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Cellular immune response, stress resistance and competitiveness in nestling great tits in relation to maternally transmitted carotenoids

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Summary

1. Egg yolks contain carotenoids that protect biological molecules against free-radical damage and promote maturation of the immune system. Availability of carotenoids to birds is often limited. Trade-offs can thus arise in the allocation of carotenoids to different physiological functions, and mothers may influence the immunocompetence of nestlings by modulating the transfer of carotenoid to the yolk.
2. In the great tit *Parus major*, we experimentally manipulated the dietary supply of carotenoid to mothers, and partially cross-fostered hatchlings to investigate the effect of an increased availability of carotenoids during egg laying on immunocompetence of nestlings.
3. In addition, we infested half of the nests with hen fleas *Ceratophyllus gallinae* to investigate the relationship between carotenoid availability, resistance to ectoparasites and immunocompetence.
4. We found that the procedure of cross-fostering can reduce the immune response of nestlings, but this effect can be compensated by the maternally transferred carotenoids. Cross-fostered nestlings of carotenoid-supplemented females show a similar immune response to non-cross-fostered nestlings, while cross-fostered nestlings of control females mounted a weaker cell-mediated immune response. This suggests that yolk carotenoids may help nestlings to cope with stress, for example the one generated by cross-fostering and/or they may enhance nestling competitiveness.
5. There was no statistically significant interaction between parasite and carotenoid treatments, as would be expected if carotenoids helped nestlings to fight parasites. Under parasite pressure, however, lighter nestlings raised a lower immune response, while the immune response was only weakly correlated with body mass in uninfested nests.

Key-words: ectoparasite resistance, maternal effects, nestling competition, stress, yolk carotenoids.

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Introduction

Maternally derived resources in egg yolk affect embryonic development, and can subsequently influence morphological, physiological and behavioural traits correlated with offspring fitness (Desai *et al*. 1995; Schwabl 1996; Anisman *et al*. 1998; Saino *et al*. 2003; Andersson *et al*. 2004). For example, high levels of androgenic hormones in egg yolk, such as testosterone, have been shown to increase post-hatching growth and nestling competitiveness (Schwabl 1996). At elevated levels, however, androgenic hormones can also have immunosuppressive effects (Andersson *et al*. 2004).

The quantity and variety of resources deposited into the eggs may affect female condition if these resources are costly for mothers to produce or acquire (Pilz *et al*. 2003; Rutkowska *et al*. 2005). Thus, female investment into eggs will depend on the balance between the costs and benefits of this maternal investment (Pilz *et al*. 2003; Biard, Surai & Møller 2005; Rutkowska *et al*. 2005). The costs and benefits may themselves depend on both the mother's condition at breeding (Pilz *et al*. 2003; Love *et al*. 2005) and the quality of the breeding environment, as reflected by the quality of the diet (Saino *et al*. 2002a; Bortolotti *et al*. 2003; Biard *et al*. 2005). Females are thus expected to optimize offspring

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quality and reproductive value of offspring by modifying investment into eggs according to the breeding conditions and their own health status (Blount *et al.* 2002a,b, 2002b; Bortolotti *et al.* 2003; Tschirren, Richner & Schwabl 2004; Biard *et al.* 2005; Verboven *et al.* 2005).

The egg yolk of birds contains carotenoids that belong to the family of antioxidant pigments. Many studies have found that they play important physiological roles in protecting biological molecules against free radical damage and in activating the immune system (Surai & Speake 1998; Møller *et al.* 2000; Alonso-Alvarez *et al.* 2004; McGraw & Ardia 2004). They are supposedly also involved in the regulation of embryonic growth and development. More precisely, they are hypothesized to control cell differentiation (Royle *et al.* 1999), to promote maturation and functioning of the immune system (Møller *et al.* 2000; Koutsos *et al.* 2003; Saino *et al.* 2003)and to provide antioxidant protection to embryonic tissues (Surai & Speake 1998; Blount *et al.* 2002a; McGraw, Adkins-Regan & Parker 2005). Carotenoids may hence be of particular importance during the early life of birds. Exposure to increased quantities of carotenoids in the egg may directly influence nestling immunocompetence by stimulating the setting up of the immune components in the developing embryo. Furthermore it may influence immune function indirectly by modulating the programming of nestling physiology (Royle *et al.* 1999; Koutsos *et al.* 2003; Saino *et al.* 2003) and therefore nestling energetics, and the ability to assimilate and efficiently use carotenoids (Koutsos *et al.* 2003). Elevated levels of carotenoids in the yolk sac may also directly enhance the protection of hatchlings against the oxidative physiological stress generated during hatching, or attenuate the effects of the oxidative physiological stress response due to sibling competition, starvation and temperature variation in the nest right after hatching. Nestlings receiving more carotenoids via the egg may therefore develop a better performing immune system than nestlings developing from eggs with lower carotenoid concentrations.

Carotenoids are not synthesized *de novo*, but ingested with the food, and their availability is supposedly limited in a natural environment (Partali *et al.* 1987; Olson & Owens 1998; Tschirren, Fitze & Richner 2003a). Owing to their various functions (as stated above) allocation trade-offs, e.g. between immunity and plumage coloration or growth, can thus be expected. Similarly, carotenoid deposition into the eggs can also be limited by their availability, and hence be adjusted by females. An increase in the availability of carotenoids allows for a higher allocation to both the eggs and other functions that are directly relevant for female health, e.g. the level of circulating antioxidants. As expected, eggs laid by females fed with carotenoid-enriched food during the laying period were found to have higher levels of carotenoids than eggs laid by control females (Blount *et al.* 2002a,b; Biard *et al.* 2005; Karadas *et al.* 2005). However, the effect of larger quantities of maternally transmitted carotenoids on the offspring's immune performance has not been clearly demonstrated yet.

