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# Maternal carotenoid supplementation does not affect breeding performance in the Great Tit (*Parus major*)

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**Summary**

**1.** Carotenoids are micronutrients with many beneficial health-related effects. They are effective antioxidants and stimulants of the immune system. Carotenoids cannot be synthesized in animals and must be obtained from food. As such, they may limit reproductive output and performance, and on the proximate level mediate reproductive trade-offs.

**2.** We studied carotenoid limitation in wild Great Tits (*Parus major*) by supplementing prelaying and laying females with lutein, the most abundant carotenoid in this species. We followed the effects of this supplementation on egg yolk carotenoid composition, and offspring and parental performance.

**3.** Females transferred the supplemented lutein into egg yolks, increasing lutein concentration to the upper limit of naturally occurring concentrations in control pairs. Concentrations of zeaxanthin, β-carotene and α-carotene did not differ between supplemented and control pairs.

**4.** Effects on offspring and parental performance were generally absent or weak. There were no effects on timing of laying, clutch size, hatching success, nestling survival, nestling mass (day 6 and 14), tarsus length or T-cell mediated immune response. Males on supplemented nests fed their young more than those on control nests. There was no positive effect on female feeding or mass.

**5.** Negligible effects of lutein supplementation on offspring and parental performance might be explained by high natural abundance of carotenoids or other antioxidants, where additional carotenoids bear no strong advantage to the birds. Additionally, conflicting results of different studies may be explained by species-specific features of their life-histories.

*Key-words*: antioxidants, carotenoids, egg yolk, food supplementation, parental investment, resource allocation

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## Introduction

Animals are expected to allocate limited resources among competing bodily functions so as to maximize fitness. During reproduction, mothers face a fundamental decision of how much resources to invest into current reproductive bout, and how much to retain for maintenance and future reproduction. Besides elaborate postnatal parental care females also invest heavily during the prenatal period into the fabrication of eggs. Besides energy needed for embryo development, eggs are packed with many valuable resources, including antibacterial enzymes, antibodies, hormones and carotenoids (Blount, Houston & Møller 2000).

Carotenoids are a group of several hundred biologically active compounds with many important biological functions in signalling and physiology (Møller *et al.* 2000). They are widely used in the colouring of bird plumage and bare parts (Olson & Owens 2005). Carotenoids enhance the intensity of both cell-mediated and humoral immune response (Chew & Park 2004). They are also effective scavengers of reactive oxygen species (ROS) that arise during metabolic processes (Krinsky 2001). ROS are free radicals and non-radical oxygen-containing molecules that are able to damage proteins, lipids and DNA (de Zwart *et al.* 1999), a condition called oxidative stress that has been implicated

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in the etiology of many diseases and ageing (Crimi *et al*. 2006). ROS are removed by the multifaceted antioxidant system that includes enzymes (e.g. catalase, superoxide dismutase), water-soluble antioxidants (ascorbic acid, glutathione) and fat-soluble antioxidants (vitamin E, carotenoids; Sies & Stahl 1995). Carotenoids can be synthesized only by plants, certain bacteria and fungi, while animals must ingest them with their food. As a 'diet-dependent' resource used in both signalling and physiology, they are a good candidate for mediating life-history trade-offs (Blount 2004).

Birds deposit carotenoids into egg yolks and the amount varies markedly within and among species (Hargitai *et al*. 2006). Some variation among yolks in the concentration of carotenoids can be explained by laying order (e.g. Blount *et al*. 2002a; Saino *et al*. 2002), year (Hargitai *et al*. 2006) or habitat (Hõrak, Surai & Møller 2002; Cassey *et al*. 2005). Studies on both domestic and wild birds demonstrated higher yolk carotenoid concentrations in mothers supplemented by carotenoid-rich diet (Blount *et al*. 2002b; Bortolotti *et al*. 2003; Biard, Surai & Møller 2005; McGraw, Adkins-Regan & Parker 2005; Ewen *et al*. 2006; Berthouly, Helfenstein & Richner 2007). However, we still do not understand whether these patterns represent active deposition of carotenoids by mothers (Blount *et al*. 2002a, Blount *et al*. 2002b; Royle, Surai & Hartley 2003) or simply reflect their supply in the diet (Partali *et al*. 1987).

