THE ROYAL SOCIETY

Received 5 May 2001
Accepted 15 August 2001
Published online 30 November 2001

Carotenoids and egg quality in the lesser black-backed gull *Larus fuscus*: a supplemental feeding study of maternal effects

Jonathan D. Blount[1*], Peter F. Surai[2], Ruedi G. Nager[1], David C. Houston[1], Anders Pape Møller[3], Michael L. Trewby[1] and Malcolm W. Kennedy[4]

[1]*Division of Environmental and Evolutionary Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK* (*r.nager@bio.gla.ac.uk*, *d.houston@bio.gla.ac.uk*)
[2]*Avian Science Research Centre, Scottish Agricultural College, Ayr KA6 5HW, UK* (*p.surai@au.sac.ac.uk*)
[3]*Laboratoire d'Ecologie, CNRS UMR 7625, Université Pierre et Marie Curie, 7 quai St Bernard, Case 237, F-75252 Paris Cedex 05, France* (*amoller@hall.snv.jussieu.fr*)
[4]*Division of Infection and Immunity, Institute of Biomedical and Life Sciences, Joseph Black Building, University of Glasgow, Glasgow G12 8QQ, UK* (*malcolm.kennedy@bio.gla.ac.uk*)

Egg quality is a phenotype of, and can profoundly influence fitness in, both mother and offspring. However, the physiological mechanisms that underlie this maternal effect are poorly understood. Carotenoids are hypothesized to enhance antioxidant activity and immune function, and are responsible for the pigmentation of egg yolk. The proximate basis and consequences of this maternal investment, however, have not previously been studied in wild birds. In this supplemental feeding study of lesser black-backed gulls, *Larus fuscus*, carotenoid-fed females are shown to have increased integument pigmentation, higher plasma concentrations of carotenoids and antioxidant activity, and lower plasma concentrations of immunoglobulins (Igs) in comparison with controls. In turn, carotenoid-fed females produced eggs containing high carotenoid but low Ig concentrations (i.e. passive immunity), whereas control females produced eggs containing low carotenoid but high Ig concentrations. Within-clutch patterns of these resources varied over the laying sequence in a similar manner in both carotenoid-fed and control nests. Our results suggest that carotenoids could be one resource responsible for egg quality maternal effects in birds. We discuss the possible implications of carotenoid-mediated effects on phenotype for fitness in mothers and their offspring.

Keywords: antioxidant activity; egg quality; maternal effects; passive immunity

1. INTRODUCTION

Parental condition at the time of offspring production can have long-lasting effects on phenotypic variation in offspring (reviewed by Bernardo (1996*a*) and Mousseau & Fox (1998)). Such 'maternal effects', where offspring phenotype is influenced by parental condition rather than genetic inheritance, have presumably evolved to enable parents to translate their environmental experience into adaptive variation in their offspring (Bernardo 1996*a*; Mousseau & Fox 1998). Maternal effects are thought to be widespread and potentially of great significance in shaping life-history evolution (e.g. as in collared flycatchers *Ficedula albicollis* (Schluter & Gustafsson 1993)), but their underlying proximate mechanisms are poorly understood (Bernardo 1996*a*).

Egg quality is an important maternal effect; it is a phenotype of, and can profoundly influence fitness in, both mother and offspring (Bernardo 1996*b*; Mousseau & Fox 1998). In birds (as in other oviparous animals), females must invest all the resources required for embryonic development in one self-contained package. The causal mechanisms and defining features of a good egg are still poorly understood (Bernardo 1996*b*). Several studies have shown that hatchability, growth, and survival of offspring correlate positively with egg size, and egg size correlates positively with maternal condition; however, other studies have found no such relationships (reviewed by Williams (1994)). Recently, it has been shown that specific egg components, including hormones (e.g. Schwabl 1993) and lipids (Nager *et al.* 2000), can influence fitness-related traits in offspring, independently of egg size.

Carotenoids have been hypothesized to be responsible for egg-quality maternal effects in wild birds (Royle *et al.* 1999; Blount *et al.* 2000). Carotenoids are lipid-soluble hydrocarbons that are synthesized only by photosynthetic plants and bacteria; all animals must obtain them through their diet (Goodwin 1984). Carotenoids are widely used by animals as red and yellow pigments (recently reviewed by Møller *et al.* (2000)), but can also act as antioxidants and immunostimulants (for biochemical and immunological reviews see, for example, Chew (1996) and Stahl & Sies (1999)). In hens, *Gallus domesticus*, dietary carotenoids are deposited into yolk where they reduce the susceptibility of embryonic tissues to free radical attack (Surai & Speake 1998), and enhance hatchling immune function (McWhinney *et al.* 1989; Haq *et al.* 1996). Free radicals are highly unstable atoms or molecules with unpaired elec-

*Author for correspondence (j.d.blount@udcf.gla.ac.uk).

trons, which arise as metabolic by-products. Free radicals seek to pair with other electrons and, in so doing, damage other molecules. If not under control, free-radical-induced changes in membrane properties (fluidity, flexibility) and functions (intercellular signalling, enzymatic activities) can result in impaired immune function (Chew 1996). It has been hypothesized that bird embryos and hatchlings are particularly in need of antioxidants because their rapid metabolism incurs high rates of free radical production, and their tissues are rich in unsaturated lipids that are susceptible to free radical attack (Surai *et al.* 2001). Female birds also deposit immunoglobulins (Igs) into yolk to provide offspring with passive immunity (Kowalczyk *et al.* 1985). Ig is the class of glycoproteins to which antibody belongs (Roitt *et al.* 1998). However, the possibility that any influence of carotenoids on maternal immune function, thereby modulating the levels of Ig in circulation, has consequences for the passive immunity imparted to offspring has not been studied in any species.