In a supplemental feeding experiment in a wild population of great tits *Parus major*, we tested the hypothesis that elevated quantities of maternally derived carotenoids improve nestling immunity. To disentangle the effects of maternally derived carotenoids from the effects of post-hatching environment on nestling immunity, we partially cross-fostered chicks at hatching. We predicted that nestlings hatched from eggs laid by carotenoid-fed females should show a stronger response to an immune challenge than nestlings hatched from eggs laid by control females. Additionally, in order to investigate the relationship between carotenoid availability, resistance to ectoparasites and immunocompetence, we infested half of the nests with a common nest ectoparasite, the hen flea *Ceratophyllus gallinae*, in a 2×2 factorial design. Fleas have been shown to affect nestling growth rate (Nilsson 2003), body condition (Richner, Oppliger & Christe 1993; Tschirren, Fitze & Richner 2003b) and plumage coloration in the great tit (Tschirren *et al.* 2003a). Ectoparasites can also activate a costly immune response, as shown in house martin nestlings (de Lope, Møller & de la Cruz 1998). Defence against ectoparasites may hence compete with other functions for limited resources and lead to allocation trade-offs for essential resources. We predicted that flea infestation should impair the capacity of control nestlings to respond to an immune challenge to a greater extent, than nestlings of carotenoid-supplied mothers. This should translate into exacerbated differences in immune response between control and carotenoid nestlings in parasitized nests.

Methods

FOOD SUPPLEMENTATION AND PARASITE TREATMENT

The study was carried out in 2004 in a nest-box breeding population of great tits in the Bremgartenwald, a forest near Bern, Switzerland. In the great tit, the yellow plumage coloration is carotenoid-based and hen fleas tend to reduce colour expression (Tschirren *et al.* 2003a), suggesting a trade-off in the allocation of carotenoids to different physiological functions (i.e. immune function and coloration in that study). Carotenoids thus seem to be a limited resource in this species and, it may be costly for females to deposit high quantities of carotenoids into their eggs. Great tits therefore appear to be an appropriate model to investigate the effects of carotenoid availability and maternally transmitted carotenoids on nestling immunity.

The study area contained 336 nest boxes distributed over 24 plots (14 nests per plot). In March, the nest boxes were cleaned by removing old nest material and brushing to eliminate all parasites. At the same time we suspended an inverted flowerpot near each nest box. We then randomly assigned a food and a flea treatment

to each plot, resulting in four groups of plots: flea-infested nests supplemented with either carotenoid-enriched (F+ C+) or nonenriched (F+ C–) food, and similarly parasite-free nests supplemented with either carotenoid-enriched (F– C+) or nonenriched (F– C–) food. We supplemented the birds food with standard, commercial fatballs mixed with sunflower seeds that were first crumbled, and heated to 60 °C to denaturate the carotenoids naturally present in the mixture (advised by a representative of Hoffmann-La Roche). After cooling, we added carotenoids (supplied by Hoffmann-La Roche) to half of the mixture at a concentration 30 times greater than in the natural diet and at an approximate ratio found in the natural diet of great tits, i.e. 80% of lutein, 3% of zeaxanthin, and 17% of β-carotene (Partali *et al.* 1987). In fact, we added 0·08 mg of lutein, 0·003 mg of zeaxanthin and 0·017 mg of β-carotene per gram of the food mixture. The fatballs contain mainly fat (plus a few seeds) and are especially used for winter feeding. In spring, birds eat mainly insects and seeds that contain both proteins and carotenoids. Thus, we added carotenoids to fatballs at elevated concentrations compared with natural concentrations to ensure that carotenoid-supplemented birds would ingest more carotenoids than the control birds. Finally, we moulded the mixtures into either enriched or nonenriched balls. In order to minimize the influence of our food treatment on breeding habitat choice, we started supplementation on the 20 March after their breeding territories had been established. Laying date, clutch size and brood size were not significantly different between the two groups (respectively: $P = 0{\cdot}832$; $P = 0{\cdot}434$; $P = 0{\cdot}269$), and thus supports the notion that female quality was randomized over the two treatments. While the provided food was eaten by the target pairs as confirmed by direct observation, it was partly also consumed by other bird species and the exact amount eaten by each individual could thus not be determined precisely. However, we noticed that C+ and C– fatballs were consumed at similar rates. Nest boxes were visited every fourth day to monitor the start of nest building and to replenish the food stock. We stopped supplying nests with food on the first day of incubation.

When nest cup construction was at an advanced stage, we introduced 40 fleas (*Ceratopyllus gallinae*) collected from old nest material into the nests of the plots assigned to be infested with parasites. This number of fleas was known to be sufficient for the establishment of a flea population (Heeb *et al.* 1996; Tripet & Richner 1999) and within the range of natural infestation levels. The laying date did not vary between infested and parasite-free pairs ($P = 0{\cdot}09$), suggesting that our experimental design was not confounded by the redistribution of individuals after experimental parasite infestation. After the laying of the fourth egg in a clutch, we visited the nest daily to determine the start of incubation and thus predict the hatching date. From the first expected day of hatching, nests were inspected daily to determine the hatching date.

