sex	**−0.006±0.013**	**0.23**	**1, 187**	**0.63**
Hatching date	**−0.011±0.001**	**63.6**	**1, 187**	**<0.0001**
Carotenoid	**−0.008±0.02**	**0.16**	**1, 83**	**0.69**
Vitamin	**−0.029±0.019**	**2.33**	**1, 83**	**0.13**
Carotenoid × sex	**0.014±0.02**	**0.49**	**1, 187**	**0.49**
Vitamin × sex	**0.031±0.019**	**2.73**	**1, 187**	**0.1**
Carotenoid × hatching date	−0.002±0.003	0.71	1, 80	0.4
Vitamin × hatching date	0.002±0.003	0.27	1, 79	0.6
Carotenoid × vitamin	**0.038±0.026**	**2.15**	**1, 83**	**0.15**
Carotenoid × vitamin × sex	**−0.054±0.027**	**3.99**	**1, 187**	**0.047**
Carotenoid × vitamin × hatching date	−0.002±0.006	0.12	1, 78	0.73

F- and *P*-values of non-significant terms are those just before removal from the model. Terms retained in the final model are highlighted in bold. Reference level for coefficients is a female not supplemented with carotenoid and vitamin.

barn swallows, *Hirundo rustica*, and red-winged blackbirds, *Agelaius phoeniceus* (de Ayala et al., 2006; Hall et al., 2010). We did not detect any direct effect of vitamins on oxidative damage, as also found in other studies on wild birds (e.g. Hall et al., 2010; Larcombe et al., 2010; Noguera et al., 2011; Kim et al., 2013). It appears that nestlings allocated extra vitamins to increase growth rate rather than to limit oxidative damage, as reported in red-winged blackbird nestlings (Hall et al., 2010). This might be explained by the well-known positive relationship between body mass at fledging and post-fledging survival (e.g. Tinbergen and Boerlijst, 1990). Because oxidative stress is supposed to limit growth rates (Alonso-Alvarez et al., 2007), the faster growth rate in vitamin-treated nestlings likely reflects the antioxidant properties of vitamins. However, we cannot entirely exclude potential effects of other roles of the vitamins, such as the regulation of enzymatic activity and gene expression.

Fledging success was positively influenced by the vitamin treatment. This is likely explained by the fact that vitamin supplementation increased mass gain, which is correlated with fledging success in our study population (Losdat et al., 2013).

Nestlings of the control group showed a decrease in the expression of breast plumage colouration during the breeding

Table 2. Linear mixed-effect models testing the effect of the vitamin and carotenoid treatments on change in body mass between days 3 and 8 and between days 8 and 14

Effect	Estimate ± s.e.	*F*	d.f	*P*
Change in body mass (day 3–8)				
Intercept	9.21±0.51	–	–	–
Brood size	−0.18±0.05	10.7	1, 89	0.002
Sex	**0.35±0.11**	**9.59**	**1, 550**	**0.002**
Hatching date	**−0.048±0.023**	**4.52**	**1, 89**	**0.036**
Carotenoid	**0.11±0.29**	**0.14**	**1, 89**	**0.7**
Vitamin	**0.35±0.28**	**1.54**	**1, 89**	**0.22**
Carotenoid × sex	**−0.042±0.16**	**0.07**	**1, 550**	**0.79**
Vitamin × sex	**−0.18±0.16**	**1.26**	**1, 550**	**0.26**
Carotenoid × hatching date	−0.010±0.046	0.048	1, 88	0.83
Vitamin × hatching date	0.011±0.049	0.049	1, 87	0.83
Carotenoid × vitamin	**−0.39±0.39**	**1**	**1, 89**	**0.32**
Carotenoid × vitamin × sex	**0.42±0.22**	**3.76**	**1, 550**	**0.053**
Carotenoid × vitamin × hatching date	−0.061±0.096	0.4	1, 86	0.53
Change in body mass (day 8–14)				
Intercept	5.37±0.75	–	–	–
Brood size	**−0.31±0.09**	**12**	**1, 86**	**0.0008**
Sex	**0.32±0.08**	**15.5**	**1, 465**	**0.0001**
Hatching date	−0.02±0.04	0.28	1, 85	0.6
Carotenoid	**0.20±0.31**	**0.44**	**1, 86**	**0.51**
Vitamin	**0.77±0.31**	**6.2**	**1, 86**	**0.014**
Carotenoid × sex	0.033±0.16	0.04	1, 463	0.84
Vitamin × sex	−0.12±0.17	0.5	1, 464	0.48
Carotenoid × hatching date	0.012±0.077	0.026	1, 83	0.87
Vitamin × hatching date	0.007±0.08	0.008	1, 82	0.93
Carotenoid × vitamin	−0.20±0.62	0.099	1, 84	0.75
Carotenoid × vitamin × sex	−0.008±0.33	0.001	1, 462	0.98
Carotenoid × vitamin × hatching date	0.03±0.16	0.036	1, 81	0.85