It has been hypothesized that carotenoids are a scarce, limiting resource to wild animals, such that their availability can constrain the expression of antioxidant activity, immune function and also integument pigmentation (Lozano 1994; von Schantz *et al.* 1999). However, we are not aware of any study that has experimentally tested whether the availability of carotenoids in wild birds might influence the levels of antioxidant activity and Ig in the eggs that they produce. Here we carry out such a test in a supplemental feeding study of lesser black-backed gulls, *Larus fuscus*. We hypothesized that body carotenoid levels (plasma, integument pigmentation) would be higher in carotenoid-supplemented females and, consequently, such females would have enhanced antioxidant protection and immune function, which would be reflected in the composition of their eggs.

2. MATERIAL AND METHODS

(a) *Site and supplemental feeding*

Data were collected at Walney Island, northwest England, where about 24 000 pairs of lesser black-backed gulls breed (Monaghan *et al.* 1998). In mid-April, shortly after the arrival of the birds at the colony and around four weeks before laying started, we randomly allocated breeding pairs, in a central part of the colony, to a feeding treatment. Thirty-seven nests were given 2 mg of total carotenoids in 20 g of solid vegetable fat (Van den Bergh Foods Ltd, Crawley, UK), and 37 other nests were given an equal amount of fat (control group), daily. Previous studies have shown that daily supplementation with a considerably larger amount of fat (120 g) does not enhance female condition or egg size in this species (Bolton *et al.* 1992). We used vegetable fat because it facilitates the gut absorption of carotenoids (Klasing 1998; Stahl & Sies 1999). To minimize the risk of theft by non-target birds, food was delivered at night and placed inside a length of PVC pipe (4.5 cm diameter, 8 cm depth) that had been buried vertically in the ground, level with the surface and next to the nest, at the start of the experiment.

The daily carotenoid dose placed at each nest equated to twice the amount of carotenoids in an average first-laid egg (= 0.98 mg; P. F. Surai, unpublished data). No data are available on the total carotenoid intake of any wild bird species, although high consumption does not cause toxicity in domestic hens (Klasing 1998). Dietary grade carotenoids were mixed in approximately the same ratio as they occur in gull eggs at the Walney colony (40% yellow xanthophylls, 25% red xanthophylls and 35% β-carotene; P. F. Surai, unpublished data). Thus, on a daily basis, each nest was given 40 mg of Oro Glo Layer (containing 1.8% lutein and 0.2% zeaxanthin; Kemin Europa NV, Herentals, Belgium), 5 mg of Carophyll Red (10% canthaxanthin; Hoffmann-La Roche, Basel, Switzerland) and 7 mg of Rovimix (10% β-carotene; Hoffmann-La Roche, Basel, Switzerland). Supplements were prepared daily. Fat was heated at 150 °C until molten, left to cool until viscous (12–14 °C; at which point carotenoids were added), then hardened at −20 °C for 2–3 h before delivery to the gulls.

The proportion of supplementally fed nests that yielded eggs did not differ significantly between treatment groups (controls, 34 of 37 nests; carotenoid-fed, 35 of 37 nests; Yates-corrected χ^2-test, $\chi^2 = 0.01$, $p = 0.936$). In relation to nests that yielded eggs, the date on which supplemental feeding began did not differ significantly between treatment groups (controls, 17.21 ± 0.50 April (mean ± s.e.); carotenoid-fed, 17.97 ± 0.74 April (mean ± 1 s.e.); Mann–Whitney *U*-test, $z = 0.261$, $p = 0.794$), nor the period of supplementation prior to laying (controls, 27.76 ± 1.01 days; carotenoid-fed, 28.66 ± 1.42 days; Mann–Whitney *U*-test, $z = 0.403$, $p = 0.687$). Hence, control and carotenoid-fed birds did not differ in their timing of laying (Mann–Whitney *U*-test, $z = 1.037$, $p = 0.300$). Supplemental feeding continued daily throughout laying.

(b) *Measurement of maternal phenotype*

Aspects of maternal phenotype were measured for a sample of supplementally fed nests that had yielded a clutch of three eggs (see § 2c). We attempted to catch all such females at the nest, using a walk-in trap, within one day of clutch completion; we were able to catch 6 controls and 10 carotenoid-fed females. The yellow colour of the bill and tarsus was measured by visual comparison with a Roche Yolk Colour Fan (RYCF; Hoffman-La Roche, Basel, Switzerland), whereas the orange-red colour of the bill spot, gape flange and orbital ring was measured using a Dulux Trade Colour Palette (DTCP; Dulux, Slough, UK). These objectively defined colour standards comprise consecutive steps in unique combinations of hue (colour in the colloquial sense, i.e. red, blue, etc.), value (brightness) and chroma (degree of saturation with hue). Observed RYCF scores ranged from 8 to 15. We numbered consecutive steps in the DTCP that characterized the colours found in our study population (scores ranged from 1 to 11). Birds were caught by M.L.T., and all measurements were made by J.D.B. who did not know the treatment group of origin. Measurements were made indoors in indirect natural light and based on the lateral right-hand aspect of each integument trait. Carotenoid pigmentation reflects, predominantly, in the visible part of the spectrum, and under standardized conditions visual colour measurements have been shown to correlate strongly with scores obtained by spectrophotometry (Hill 1998). Immediately after colour measurements, 0.5 ml of blood were collected from the tarsal vein into a heparin-rinsed syringe (under statutory license from the British Home Office), centrifuged at 14 000 *g* for 5 min, and plasma stored at −20 °C until analysis (see below).

