

# Experimental manipulation of egg carotenoids affects immunity of barn swallow nestlings

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The yolk of bird eggs contains maternal carotenoids that may act as antioxidants thus influencing offspring performance and survival. However, to our knowledge, this hypothesis has not been subjected to experimental tests and the function of transmission of carotenoids to the egg is largely unknown. We directly manipulated the concentration of the main carotenoid (lutein) in the eggs of barn swallows (*Hirundo rustica*) and analysed the effect of experimental manipulation on growth of nestlings and two fundamental components of their acquired immunity. Nestlings hatched from lutein-inoculated eggs had larger T-cell-mediated immune response compared with those of two control groups. T-cell-mediated immune response predicted nestling survival until fledging. However, lutein inoculation did not affect antibody response to an immunogen, body mass, tarsus length or plumage development. Nestling body mass and plumage development declined with egg laying order, but the effects of lutein inoculation were independent of egg laying order for all traits. Our results show that maternal yolk carotenoids can have a major effect in promoting a fundamental component of immunity that predicts offspring survival and suggests that adaptive early maternal effects can be mediated by transmission of antioxidants to eggs.

**Keywords:** carotenoids; early maternal effects; egg; growth; *Hirundo rustica*; immunity

## 1. INTRODUCTION

The quality of eggs has been thought to affect embryonic development, subsequent offspring performance and ultimately fitness (Mousseau & Fox 1998). However, support for this hypothesis is based on correlations that cannot discriminate the effects of egg quality from those of parental phenotype. Experimental manipulations have shown that the size of offspring is influenced by manipulation of egg size (Sinervo 1990) but these experiments do not identify the factors limiting offspring performance.

Metabolic processes and functioning of the immune system of vertebrates normally result in production of compounds with high oxidizing potential that can damage biological molecules (Halliwell & Gutteridge 1999). Because oxidative stress is a ubiquitous phenomenon, antioxidant defences are likely to be crucial for development and performance of vertebrates (Møller *et al.* 2000). Carotenoids are a large family of organic compounds that are acquired by animals from food (Goodwin 1984) and act as scavengers of reactive oxygen species thus conferring protection from oxidative damage (Edge *et al.* 1997; Surai *et al.* 2001). Partly as a result of their antioxidant action, carotenoids exert a role in regulation and stimulation of immune function (Chew 1996; Møller *et al.* 2000). In addition, carotenoids regulate embryonic development by regulating gene expression, and the synthesis and activity of growth factors and hormones (Hofmann & Eichele 1994).

The yolk of bird eggs contains carotenoids in larger concentrations than maternal plasma (Surai *et al.* 2001; Saino *et al.* 2002a) and females undergo a decline in concentration of circulating carotenoids during egg laying (A. P. Møller, unpublished data), suggesting that carotenoids are actively concentrated before being transferred to eggs, and that limited amounts of dietary carotenoids are available to laying females. Thus mothers may influence their offspring phenotype by allocating carotenoids differentially to the eggs. However, there is almost no information about the effects of egg yolk carotenoids on performance of offspring, and we are aware of no studies where the effect of egg carotenoids on growth and immunity has been investigated by directly manipulating yolk carotenoids. Under the assumption that carotenoid supply is limited for laying females, experimentally increasing the concentration of carotenoids in eggs should result in better performance of offspring soon after hatching.

In the present study we tested this prediction by injecting lutein, which is the main carotenoid in barn swallow eggs (Saino *et al.* 2002a), into the yolk, thereby increasing its concentration within the natural range of variation. Morphology of the nestlings hatched from eggs inoculated with lutein was compared with that of two control groups of siblings sharing the same environment during the incubation and nestling stages. In addition, we analysed the effect of lutein inoculation on two components of acquired immunity, i.e. T-cell-mediated immunity by the phytohaemagglutinin (PHA) test (see Tella *et al.* 2002), and humoral immune response by measuring antibody production in response to vaccination against the Newcastle disease virus (Alexander 1997).