F- and *P*-values of non-significant terms are those just before removal from the model. Terms retained in the final model are highlighted in bold. Reference level for coefficients is a female not supplemented with carotenoid and vitamin.

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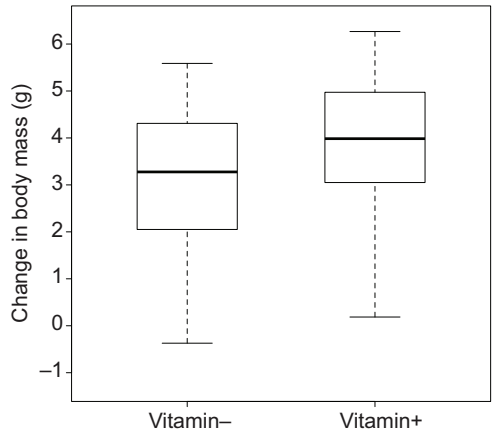

Fig. 2. Predicted change in body mass (g) from day 8 to 14 in relation to vitamin treatment. Horizontal lines are 25th, 50th (bold) and 75th percentiles; whiskers show the maximum and minimum values.

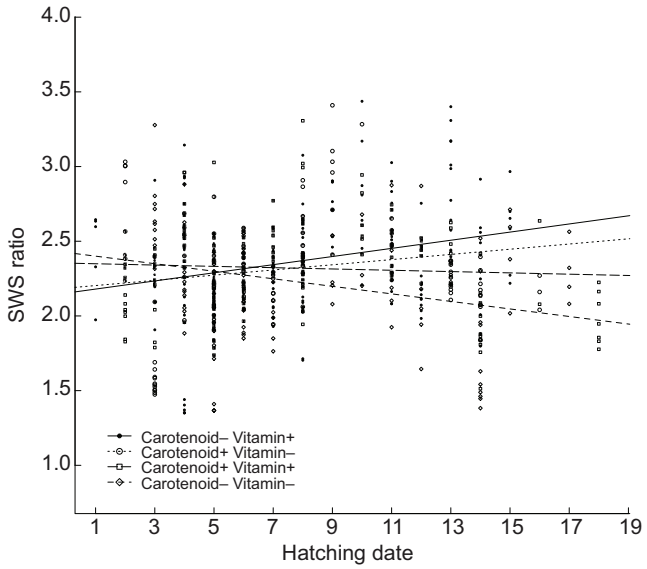

Fig. 3. Short-wavelength sensitive (SWS) ratio (an index of chromatic reflectance) in relation to hatching date in nestlings of the four experimental groups. The lines are the linear regression lines.

season. This could reflect a decrease of caterpillar and food availability (Arnold et al., 2010), a decrease of parental quality (Verhulst and Nilsson, 2008) or an increase in the number and type of parasites. Interestingly, vitamin-treated nestlings showed the opposite trend, with an increase in intensity of the yellow colouration over the breeding season. These results suggest that vitamins compensated for the possible deterioration of environmental condition. Contrary to the predictions of the protection hypothesis, there was neither a significant effect of carotenoids on the yellow plumage colouration nor any synergistic effect when carotenoids and vitamins were supplemented in combination. The absence of an effect of carotenoids on plumage colouration could be explained if the doses provided were too low to detect any significant change. The same result could be predicted if carotenoids were not limiting for nestling plumage colouration. However, some studies found a positive effect of carotenoids on nestling plumage colouration and thus suggest a limitation (Fitze et al., 2003b; Tschirren et al., 2003; but see Larcombe et al., 2010). It is also possible that the assimilation and transportation of carotenoids are the more limiting factors than their availability per se. Finally, carotenoids could have been invested into functions other than plumage colouration. However, we did not find any positive effect on other traits, such as body mass, oxidative stress and fledging success. If the carotenoid-based colouration does not function as a signal during the juvenile or post-fledging stage, the protection hypothesis might apply just to adults.