At hatching, nestlings were weighted and partially cross-fostered to separate the effects of maternally deposited carotenoids and post-hatching environment (Fig. 1). Hatchlings were exchanged between two nests of similar clutch size, from different food treatments (C+ ↔ C–) but sharing the same flea treatment (C+ F+ ↔ C– F+ and C+ F– ↔ C– F–). Hatchlings were ranked according to their body mass. The lightest hatchling was randomly assigned either to stay in the nest of origin or to be exchanged. Cross-foster treatment was then alternated through the mass-based rank list of hatchlings. Control nestlings that were cross-fostered were raised by a female that was carotenoid-fed during egg laying, while carotenoid nestlings that were cross-fostered were reared by a control female. As there is some hatching asynchrony in the great tit, on average only two or three of the eggs of a nest had hatched at the time of cross-fostering. Thus, nestlings hatched 1 or 2 days after the first hatchlings, stayed in their nest of origin. Our design did not modify the original brood size. For identification we marked hatchlings individually by partially removing tuft feathers from their heads, backs and wings. Nestlings were banded when 9 days old with numbered aluminium rings. A total of 435 nestlings from 98 nests were identified, sexed by molecular methods (Griffiths *et al.* 1998) and measured.

IMMUNITY ASSESSMENT

We assessed cellular immune performance by an injection of phytohaemagglutinin (PHA). PHA is a

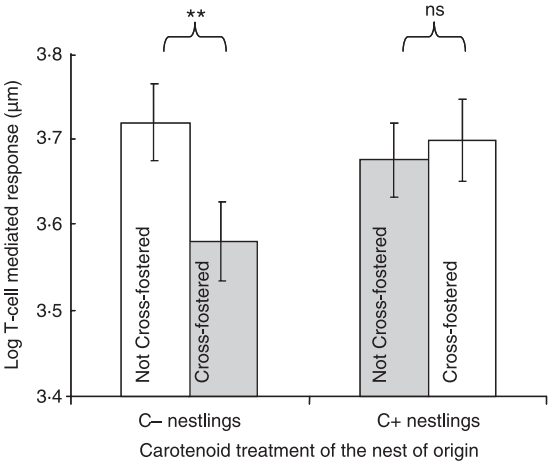

Fig. 1. Mean (± 1 SE) change in wing-web thickness 24 h after the injection of phytohaemagglutinin into the wing-web of nestlings hatched from eggs laid by carotenoid-fed mothers (C+ nestlings) or from eggs laid by control mothers (C– nestlings), and raised either by a mother that was carotenoid-fed (grey bars) during egg laying or a by control mother (white bars). In each group (C+ and C– nestlings) more than half of the nestlings were raised by their genetic mother (not cross-fostered) and less than half of the nestlings were raised by a foster mother (cross-fostered). The difference between C+ and C– nestlings was not significant.

lectin that induces a T-cell proliferation and aggregation at the site of injection. Responsiveness to an injection of PHA has been commonly used as an indicator of the immunocompetence of an individual (Wayland *et al*. 2002; Blount *et al*. 2003; El-Lethey, Huber-Eicher & Jungi 2003; Saino *et al*. 2003; Tschirren *et al*. 2003b). We injected the wing web of the nestlings 14 days after hatching with 0·01 mg of PHA (PHA-P, Sigma-Aldrich, Basel, Switzerland) dissolved in 0·02 mL of phosphate-buffered saline. We assessed the individual ability to raise a cellular immune response by measuring the wing web thickness before and 24 h after the injection to the nearest 0·01 mm with a constant-tension dial micrometer (Mitotuyo, Type 2046FB-60). A greater swelling reflects a better cellular immune response (Smits, Bortolotti & Tella 1999). We also weighted nestlings to the nearest 0·1 g in order to statistically control for the effect of body mass on the immune response.

YOLK CAROTENOIDS

We did not collect eggs in 2004 in order to keep broods at their original size. In 2005 and 2006 we repeated a similar food supplementation experiment and collected the third and the fifth (minimal clutch size) egg in a random sample of nests (2005: $n = 23$; 2006: $n = 107$). Total carotenoid concentrations were assessed using high-performance liquid chromatography following the protocol described in Surai & Speake (1998) and Karadas *et al*. (2005).

STATISTICAL PROCEDURES

We used mixed models to analyse the effects of carotenoid supplementation on the T-cell-mediated immune response in relation to the presence of fleas. The parasite and the carotenoid treatments of the nest of origin, and the carotenoid treatment of the nest of rearing were the main factors in the models. We also included the following fixed effects and covariates: nestling sex, brood size of the nest of rearing, hatching date and nestling body mass at 14 days post-hatching. To account for the hierarchical structure of our experimental design we included the following random effects: the plot nested within the parasite and carotenoid treatments, the nest of origin and the nest of rearing nested within the plot, and the interaction between the nest of origin and the nest of rearing.

The effect of our carotenoid supplementation on total yolk carotenoid concentration was assessed using a mixed model, including the nest and rank as random factors. The carotenoid treatment, the clutch size, the laying date and the egg rank were included as fixed factors and covariates in the model.

Analyses of the immune response were performed using SAS (SAS® 2003). The MIXED procedure was used to fit this model, using a compound symmetry structure for the variance–covariance matrix. Immune response was log-transformed in order to meet the assumption of normal distribution of residuals. Analyses of egg carotenoids were performed using the software R (Maindonald & Braun 2003; R Development Core Team 2006). We used restricted maximum likelihood estimation in all mixed effect models. Nonsignificant interactions were backward eliminated using a stepwise elimination procedure. Tests are two-tailed with a significance level set to $\alpha = 0{\cdot}05$.