(c) *Measurement of egg phenotype*

Three is the modal clutch size in lesser black-backed gulls (e.g. Bolton *et al.* 1992). A random sample of three-egg clutches was collected for analyses of yolk composition (7 control clutches; 11 from the carotenoid-fed group). We had planned

to collect 12 such clutches from each feeding treatment; our sample sizes are smaller and unbalanced because of predation of eggs. The remaining eggs were used in different experiments, the results of which will be published elsewhere. Eggs were collected on the day of laying, replaced with dummies, weighed (± 0.1 g) using an electronic balance and measured (length and breadth, ± 0.1 mm) using a sliding calliper. The yolk was separated from the albumen with a domestic egg separator sieve, then rolled on damp filter paper to remove any remaining traces of albumen, weighed (± 0.1 g), homogenized and stored at −20 °C until analysis (see below). Clutches sampled for yolk composition analysis were produced during the first half of the laying period (because three-egg clutches are produced mostly by early layers), and therefore all experimental birds were of relatively high quality. In respect of total clutch volume (equation for counting egg volume in Bolton *et al.* (1992)), an estimate of maternal investment in the clutch, the subset of clutches sampled for yolk composition analysis did not differ from the remaining three-egg clutches either in control (*t*-test, $t_{16} = 1.478$, $p = 0.159$) or carotenoid-fed groups ($t_{25} = 0.783$, $p = 0.441$). Hereafter, first-laid eggs will be referred to as a-eggs, second-laid eggs as b-eggs, etc.

(d) *Biochemical assays*

Total carotenoid concentrations in female plasma and yolk were determined using HPLC as described in Surai & Speake (1998), using mobile phases of acetonitrile/methanol (85 : 15) and acetonitrile/dichloromethane/methanol (70 : 20 : 10) in gradient elution (see Granado *et al.* 1998). Detection was by absorbance at 445 nm. Total carotenoids are reported as μg ml^{-1} plasma or μg g^{-1} yolk.

Antioxidant activity was measured in samples of plasma and a-egg yolk using a decolorization assay (Re *et al.* 1999). Assays were performed on the same yolk lipid-phase used for the measurement of carotenoids. The assay measures the rate at which a pre-formed radical monocation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\cdot+}$) is quenched by antioxidants in the test sample. Trolox was used as a standard (Hoffmann-La Roche, Basel, Switzerland). Results are expressed as μmol of Trolox equivalent per ml or g of test sample.

(e) *Immunological assays*

The total Ig concentrations in blood plasma and yolk were determined using a single radial immunodiffusion assay (Roitt *et al.* 1998). Sheep anti-lesser black-backed gull Ig was produced for us at the Scottish Antibody Production Unit (Carluke, UK) by serially injecting a sheep with gull Ig (obtained by precipitation from yolk). Sheep plasma, containing an antibody against gull Ig, was collected and stored at −20 °C until use as an antiserum. Antiserum was mixed into a preparation of 2% agar in barbitone buffer at a ratio of 1 : 14 (v/v) at 56 °C, 3 ml of which were poured evenly onto microscope slides and allowed to set. Circular wells (4 mm diameter) were punched into the agar at an equal spacing, and 10 μl of test antigen was added (samples of egg yolk were first diluted 1 : 1 w/v in phosphate-buffered saline (PBS)). Plates were left for 72 h for the antigen to diffuse out of the wells and bind with the antiserum, precipitating in a ring. The ring diameter was measured using a magnifying lens and sliding calliper (to the nearest 0.02 mm); (ring diameter)^2 is proportional to the antigen (i.e. gull Ig) concentration (Roitt *et al.* 1998). Unknowns were determined by interpolation from a standard curve based on six serial dilutions of a pool of non-test samples of gull plasma in PBS.

Samples from control and carotenoid-fed females were allocated among slides at random. Each test sample was included on two slides for calculation of repeatability (Lessells & Boag 1987). The ring diameter was measured twice in different directions. There was significant repeatability between mean ring diameters of the same test antigen on two different slides (yolk: $r = 0.536$, $F_{53,54} = 3.312$, $p < 0.001$; plasma: $r = 0.707$, $F_{15,16} = 5.810$, $p < 0.001$). Means from the two assays were used in subsequent analyses.

(f) *Data analyses*

Principal components analysis (PCA) was used to generate an index of female integument coloration from the five colour measurements made on each bird. The variation in egg mass, yolk carotenoid and Ig concentrations was assessed using repeated measures analysis of covariance (rmANCOVA) with laying sequence (a-, b-, c-egg) as a within-subjects variable, feeding treatment as a between-subjects factor and female coloration index as a covariate. We used female coloration index as a covariate to represent body carotenoid levels in general, because plasma carotenoid concentrations may reflect relatively ephemeral patterns, as affected by the time since the last meal for example. Within-subjects effects were evaluated according to the multivariate approach; tests based on Pillai's Trace, Wilks's Lambda, Hotelling's Trace and Roy's Largest Root always gave identical *F*-values. Significant laying sequence effects were followed by *post hoc* contrasts (a- versus b-egg; b- versus c-egg; Bonferroni adjusted alpha = 0.025). Where necessary, data were $\log_{10}$-transformed before analysis. Data for Ig indices in yolk remained heteroscedastic after transformation. We only present results of the analysis based on untransformed data, because ANOVA is robust for heteroscedasticity if results are statistically significant (Ito 1980). Information about egg size together with yolk concentrations of resources may directly reflect resource availability to offspring only if yolk size varies proportionately (i.e. isometrically) with egg size. To compare differences in yolk mass relative to egg mass between treatment groups, we tested the allometric relationship between clutch mean yolk mass and egg mass using $\log_{10}$: $\log_{10}$ regression (e.g. see Williams 1994), including feeding treatment and female coloration index as factors (i.e. ANCOVA). In all models *p*-values correspond to type III sums of squares. Models were developed using backwards elimination starting with the highest order interaction. Other statistical tests are introduced in § 3 (two-tailed alpha = 5%). Values are reported as mean ± 1 s.e.