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## 2. METHODS

The experiment was done in Spring 2002 at five colonies east of Milan (northern Italy). Eggs were marked according to laying order and assigned to one of three experimental groups. The yolk of the eggs of the first group was injected with 7.5 µg lutein dissolved in 5 µl corn oil. This amount of lutein corresponds to *ca.* 1 s.d. of the mean amount of lutein contained in a sample of eggs from the same population (mean ± s.d. = 17.4 µg ± 7.36) (see also Saino *et al.* 2002a). The eggs of the second group were injected with the same amount of corn oil. The eggs of the third group were just removed from the nest and returned. Injection was done in the field using 25 µl Hamilton syringes with 26 g needles. The egg was placed above a  $1 \times 10^6$  candle light source in a dark place. This allowed us to identify the position of the yolk inside the egg. Injection was made from the acute pole after disinfecting the eggshell. The hole was sealed with a superoxide glue with a small piece of swallow eggshell superimposed. To check whether the injection actually occurred in the yolk, we injected five eggs with a green food dye and dissected them after they had been deep frozen following injection. In all cases the green dye was in the yolk. Eggs were injected within the day following that of laying. In the barn swallow, incubation starts with laying of the penultimate egg (Møller 1994). Thus, all eggs were injected when they were unincubated or in a very early stage of embryonic development. We attempted to establish all experimental groups in all clutches while randomizing treatment with respect to laying order. We *a priori* assumed that all clutches would have five eggs, the modal clutch size in our study population. We then assigned the first (putative) five eggs to different treatments according to predetermined schemes where all experimental groups were represented in a proportion of 2 : 2 : 1 and treatments were assigned randomly according to laying order. Schemes were assigned randomly to clutches. If the final clutch size was smaller than five, at least one experimental group could be missing. However, we retained the clutch in the analyses if at least two experimental groups were represented. In cases of clutches of six, the last egg was randomly assigned to one of the three groups. Around the estimated hatching date we made frequent visits to the nests to assign each nestling to its original egg. Nestlings that could not be assigned were neglected in subsequent analyses.

Nestlings were weighed at days 4, 7, and 12 after hatching and tarsometatarsus (tarsus) length was measured at days 7 and 12. Length of the left innermost tail feather (rectrix) was measured at day 12. On day 4 after hatching, all nestlings were injected subcutaneously with 10 µl Newcastle disease virus (NDV) vaccine (Nobivac Paramyxo, Intervet) and a blood sample was taken 13 or 14 days post-vaccination to measure the humoral response to the vaccine (see also Saino *et al.* 2002b). Anti-NDV antibody concentration was measured by monoclonal antibody-blocking enzyme-linked immunosorbent assay using commercial kits (Svanovir NDV-Ab, SVANOVA Biotech, Uppsala, Sweden) (Czifra *et al.* 1996). Optical density (OD) values of test plasma were compared with OD values of a pool of plasma collected from 18 day-old unvaccinated nestlings from other broods. ODs were used to calculate percentage inhibition (PI) ( $PI = (OD_{(negative\ control)} - OD_{(sample)}) / OD_{(negative\ control)}$ ) values. Large PI values indicate large NDV-specific antibody concentration in the plasma. Vaccination elicited an antibody response because PI values were larger than 0 (mean PI = 16.49 (0.44 s.e.);  $t_{226} = 37.31$ ,  $p < 0.001$ ).

On day 12 we started a cutaneous test to measure T-cell-mediated immune response by injecting 0.2 mg of a lectin (PHA) dissolved in 0.04 ml phosphate buffered saline (PBS) in the right wing web. The left wing web was injected with 0.04 ml PBS to serve as a control, after the thickness of the wing web at the inoculation sites had been measured using a pressure sensitive micrometer (Alpa S.p.A., Milano). The change in thickness of the right wing web measured 24 h after PHA injection minus the change in thickness of the control wing web is an index of T-cell-mediated immune response (Lochmiller *et al.* 1993; Saino *et al.* 1997; Tella *et al.* 2002). Repeatability of wing web thickness measures was as high as 0.93, similar to previous studies (see Saino *et al.* 1997).