Our study supports the idea that vitamins E and/or C regulate the trade-off between growth and self-maintenance. Moreover, our findings are consistent with the idea that carotenoids are minor antioxidants in birds (Costantini and Møller, 2008), but do not support the protection hypothesis of Hartley and Kennedy (Hartley and Kennedy, 2004).

MATERIALS AND METHODS

The experiment was carried out during spring 2011 in a free-ranging population of great tits, *Parus major*, breeding in nest-boxes in the Forst and Bremgartenwald forests, near Bern, Switzerland (46°7′N, 7°8′E; 46°57′N, 7°24′E). Nest-boxes were visited regularly from the beginning of the breeding season to determine the day of the start of incubation and the day of hatching (day 0).

This work was conducted under licence of the Ethical Committee of the Agricultural Office of the Canton Bern (BE23/11).

Table 3. Linear mixed-effect model testing the effect of the vitamin and carotenoid treatments on SWS ratio (an index of chromatic reflectance), 14 days after hatching

Effect	Estimate ± s.e.	*F*	d.f.	*P*
Intercept	2.24±0.28	–	–	–
Brood size	−0.012±0.019	0.39	1, 78	0.53
Sex	**0.12±0.02**	**38.03**	**1, 432**	**<0.0001**
Hatching date	**−0.025±0.017**	**2.28**	**1, 79**	**0.13**
Carotenoid	**−0.24±0.21**	**1.29**	**1, 79**	**0.26**
Vitamin	**−0.27±0.21**	**1.76**	**1, 79**	**0.19**
Carotenoid × sex	−0.072±0.041	3.04	1, 430	0.082
Vitamin × sex	0.015±0.04	0.15	1, 429	0.7
Carotenoid × hatching date	**0.043±0.024**	**3.23**	**1, 79**	**0.08**
Vitamin × hatching date	**0.052±0.023**	**5.35**	**1, 79**	**0.02**
Carotenoid × vitamin	**0.44±0.28**	**2.44**	**1, 79**	**0.12**
Carotenoid × vitamin × sex	−0.15±0.084	3.2	1, 428	0.074
Carotenoid × vitamin × hatching date	**−0.074±0.031**	**5.56**	**1, 79**	**0.02**

F- and *P*-values of non-significant terms are those just before removal from the model. Terms retained in the final model are highlighted in bold. Reference level for coefficients is a female not supplemented with carotenoid and vitamin.

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Antioxidant supplementation

Nest-boxes were randomly assigned to four treatments: control group (N=23), carotenoid group (N=23), vitamin group (N=25) and combined carotenoid plus vitamin group (N=27). Thus the nests were the independent units in the statistical analyses (see below). We supplemented nestlings with one larva of *Calliphora* spp. on days 3, 5 and 7 after hatching. This larva was coated with corn oil for control nestlings and with carotenoids, vitamins or carotenoids plus vitamins for treated nestlings. We added the different compounds to fresh living larvae in a jar that was kept in the dark overnight before supplementation. Because vitamin E and C are, respectively, fat and water soluble, they were not mixed together in the same solution but were added subsequently to the larvae mixture [for more details of the method, see Helfenstein et al. (Helfenstein et al., 2008)]. We provided a dose of carotenoids and vitamins aiming at doubling the daily amount that individuals naturally obtain from food between days 3 and 8 post-hatch. We calculated the daily food intake (DFI) for great tit nestlings, according to Crocker et al. (Crocker et al., 2002) and taking into account a surplus of food due to growth (de Ayala et al., 2006). To obtain an estimated daily antioxidant intake, we multiplied the DFI by the concentration of antioxidants in caterpillar, the main source of food for great tit nestlings (Gosler, 1993). We calculated age-specific doses for the three supplementations because in nestlings the DFI changes rapidly as they grow.

First, the average daily intake of carotenoids was estimated using the value of 3.3 μg g^{-1} reported by Partali et al. (Partali et al., 1987). This carotenoid supplement has previously proved to be biologically relevant (e.g. Fitze et al., 2007; Losdat et al., 2011a). We fed nestlings with lutein and zeaxanthin only [following the proportion described by Partali et al. (Partali et al., 1987)] but not with β-carotene as it is not deposited into the feathers. We supplemented nestlings with the following quantities of lutein and zeaxanthin, respectively (DSM, Nutritional Products Ltd, Lupsingen, Switzerland): 0.0576 mg and 0.00216 mg on day 3; 0.0848 mg and 0.00318 mg on day 5; and 0.1088 mg and 0.00408 mg on day 7.