Carotenoids are responsible for the typical yellow to orange colour of yolks and have important physiological functions. They reduce the susceptibility of yolk lipids to peroxidative damage (Blount *et al*. 2002b) and later protect developing embryo from oxidative stress (Surai, Noble & Speake 1996). This is important because birds grow very fast and their intense metabolism makes them especially vulnerable to oxidative damage (Blount *et al*. 2000). Upon hatching, yolk-derived carotenoids can affect the susceptibility of hatchling tissues to oxidative damage (Surai *et al*. 1996), the ability of chicks to accumulate dietary carotenoids in their body (Koutsos *et al*. 2003), or parameters of their immune function (Haq, Bailey & Chinnah 1996; Koutsos, López & Klasing 2006). All these studies were on domestic hens. Three studies on passerines suggest that similar effects may exist in this group of birds. McGraw *et al*. (2005) found that carotenoid supplementation of females enhances hatching and fledging success in captive Zebra Finches (*Taeniopygia guttata*). Under wild conditions Biard *et al*. (2005) found that young hatching from the eggs of carotenoid-supplemented females had longer tarsi at hatching and more leukocytes in their blood during growth. Berthouly *et al*. (2007) found out that maternally derived carotenoids can help nestlings cope with stress.

To advance our understanding of potential carotenoid limitation in wild birds, we performed a carotenoid-supplementation study in wild Great Tits (*Parus major* Linnaeus 1758). Reproducing parents face a trade-off of how many limited resources to allocate into current reproductive bout *vs* self-maintenance and future reproduction. In this study we focused on the potential for carotenoid limitation in the current reproductive bout. We provided Great Tit pairs with a lutein-rich supplement before and during egg laying and followed the effects of this supplementation on yolk carotenoid concentrations, and reproductive and parental performance. We tested three possible scenarios: (1) Supplemented females do not increase yolk lutein concentration and the intensity of parental care does not change. This would mean that parents are not limited during current reproduction. (2) Supplemented females increase yolk lutein with no effects on offspring performance and no effects on the intensity of parental care. In this scenario, parents are not limited in their current reproductive bout and the female bird just channels surplus micronutrients into the eggs. (3) Supplemented females do increase yolk lutein concentration with positive effects on offspring performance and/or parents care more intensely. This would demonstrate carotenoid limitation during current reproduction.

## Methods

### FIELD WORK

Great Tits are small, insectivorous, resident passerines that breed in nest holes during April–June in various woodland types. We studied them in 2004 on six nest-box plots (400 nest-boxes in total) in the Velký Kos'ř area in the eastern Czech Republic (49°32′N, 17°04′E, 300–450 m a.s.l.). Three plots were in a sessile oak (*Quercus petraea*) forest, the other three in a Norway spruce (*Picea abies*) forest. Before birds started breeding nest-boxes were checked and cleaned.

We visited nest-boxes daily to determine the start of nest building and egg laying. We marked eggs daily by a water-proof pen. Before and during egg laying we supplemented experimental tit pairs with 25 mg of CWS lutein (DSM Nutritional Products (Basel, Switzerland), composition: 7% of lutein, 1% DL-α-tocopherol, 1% ascorbyl palmitate, 18% fish gelatine, 46% sucrose, 2% sodium ascorbate and 25% corn starch), which means 1·75 mg of lutein daily. According to the information given in Partali *et al*. (1987; *c.* 3·3 μg of carotenoids per one lepidopteran larva) this makes daily increase in carotenoid intake equivalent to *c.* 530 lepidopteran larvae. Control pairs were supplemented with a placebo lacking lutein with otherwise identical composition. We started with 33 experimental and 27 control pairs. Both lutein and placebo were enclosed in a pill made from animal fat (*c.* 0·6 g) and put into a plastic cup (diameter 3 cm, height 2 cm). It was put inside the nest-box inhabited by the focal tit pair, *c.* 5 cm above the nest rim on one side of the nest-box. Supplemental units were freshly prepared every evening and

stored at –20 °C and in the dark until use the following day. To increase the attractiveness of the pill, we always added five meal-worms into the plastic cup. A pill was supplemented daily until egg laying was terminated. We started the supplementation on the day when tits started to bring animal fur into the moss base of the nest being built. We did not start the supplementation earlier, because females may switch between nest-boxes in the earlier phases of nest building. All the pairs which we started to supplement continued in breeding. On average 2·4 pills (SD = 2·6, $N = 60$) had already been eaten on the day the first egg of the clutch was laid (supplemented: 2·4 ± 2·8, $N = 33$; control: 2·5 ± 2·3, $N = 27$; $F < 0{\cdot}1$, $P = 0{\cdot}818$). Supplementation was regularly taken by birds, only 7 out of 812 pills remained uneaten the next day. We did not monitor the nest-boxes and thus we do not know the relative share of the sexes in the consumption of the supplement. When incubation commenced, apart from seven cases we collected the egg laid on that day (i.e. the last egg in the laying sequence that could have been collected without having been incubated for more than a few hours) and stored it at –20 °C before further analysis. On average, 11·5 (SD = 2·8, $N = 53$) pills had been eaten by the birds in each nest before the collected egg was laid (supplemented: 11·5 ± 3·1, $N = 28$; control: 11·5 ± 2·5, $N = 25$; $F < 0{\cdot}1$, $P = 0{\cdot}984$). Average position in the laying sequence of this egg was 10·0 (SD = 2·0, $N = 53$; supplemented: 10·1 ± 2·1, $N = 28$; control: 9·9 ± 1·9, $N = 25$; $F = 0{\cdot}1$, $P = 0{\cdot}726$).