We manipulated the concentration of carotenoids in the eggs of 94 clutches. Owing to hatching failures, nestling predation and nest destruction, and our failure to assign hatchlings to their egg, a total of 316 of 470 nestlings could be included in the analyses.

As a part of a long-term study of interannual variation in barn swallow nestling phenotype in the same study area we measured immune response to PHA injection at age 12 days in an additional set of nests with unmanipulated eggs, according to the same protocol described above (see also Saino *et al.* 1997). We used data on nestling mortality from these nests to increase the sample size in a comparison of PHA response of nestlings that died before fledging and the mean immune response of their siblings that fledged successfully.

### (a) *Statistical analyses*

We used generalized linear models (GLM) to analyse the effect of lutein inoculation on phenotype of individual nestlings at different ages with brood (random effect) and experimental treatment of their egg (fixed effect) as factors, and laying order as a continuous covariate. This analysis allows us to test the effect of treatment while controlling for any egg laying order effect and, reciprocally, the effect of egg laying order while partitioning out the variance in nestling phenotypic data due to the effect of treatment. Appropriate *F*-values and degrees of freedom were computed according to Zar (1999). Analyses were run using the SPSS.10 package.

We adjusted the significance level for simultaneous tests on five phenotypic variables according to sequential Bonferroni procedure. We used Bonferroni tests to analyse the pairwise differences between experimental groups in GLM analyses. We corrected the significance level of pairwise comparisons between within-brood mean values of the experimental groups for three comparisons. In practice, this resulted in tests of between-experimental group differences where the significance level was set at  $p = 0.05 / (\text{five variables} \times \text{three comparisons}) = 0.0033$ . Probability values that were significant after Bonferroni correction are marked with an asterisk.

## 3. RESULTS

Our results are based on 316 nestlings from 78 broods (see figure 1 for sample sizes). The proportion of hatching failures due to injection could not be measured precisely because we could not assign some nestlings to eggs and in some cases unhatched eggs were ejected before they could be identified. However, we estimate that *ca.* 20–22% of the injected eggs failed to hatch. Because the overall proportion of eggs failing to hatch has been repeatedly estimated to be *ca.* 10% of the eggs laid, we infer that

