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Carotenoid concentration in barn swallow eggs is influenced by laying order, maternal infection and paternal ornamentation

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Carotenoids are critical to embryonic development, immunity and protection from oxidative stress. Transmission of carotenoids to the eggs may affect development and maturation of immunity in offspring, but carotenoids may be available to females in limiting amounts. Females may thus transfer carotenoids to the eggs differentially in relation to the reproductive value of the offspring as affected by sexual ornamentation of their father. In this study of maternal allocation of carotenoids to the eggs in the barn swallow (*Hirundo rustica*), females whose immune system had been experimentally challenged with an antigen had smaller lutein concentrations in their eggs than controls. We manipulated the size of a secondary sexual character (tail length) of males, and analysed the effect of manipulation on allocation of lutein to eggs by their vaccinated mates. Contrary to our prediction based on parental allocation theory, mates of tail-shortened males had a larger lutein concentration in their eggs compared with those of control and tail-elongated males. According to previous studies, offspring of short-tailed males have larger exposure and/or susceptibility to parasites. A larger lutein concentration in the eggs of females mated to males with experimentally reduced ornaments may thus reflect adaptive maternal strategies to enhance offspring viability.

Keywords: carotenoids; egg; *Hirundo rustica*; immunity; laying order; secondary sexual characters

1. INTRODUCTION

Carotenoids are synthesized by plants, fungi and bacteria, and they must be assimilated by animals from food (Brush 1978; Goodwin 1984). These molecules can act as biochromes, being the main determinants of yellow to red coloration of bird plumage (Stradi 1998). Several physiological processes depend on or are regulated by carotenoids. Carotenoids act as scavengers of reactive oxygen molecules, protecting biological molecules (e.g. DNA and lipids) from oxidative damage, and they play an important role in the regulation of immune function (reviewed in Chew 1993; Edge *et al.* 1997; Olson & Owens 1998; Møller *et al.* 2000; Surai *et al.* 2001). In addition, they are precursors of vitamin A, whose derivatives (retinoids) are required for normal development of vertebrates (Gudas *et al.* 1994). However, absorption and accumulation of carotenoids are depressed by parasites (McGraw & Hill 2000; Møller *et al.* 2000).

Several studies have investigated the role of carotenoids as mediators of sexual communication systems because they possibly act as reliable signals of health or parenting ability (see Lozano 2001; Shykoff & Widmer 1996; Hill 1999; Møller *et al.* 2000). The basis for such reliable signalling is derived from the physiological actions of carotenoids, and from the trade-off that may exist between the use of carotenoids for physiological functions and the expression of sexual signals. However, the function of maternally transmitted carotenoids to eggs has rarely been investigated (Blount *et al.* 2000; Royle *et al.* 2001).

Carotenoids may play a role in protection from oxidative stress during the embryo and the early post-hatching stages, and they may promote maturation and functioning of the embryo immune system, which is likely to be crucial to offspring survival because immune defence tends to be weak soon after birth (Tizard 1991; Pastoret *et al.* 1998). Hence, carotenoids may mediate early maternal effects (Mousseau & Fox 1998), as mothers may influence the phenotype of their offspring by varying the amount of carotenoids transmitted to eggs in relation to ecological and socio-sexual factors. Natural selection may have favoured the evolution of strategies that allow for optimal allocation of such critical micronutrients to the eggs and the competing demands of maternal physiological processes (Sheldon 2000).

Evolutionary theory of parental investment posits that effort spent on parental care by iteroparous species should be adjusted to the reproductive value of current progeny because a trade-off may exist between current and future reproduction (Stearns 1992). In particular, females are predicted to invest more in their offspring when these are sired by a male with large secondary sexual characteristics, because their sons will be preferred as mates and/or their offspring will inherit the desirable genetic qualities (e.g. resistance to parasites) that are reliably advertised by male ornaments (Andersson 1994).

In the present study of the barn swallow (*Hirundo*

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rustica), we analysed carotenoid concentration in the egg yolk in relation to an experimental challenge to the immune system of mothers and manipulation of the expression of a secondary sexual character of their mates. Under the assumption that carotenoids are needed by maternal immunity, we predicted that females that were challenged with an antigen (the vaccine against the Newcastle disease virus) before egg laying produced eggs with smaller concentrations of the main carotenoid in barn swallows, lutein (Saino *et al.* 1999), compared with control females. An experimental increase in male ornamentation was predicted to result in a larger concentration of carotenoids in eggs, whereas the opposite effect was predicted for a reduction of male ornamentation. To the best of our knowledge, this is the first study where allocation of carotenoids to eggs in relation to maternal infection and paternal ornamentation is investigated.