To recognize the young hatching from late eggs that had the greatest probability of being affected by the supplementation, we frequently visited nests around the expected time of hatching. At most nests, we identified and marked nestlings hatched from late eggs in the laying sequence by clipping their dawn feathers. We weighed all the young when they were 6 days old and again when they were 14 days old. On day 14 we also measured their tarsi. On day 13, we measured the thickness of the right wing web of three young per nest (those that hatched from late eggs, if known) with a pressure-sensitive gauge (model PK-1012E, Mitutoyo, Tokyo, Japan) and then injected it with 0·09 mg of phytohaemagglutinin (L-8754, Sigma-Aldrich, St. Louis, MO, USA) in 25 µL of phosphate buffered saline. We re-measured the wing web 24 h later (± 2 h). We always measured the wing web twice and took the average. T-cell mediated immune response was quantified as the difference in the wing web thickness measured 1 day after the injection and a day before. On average 8·8 pills (SD = 3·0, $N = 46$; supplemented: 9·2 ± 3·0, $N = 25$; control: 8·1 ± 2·9, $N = 21$; $F = 1{\cdot}7$, $P = 0{\cdot}194$) had been eaten by parents before the eggs from which the young that were scored for immune response originated were laid (mean position in the laying sequence = 7·4, SD = 2·1, $N = 46$; supplemented: 7·3 ± 2·0, $N = 25$; control: 7·4 ± 2·2, $N = 21$; $F < 0{\cdot}1$, $P = 0{\cdot}985$). In some nestlings, we did not know their exact position in the laying order. In such cases, we assigned the average of the possible positions for the chick (e.g. if we knew that the chick hatched either from egg 7 or 8, its position in the laying order was assigned to be 7·5).

During incubation we captured females, weighed them on a spring Pesola balance (to the nearest 0·25 g), and measured their tarsus with a digital caliper (to the nearest 0·01 mm). We also quantified male and female feeding rate per hour when nestlings were 7–11 days old (median = 9 days). We set up a camera *c.* 5–10 m from the nest-box and filmed feeding activity for 75 min. We then discarded the first 15 min of the recording and counted number of feeds provided by male and female during subsequent 60 min.

### ANALYSIS OF CAROTENOIDS

In the yolk of the collected eggs we determined concentrations of lutein, zeaxanthin, α-carotene and β-carotene. To extract carotenoids, weighted amount of egg yolk (on an average of 200 µL) was homogenized with 2 mL of a mixture of 4% NaCl solution and ethanol (1 : 1, v/v) followed by sonication for 7 min. We then added 3 mL of hexane and further homogenized for 5 min. Yolk was then drawn into the tubes and centrifuged at 6000 r.p.m. for 5 min. After centrifugation hexane was collected and the extraction was repeated three times. Hexane extracts were combined and evaporated under $N_2$ at room temperature, and the residue was dissolved in 1·5 mL of acetonitrile : dichloromethane (1 : 1, v/v) and centrifuged. The supernatant was used for carotenoid determination. Carotenoids were determined by high performance liquid chromatography equipped with a binary LC pump Model 250 (Perkin Elmer, Norwalk, CT, USA), using two sequential LICHROCARTTM PUROSPHERTM RP18 columns (250 × 4nn I.D) maintained at 40 °C by a column block heater. A mobile phase of acetonitrile : methanol (85 : 15) and acetonitrile : dichloromethane : methanol (70 : 20 : 10, v/v) in linear gradient elution with PDA detection (Series 200, Perkin Elmer) at 450 nm was used. Peaks were identified and quantified using reference carotenoids kindly supplied by Carotenature (Lupsingen, Switzerland).