3. RESULTS

(a) *Carotenoid effects on maternal phenotype*

Carotenoid supplementation did not affect the frequency of three-egg clutches (controls, 24 of 34 females; carotenoid-fed, 29 of 35 females; Yates-corrected χ^2-test, $\chi^2 = 0.06$, $p = 0.799$). The five integument colour measurements made on each bird were significantly intercorrelated (all $p < 0.015$). In a PCA the first factor explained 59.65% of the variance in female coloration, with a large positive loading on colour scores for gape, orbital ring, bill and leg (eigenvectors of 0.87, 0.84, 0.81 and 0.71, respectively), and a negative loading on bill spot colour (−0.60). Only the first factor accounted for variance greater than 1 (i.e. eigenvalue > 1), and first factor

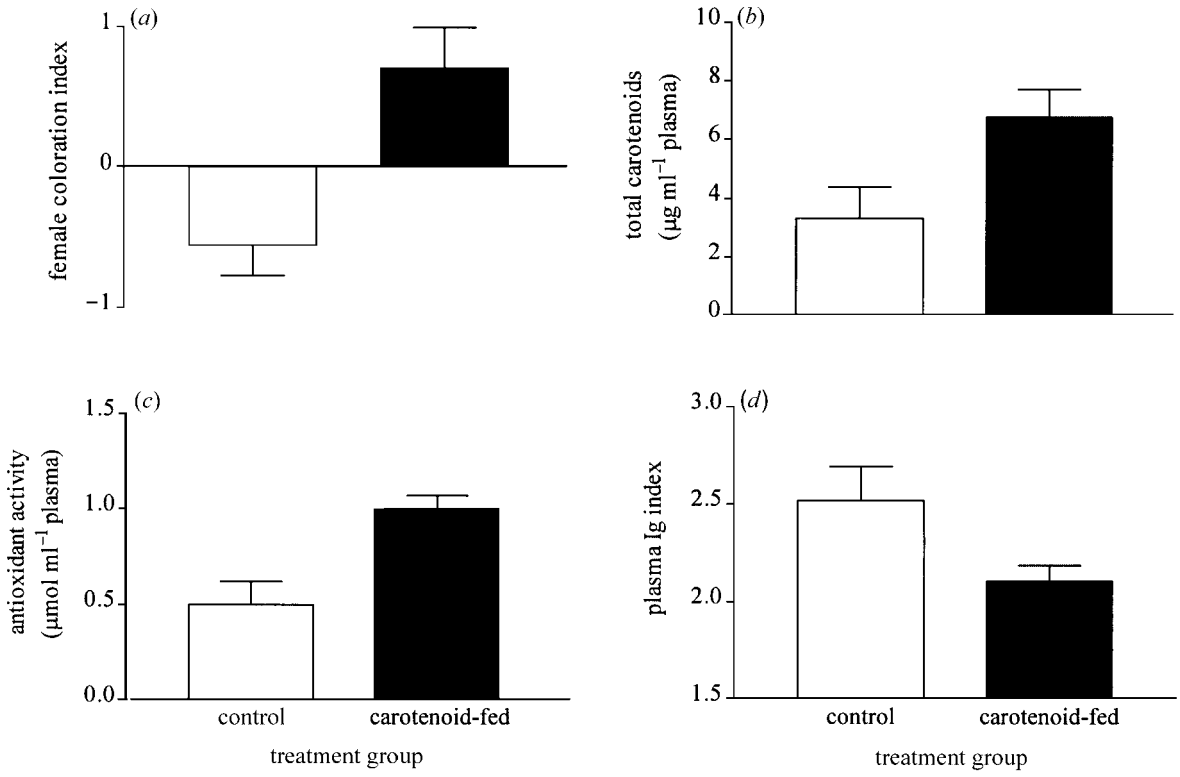

Figure 1. Effects of supplemental feeding on maternal phenotype in control ($n = 6$) and carotenoid-fed lesser black-backed gulls ($n = 10$, except in (*c*) where $n = 9$ because there was insufficient plasma to assay antioxidant activity for one individual). Mean (± 1 s.e.) values are shown. (*a*) Female coloration index (first principal component; see § 3), which differed significantly between feeding treatments (*t*-test, $t_{14} = 2.88$, $p = 0.012$). (*b*) Plasma carotenoid concentration, which was significantly higher in carotenoid-fed females (ANCOVA: feeding treatment, $F_{1,14} = 5.52$, $p = 0.034$; female coloration index, $F_{1,13} = 0.02$, $p = 0.894$; interaction n.s.). (*c*) Plasma antioxidant activity, which was significantly higher in carotenoid-fed females (ANCOVA: feeding treatment, $F_{1,13} = 14.98$, $p = 0.002$; female coloration index, $F_{1,12} = 0.00$, $p = 0.994$; interaction n.s.). (*d*) Plasma Ig index, which was significantly lower in carotenoid-fed females (ANCOVA: feeding treatment, $F_{1,14} = 6.11$, $p = 0.027$; female coloration index, $F_{1,13} = 0.01$, $p = 0.936$; interaction n.s.).

scores were extracted and used as a female coloration index. Carotenoid-fed females had significantly higher coloration indices (figure 1*a*), plasma carotenoid concentrations (figure 1*b*) and antioxidant activity (figure 1*c*), but significantly lower plasma Ig indices compared with control females (figure 1*d*).

(b) *Maternal effects on egg phenotype*

The egg mass declined over the laying sequence in a similar manner in both feeding treatments, and did not differ significantly between control and carotenoid-fed groups (figure 2*a* and table 1). The yolk mass increased proportionately with egg mass, and this isometric relationship did not differ significantly between control and carotenoid-fed groups (ANCOVA, feeding treatment, $F_{1,15} = 0.00$, $p = 0.966$; female coloration index, $F_{1,12} = 0.02$, $p = 0.884$; $\log_{10}$(clutch mean egg mass), $F_{1,16} = 36.72$, $p < 0.0001$; all interactions, n.s.; $\log_{10}$(clutch mean yolk mass) = −0.622 (± 0.311) + 1.003 (± 0.165) $\log_{10}$(clutch mean egg mass); p (slope = 1) > 0.5).