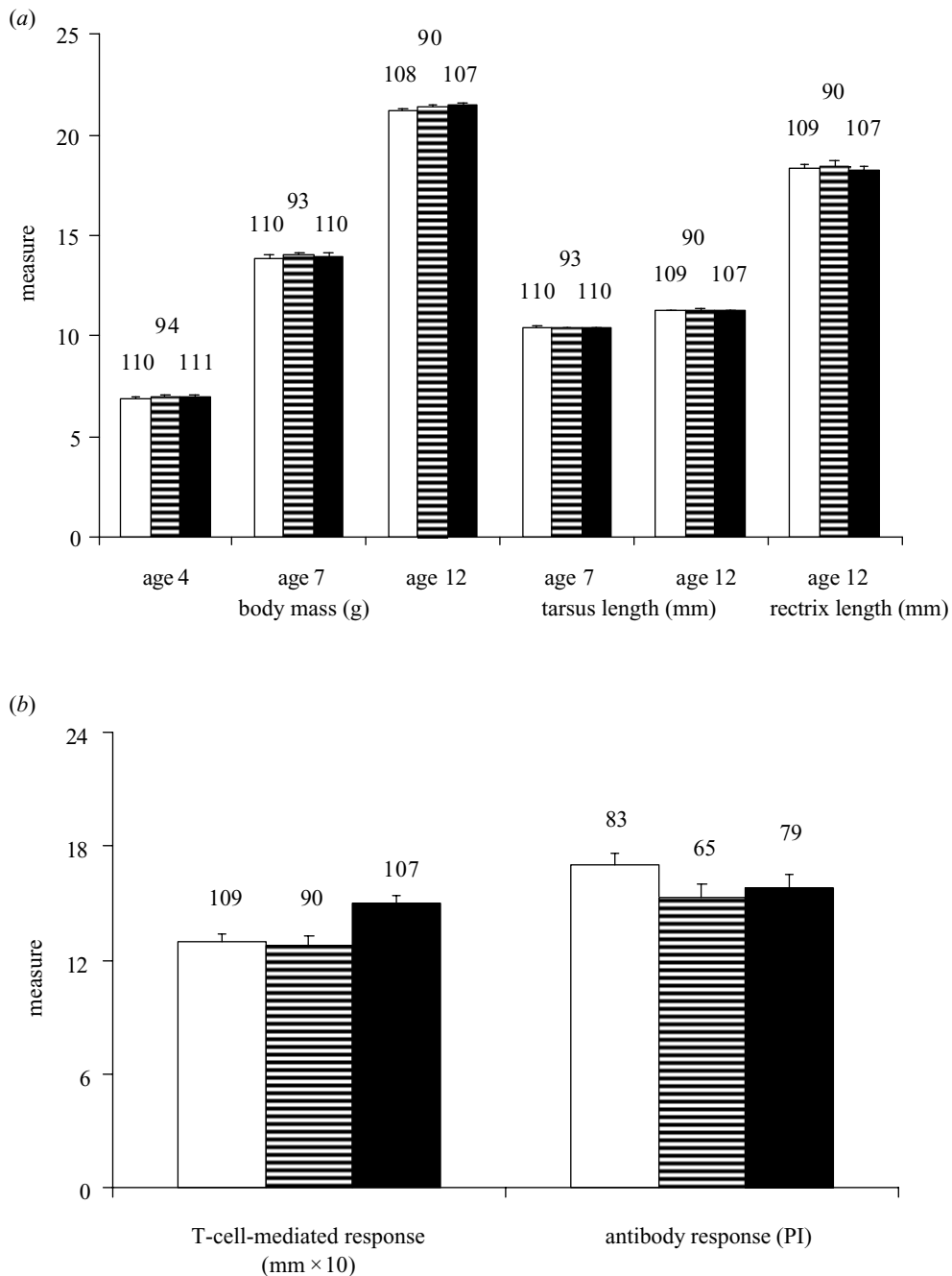


Figure 1. (a) Mean (+ s.e.m.) body mass, tarsus length, and rectrix length; and (b) index of T-cell-mediated immune response and humoral response (percentage inhibition; see § 2) of nestlings originating from eggs that were just handled (unmanipulated, white bars), injected with the solvent of lutein (sham-inoculated, horizontal striped bars), or injected with lutein (lutein-inoculated, black bars). Numbers in the figure are sample sizes. Anti-NDV vaccine antibody response was measured in a subset of nestlings.

10–12% of the eggs failed to hatch because of injection. In a logistic regression analysis of injected eggs whose hatching success could be determined where we included clutch as a categorical predictor, chances of hatching were not affected by egg treatment (Wald statistic = 0.01, d.f. = 1,  $p > 0.90$ ). Mean laying order of the eggs of the nestlings considered did not differ among the experimental groups (laying order of unmanipulated eggs: 3.06 (0.14 s.e.m.); sham-inoculated: 2.91 (0.15 s.e.m.); lutein-inoculated: 2.96 (0.14 s.e.m.);  $F_{2,313} = 0.32$ ,  $p = 0.73$ ; see figure 1 for sample sizes).