### DATA ANALYSIS

Data were analysed by general linear models. For every response variable (i.e. offspring and parental traits), we fit a separate model with treatment as the main predictor of interest and other explanatory variables that have been previously shown to be important as covariates. Initially, we included habitat (oak *vs* spruce) and season (Julian date of the first egg, date 1 = 1 January) as covariates to all models. We analysed these response variables (additional covariates in parentheses): carotenoid concentration, hatching success (clutch size), nestling survival until day 14 and nestling mass at day 6 (brood size at hatching), nestling

tarsus length at day 14 (brood size at day 14, female tarsus length), nestling body mass at day 14 and T-cell mediated immunocompetence (brood size at day 14, tarsus length at day 14), clutch size (female tarsus length), female body mass (brood size, female tarsus length, day of the nest cycle when captured), and male and female feeding rate per hour (brood size at feeding, hour of day, age of the young). In the case of nestling mass and tarsus length we used the young originating from late eggs that had the greatest chance to be affected by supplementation (the three nestlings used for the PHA test, see above). However, analyses using mean values for all the young in the nest generated identical results (results not shown). Initially, we also included interactions between the treatment and all other factors. We gradually removed non-significant predictors beginning with interactions until only significant factors remained in the model (at $\alpha = 0{\cdot}05$), with the exception of treatment. It was always retained as the main factor of interest. Variables were checked for departures from normality and appropriately transformed if necessary. We checked the reliability of our results by calculating standardized effect sizes (difference in least squares means of the dependent variable between supplemented and control groups/SD of the total sample) with their 95% confidence limits. All tests were performed in JMP software of SAS Institute, Cary, NC, USA.

## Results

### EGG YOLK CAROTENOIDS

Egg yolk concentration of lutein was significantly increased in experimental nests ($F_{1,51} = 20{\cdot}4$, $P < 0{\cdot}001$; Fig. 1). This increase was within physiological levels experienced by birds in this population: average concentration in experimental eggs was 54·6 μg g$^{-1}$, whereas one-third (8 of 25) of control nests had lutein concentration very close to this value (49 μg g$^{-1}$ or higher, maximum value in control eggs was 58·9 μg g$^{-1}$). Although zeaxanthin tended to have higher concentration in experimental pairs, no other carotenoid differed between experimental and control pairs: zeaxanthin ($F_{1,51} = 3{\cdot}4$, $P = 0{\cdot}073$), α-carotene ($F_{1,47} = 2{\cdot}2$, $P = 0{\cdot}145$) and β-carotene ($F_{1,47} < 0{\cdot}1$, $P = 0{\cdot}944$). Surprisingly, there was a negative relationship between the number of supplementation units eaten and lutein concentration in yolk in experimental pairs ($r = -0{\cdot}48$, $P = 0{\cdot}009$, $N = 28$; Fig. 2). However, the significance of this relationship was caused by one outlying nest and disappeared after its exclusion ($r = -0{\cdot}33$, $P = 0{\cdot}097$, $N = 27$). There was no significant relationship in control pairs ($r = 0{\cdot}13$, $P = 0{\cdot}535$, $N = 25$; Fig. 2). Since the overall effect of supplementation was positive (i.e. increased carotenoid concentration in experimental as compared to control pairs; Fig. 1), it is rather difficult to explain this negative correlation. It might be possible that in females that ate too many units and therefore had ingested a greater amount of lutein this interfered in some way with the incorporation into the egg yolk. However, this is only speculation and further research with precise doses of lutein would be needed to solve this puzzle.

### OFFSPRING TRAITS

There was no effect of supplementation on offspring performance-related traits, including hatching success ($F_{1,49} = 0{\cdot}3$, $P = 0{\cdot}597$), nestling survival from hatching to day 14 of age ($F_{1,47} = 0{\cdot}2$, $P = 0{\cdot}684$), nestling mass at day 6 ($F_{1,47} = 0{\cdot}7$, $P = 0{\cdot}402$), nestling mass at day 14 ($F_{1,42} = 0{\cdot}3$, $P = 0{\cdot}571$), nestling tarsus length at day 14 ($F_{1,42} = 0{\cdot}5$, $P = 0{\cdot}478$) and T-cell mediated immunocompetence ($F_{1,42} = 0{\cdot}5$, $P = 0{\cdot}484$; Table 1).