The barn swallow is a socially monogamous, insectivorous passerine. Females alone incubate from two to seven eggs of one to three clutches per breeding season. Females are known to prefer males with long outermost tail feathers as social as well as extra-pair mates (Møller 1994; Saino *et al.* 1997*a*).

2. METHODS

We studied barn swallows in four colonies located east of Milano (northern Italy) in 2001. Adults were captured and individually marked to allow recognition and assignment to breeding pairs. Males were sequentially assigned to one of four experimental treatments to alter the expression of their tail ornaments: tail-shortening by 20 mm, no tail manipulation, tail cutting and re-glueing with no alteration of tail length, or tail elongation by 20 mm (see Møller 1994; Saino *et al.* 1997*b*). Before sequential assignment started, males were arranged in random order. In some capture sessions or parts of them, however, we did not manipulate tail length. Pairs were already formed when we manipulated tail length and no divorce occurred afterwards.

Females were injected subcutaneously with 20 µl commercial NDV vaccine (Nobivac Paramyxo, Intervet). Blood samples were collected from all individuals at the time of tail manipulation (males) or vaccination (females) and also 14 days later. Plasma was stored at −20 °C for analysis of anti-NDV antibodies. A group of females was sham-inoculated with the solvent of NDV vaccine to test for the effect of injection on lutein concentration in the egg yolk. All males had their tail manipulated more than nine days before the start of laying by their mate and all females were vaccinated more than nine days before laying. There was no significant variation in time elapsed between vaccination, sham-inoculation or capture among females used to test the effect of vaccination on egg lutein concentration (vaccinated females: 16.5 (1.76, $n = 11$) days, sham-inoculated: 17.0 (2.00, $n = 5$), non-injected: 16.4 (1.95, $n = 7$); ANOVA, $F_{2,20} = 0.02$, $p = 0.98$). There was also no significant variation in stage in the breeding cycle (time elapsed from tail treatment (males) or vaccination (females) and start of laying) at tail manipulation (tail-shortened males: 20.5 (2.73) days, $n = 8$, control: 19.4 (1.73), $n = 11$, cut and re-glued: 18.9 (1.65), $n = 10$, elongated: 25.9 (2.11), $n = 10$; ANOVA, $F_{3,35} = 2.50$, $p = 0.08$) or vaccination (females mated to tail-shortened males: 16.0 (2.16), control: 16.5 (1.76), cut and re-glued: 16.7 (1.54), elongated: 16.7 (2.14); sample sizes as for males, ANOVA, $F_{3,35} = 0.03$, $p = 0.99$). The four experimental groups of males were homogeneous with respect to pre-manipulation tail length (ANOVA, $F_{3,35} = 0.38$, $p = 0.77$). In the analyses of the effect of vaccination and tail manipulation on egg lutein concentration we first included experimental treatment as well as stage in the breeding cycle at treatment of male and female parents, and pre-manipulation male tail length as covariates, while allowing for the interactions. Non-significant interactions and covariates were removed from the ANCOVA models starting from those associated to the smallest effect size. Main effects were removed after the corresponding interactions. This removal procedure ended when only significant terms were included in the model. Lutein concentration data were log transformed to achieve homoscedasticity and normality where necessary (see § 3). Throughout the paper, standard errors of the mean are reported in parentheses.

Eggs were marked according to laying order. First and fourth eggs from clutches of females included in the tail manipulation experiment were considered in the present study. From five clutches of sham-inoculated females, only the first egg was collected to minimize the impact on reproductive output of the population. From seven clutches of non-injected females we collected all eggs. Eggs were collected the second day after laying of the last egg, in order to ensure that the clutch was complete, but before embryonic development could affect egg yolk composition. Yolks were stored at −20 °C and thoroughly mixed before analysis.

(a) *Anti-NDV antibody assay*

Anti-NDV antibody concentration was measured by monoclonal antibody-blocking ELISA (Svanovir NDV-Ab; SVANOVA Biotech, Uppsala, Sweden) (Czifra *et al.* 1996). Optical density (OD) values of female plasma and yolks were compared with the OD value of the kit NDV-negative control. Plasma samples at the time of vaccination were used to assess effectiveness of antigen inoculation in elicting an antibody response. ODs were used to calculate percentage inhibition (PI) ($\mathrm{PI} = (\mathrm{OD}_{\text{negative control}} - \mathrm{OD}_{\text{sample}})/\mathrm{OD}_{\text{negative control}}$); large PI values indicating large anti-NDV antibody concentration (see Saino *et al.* (2002) for details).