Second, for the concentration of vitamin E, we used a weighted mean between the quantities reported by Catoni et al. (Catoni et al., 2008) and Arnold et al. (Arnold et al., 2010), i.e. 24.4 μg g^{-1}. We used the same estimation for vitamin C. The final supplemented doses of α-tocopherol acetate and L-ascorbic acid (Sigma-Aldrich, Basel, Switzerland) were, respectively: 0.394 mg (0.536 IU) and 0.536 mg on day 3; 0.00408 mg and 0.735 mg (1 IU) on day 5; and 0.00408 mg and 0.735 mg (1 IU) on day 7.

Morphological measurement

On day 3 post-hatch we measured nestling body mass with an electronic balance (±0.1 g), and took 1 μl of blood from the metatarsal vein. This blood was then stored in ethanol 96% until later analyses to determine sex [see Griffiths et al. (Griffiths et al., 1998) for the sexing technique]. On days 8 and 14 post-hatch, we measured body mass. On day 8 post-hatch, we took a blood sample from the brachial vein: 7 μl were used to assess the resistance to oxidative stress while the remaining blood was centrifuged and the plasma was stored at −20°C before analysis of oxidative damage.

Oxidative damage

We measured the concentrations of MDA, a by-product of lipid peroxidation, caused by β-scission of peroxidized fatty acids, to assess oxidative damage. This method has already been described and used successfully in several studies (e.g. Mougeot et al., 2009; Losdat et al., 2011b).

All chemicals were HPLC grade and chemical solutions were prepared using ultra pure water (Milli-Q Synthesis; Millipore, Watford, UK). In a 2 ml plastic centrifuge tube we pipetted 5 μl of sample or standard (1,1,3,3-tetraethoxypropane), 5 μl of butylated hydroxytoluene solution (0.05% w/v in 95% ethanol), 40 μl of phosphoric acid solution (0.44 mol l^{-1}) and 10 μl of thiobarbituric acid (TBA) solution (42 mmol l^{-1}). Samples were vortexed for 5 s, heated at 100°C for 1 h in a dry bath incubator to allow formation of MDA–TBA adducts, and then cooled on ice for 5 min. To extract the MDA–TBA complex, 80 μl of *n*-butanol were added to each tube, and samples were vortexed for 20 s and centrifuged for 3 min at 4°C and 13.8 ***g*** to separate the two phases. Fifty-five microlitres of the upper phase were transferred to an HPLC vial for analysis. Then, 40 μl of the sample were injected into Dionex HPLC system (Dionex Corporation, CA, USA) fitted with a Hewlett-Packard Hypersil 5 μm ODS 100×4.6 mm column and a 5 μm ODS guard column maintained at 37°C. The mobile phase was methanol buffer (40:60, v/v), the buffer being a 50 mmol l^{-1} anhydrous solution of potassium monobasic phosphate at pH 6.8 (adjusted using 5 mol l^{-1} potassium hydroxide solution), running isocratically over 3.5 min at a flow rate of 1 ml min^{-1}. Data were collected with a fluorescence detector (RF2000; Dionex) at 515 nm (excitation) and 553 nm (emission). For calibration, a standard curve was prepared using a 1,1,3,3-tetraethoxypropane stock solution (5 μmol l^{-1} in 40% ethanol) serially diluted using 40% ethanol. The repeatability of the method was high (r=0.87, P<0.0001, n=80).

Antioxidant capacity

To assess antioxidant capacity, we used the Kit Radicaux Libres® (KRL) test (Brevet Spiral V02023, Couternon, France) adapted to bird physiological parameters (Alonso-Alvarez et al., 2004). This test measures the time needed to hemolyse 50% of the red blood cells when exposed to a controlled free-radical attack. After sampling, 7 μl of the whole blood were immediately diluted in 255.5 μl of KRL buffer (150 mmol l^{-1} Na^{+}, 120 mmol l^{-1} Cl^{-}, 6 mmol l^{-1} K^{+}, 24 mmol l^{-1} HCO^{3-}, 2 mmol l^{-1} Ca^{2+}, 340 mOsm, pH 7.4) and stored at 4°C before analysis was conducted within 12 h of blood collection. Eighty microlitres of the KRL-diluted blood were pipetted into a 96-well microplate together with 136 μl of a 150 mmol l^{-1} solution of 2,2-azobis-(amidinopropane) hydrochloride, a free-radical generator, and incubated at 40°C. The rate of hemolysis was assessed with a microplate reader spectrophotometer (PowerWave XS reader, Witec Ag, Switzerland) that measures the change in optical density at 540 nm. Readings were made every 3.5 min for 80 min. The repeatability of the method, assessed using samples from individual great tits that were not included in the present study, was high and significant (r=0.78, P<0.001, n=80).