On the other side, these traits were significantly related to some covariates. Hatching success was

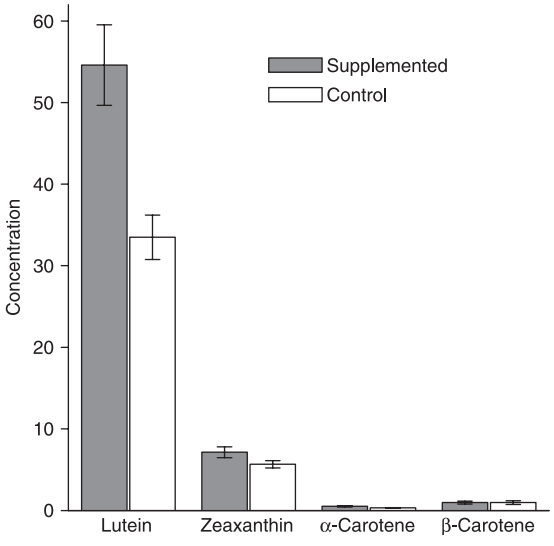


**Fig. 1.** Mean concentration (± 1 SE, in μg/g) of egg yolk carotenoids in the Great Tit in lutein-supplemented and control pairs.

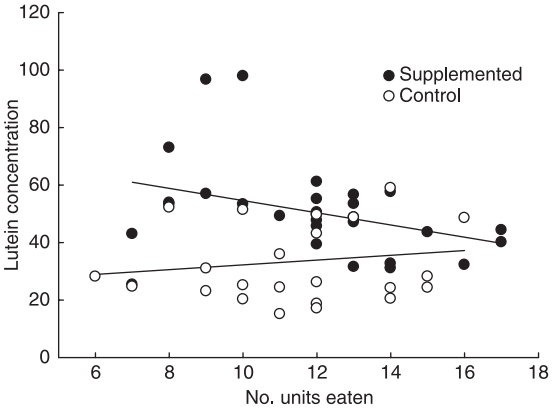


**Fig. 2.** Relationship between lutein concentration in egg yolk (μg/g) and number of supplemental units eaten fit separately for lutein-supplemented and control pairs. One extreme value (lutein concentration = 156·7 μg g$^{-1}$, no. of units eaten = 5) is omitted from the figure (see Results).

**Table 1.** Least squares means (1SE) of offspring and parental traits in lutein-supplemented and control pairs. LS means are from final models with only significant covariates retained

| | LS means (SE) | |
|---|---|---|
| | Supplemented | Control |
| Offspring traits | | |
| Hatching success (proportion hatched) | 0·86 (0·012) | 0·88 (0·014) |
| Nestling survival (per 14 days) | 0·75 (0·055) | 0·78 (0·058) |
| Nestling mass day 6 (g) | 8·9 (0·20) | 9·2 (0·22) |
| Nestling mass day 14 (g) | 16·9 (0·22) | 17·0 (0·22) |
| Nestling tarsus length (mm) | 22·6 (0·11) | 22·7 (0·12) |
| Nestling T-cell immunocompetence (mm) | 0·52 (0·026) | 0·54 (0·026) |
| Parental traits | | |
| Clutch size (no. of eggs) | 10·2 (0·30) | 10·3 (0·31) |
| Laying date (Julian date) | 107·2 (0·66) | 108·6 (0·73) |
| Female feeding rate (per hour) | 9·2 (1·23) | 8·9 (1·31) |
| Male feeding rate (per hour) | 16·2 (1·61) | 11·1 (1·72) |
| Female body mass (g) | 19·0 (0·17) | 19·3 (0·17) |

positively related to clutch size ($F_{1,49} = 6{\cdot}2$, $P = 0{\cdot}016$; whole model: $F_{2,49} = 3{\cdot}3$, $P = 0{\cdot}045$, $R^2 = 0{\cdot}12$), nestling survival was higher in the oak than in the spruce habitat ($F_{1,47} = 15{\cdot}0$, $P < 0{\cdot}001$; whole model: $F_{2,47} = 7{\cdot}5$, $P = 0{\cdot}001$, $R^2 = 0{\cdot}24$) and body mass at day 6 was negatively related to brood size ($F_{1,47} = 4{\cdot}3$, $P = 0{\cdot}043$; whole model: $F_{2,47} = 2{\cdot}6$, $P = 0{\cdot}089$, $R^2 = 0{\cdot}10$). Further, body mass at day 14 was higher in the oak than in the spruce habitat ($F_{1,42} = 9{\cdot}2$, $P = 0{\cdot}004$) and positively related to tarsus length ($F_{1,42} = 42{\cdot}7$, $P < 0{\cdot}001$; whole model: $F_{3,42} = 19{\cdot}3$, $P < 0{\cdot}001$, $R^2 = 0{\cdot}58$), tarsus length at day 14 was positively related to both brood size ($F_{1,42} = 5{\cdot}9$, $P = 0{\cdot}019$) and female tarsus length ($F_{1,42} = 6{\cdot}7$, $P = 0{\cdot}013$; whole model: $F_{3,42} = 5{\cdot}5$, $P = 0{\cdot}003$, $R^2 = 0{\cdot}28$) and T-cell immunocompetence was positively related to brood size ($F_{1,42} = 11{\cdot}7$, $P = 0{\cdot}001$; whole model: $F_{2,42} = 5{\cdot}9$, $P = 0{\cdot}006$, $R^2 = 0{\cdot}22$).