Yolk carotenoid concentrations declined over the laying sequence in a similar manner in both feeding treatments, but were significantly higher in eggs laid by carotenoid-fed females (figure 2*b* and table 1). This within-clutch pattern varied according to female integument pigmentation (table 1): in more brightly coloured females the decline in carotenoids between the last two eggs was smaller than in dull females (correlation of female coloration index with $\log_{10}$(difference in carotenoid concentration between b- and c-eggs): $r = -0.522$, $n = 16$, $p = 0.038$). The concentration of carotenoids in maternal circulation correlated with the mean concentration of carotenoids in the clutch ($r = 0.598$, $n = 16$, $p = 0.014$).

Yolk Ig indices were significantly higher in a-eggs compared with b- and c-eggs, and this within-clutch pattern did not differ significantly among feeding treatments (figure 2*c* and table 1). However, eggs produced by carotenoid-fed females contained significantly lower concentrations of Ig compared with controls (figure 2*c* and table 1). Antioxidant activity in a-egg yolk did not differ significantly among feeding treatments (controls, 0.59 ± 0.04 μmol g^{-1} yolk; carotenoid-fed, 0.65 ± 0.05 μmol g^{-1} yolk; ANCOVA: feeding treatment, $F_{1,14} = 2.72$, $p = 0.121$; female coloration index, $F_{1,13} = 2.00$, $p = 0.181$; interaction n.s.), but was positively correlated with the a-egg yolk carotenoid concentration ($r = 0.521$, $n = 18$; $p = 0.039$). As for carotenoids, there was a correlation between the concentration of Ig in maternal circulation and the mean concentration of Ig in the clutch ($r = 0.651$, $n = 16$, $p = 0.006$).

4. DISCUSSION

This study has shown that dietary supplementation with carotenoids resulted in almost twofold increases in carot-

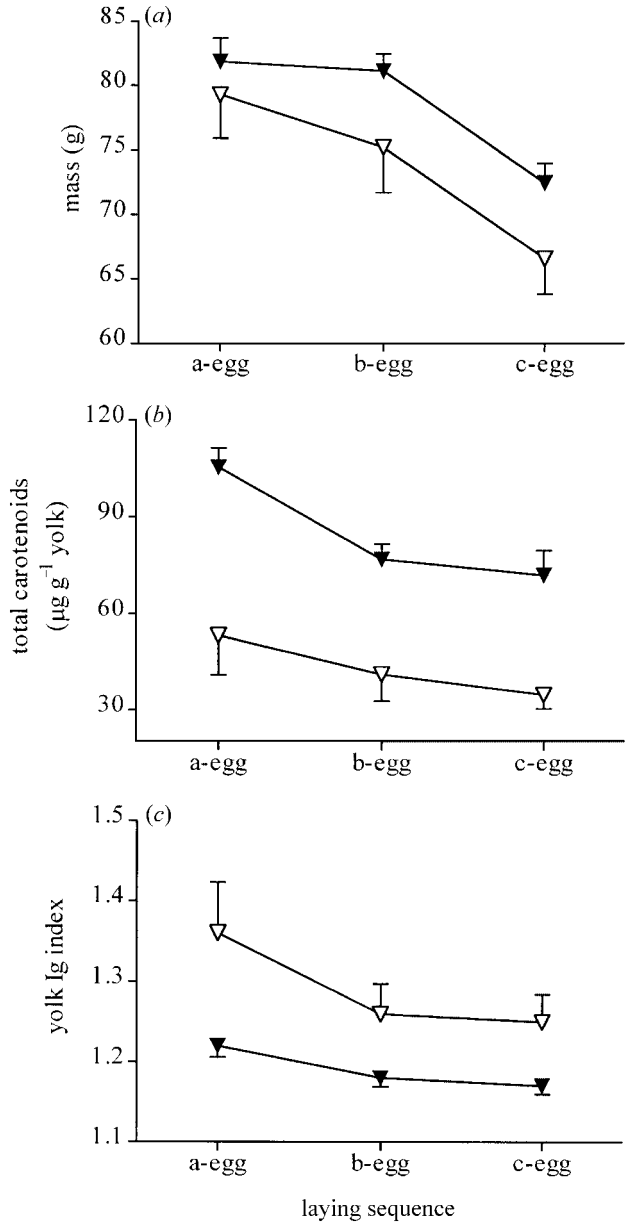

Figure 2. Effects of supplemental feeding on egg phenotype in the control (▽; $n = 7$ clutches) and carotenoid-fed groups (▼; $n = 11$ clutches). Mean (± 1 s.e.) values are shown. (*a*) Egg mass. (*b*) Yolk carotenoid concentration. (*c*) Yolk Ig indices. See table 1 for results of statistical analyses.

enoid concentrations and free radical trapping activity in plasma, and carotenoid pigmentation of integument, in breeding female lesser black-backed gulls. The females in the two treatments also differed in their immune function: carotenoid-supplemented birds had significantly lower plasma Ig concentrations. The effects of carotenoid supply on maternal phenotype were reflected in the phenotypic variation among the eggs that they laid. Carotenoid-fed birds produced eggs that contained significantly more carotenoids, but less Ig, in comparison with controls. Antioxidant activity in yolk was correlated with its carotenoid concentration. These results suggest that carotenoid supply can profoundly influence maternal phenotype, with consequences for the composition of the eggs that they lay. But do these results indicate that carotenoids are a scarce, limiting resource to breeding females?