Phenotypic nestling values at different ages in relation to egg manipulation and egg laying order were subjected

to analyses of variance where we also included the effect of brood (see § 2a). The effect of the interaction term between egg treatment and laying order did not achieve statistical significance in any analysis and was therefore excluded from the models. Body mass was not affected by egg treatment (age 4 days:  $F_{2,234} = 0.35$ ,  $p = 0.70$ ; age 7 days:  $F_{2,232} = 0.25$ ,  $p = 0.78$ ; age 12 days:  $F_{2,224} = 1.61$ ,  $p = 0.20$ ; see figure 1), whereas a significant decline of body mass with laying order existed at all ages (age 4 days:  $F_{1,234} = 208.26$ ,  $p < 0.001^*$ , slope =  $-5.21$  (0.36 s.e.m.); age 7 days:  $F_{1,232} = 169.98$ ,  $p < 0.001^*$ , slope =  $-7.21$  (0.55 s.e.m.); age 12 days:  $F_{1,224} = 24.53$ ,  $p < 0.001^*$ , slope =  $-2.31$  (0.47 s.e.m.)). Tarsus length at

7 days did not vary among treatments (age 7 days:  $F_{2,232} = 0.17$ ,  $p = 0.84$ ; figure 1), but significantly declined with laying order ( $F_{1,232} = 53.91$ ,  $p < 0.001^*$ , slope =  $-12.88$  (1.75 s.e.m.)), whereas the effects of both egg treatment and laying order on tarsus length were non-significant at 12 days (treatment:  $F_{2,225} = 0.80$ ,  $p = 0.45$ ; laying order:  $F_{1,225} = 0.63$ ,  $p = 0.43$ ). Rectrix length at 12 days did not vary in relation to egg treatment ( $F_{2,225} = 0.25$ ,  $p = 0.78$ ; figure 1) and significantly declined with laying order ( $F_{1,225} = 84.07$ ,  $499 < 0.001^*$ , slope =  $-0.85$  (0.09 s.e.m.)). Repeated-measures analyses of variance were nestling phenotypic values at all ages were considered simultaneously led to qualitatively similar results.

T-cell-mediated immune response was significantly affected by egg treatment ( $F_{2,225} = 9.09$ ,  $p < 0.001^*$ ). Pairwise comparisons between experimental groups showed that T-cell-mediated immune response was significantly larger in nestlings from eggs inoculated with lutein compared with the two control groups ( $p < 0.001^*$  in both cases; figure 1), whereas no significant difference existed between nestlings from sham-inoculated and unmanipulated eggs ( $p > 0.99$ ). In this analysis, the effect of laying order was not significant ( $F_{1,225} = 3.01$ ,  $p = 0.08$ ). The concentration of anti-NDV antibodies did not vary in relation to egg manipulation ( $F_{1,163} = 1.85$ ,  $p = 0.16$ ; figure 1) or laying order ( $F_{1,163} = 0.01$ ,  $p = 0.92$ ).

To test whether lutein inoculation had a different effect on nestling T-cell immune response in relation to clutch size we analysed the correlation between the number of eggs in a clutch and the difference in mean within-brood T-cell response between nestlings from eggs inoculated with lutein and those from control eggs. In this analysis we pooled the nestlings originating from unmanipulated and sham-inoculated eggs because sham-inoculation did not affect T-cell response. The difference in immune response between nestlings from lutein-inoculated and control eggs significantly increased with clutch size ( $r = 0.27$ , d.f. = 68,  $p = 0.025$ ).

To test whether T-cell response predicted survival of nestlings, we identified the broods where mortality occurred after the day of measurement of T-cell response and contained one or two surviving nestlings of the same experimental group as the nestling that died. We then compared T-cell response of the dying nestlings with mean within-brood T-cell response of their surviving siblings. Surviving nestlings had non-significantly larger T-cell response than those that died (Wilcoxon test;  $z = -1.57$ ,  $n = 6$ ,  $p = 0.12$ ). However, when in this analysis we included data from other broods not included in the main experiment (see § 2), T-cell-mediated immune response positively predicted survival (mean of survivors: 139.4 mm (5.96 s.e.m.); non-survivors: 114.9 mm (5.28 s.e.m.);  $t = 3.28$ , d.f. = 19,  $p = 0.004$ ).