(b) *Lutein concentration assay*

Weighed amounts of egg yolk were sonicated for 3 min in the presence of acetone (1.5 ml), the suspension obtained was centrifuged for 5 min at 14 000 rpm and the supernatant was ready for carotenoid identification by high-performance liquid chromatography (HPLC) (lutein standard). HPLC conditions were as follows—mobile phase: acetonitrile–methanol (70 : 30); flow: 0.6 ml min^{-1}; column: purosphere RP18e, 25 cm, 5 µm; 37 °C. Total carotenoid content, and thus concentration, was calculated by spectrophotometric maximum absorbance value (448 nm; LambdaEZ201 with 1 cm quartz path-cuvette) (Britton 1985). Samples were analysed in a random sequence. The absorption coefficient ($A^{1\%}_{1\,\mathrm{cm}}$) used was 2500 (Schiedt *et al.* 1995). Repeatability of lutein concentration measures is known to be high ($R = 0.94$; Saino *et al.* 1999). The coefficient of variation of lutein concentration measured in six subsamples of the same yolk was 5.1%.

3. RESULTS

(a) *Effect of NDV-injection on lutein concentration in the yolk*

PI of maternal plasma was significantly larger at recapture compared with that recorded at NDV-inoculation (paired *t*-test: $t = 6.38$, d.f. $= 38$, $p < 0.001$; mean change in PI $= 21.61$ (3.39)), indicating that females produced anti-NDV antibodies (Saino *et al.* 2002). The lutein concentration in first eggs showed a significant variation among vaccinated, sham-inoculated and non-injected females (mean $= 23.3$ (1.92) µg g^{-1}, $n = 11$, 37.3 (6.13, $n = 5$), 36.6 (5.21, $n = 7$), respectively; ANOVA on log-transformed data: $F_{2,20} = 4.67$, $p = 0.022$). There was no difference between non-injected and sham-inoculated females ($t = 0.16$, d.f. $= 10$, $p = 0.97$), whereas lutein concentration in the eggs of vaccinated females was significantly smaller than in the other groups (vaccinated versus non-injected: $t = 2.58$, d.f. $= 16$, vaccinated versus sham-inoculated: $t = 2.78$, d.f. $= 14$, $p < 0.05$ in both cases after sequential Bonferroni correction). Hence, vaccination but not injection *per se* caused a reduction in lutein concentration in first eggs. The lutein concentration in fourth eggs of non-injected and vaccinated females did not differ (non-injected: 24.1 (4.20) µg g^{-1}, $n = 7$; vaccinated: 18.3 (2.25, $n = 11$); $t = 1.23$, d.f. $= 16$, $p = 0.24$).

A repeated-measures analysis of variance of first and fourth eggs of non-injected and vaccinated females showed that lutein concentration was significantly reduced by vaccination ($F_{1,15} = 5.07$, $p = 0.036$). In this analysis, first eggs were found to have significantly larger concentrations of lutein compared with fourth eggs (effect of laying order: $F_{1,15} = 11.30$, $p = 0.004$, see also analyses of non-injected females below) but no significant interaction existed between egg laying order and maternal treatment ($F_{1,15} = 1.25$, $p = 0.28$). A marginally significant, positive effect of stage in the breeding cycle at treatment of the mother existed ($F_{1,15} = 4.55$, $p = 0.050$).

(b) *Lutein concentration in relation to egg laying order*

An analysis of covariance with clutch as a factor and both linear and quadratic terms of egg laying order as covariates showed that a highly significant variation existed in mean lutein concentration among eggs laid by different non-injected females ($F_{6,19} = 26.66$, $p < 0.001$). Lutein concentration declined with laying order of the eggs, but the decline was not linear, as the quadratic term of laying order also significantly contributed to the ANCOVA model (linear term: $F_{1,19} = 12.25$, $p = 0.002$, coefficient $= -14.78$; quadratic term: $F_{1,19} = 6.47$, $p = 0.02$, coefficient $= 2.11$; figure 1). The function fitted to lutein concentration reached an estimated minimum for laying order of 3.50. Hence, the decline in lutein concentration was largest between first and second eggs and was null between the third and the fourth eggs.