Colour measurement

On day 14 post-hatch, we collected from each nestling six feathers from two patches on both sides of the chest, and stored them in small plastics bags kept in the dark. After the breeding season, we superposed the feathers in a stack placed on a black velvet surface before proceeding with spectrometric measurements. We recorded the feather reflectance with a spectrophotometer (USB 4000, Ocean Optics, Duiven, The Netherlands), a bifurcated reflectance probe with a 200 μm fibre core diameter (FCR-7UV200-2-ME) and a balanced deuterium tungsten-halogen light source (DH-2000-BAL, Ocean Optics). The probe was fitted with a black cylinder to standardize the measuring distance and exclude ambient light. We calibrated the spectrophotometer every three measurements with a diffuse reflectance standard (WS-1, Ocean Optics) and measured the reflectance spectra holding the probe perpendicular to the surface of the feathers. We recorded three reflectance spectra (each of these is the average of four scans with a 100 ms integration time) per patch using Spectrasuite software version 1.0 (Ocean Optics). We averaged the measurements per repetition and then per patch to describe each individual (intra-patch repeatability: r=0.77, P<0.0001, inter-patch repeatability: r=0.75, P<0.0001).

Using Hadfield's SPEC package (Hadfield, 2005), we estimated the amount of light captured by each of the avian single cones: UV sensitive, short-wavelength sensitive (SWS), medium-wavelength sensitive and long-wavelength sensitive. This method calculates quantum cone catches taking into account the sensitivity of the retinal cones, the transmittance properties of the ocular media and ambient light. We used the cone spectral sensitivities and ocular media transmittance reported for the blue tit (Hart et al., 2000), the standard forest shade irradiance spectrum (Endler, 1993), and we applied the von Kries algorithm to account for colour constancy. Following the method described in Evans et al. (Evans et al., 2010), we then calculated the SWS ratio, an index of chromatic reflectance based on opponent processing that compares the quantum catch of the SWS single cones with the mean of the other three. The SWS ratio is increasing with higher carotenoid content of feathers, because it estimates the size of the trough in the violet–blue

region of the reflectance spectra, caused by the selective absorption of these pigments. The SWS ratio takes into account how the colour would be perceived via a tetrachromatic visual system and is highly positively correlated with carotenoid chroma (r=0.83, P<0.0001, n=540), a known measure of the amount of pigment deposited in the feathers (Saks et al., 2003).

Statistical analyses

The effects of carotenoid and vitamin treatments on nestlings' yellow feather colouration, antioxidant capacity, oxidative damage, change in body mass both from day 3 to 8 and day 8 to 14 were analysed using linear mixed-effect models with restricted maximum-likelihood estimation. To normalize data, we transformed MDA values using a Box–Cox transformation with λ=1, and square-root-transformed antioxidant capacity values. While nests were the independent units, as described above, we controlled for the non-independence of nestlings within each nest by including in all the models nest identity as a random factor. Our initial models included the three-way interactions carotenoid treatment×vitamin treatment×sex and carotenoid treatment×vitamin treatment×hatching date. Hatching date refers to the date of the first nest hatching in the population, entered as day 1, thereby adjusting for seasonal effects. To interpret significant interactions we split the models according to treatment levels and examined the model summaries. We included as covariates brood size on day 3 in all mixed-effect models and haematocrit, estimated as the initial optical density of the KRL test, in the model of antioxidant capacity.

Fledging success, calculated as the proportion of hatchlings that fledged, was analysed using a generalized linear model with a quasi-binomial distribution to account for overdispersion. Hatching date and brood size were included as covariates. Models were simplified following a backward stepwise elimination procedure based on Akaike's information criterion. All the analyses were performed with R version 2.15.1 (R Development Core Team, 2010), using nlme and car library.

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Competing interests

The authors declare no competing financial interests.

Author contributions

V.M. and H.R. designed the study and wrote the paper. V.M. collected and analysed the data.

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