Results

YOLK TOTAL CAROTENOIDS

Carotenoid supplementation significantly enhanced total carotenoid concentration in the egg yolk (Table 1), as shown by the additional experiments performed in 2005 and 2006 to test this assumption. Carotenoid-fed mothers deposited a higher quantity of carotenoid into the egg yolk than control mothers (2005: C+ females: $26{\cdot}16 \pm 11{\cdot}08$ μg g^{-1} of egg yolk; C– females: $16{\cdot}40 \pm 8{\cdot}7$ μg g^{-1} of egg yolk; 2006: C+ females: $25{\cdot}79 \pm 9{\cdot}91$ μg g^{-1} of egg yolk; C– females: $20{\cdot}27 \pm 9{\cdot}21$ μg g^{-1} of egg yolk). The concentrations found in the eggs collected in 2005 and 2006 were well within the range measured in other great tit populations (Partali *et al*. 1987). Thus the carotenoid dosing did not induce unusually high carotenoids concentrations in the eggs.

IMMUNE RESPONSE

The effect of maternally transmitted carotenoids on the immune response depended on whether nestlings had been cross-fostered or not, as shown by the significant interaction between the carotenoid treatments of the genetic mother and that of the rearing mother (Table 2). Control nestlings (C– nestlings) that were cross-fostered had a significantly reduced immune response compared with control nestlings that were not cross-fostered ($F_{1,155} = 8{\cdot}30$, $P = 0{\cdot}004$, cross-fostered: $38{\cdot}69 \pm 1{\cdot}74$, not cross-fostered: $43{\cdot}49 \pm 1{\cdot}714$). In contrast, cross-fostered nestlings originating from eggs laid by carotenoid-fed mothers (C+ nestlings) showed the same immune response as their nontranslocated siblings ($F_{1,168} = 0{\cdot}05$, $P = 0{\cdot}82$, exchanged: $42{\cdot}38 \pm 1{\cdot}82$, not exchanged: $42{\cdot}28 \pm 1{\cdot}64$) (Fig. 1). Thus, compared with control nestlings, carotenoid supplementation

Table 1. Summary of the linear mixed effect models for log transformed carotenoid concentration in egg yolk to test, in 2005 and 2006, the assumption that the food-supplemented carotenoids lead to higher carotenoid concentrations in the egg yolk

Year	Estimation ± 1 SE (C+)	*F*-value	*P*-value
2005	$0{\cdot}318 \pm 0{\cdot}087$	$13{\cdot}17_{1,13}$	0·0031
2006	$0{\cdot}119 \pm 0{\cdot}044$	$7{\cdot}08_{1,60}$	0·0100
2005–06 jointly	$0{\cdot}162 \pm 0{\cdot}042$	$14{\cdot}92_{1,15}$	0·0002

Table 2. Summary of the linear mixed-model for the log transformed T-cell immune response. The study plot was defined as a random effect and nested within the parasite and carotenoid treatments. Nest of origin (NoO) and nest of rearing (NoR) were also included as random factors and nested within the study plot. The model also included interaction between the nest of rearing and the nest of origin. *F*- and *P*-values associated with non-significant terms given here arise from the full model before their backward elimination

Parameter	Estimation ± 1 SE	*F*-value	*P*-value
Carotenoid treatment (C+)		$0{\cdot}18_{1,75{\cdot}8}$	0·6752
Flea treatment (F+)	–1·6424 ± 0·363	$20{\cdot}53_{1,348}$	< 0·0001
Hatching date		$0{\cdot}00_{1,99}$	0·9992
Cross-fostering		$1{\cdot}33_{1,73{\cdot}7}$	0·2530
Body mass 14 days old	0·012 ± 0·015	$24{\cdot}74_{1,359}$	< 0·0001
Brood size	–0·038 ± 0·017	$4{\cdot}73_{1,118}$	0·0316
Sex		$0{\cdot}37_{1,360}$	0·5448
Body mass 14 days old × flea treatment	0·1535 ± 0·069	$16{\cdot}87_{1,370}$	< 0·0001
Carotenoid treatment × cross-fostering	0·092 ± 0·022	$4{\cdot}95_{1,263}$	0·0270
Carotenoid treatment × flea treatment		$0{\cdot}04_{1,71{\cdot}3}$	0·8366

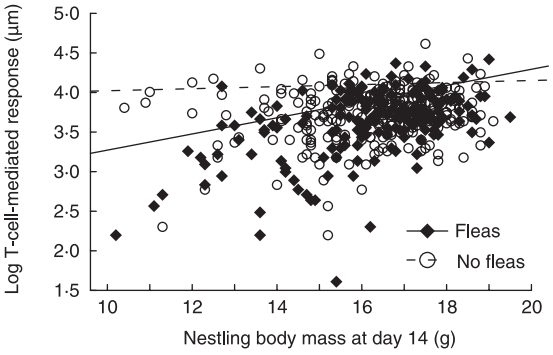

Fig. 2. Relationship between the log-transformed T-cell-mediated immune response and body mass of nestlings at the age of 14 days for ectoparasite-infested (filled squares, slope = 0·10217) and noninfested nests (open circles, slope = 0·01295).