### PARENTAL TRAITS

There was a significant effect of lutein supplementation on clutch size but it depended on season (interaction: $F_{1,50} = 8{\cdot}3$, $P = 0{\cdot}006$). In control nests, clutch size decreased with season whereas in experimental nests it changed nonlinearly – at first it increased, whereas later (after Julian day 105) it decreased in a similar way to control clutches (Fig. 3). Clutch size was furthermore positively affected by female tarsus length ($F_{1,50} = 6{\cdot}2$, $P = 0{\cdot}017$) and was larger in the oak as compared to the spruce forest ($F_{1,50} = 16{\cdot}1$, $P < 0{\cdot}001$; whole model: $F_{5,50} = 6{\cdot}1$, $P < 0{\cdot}001$, $R^2 = 0{\cdot}38$). Experimental and control pairs did not differ in their timing of breeding ($F_{1,58} = 2{\cdot}1$, $P = 0{\cdot}157$).

Female feeding rate did not differ between treatments ($F_{1,45} < 0{\cdot}1$, $P = 0{\cdot}869$, $R^2 < 0{\cdot}01$) whereas males on experimental nests fed more frequently than males on control nests ($F_{1,45} = 4{\cdot}7$, $P = 0{\cdot}036$, $R^2 = 0{\cdot}09$; Table 1). No other factors were significant in the analysis of feeding rates. Male and female feeding frequencies were not intercorrelated ($r = -0{\cdot}17$, $P = 0{\cdot}242$, $N = 47$). Female body mass did not differ between treatments ($F_{1,50} = 1{\cdot}8$, $P = 0{\cdot}184$), whereas it was higher in the oak habitat than in the spruce habitat ($F_{1,50} = 10{\cdot}5$, $P = 0{\cdot}002$), scaled positively with female tarsus length ($F_{1,50} = 20{\cdot}5$, $P < 0{\cdot}001$), and negatively with the day of the nest cycle at capture ($F_{1,50} = 32{\cdot}6$, $P < 0{\cdot}001$; whole model: $F_{4,50} = 19{\cdot}9$, $P < 0{\cdot}001$, $R^2 = 0{\cdot}61$).

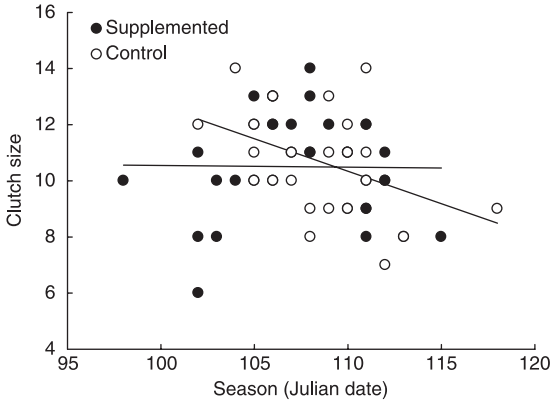


**Fig. 3.** Relationship between clutch size and season (Julian date of the first egg, date 1 = 1 January) fit separately for lutein-supplemented and control pairs.

### CHECKING THE RELIABILITY OF THE RESULTS

In four experimental nests, the concentration of egg yolk lutein was higher than the highest value in any control nest. In these nests, unnaturally high doses of lutein could have toxic effects on nestlings. Then, mixing of beneficial (physiologically high levels) and toxic (pharmacological levels) effects of egg carotenoids in one analysis could have prevented any beneficial effects showing up. Thus, we repeated all the above analyses without those four nests. However, the results did not change. It seems that any potentially harmful effects of very high doses of carotenoids did not compromise our analyses.

For the standardized effect sizes (with confidence intervals) of carotenoid supplementation treatment on offspring and parental performance traits see Fig. 4.