It has been suggested that individual wild animals differ in their supplies of carotenoids because of variation in foraging efficiency (access to carotenoids; Endler (1980), Kodric-Brown (1985) and Hill (1990)), or parasitism and diseases (e.g. Milinski & Bakker 1990; Houde & Torio 1992), which, in some instances, may directly inhibit carotenoid absorption, or place demands on carotenoid supply for antioxidant activity and immune function (Lozano 1994; von Schantz *et al.* 1999; reviewed by Olson & Owens (1998) and Møller *et al.* (2000)). The present study indicates that increased carotenoid supply, resulting in higher blood carotenoid levels, can translate into increased antioxidant activity *in vitro*. Several studies of humans and domesticated animals have implicated free radicals in the aetiology of diseases, and antioxidant activity has been shown to play an important role in disease resistance (reviewed by Chew (1996), Stahl & Sies (1999) and Møller *et al.* (2000)). However, because we did not measure whether free radical production exceeded the availability of carotenoids to neutralize them in control birds *in vivo*, it remains unclear whether carotenoid supply is limiting for protection against free radicals in gulls (see also Hill 1999). Our results also show that carotenoid supplementation resulted in lower levels of Ig in circulation. Previous (observational) studies of wild birds that have related body carotenoid levels to indirect measures of immune system activity, such as circulating levels of Ig or leucocytes, have reported both positive and negative correlations (reviewed by Møller *et al.* (2000)). This may seem puzzling, given that studies of humans and domesticated animals have shown that carotenoids can stimulate the production of immune cells, and prevent them being damaged by free radical attack (reviewed by Chew (1996), Stahl & Sies (1999) and Møller *et al.* (2000)). But levels of Ig in circulation partly reflect the current requirement for antibody (i.e. exposure to antigens). Higher antibody titres should result in faster clearance of antigen, but titres will not remain high unless infection persists (Roitt *et al.* 1998) because Ig turnover is very rapid in birds, being about 1.5 days in adult domestic hens (Patterson *et al.* 1962). Thus, the low plasma Ig indices in carotenoid-fed females possibly reflected their enhanced efficiency at clearing infections over the preceding four weeks, during the period of supplemental feeding. We cannot rule out the alternative possibility that carotenoid supplementation inhibited immune function. There is limited evidence of an association between high carotenoid consumption and disease in humans, possibly because certain carotenoids switch from being antioxidants to prooxidants above threshold concentrations (Mayne 1996). However, this seems an unlikely explanation for our results because carotenoid-fed females had markedly enhanced plasma antioxidant activity. Ultimately, the issue of whether carotenoid supply is limiting for immune function in wild animals will only be resolved by experimentally varying exposure to antigens and then measuring specific responses in individuals with different circulating levels of carotenoids.

It is not possible to conclude whether there were causal relationships between female integument coloration and plasma concentrations of carotenoids or Ig. However, females producing clutches with the largest decline in carotenoids over the laying sequence had the dullest integu-

Table 1. Effects of supplemental feeding on egg phenotype in control ($n = 7$ clutches) and carotenoid-fed ($n = 11$ clutches) lesser black-backed gulls.
(Statistical results are from rmANCOVAs with laying sequence as a within-subjects factor, feeding treatment as a between-subjects factor and female coloration index as a covariate. Models were developed using backwards elimination (see text for details). Only main effects and significant interactions are shown. All *post hoc* contrasts, Bonferroni adjusted alpha = 0.025.)

source	egg mass			$\log_{10}$ (total carotenoids)			total immunoglobulins		
	F	d.f.	p	F	d.f.	p	F	d.f.	p
within-subjects									
laying sequence	43.91	2,16	<0.001[a]	80.47	2,13	<0.001[b]	7.18	2,16	0.006[c]
laying sequence × female coloration index	—	—	—	11.65	2,13	0.001	—	—	—
between-subjects									
feeding treatment	0.58	1,14	0.459	8.32	1,13	0.013	10.01	1,14	0.007
female coloration index	0.03	1,13	0.864	0.27	1,13	0.613	1.38	1,13	0.261

[a] *Post hoc* contrasts: a-egg versus b-egg, $F_{1,17} = 9.48$, $p = 0.007$; b-egg versus c-egg, $F_{1,17} = 67.29$, $p < 0.001$.
[b] *Post hoc* contrasts: a-egg versus b-egg, $F_{1,14} = 101.04$, $p < 0.001$; b-egg versus c-egg, $F_{1,14} = 8.12$, $p = 0.013$.
[c] *Post hoc* contrasts: a-egg versus b-egg, $F_{1,17} = 12.79$, $p = 0.002$; b-egg versus c-egg, $F_{1,17} = 1.65$, $p = 0.216$.

ment pigmentation on clutch completion, consistent with the explanation that carotenoid pigmentation of integument and yolk are associated. Carotenoid investment in eggs has been linked to declining carotenoid pigmentation of integument in domestic hens (Klasing 1998; and see also Burley *et al.* (1992) and Negro *et al.* (1998)). There is increasing interest in the possibility that female ornaments reveal aspects of quality to males (Amundsen 2000). In passerine species of birds, there is some evidence that male mate choice can be influenced by female carotenoid pigmentation (Burley & Coopersmith 1987), but there is presently no evidence that such choices are related to laying date, clutch size or other measures of female reproductive success (Hill 1993). Similarly, in the present study carotenoid supply was not a proximate constraint on laying date, egg or clutch size. However, under natural feeding conditions gulls presumably do not obtain carotenoids independently of other nutrients, such as amino acids, that have been shown to be limiting for egg production (Bolton *et al.* 1992). Therefore, the possibility that carotenoid pigmentation of integument could predict female quality in gulls deserves further study.