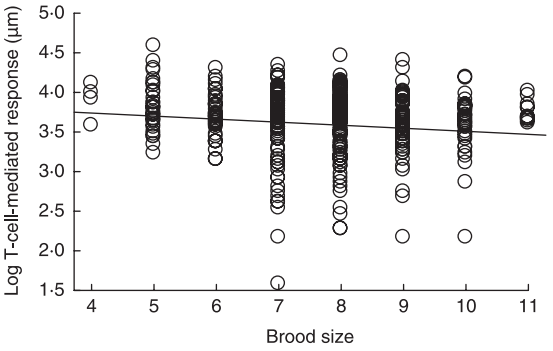

Fig. 3. Relationship between brood size and the log-transformed T-cell-mediated immune response (slope = –0·03837).

prevented a reduction in immune response in cross-fostered nestlings. We also found that flea infestation reduced the immune response of the lightest nestlings more strongly than the immune response of heavy nestlings, as shown by a significant interaction of flea treatment and body mass 14 days after hatching (Fig. 2). However, the interaction between the flea treatment and the carotenoid treatment of the nest of origin was not significant (Table 2): the difference in immune response between C+ nestlings and C– nestlings raised in parasitized nests was less than the difference between C+ nestlings and C– nestlings raised in parasite-free nests. The immune response significantly decreased with increasing brood size of the rearing nest (Table 2) (Fig. 3). Male and female nestlings did not differ in their capacity to respond to the immune challenge (Table 2).

Discussion

The two main findings from this investigation are that carotenoid supplementation compensated for the immunosuppressive consequence of nestling translocation, and that flea infestation impaired the immune response of the smaller nestlings. These results suggest that under harsh conditions such as parasite infestation, sibling competition and/or stress arising from nest translocation, the development of the nestlings' cellular immune system is influenced in different ways. On the one hand, when parasite exposure is high, body mass and supposedly overall nutritional status appear to influence a nestling's ability to raise a cellular immune response. However, following transient stress (i.e. cross-fostering) early in life or when nestling competition is high, the amount of carotenoids deposited into the eggs seem to be more important for the development of competent cellular immunity in nestlings than their overall nutritional status.

C– nestlings suffered from translocation between nests, as reflected by their reduced cellular immune response: C– nestlings that were cross-fostered (i.e. raised by a carotenoid-fed female) had a lower cellular immune response than C– nestlings that were not translocated. Contrary to C– nestlings, translocation had no effect on the immune response of C+ nestlings. Thus, two main scenarios could explain this result, the stress induced by translocation and/or competitive differences between nestlings of the two groups.

First, C+ hatchlings may have been less affected by the translocation than C– hatchlings. During and after translocation, hatchlings are likely to be subjected to

stress. Translocated hatchlings may have to cope with temporary starvation that could induce the use of larger amounts of yolk sac nutrients including carotenoids. In addition, their body temperature may be temporarily lowered and induce a number of physiological responses. Finally, translocated nestlings are placed in a new environment (i.e. new siblings, new parents, new pathogens and antigenic environments). Such physiological stresses may cause stress-related 'disorders' such as increased metabolic rate (i.e. oxidative stress), and stimulation of the hypothalamic–pituitary–adrenal axis (HPA) (Romero 2004). The activation of the HPA leads to an increased release of major stress hormones (i.e. hormonal stress response) (Kitaysky *et al.* 2001; Romero 2004) that can initiate an acute phase response (APS) resulting in an inflammatory response (Romero 2004). The additional carotenoids may thus have helped hatchlings to cope with the stress generated by the translocation (e.g. starvation, thermic shock, new antigenic environment) by: (1) protecting them against damage caused by reactive oxygen species (Alonso-Alvarez *et al.* 2004) produced at elevated rates under high metabolic rates and during the inflammatory response; and/or (2) decreasing the inflammatory response and hence the production of reactive oxygen species (Koutsos, Lopez & Klasing 2006); and/or (3) compensating for the immunosuppressive effects of stress hormones such as glucocorticoids (Romero 2004) produced at high levels under stress.

Second, C+ nestlings may be more competitive than C– nestlings, a scenario that is supported by the finding that C+ nestlings show more intensive begging behaviour than C– nestlings (Helfenstein *et al.* unpublished). C– nestlings raised by carotenoid-fed females in nests where more than half of the nestlings are C+ nestlings, could then have suffered disproportionally from competition simply because of their lower number. In contrast, C– nestlings raised in their own nest, where more than half of the nestlings were C– nestlings, may not have suffered from the presence of potentially more competitive C+ nestlings. Cross-fostered C– nestlings may thus have received less food than other nestlings (i.e. C+ nestlings and non-cross-fostered C– nestlings), and thereby invest less energy reserves in the building up of the immune system. Both explanations are not mutually exclusive: the result can be a consequence of a reduced competitiveness of translocated C– nestlings following the impairment of nestling condition due to the stress induced by the cross-fostering. It could also be an additive effect of the beneficial influence of the carotenoids on both the susceptibility to the translocation and the nestling competitiveness. Elevated levels of carotenoids in the eggs could have directly aided hatchlings to cope with the stress imposed by cross-fostering, as hatchlings could have benefited from these additional carotenoids via the yolk sac reserves. However, the beneficial effects of additional carotenoids from the yolk sac on stress resistance and also on competitiveness, may be mediated via their potentially stimulating effects on embryonic growth and development, resulting for example in different energetic metabolisms and/or in an enhanced ability of nestlings to assimilate carotenoids (Koutsos *et al.* 2003). In summary, our new finding suggests that the beneficial effects of maternally transmitted carotenoids on cellular immunity may be mediated via their positive direct or indirect influence on physiological and behavioural processes involved in stress resistance and competitiveness. In addition, a differential transfer of others substances than carotenoids in the egg yolk may also enhance competitiveness and stress resistance of C+ nestlings. Carotenoid intake of supplemented birds might have influenced the mothers' condition and thereby the size or the composition of their eggs, as for example the content of vitamins, antibodies or hormones (Royle *et al.* 1999; Grindstaff, Brodie & Ketterson 2003; Saino *et al.* 2004; Tschirren *et al.* 2004). We found, however, that carotenoid-fed mothers transferred higher quantities of carotenoids into their eggs, and thus the patterns observed may at least be due partially to a higher carotenoid transfer into the eggs. Further experiments would be necessary to elucidate the mechanisms proposed above. Carotenoids should be injected directly into the eggs and immunocompetence of nestlings raised under different conditions of stress and competition measured.