(c) *Lutein concentration of eggs in relation to tail manipulation of males*

A repeated-measures analysis of variance on first and fourth eggs showed significant variation in mean lutein concentration among the four experimental groups of females ($F_{3,34} = 3.34$, $p = 0.033$; figure 2), but no significant interaction between egg laying order and male tail manipulation ($F_{3,34} = 2.37$, $p = 0.09$). In this model, stage in the breeding cycle at vaccination was retained, although its effect was not significant ($F_{1,34} = 1.15$, $p = 0.29$), because of a significant interaction between egg laying order and stage in the breeding cycle at vaccination ($F_{1,34} = 9.88$, $p = 0.003$). Females mated to tail-shortened males had larger lutein concentrations in their eggs compared with females of the two control groups, whereas the mean values of the two control groups were only slightly larger than the mean for females mated to tail-elongated males (figure 2). Tail manipulation had a highly significant effect on lutein concentration in first eggs ($F_{3,35} = 4.81$, $p = 0.007$). Females mated to tail-shortened males had a significantly larger lutein concentration compared with those of the other three groups ($p < 0.05$ in all pairwise comparisons after sequential Bonferroni correction), whereas no significant differences existed among the other three groups ($p > 0.99$ in all pairwise comparisons after Bonferroni correction). No significant variation in lutein concentration in relation to tail manipulation existed among fourth eggs ($F_{3,35} = 0.52$, $p = 0.67$).

PI, measuring the concentration of anti-NDV antibodies in the yolks relative to a pool of egg yolks from unvaccinated females was significantly larger than zero (first eggs: PI $= 12.9$ (1.89), $t = 6.82$, d.f. $= 38$, $p < 0.001$;

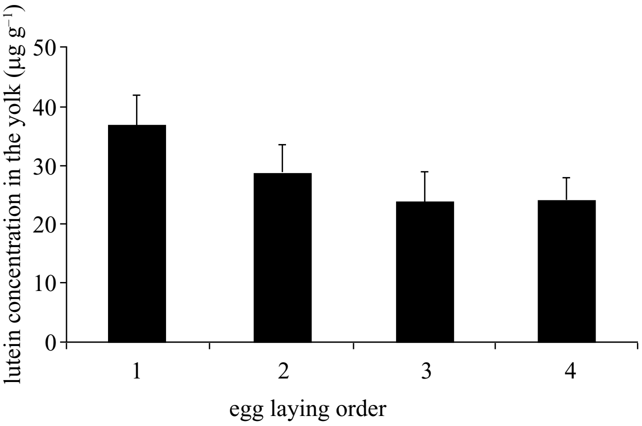

Figure 1. Mean (+s.e.) lutein concentration (µg g^{-1} yolk) in the eggs of non-injected female barn swallows whose mate received no tail manipulation in relation to laying order. $n = 7$ in all cases.

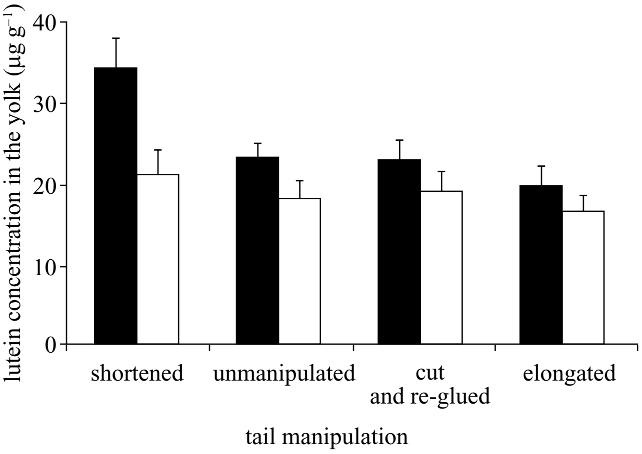

Figure 2. Mean (+s.e.) lutein concentration (µg g^{-1} yolk) in first (black bars) and fourth (white bars) eggs of females vaccinated with NDV-vaccine mated to males that had their ornamental tail shortened by 20 mm ($n = 8$ clutches), not manipulated ($n = 11$), cut and re-glued without altering original length ($n = 10$) or elongated by 20 mm ($n = 10$).

fourth eggs: 12.7 (2.29), $t = 5.56$, d.f. = 34, $p < 0.001$; anti-NDV antibody concentration was not available for four eggs), indicating that maternal anti-NDV antibodies were actually transferred to the eggs. Lutein concentration in first eggs positively covaried with the concentration of anti-NDV antibodies in the egg in an analysis of covariance with tail manipulation as a factor (effect of anti-NDV antibodies: $F_{1,34} = 4.37$, $p = 0.044$, coefficient = 0.302; effect of tail treatment: $F_{3,34} = 5.59$, $p = 0.003$), after that breeding stage at vaccination and tail manipulation, and original tail length of males had been excluded from the ANCOVA model because they did not significantly contribute to it (see § 2). Covariation between lutein concentration in fourth eggs and anti-NDV antibody concentration was not significant ($F_{1,30} = 0.34$, $p = 0.57$).