## Discussion

To investigate carotenoid limitation on egg formation and reproduction in wild birds, we supplemented prelaying and laying female Great Tits with lutein, the most abundant egg yolk carotenoid in this species (Partali *et al.* 1987). We showed that this supplementation had a clear effect on egg composition, because yolks of supplemented females had significantly more lutein than those of control females (Fig. 1). Strong effect of lutein supplementation on its concentration in egg yolk is not surprising. Increased concentrations of yolk carotenoids in females supplemented with carotenoids in their diet were demonstrated in both captive

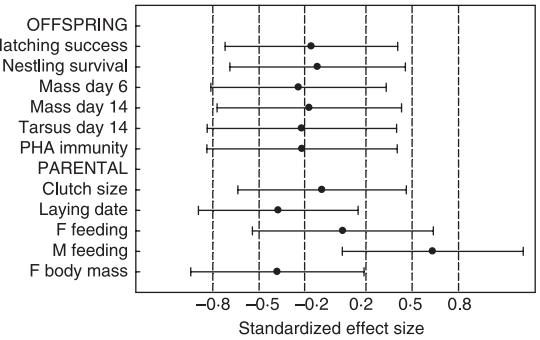


**Fig. 4.** Standardized effects (with 95% CIs) of treatment on offspring and parental, performance-related traits. Vertical dashed lines denote small (0·2), medium (0·5) and large (0·8) effects, respectively, according to Cohen (1988). For the definition of the traits, see Methods.

(summarised in Bortolotti *et al.* 2003; McGraw *et al.* 2005) and wild birds (Blount *et al.* 2002b; Biard *et al.* 2005; Ewen *et al.* 2006; Berthouly et al. 2007).

On the other hand, subsequent effects on offspring performance were negligible, whereas the effects on parental traits were slightly stronger. Clutch size and male feeding responded significantly to the supplementation, and although non-significant, confidence intervals for timing of laying included a strong negative effect (i.e. advancement of laying date; see Fig. 4), which suggests that it might have gone undetected because of low statistical power. The effect on clutch size was only apparent in the interaction with season (Fig. 3). These results by and large conform to the scenario 2 (see Introduction). In this scenario, parents are not carotenoid limited in their current reproductive bout and the female bird just channels surplus micronutrients into the eggs. However, males on supplemented nests fed their offspring more than males on control nests. This could mean that males might have been limited in the intensity of parental care, which would conform to the scenario 3. Parents may also have been limited by carotenoids in their self-maintenance and future reproduction. However, based on our data we were not able to test this possibility and it remains an interesting challenge for future work.

We showed that clutch size decreased with the advancement of the laying season in control pairs whereas it changed nonlinearly in supplemented pairs (Fig. 3). This pattern of clutch size change with the season is quite puzzling. Whereas one study found a beneficial effect of carotenoid supplementation on laying potential of females (in Lesser Black-backed Gulls, *Larus fuscus*, Blount *et al.* 2004) other experiments did not demonstrate any effects (Biard *et al.* 2005; McGraw *et al.* 2005). Moreover, since there was no significant effect of carotenoid supplementation on laying date, we have currently no explanation for the pattern found.

Another interesting result is the higher feeding rate of males on supplemented nests as compared to control nests. This may have been caused by better male condition if they also consumed the supplement, in which case they would be carotenoid limited in their current reproductive bout. Alternatively, they may have been willing to increase paternal investment in supplemented broods where supplementation may have made either females or offspring more attractive and worthy of increased investment. In this case this result would not be indicative of carotenoid limitation in males but rather of differential allocation of parental effort (see Sheldon 2000). However, the plausibility of this explanation is decreased by the finding in a recent study of the Great Tit that the young supplemented by carotenoids are not more attractive to parents and the parents do not increase their investment (Tschirren, Fitze & Richner 2005). If proved by further studies, higher feeding rate of males on supplemented nests would be an interesting observation since we currently know virtually nothing about possible relationships between male carotenoid supply, health and physiology, and paternal investment in birds (Blount 2004).

There are several nonexclusive explanations for weak to absent positive effects of our supplementation on offspring performance. Confidence intervals for the effect of supplementation on offspring traits did not embrace either middle or large positive effects (standardised effects of 0·5 and 0·8, respectively, according to Cohen 1988; see Fig. 4). There is a possibility that there were small positive effects (standardized effect size of 0·2) that we were not able to detect with our sample size. However, if there were any important (i.e. middle or large) positive effects of extra carotenoids in eggs on offspring performance, we would have been able to detect them.

Three biologically interesting explanations seem to be worth discussing. First, the detectability of potential effects may depend upon the amount of carotenoids already present in the egg. All eggs may have been supplied with carotenoids to such an extent that any increase brought about by our supplementation had no detectable health and performance related benefits for the offspring. It is known that beneficial effects of carotenoids are dose-dependent, increasing with increasing amounts supplemented but later reaching a plateau (Alonso-Alvarez *et al.* 2004).