In infested nests, only heavy nestlings were able to raise a strong response to the PHA challenge. This result supports the idea that producing an immune defence against parasites is costly (Gonzalez *et al.* 1999), and that energy requirements increase under parasite pressure, and may lead to different allocation strategies (Lochmiller & Deerenberg 2000). In infested nests energy requirements are larger because nestlings have to cope with the parasites' deleterious effects (Simon *et al.* 2004) resulting in increased competition between nestlings. In such a situation, light nestlings raised in infested nests may receive less food compared with light nestlings raised in noninfested nests. Under such resource limitations, one might expect that light nestlings may have to allocate proportionally more energy to growth in order to exceed the critical body mass at fledging that will maximize their survival after leaving the nest (Naef-Daenzer, Widmer & Nuber 2001). Consequently, light nestlings, may be unable to allocate as much energy to immune function as their heavier siblings. Two other studies conducted on great tits (Brinkhof *et al.* 1999; Tschirren *et al.* 2003b) and one on house sparrows (Westneat *et al.* 2004) did not show a relationship between flea infestation and cellular immunity, suggesting that the effect of flea infestation may also depend on a combination of various environmental conditions at breeding.

We did not find an exacerbated effect of carotenoid treatment on immunity in the infested group (i.e. no interaction between carotenoid treatment and flea treatment). It is well known that in birds, mothers can

modulate the composition of their eggs depending on the presence or absence of ectoparasites in order to help nestlings fight against deleterious effects of parasites in environments where ectoparasite prevalence is high (Heeb *et al*. 1998; Buechler *et al*. 2002; Gasparini *et al*. 2002; Tschirren *et al*. 2004). Mothers can for example protect their nestlings by transferring elevated levels of antibodies to the eggs as a form of passive immunization of nestlings (Heeb *et al*. 1998; Buechler *et al*. 2002; Gasparini *et al*. 2002). Moreover, it has been found in the lesser black-backed gull, that carotenoid-fed females deposit high levels of carotenoids into the eggs, but low levels of immunoglobulin, whereas control females do the opposite (Blount *et al*. 2002b). Thus, in our experiment, flea-exposed control females may have deposited high levels of immunoglobulin into their eggs compared with flea exposed carotenoid-fed females. This would help C– nestlings fight parasites and thereby compensate for lower levels of maternally transmitted carotenoids. It may then be interesting to infest nests after laying to detect the expected positive effects of maternally transmitted carotenoids in their defence against fleas.

In summary, we show here that maternally transmitted carotenoids aid nestlings to cope with the deleterious effects of stress and/or increase their competitiveness, resulting in a competent cellular immune response (Alonso-Alvarez *et al*. 2004). We also show that under parasite exposure, body mass is of prime importance for the development and activation of active cellular immunity. It suggests that in stressful environments (e.g. resource limitation, intense competition, high parasitism) females may transfer higher quantities of carotenoids into the eggs to enhance competitiveness and stress resistance of offspring. Such an allocation strategy is expected to be more pronounced in species without parental care and protection after birth. Future experiments should: (1) investigate whether females modulate the transfer of antioxidants into their eggs in relation to environmental stressors, and (2) focus on the mechanisms that underlay the positive effect of carotenoids on stress resistance (Koutsos *et al*. 2006) and nestling competitiveness.

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References

Alonso-Alvarez, C., Bertrand, S., Devevey, G., Gaillard, M., Prost, J., Faivre, B. & Sorci, G. (2004) An experimental test of the dose-dependent effect of carotenoids and immune activation on sexual signals and antioxidant activity. *American Naturalist* **164**, 651–659.

Andersson, S., Uller, T., Lohmus, M. & Sundstrom, F. (2004) Effects of egg yolk testosterone on growth and immunity in a precocial bird. *Journal of Evolutionary Biology* **17**, 501–505.

Anisman, H., Zaharia, M.D., Meaney, M.J. & Merali, Z. (1998) Do early-life events permanently alter behavioral and hormonal responses to stressors? *International Journal of Developmental Neuroscience* **16**, 149–164.

Biard, C., Surai, P.F. & Møller, A.P. (2005) Effects of carotenoid availability during laying on reproduction in the blue tit. *Oecologia* **144**, 32–44.

Blount, J.D., Surai, P.F., Houston, D.C. & Møller, A.P. (2002a) Patterns of yolk enrichment with dietary carotenoids in gulls: the roles of pigment acquisition and utilization. *Functional Ecology* **16**, 445–453.

Blount, J.D. Surai, P.F. Nager, R.G. Houston, D.C. Møller, A.P. Trewby, M.L. & Kennedy, M.W. (2002b) Carotenoids and egg quality in the lesser black-backed gull *Larus fuscus*: a supplemental feeding study of maternal effects. *Proceedings of the Royal Society of London, B* **269**, 29–36.