4. DISCUSSION

Lutein concentration in the eggs of females whose immune system had been challenged with an antigen that elicited a humoral immune response was smaller than in the eggs of unchallenged females. The concentration of lutein in the egg yolk of non-injected females was more than four times larger than in maternal plasma (7.1 µg ml^{-1}, $n = 31$; A. P. Møller, unpublished data), and the total amount of lutein contained in an average clutch of 4.7 eggs is *ca.* 62 µg (assuming an average yolk mass of 0.45 g), approximately nine times the amount of lutein instantaneously present in maternal plasma (*ca.* 7 µg assuming 1 g of plasma). Female barn swallows undergo a steep decline in circulating carotenoids before egg laying (P. Ninni and A. P. Møller, unpublished data) and, in this study, lutein concentration declined with laying order. Hence, female barn swallows may actively allocate carotenoids to the eggs, because the concentration in the eggs was larger than in their plasma, but the amount of lutein declined with egg laying order, indicating that maternal carotenoids were progressively depleted as more eggs were laid, and that carotenoids may be limiting in barn swallow diet. Differential allocation of carotenoids in relation to egg laying order may contribute to establishing a hierarchy of reproductive value among the offspring (Saino *et al.* 2001).

Evolutionary theories of parental investment and sexual selection lead to the prediction that parents should invest more in offspring when they are sired by more attractive males, and empirical studies have tested and found support for the prediction that maternal effort is larger when offspring are sired by highly ornamented males (Cunningham & Russell 2000; Sheldon 2000; but see Reyer *et al.* 1998). The prediction of allocation of carotenoids to eggs in direct proportion to experimental tail ornamentation was therefore based on the assumption that a positive relationship exists between carotenoids and offspring viability, because embryos and nestlings that receive more carotenoids are predicted to have better immune and antioxidant functions (Blount *et al.* 2000). However, the effect of tail manipulation on egg carotenoids was opposite to our expectation.

If carotenoids promote immune defence (Chew 1993; Edge *et al.* 1997; Olson & Owens 1998; Møller *et al.* 2000; Surai *et al.* 2001), but offspring are differentially harmed by virulent parasites because of additive genetic variation in resistance to parasites, then females may adaptively allocate more carotenoids to offspring of males with poor genetically based parasite resistance. In the barn swallow, naturally long-tailed males have fewer parasites than short-tailed ones, and the same applies to their nestlings, when these are cross-fostered (Møller 1994). If this relationship is determined by genetically based greater susceptibility to parasites of short-tailed compared with long-tailed males, more carotenoids may have been allocated to the offspring of tail-shortened males because these may appear to have greater expenditure due to greater activity of their immune system, and/or due to protection from larger oxidative stress arising as a consequence of the level of functioning of their immune system (Blount *et al.* 2000). Even in the case that the negative correlation between male ornamentation and parasite infestation is merely phenotypic, arising because males that have not been exposed to parasites produce larger, more costly sexual ornaments, females mated to less ornamented males may allocate more immune factors to their offspring to compensate for their larger exposure to vertically transmitted parasites. Larger allocation of carotenoids would still be adaptive in the presence of a physiological trade-off between egg quality and maternal viability because of a differentially greater beneficial effect of allocation by females mated to males with small compared with large sexual ornaments.

Anti-NDV antibody concentration in egg yolk positively covaried with lutein concentration. A positive covariation could be expected because maternally derived antibodies may have to be protected from catabolic degradation and this action is accomplished by carotenoids or their derivatives (Møller *et al.* 2000).

In conclusion, we found evidence consistent with the hypothesis that female barn swallows have limited availability of carotenoids for competing processes of immune defence and egg production. Allocation of carotenoids to eggs depended on the expression of secondary sexual characters of their father, but mothers transferred more carotenoids to the eggs when mated to a male with reduced, rather than enlarged, ornaments. This response may be interpreted as an adaptive modulation of early maternal effects compensating for larger susceptibility and/or exposure to parasites of offspring sired by poor quality males.

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