Second, the antioxidant system of birds consists of an integrated system of substances, including enzymes, water-soluble and fat-soluble antioxidants. Vitamin E is a fat-soluble antioxidant present in bird egg yolk, including the Great Tit (Hõrak *et al.* 2002). It is transferred from egg yolk to the developing young and increases resistance to oxidative damage of tissues (Surai, Noble & Speake 1999). Our supplementation included small amounts of α-tocopherol (see Methods). It could be possible that α-tocopherol enhanced the antioxidant system of developing chicks in both experimental and control nests to such an extent that its further enhancement by lutein in experimental nests was not detectable. However, in such a case, young

birds would have to be much more sensitive to α-tocopherol than to lutein. Great Tit yolk (*c.* 0·35 g, V. Remeš, unpublished data) contains about 54 μg of α-tocopherol (*c.* 155 μg $g^{-1}$; Hõrak *et al.* 2002) and about 13 μg of lutein (*c.* 35–38 μg $g^{-1}$; this study, average for control pairs; Hõrak *et al.* 2002). We supplemented about 250 μg of α-tocopherol (4·6 × the amount in one yolk) and 1750 μg of lutein (135 × the amount in one yolk) daily. Thus, we supplemented about 29 times more intensely with lutein than with α-tocopherol. Accordingly, this explanation does not seem likely. However, factorial experiments supplementing laying mothers with different antioxidant system-enhancing micronutrients (e.g. Surai 2000) in the wild will be needed to resolve this issue.

Third, conflicting results of our study and previous ones could be explained by different study organisms. For instance, Biard *et al.* (2005) studied Blue Tits (*P. caeruleus*). In these smaller birds clutch mass comprises a relatively larger proportion of female body mass than in the closely related Great Tit. Thus, these authors suggest that laying females in this species need relatively more carotenoids and are thus more carotenoid limited than Great Tits (see also Biard, Surai & Møller 2006). This view concerns limitation during acquisition of resources. On the other hand, species differ in their resolution of the trade-off between current and future reproduction based on their position on the slow-fast life-history continuum (Ghalambor & Martin 2001). This might drive the species-specific patterns of allocation of acquired (supplemented) micronutrients between offspring and self-maintenance. Life-history differences between species together with carotenoid supply in the environment might thus be responsible for conflicting results of carotenoid-supplementation experiments. For further development of this area, it will be critical to perform similar supplementation studies on various species differing in their life-history strategies, while at the same time also following allocation of supplemented carotenoids to self-maintenance and future reproduction.

In general, carotenoid-supplementation studies that generated clear and strong positive effects on offspring performance were either performed in captivity (McGraw *et al.* 2005) or carotenoids were injected directly into the eggs in the wild (Saino *et al.* 2003). Food supplementation studies made in the wild up to now generated weak and unconvincing results (Biard *et al.* 2005; Berthouly *et al.* 2007; this study). Decisiveness of these weak results becomes even lower in the light of the number of statistical tests often performed with inherently increased probability of statistical error and finding false relationships (see also de Ayala, Martinelli & Saino 2006). This is surprising given the many beneficial health-related effects of carotenoids (see above, but see McCall & Frei 1999). It may be more difficult to detect beneficial effects of carotenoids in the wild because of less well controlled experimental conditions. Wild birds may also have enough carotenoids to provide to their young with resulting sufficient antioxidant protection. Further experimental increase of carotenoids may then operate in the plateau region of the dose-dependent relationship between carotenoid concentration and beneficial effects (Alonso-Alvarez *et al.* 2004). Experiments with carotenoid-deplete and carotenoid-replete eggs in semi-natural conditions could help to resolve this issue (e.g. Koutsos *et al.* 2003). Alternatively, the antioxidant system of the young may be supported by other antioxidants to a sufficient level, again precluding any potentially beneficial effects of carotenoids to show up. These thoughts are in line with weak beneficial effects of direct supplementation of nestling food with dietary carotenoids (Biard *et al.* 2006) and with vitamin E (de Ayala *et al.* 2006) in the wild. Moreover, the effects detected by Biard *et al.* (2006) differed between species, and the authors suggested that the potential for beneficial effects of supplemental antioxidants might vary with the life-history strategy of the particular species. Similar species-specific effects were found in the relationships between carotenoids, immune function and male ornamentation in birds (summarized by Blount 2004). More studies on diverse species taking into account broader spectrum of antioxidants and employing more sophisticated study design are clearly needed to resolve these interesting issues.




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