Blount, J.D., Metcalfe, N.B., Birkhead, T.R. & Surai, P.F. (2003) Carotenoid modulation of immune function and sexual attractiveness in zebra finches. *Science* **300**, 125–127.

Bortolotti, G.R., Negro, J.J., Surai, P.F. & Prieto, P. (2003) Carotenoids in eggs and plasma of red-legged partridges: effects of diet and reproductive output. *Physiological and Biochemical Zoology* **76**, 367–374.

Brinkhof, M.W.G. Heeb, P. Kölliker, M. & Richner, H. (1999) Immunocompetence of nestling great tits in relation to rearing environment and parentage. *Proceedings of the Royal Society of London, B* **266**, 2315–2322.

Buechler, K., Fitze, P.S., Gottstein, B., Jacot, A. & Richner, H. (2002) Parasite-induced maternal response in a natural bird population. *Journal of Animal Ecology* **71**, 247–252.

Desai, M., Crowther, N.J., Ozanne, S.E., Lucas, A. & Hales, C.N. (1995) Adult glucose and lipid-metabolism may be programmed during fetal life. *Biochemical Society Transactions* **23**, 331–335.

El-Lethey, H., Huber-Eicher, B. & Jungi, T.W. (2003) Exploration of stress-induced immunosuppression in chickens reveals both stress-resistant and stress-susceptible antigen responses. *Veterinary Immunology and Immunopathology* **95**, 91–101.

Gasparini, J., McCoy, K.D., Tveraa, T. & Boulinier, T. (2002) Related concentrations of specific immunoglobulins against the Lyme disease agent *Borrelia burgdorferi sensu lato* in eggs, young and adults of the kittiwake (*Rissa tridactyla*). *Ecology Letters* **5**, 519–524.

Gonzalez, G., Sorci, G., Møller, A.P., Ninni, P., Haussy, C. & De Lope, F. (1999) Immunocompetence and condition-dependent sexual advertisement in male house sparrows (*Passer domesticus*). *Journal of Animal Ecology* **68**, 1225–1234.

Griffiths, R., Double, M.C., Orr, K. & Dawson, R.J.G. (1998) A DNA test to sex most birds. *Molecular Ecology* **7**, 1071–1075.

Grindstaff, J.L. Brodie, E.D. & Ketterson, E.D. (2003) Immune function across generations: integrating mechanism

and evolutionary process in maternal antibody transmission. *Proceedings of the Royal Society of London, B* **270**, 2309–2319.

Heeb, P., Werner, I., Richner, H. & Kölliker, M. (1996) Horizontal transmission and reproductive rates of hen fleas in great tit nests. *Journal of Animal Ecology* **65**, 474–484.

Heeb, P., Werner, I., Kolliker, M. & Richner, H. (1998) Benefits of induced host responses against an ectoparasite. *Proceedings of The Royal Society of London B* **265**, 51–56.

Karadas, F., Pappas, A.C., Surai, P.F. & Speake, B.K. (2005) Embryonic development within carotenoid-enriched eggs influences the post-hatch carotenoid status of the chicken. *Comparative Biochemistry and Physiology B* **141**, 244–251.

Kitaysky, A.S., Kitaiskaia, E.V., Wingfield, J.C. & Piatt, J.F. (2001) Dietary restriction causes chronic elevation of corticosterone and enhances stress response in red-legged kittiwake chicks. *Journal of Comparative Physiology B* **171**, 701–709.

Koutsos, E.A., Clifford, A.J., Calvert, C.C. & Klasing, K.C. (2003) Maternal carotenoid status modifies the incorporation of dietary carotenoids into immune tissues of growing chickens (*Gallus gallus domesticus*). *Journal of Nutrition* **133**, 1132–1138.

Koutsos, E.A., Lopez, J.C.G. & Klasing, K.C. (2006) Carotenoids from in ovo or dietary sources blunt systemic indices of the inflammatory response in growing chicks (*Gallus gallus domesticus*). *Journal of Nutrition* **136**, 1027–1031.

Lochmiller, R.L. & Deerenberg, C. (2000) Trade-offs in evolutionary immunology: just what is the cost of immunity? *Oikos* **88**, 87–98.

de Lope, F., Møller, A.P. & de la Cruz, C. (1998) Parasitism, immune response and reproductive success in the house martin *Delichon urbica*. *Oecologia* **114**, 188–193.

Love, O.P., Chin, E.H., Wynne-Edwards, K.E. & Williams, T.D. (2005) Stress hormones: a link between maternal condition and sex-biased reproductive investment. *American Naturalist*, **166**, 751–766.

Maindonald, J. & Braun, J. (2003) *Data Analysis and Graphics Using R – An Example-based Approach*. Cambridge University Press.

McGraw, K.J. & Ardia, D.R. (2004) Immunoregulatory activity of different dietary carotenoids in male zebra finches. *Chemoecology* **14**, 25–29.

McGraw, K.J., Adkins-Regan, E. & Parker, R.S. (2005) Maternally derived carotenoid pigments affect offspring survival, sex ratio, and sexual attractiveness in a colorful songbird. *Naturwissenschaften* **92**, 375–380.

Møller, A.P., Biard, C., Blount, J.D., Houston, D.C., Ninni, P., Saino, N. & Surai, P.F. (2000) Carotenoid-dependent signals: indicators of foraging efficiency, immunocompetence or detoxification ability? *Avian and Poultry Biology Reviews* **11**, 137–159.