As in female plasma, high carotenoid concentrations in yolk were associated with high antioxidant activity, but low levels of Ig. Thus it appears that the level of carotenoid and Ig deposition into yolk can simply be a function of the level of carotenoids and Ig circulating in the females themselves. The significance, for the offspring, of this inverse relationship is not known, because both these resources would seem independently likely to benefit chicks. Embryos and hatchlings are incapable of synthesizing Ig, so they rely on passive immunity before their own immune system becomes effective (Kowalczyk *et al.* 1985). It has recently been shown that kittiwakes, *Rissa tridactyla*, breeding in areas that have a high prevalence of specific parasites deposit relevant antibody into their eggs, consistent with the suggestion that Ig deposition into yolk is adaptive (Gasparini *et al.* 2001; and see Heeb *et al.* (1998)). There is some evidence from captive studies that passive immunity enhances disease resistance (e.g. Fadly & Smith 1991). However, domestic hen chicks with high tissue concentrations of maternally derived carotenoids have enhanced antioxidant protection (Surai & Speake 1998) and lymphocyte synthesis (Haq *et al.* 1996).

Consistent with earlier studies of this species, egg size declined sequentially through the clutch (e.g. Bolton *et al.* 1992; Royle *et al.* 1999; Nager *et al.* 2000), and as the relationship between egg mass and yolk mass was isometric, our results indicate that the absolute amounts of carotenoids and Ig covaried with egg size. Thus, the mechanisms that bring about a within-clutch hierarchy in carotenoid (*sensu* Royle *et al.* 1999) and Ig investment into offspring appear to be independent of maternal supplies of carotenoids. The costs of producing young (rather than eggs *per se*) could potentially explain the evolution of such mechanisms. It was recently hypothesized that low antioxidant reserves in c-eggs comprised a maternal strategy to facilitate brood reduction in gulls (Royle *et al.* 1999). Our results complement this suggestion, because c-eggs are also low in passive immunity.

The question arises as to whether deposition of carotenoids and passive immunity into eggs could be costly for females. Costs incurred by females solely during egg production have been linked to increased parasitism (Oppliger *et al.* 1996) and impaired ability to rear chicks (Monaghan *et al.* 1998), but a physiological explanation has not been elucidated. Endocytosis of circulating Ig by developing oocytes means that a domestic hen loses 30–40% of circulating Ig per day in addition to normal turnover (Kowalczyk *et al.* 1985). Could such investment cause increased maternal susceptibility to parasites and diseases, and does maternal deposition of carotenoids into eggs reduce her antioxidant protection? Similarly, it remains to be established whether maternally derived carotenoid supply is limiting to offspring in wild birds. Our results suggest that when carotenoids are in abundant supply to breeding females, this potential benefit can be transferred to their offspring via the egg. But could high antioxidant protection compensate for low levels of passive immunity in determining chick fitness? In conclusion, in lesser black-backed gulls, aspects of maternal and egg phenotypes that seem likely to have potential importance

for reproductive success are influenced by the carotenoid supply in the maternal diet. Questions that address the fitness consequences of carotenoid-induced maternal effects are the focus of our continuing studies.

The authors thank the Cumbria Wildlife Trust for assistance in the field, Stuart Humphries, Francois Mougeot and Paola Ninni for advice on colour measurements, Andrea Fidgett for helping to develop the Ig assay, Kemin Europa NV and Roche Products Ltd for the donation of carotenoids, and Geoffrey Hill, Kate Lessells, Pat Monaghan, Graeme Ruxton and an anonymous reviewer for helpful critiques of the manuscript. J.D.B. was supported by a postgraduate scholarship from the University of Glasgow, and P.F.S. by a grant from the Scottish Executive Rural Affairs Department.

REFERENCES

Amundsen, T. 2000 Why are female birds ornamented? *Trends Ecol. Evol.* **15**, 149–155.

Bernardo, J. 1996*a* Maternal effects in animal ecology. *Am. Zool.* **36**, 83–105.

Bernardo, J. 1996*b* The particular maternal effect of propagule size, especially egg size: patterns, models, quality of evidence and interpretations. *Am. Zool.* **36**, 216–236.

Blount, J. D., Houston, D. C. & Møller, A. P. 2000 Why egg yolk is yellow. *Trends Ecol. Evol.* **15**, 47–49.

Bolton, M., Houston, D. & Monaghan, P. 1992 Nutritional constraints on egg formation in the lesser black-backed gull: an experimental study. *J. Anim. Ecol.* **61**, 521–532.

Burley, N. & Coopersmith, C. B. 1987 Bill color preferences of zebra finches. *Ethology* **76**, 133–151.

Burley, N. T., Price, D. K. & Zann, R. A. 1992 Bill color, reproduction and condition effects in wild and domesticated zebra finches. *Auk* **109**, 13–23.

Chew, B. P. 1996 Importance of antioxidant vitamins in immunity and health in animals. *Anim. Feed Sci. Technol.* **59**, 103–114.

Endler, J. A. 1980 Natural selection on color patterns in *Poecilia reticulata*. *Evolution* **34**, 76–91.

Fadly, A. M. & Smith, J. 1991 Influence of maternal antibody on avian leukosis virus infection in white leghorn chickens harboring endogenous virus-21 (EV21). *Avian Dis.* **35**, 443–451.

Gasparini, J., McCoy, K. D., Haussy, C., Tveraa, T. & Boulinier, T. 2001 Induced maternal response to the Lyme disease spirochaete *Borrelia burgdorferi sensu lato* in a colonial seabird, the kittiwake *Rissa tridactyla*. *Proc. R. Soc. Lond.* B **268**, 647–650. (DOI 10.1098/rspb.2000.1411.)

Goodwin, T. W. 1984 *The biochemistry of the carotenoids, II. Animals*. London: Chapman & Hall.

Granado, F., Olmedilla, B., Gil-Martínez, E. & Blanco, I. 1998 Lutein ester in serum after lutein supplementation in human subjects. *Br. J. Nutr.* **80**, 445–449.