#### 4. DISCUSSION

In this study we showed that an experimental increase in egg yolk carotenoid concentration enhanced T-cell-mediated immune response of barn swallow nestlings, consistent with our prediction, but had no effect on humoral immunity and growth.

T-cell-mediated immune response is a major component of acquired immunity of vertebrates (Pastoret *et al.* 1998). In birds, large T-cell-mediated immune response, as evaluated *in vivo* by the same test used here, has been shown to be a predictor of long-term offspring survival (Christe *et al.* 1998; Hōrak *et al.* 1999), and a positive relationship between T-cell response and viability has also been documented in the barn swallow (Merino *et al.* 2000). In the present study we showed that T-cell response also predicts survival during the nestling period. Injection with PHA results in proliferation of 30% of the T-cell lines (Elgert 1996). The PHA test therefore reflects the general ability of the challenged individual to raise a lymphoproliferative response (Tella *et al.* 2002). However, we found no effect of egg carotenoids on humoral response to a specific antigen. At present, we have no explanation for this difference in the effect of carotenoid injection on the two kinds of response. In fact, carotenoid supplementation in the diet of adult vertebrates has been demonstrated to enhance both T-cell-mediated and humoral response (see Møller *et al.* (2000) for a review). Thus, we found evidence that egg carotenoids influence a major component of the acquired immune system that predicts nestling survival whereas they have no detectable effect on humoral response to a specific immunogen.

The effect of egg carotenoids on nestling immunity could be mediated by the antioxidant effects of carotenoids during embryo development and early life-stages. Alternatively, the effect on T-cell-mediated immune response could be mediated by parental decisions on allocation of food among progeny. If nestlings with large amounts of carotenoids of maternal origin are perceived as being of relatively large reproductive value compared with their siblings, parents may provide them with larger amounts of food enhancing immune response. Gape coloration of barn swallow nestlings is carotenoid dependent (Saino *et al.* 2000). As nestlings with more red gapes are fed preferentially by their parents (Saino *et al.* 2000), and T-cell-mediated immune response is known to be dependent on nutritional conditions (Lochmiller *et al.* 1993; Dietert *et al.* 1994; Saino *et al.* 1997), larger parental effort allocated to nestlings originating from lutein-injected eggs could result in enhanced T-cell-mediated immune response. However, preferential parental care to nestlings from lutein-injected eggs, if any, did not affect body mass of nestlings, which should also be influenced by nutritional conditions. In fact, parental provisioning rates have been shown to result in enhanced nestling body mass in previous studies of the barn swallow (Saino *et al.* 1997).

Lutein inoculation in the eggs from large clutches had a greater positive effect on nestling T-cell response than in small clutches. Lutein concentration may be smaller in eggs from large compared with small clutches and a given experimental increase in lutein concentration may thus have a greater effect on nestlings from large compared with small clutches. However, no data are available at present on natural variation of lutein concentration in relation to clutch size in our study population.

Body mass and rectrix length were greater for nestlings hatched from the first compared with the last eggs. Size hierarchy probably results from the fact that the first laid eggs in a clutch hatch first (see also Saino *et al.* 2001).

The effect of lutein inoculation on nestling phenotype and particularly T-cell-mediated immune response did not vary in relation to laying order of the eggs from which they hatched. This result suggests that lutein concentration even in first eggs, which are known to contain relatively more carotenoids (Saino *et al.* 2002a), is well below the concentration that allows for maximal immune response.

In conclusion, our study for the first time, to our knowledge, provides evidence that early maternal effects mediated by carotenoid content of the eggs affect a fundamental component of nestling immunocompetence and thus viability. The physiological versus parental behavioural mechanisms mediating such effects, however, remain to be elucidated.

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