Naef-Daenzer, B., Widmer, F. & Nuber, M. (2001) Differential post-fledging survival of great and coal tits in relation to their condition and fledging date. *Journal of Animal Ecology* **70**, 730–738.

Nilsson, J.A. (2003) Ectoparasitism in marsh tits: costs and functional explanations. *Behavioral Ecology* **14**, 175–181.

Olson, V.A. & Owens, I.P.F. (1998) Costly sexual signals: are carotenoids rare, risky or required? *Trends in Ecology and Evolution* **13**, 510–514.

Partali, V., Liaaenjensen, S., Slagsvold, T. & Lifjeld, J.T. (1987) Carotenoids in food-chain studies. 2. The food-chain of *Parus* spp. monitored by carotenoid analysis. *Comparative Biochemistry and Physiology B* **87**, 885–888.

Pilz, K.M., Smith, H.G., Sandell, M.I. & Schwabl, H. (2003) Interfemale variation in egg yolk androgen allocation in the European starling: do high-quality females invest more? *Animal Behaviour* **65**, 841–850.

R Development Core Team (2006) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

Richner, H., Oppliger, A. & Christe, P. (1993) Effect of an ectoparasite on reproduction in great tits. *Journal of Animal Ecology* **62**, 703–710.

Romero, L.M. (2004) Physiological stress in ecology: lessons from biomedical research. *Trends in Ecology and Evolution* **19**, 249–255.

Royle, N.J., Surai, P.F., McCartney, R.J. & Speake, B.K. (1999) Parental investment and egg yolk lipid composition in gulls. *Functional Ecology* **13**, 298–306.

Rutkowska, J., Cichon, M., Puerta, M. & Gil, D. (2005) Negative effects of elevated testosterone on female fecundity in zebra finches. *Hormones and Behavior* **47**, 585–591.

Saino, N., Ambrosini, R., Martinelli, R. & Møller, A.P. (2002a) Mate fidelity, senescence in breeding performance and reproductive trade-offs in the barn swallow. *Journal of Animal Ecology* **71**, 309–319.

Saino, N. Bertacche, V. Ferrari, R.P. Martinelli, R. Møller, A.P. & Stradi, R. (2002b) Carotenoid concentration in barn swallow eggs is influenced by laying order, maternal infection and paternal ornamentation. *Proceedings of the Royal Society of London, Series B* **269**, 1729–1733.

Saino, N. Ferrari, R. Romano, M. Martinelli, R. & Møller, A.P. (2003) Experimental manipulation of egg carotenoids affects immunity of barn swallow nestlings. *Proceedings of the Royal Society of London, B* **270**, 2485–2489.

Saino, N., Romano, M., Ambrosini, R., Ferrari, R.P. & Møller, A.P. (2004) Timing of reproduction and egg quality covary with temperature in the insectivorous Barn Swallow, *Hirundo rustica*. *Functional Ecology* **18**, 50–57.

SAS® (2003) *SAS User's Guide*. SAS Institute Inc., Cary, NC, USA.

Schwabl, H. (1996) Maternal testosterone in the avian egg enhances postnatal growth. *Comparative Biochemistry and Physiology A* **114**, 271–276.

Simon, A., Thomas, D., Blondel, J., Perret, P. & Lambrechts, M.M. (2004) Physiological ecology of Mediterranean blue tits (*Parus caeruleus* L.): effects of ectoparasites (*Protocalliphora* spp.) and food abundance on metabolic capacity of nestlings. *Physiological and Biochemical Zoology* **77**, 492–501.

Smits, J.E., Bortolotti, G.R. & Tella, J.L. (1999) Simplifying the phytohaemagglutinin skin-testing technique in studies of avian immunocompetence. *Functional Ecology* **13**, 567–572.

Surai, P.F. & Speake, B.K. (1998) Distribution of carotenoids from the yolk to the tissues of the chick embryo. *Journal of Nutritional Biochemistry* **9**, 645–651.

Tripet, F. & Richner, H. (1999) Dynamics of hen flea *Ceratophyllus gallinae* subpopulations in blue tit nests. *Journal of Insect Behavior* **12**, 159–174.

Tschirren, B., Fitze, P.S. & Richner, H. (2003a) Proximate mechanisms of variation in the carotenoid-based plumage coloration of nestling great tits (*Parus major* L.). *Journal of Evolutionary Biology* **16**, 91–100.

Tschirren, B., Fitze, P.S. & Richner, H. (2003b) Sexual dimorphism in susceptibility to parasites and cell-mediated immunity in great tit nestlings. *Journal of Animal Ecology* **72**, 839–845.

Tschirren, B. Richner, H. & Schwabl, H. (2004) Ectoparasite-modulated deposition of maternal androgens in great tit eggs. *Proceedings of the Royal Society of London, B* **271**, 1371–1375.

Verboven, N., Evans, N.P., D'Alba, L., Nager, R.G., Blount, J.D., Surai, P.F. & Monaghan, P. (2005) Intra-specific interactions influence egg composition in the lesser black-backed gull (*Larus fuscus*). *Behavioral Ecology and Sociobiology* **57**, 357–365.

Wayland, M., Gilchrist, H.G., Marchant, T., Keating, J. & Smits, J.E. (2002) Immune function, stress response, and body condition in arctic-breeding common eiders in relation to cadmium, mercury, and selenium concentrations. *Environmental Research* **90**, 47–60.

Westneat, D.F., Weiskittle, J., Edenfield, R., Kinnard, T.B. & Poston, J.P. (2004) Correlates of cell-mediated immunity in nestling house sparrows. *Oecologia* **141**, 17–23.

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