Haq, A.-U., Bailey, C. A. & Chinnah, A. 1996 Effect of β-carotene, canthaxanthin, lutein, and vitamin E on neonatal immunity of chicks when supplemented in the broiler breeder diets. *Poultry Sci.* **75**, 1092–1097.

Heeb, P., Werner, I., Kölliker, M. & Richner, H. 1998 Benefits of induced host responses against an ectoparasite. *Proc. R. Soc. Lond.* B **265**, 51–56. (DOI 10.1098/rspb.1998.0263.)

Hill, G. E. 1990 Female house finches prefer colourful males: sexual selection for a condition-dependent trait. *Anim. Behav.* **40**, 563–572.

Hill, G. E. 1993 Male mate choice and the evolution of female plumage coloration in the house finch. *Evolution* **47**, 1515–1525.

Hill, G. E. 1998 An easy, inexpensive means to quantify plumage coloration. *J. Field Ornithol.* **69**, 353–363.

Hill, G. E. 1999 Is there an immunological cost to carotenoid-based ornamental coloration? *Am. Nat.* **154**, 589–595.

Houde, A. E. & Torio, A. J. 1992 Effect of parasitic infection on male color pattern and female choice in guppies. *Behav. Ecol.* **3**, 346–351.

Ito, P. K. 1980 Robustness of ANOVA and MANOVA test procedures. In *Handbook of statistics* (ed. P. R. Krishnaiah), pp. 199–236. Amsterdam: North-Holland Publications.

Klasing, K. C. 1998 *Comparative avian nutrition*. Wallingford, UK: CAB International.

Kodric-Brown, A. 1985 Female preference and sexual selection for male coloration in the guppy (*Poecilia reticulata*). *Behav. Ecol. Sociobiol.* **17**, 199–206.

Kowalczyk, K., Daiss, J., Halpern, J. & Roth, T. F. 1985 Quantitation of maternal–fetal IgG transport in the chicken. *Immunology* **54**, 755–762.

Lessells, C. M. & Boag, P. T. 1987 Unrepeatable repeatabilities: a common mistake. *Auk* **104**, 116–121.

Lozano, G. A. 1994 Carotenoids, parasites, and sexual selection. *Oikos* **70**, 309–311.

McWhinney, S. L. R., Bailey, C. A. & Panigrahy, B. 1989 Immunoenhancing effect of β-carotene in chicks. *Poultry Sci.* **68**(Suppl. 1), 94.

Mayne, S. T. 1996 Beta-carotene, carotenoids, and disease prevention in humans. *FASEB J.* **10**, 690–701.

Milinski, M. & Bakker, T. C. M. 1990 Female sticklebacks use male coloration in mate choice and hence avoid parasitized males. *Nature* **344**, 330–333.

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 Poultry Biol. Rev.* **11**, 137–159.

Monaghan, P., Nager, R. G. & Houston, D. C. 1998 The price of eggs: increased investment in egg production reduces the offspring rearing capacity of parents. *Proc. R. Soc. Lond.* B **265**, 1731–1735.

Mousseau, T. A. & Fox, C. W. 1998 The adaptive significance of maternal effects. *Trends Ecol. Evol.* **13**, 403–407.

Nager, R. G., Monaghan, P. & Houston, D. C. 2000 Within-clutch trade-offs between the number and quality of eggs: experimental manipulations in gulls. *Ecology* **81**, 1339–1350.

Negro, J. J., Bortolotti, G. R., Tella, J. L., Fernie, K. J. & Bird, D. M. 1998 Regulation of integumentary colour and plasma carotenoids in American kestrels consistent with sexual selection theory. *Funct. Ecol.* **12**, 307–312.

Olson, V. A. & Owens, I. P. F. 1998 Costly sexual signals: are carotenoids rare, risky or required? *Trends Ecol. Evol.* **13**, 510–514.

Oppliger, A., Christe, P. & Richner, H. 1996 Clutch size and malaria resistance. *Nature* **381**, 565.

Patterson, R., Youngner, J. S., Weigle, W. O. & Dixon, F. J. 1962 Antibody production and transfer to egg yolk in chickens. *J. Immunol.* **89**, 272–278.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. & Rice-Evans, C. 1999 Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Rad. Biol. Med.* **26**, 1231–1237.

Roitt, I., Brostoff, J. & Male, D. 1998 *Immunology*, 5th edn. London: Mosby.

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

Schluter, D. & Gustafsson, L. 1993 Maternal inheritance of condition and clutch size in the collared flycatcher. *Evolution* **47**, 658–667.

Schwabl, H. 1993 Yolk is a source of maternal testosterone for developing birds. *Proc. Natl Acad. Sci. USA* **90**, 11 446–11 450.

Stahl, W. & Sies, H. 1999 Carotenoids: occurrence, biochemical activities, and bioavailability. In *Antioxidant food supplements in human health* (ed. L. Packer, M. Hiramatsu & T. Yoshikawa), pp. 183–202. San Diego, CA: Academic Press.

Surai, P. F. & Speake, B. K. 1998 Distribution of carotenoids from the yolk to the tissues of the chick embryo. *J. Nutr. Biochem.* **9**, 645–651.

Surai, P. F., Speake, B. K. & Sparks, N. H. C. 2001 Carotenoids in avian nutrition and embryonic development. 2. Antioxidant properties and discrimination in embryonic tissues. *J. Poultry Sci.* **38**, 117–145.

von Schantz, T., Bensch, S., Grahn, M., Hasselquist, D. & Wittzell, H. 1999 Good genes, oxidative stress and condition-dependent sexual signals. *Proc. R. Soc. Lond.* B **266**, 1–12. (DOI 10.1098/rspb.1999.0597.)

Williams, T. D. 1994 Intraspecific variation in egg size and egg composition in birds: effects on offspring fitness. *Biol. Rev.* **68**, 35–59.

As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.