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Effects of β-carotene on adult immune condition and antibacterial activity in the eggs of the Grey Partridge, *Perdix perdix*

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Abstract

Carotenoids are important dietary constituents in birds. Their functions are numerous and complex, and breeding females are potentially faced with an optimal allocation of these resources between themselves and offspring. We conducted a dietary experiment (low and high supply of β-carotene) to examine the effect of β-carotene on health and immune response of 64 reproducing pairs of Grey Partridge (*Perdix perdix* L.) and on the quality of their eggs, as revealed by the measurement of biochemical components in yolk and albumen, the egg hatching rate and chick survival. We found a beneficial effect of β-carotene on the erythrosedimentation rate and immune response of females (PHA reaction), while the diet did not significantly affect these variables in males. In both sexes, the plasma level of carotenoids was not related to the quantity of β-carotene supplied. A higher quantity of β-carotene in the diet did not induce a variation of egg nutrients (proteins and lipids), nor an increase of yolk β-carotene concentration. We detected a higher concentration of lysozyme, an enzyme with antibacterial activity, in the albumen of eggs laid by females with a high supply of β-carotene. These eggs showed higher hatching rates. The present study indicates that although carotenoid supplementation does not influence blood and yolk carotenoid levels, it results in better immune conditions of females, eventually translated into increased antibacterial activity of the eggs. The broad range of beneficial effects of carotenoids is discussed.

Keywords: β-carotene; Adult health; Immunity; Egg quality; Lysozyme; Maternal effects; *Perdix*

1. Introduction

Models of optimal parental investment require knowledge of the aspects of maternal phenotype that influence propagule quality (Bernardo, 1996). In oviparous animals, maternal fitness is influenced by egg production, since this is an energetically expensive process that can influence future reproductive prospects (Williams, 1975). In turn, egg quality can profoundly influence the fitness of the offspring, so that the distribution of reserves within a clutch can be considered an evolutionarily strategic decision (Mousseau and Fox, 1998).

Carotenoids are important dietary constituents. They play numerous physiological roles in both the laying female and developing embryo, and they are resources that mothers allocate between herself and the progeny. However, factors determining the absorption of carotenoids and their utilization for different functions are numerous and complex, and causal relationships are generally poorly known (Bortolotti et al., 2003; Costantini and Dell'Omo, 2006).

Carotenoids are fat soluble components present in adult females and eggs (yolk) believed to be responsible for maternal effects in birds (Royle et al., 1999; Blount et al., 2002). Aside from their role in pigmentation (Hill et al., 2002), carotenoids are extremely important in a variety of physiological and immunological processes in adult birds (Møller et al., 2000). In a detailed study on the Lesser Black-backed Gull *Larus fuscus* (Blount et al., 2002), a supplemental feeding experiment showed that carotene-fed gulls had higher plasma antioxidant activity and higher concentrations of carotenoids. Interestingly, carotene-fed females also had a low plasma concentrations of immunoglobulins.

Oviparous animals provision their egg yolk with carotenoids. It now appears that these pigments, which give a yellow-red colour to the yolk, play various physiological roles in developing birds, thus suggesting new perspectives on reproductive trade-

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offs in females (Blount et al., 2000). Domestic hen *Gallus domesticus* embryos with high concentrations of maternally derived carotenoids have enhanced antioxidant protection (Surai et al., 2001a,b) and hatchlings show improved lymphocyte synthesis (Haq et al., 1996). Moorhen *Gallinula chloropus* and Grey Partridge chicks fed with high concentrations of carotenoids have a better immune response than controls (Fenoglio et al., 2002; Cucco et al., 2006a). In a study by Blount et al. (2000) the absolute amounts of carotenoids and immunoglobulins covaried with egg size. The β-carotene egg content also covaried with egg size in the Moorhen (Fenoglio et al., 2003). In these cases, egg size could be viewed as a concise parameter of overall egg quality and many studies have shown that heavier eggs produce heavier chicks, giving them a selective survival advantage (Christians, 2002). However, it has also been shown that egg size and egg quality do not necessarily covary. Absolute egg size is not necessarily an accurate guide to egg quality when there are large within-clutch differences (Blount et al., 2002). Indeed, hormone (Schwabl, 1993; Groothuis and Schwabl, 2002), triglyceride (Nager et al., 2000) and lysozyme concentrations (Saino et al., 2002b) may influence fitness-related traits independently of egg size. Lysozyme is a major component of maternal antibacterial immunity, which is transferred to eggs. In the Barn Swallow *Hirundo rustica*, lysozyme activity decreases with hatching failure, suggesting that this is a key maternal substance influencing egg quality. Moreover, a negative relationship between clutch size and lysozyme concentration suggests that females have limited lysozyme production (Saino et al., 2002b).

Some studies showed that dietary supplementation of carotenoids to females resulted in the production of eggs with higher yolk carotenoid concentrations than controls (Blount et al., 2002; Bortolotti et al., 2003; Karadas et al. 2005a, but see Royle et al., 2003). This suggests that concentrations of antioxidants in eggs could be regulated by females and do not simply reflect their abundance in the diet.

In the present research, we used the Grey Partridge, a precocial bird characterized by large clutches, as the study model. We used a food supplementation experiment, with two groups fed with different quantities of β-carotene, to evaluate if more carotenoids in food (a) are translated into more carotenoids in the blood and/or eggs, (b) can improve the immunocompetence of the reproducing adult birds, and (c) can affect the quality of laid eggs. Other than carotenoid yolk concentration, egg quality was also assessed by measuring their size, protein and lipid levels, and the lysozyme concentration in the albumen. Finally, hatchability and survival of chicks hatched from these eggs were assessed.

2. Materials and methods

2.1. Study area and experimental design

The study was conducted on Grey Partridges (*Perdix perdix*), reared in 2002 and 2003 at a breeding farm in S. Giuliano Nuovo, Alessandria, NW Italy (Cucco et al., 2006b). The Grey Partridge is a socially monogamous bird. As a precocial species that lays large clutches (more than 14 eggs, even in captivity), it is a good model for studies of maternal pre-hatching investment. In total, 32 breeding pairs in 2002 and another 32 pairs in 2003 were housed in individual outdoor reproduction cages (4 m long × 1 m wide × 0.5 m high). The birds experienced natural light and temperature conditions throughout the year, and were one year old. From April to June, the hens laid a total of 1040 eggs in 2002 and 1037 eggs in 2003.

The influence of dietary carotenoids on adult health and egg quality was investigated with a β-carotene supplementation experiment lasting 5 months, from late January (about 2 months before the egg laying period) to early July (at the end of the laying period). Before the experimental period, all birds had a rearing diet including a vitamin A supplement of 10,500 I.U./kg. We chose β-carotene because of its known effect on immune condition (Tengerdy et al., 1990; Haq et al., 1995). β-carotene is efficiently converted to vitamin A (Moren et al., 2002). However, since we did not use enzyme blockers or radio-labelled and tracked carotenoids to investigate the direct molecular effects, we do not know the active molecular form in the partridge body. In both years the birds were assigned to two groups raised with different food: the low-carotene group (β−) was fed with a standard partridge diet of cereal pellet plus 2.7 mg/kg of β-carotene, while the high-carotene group (β+) received the same standard food plus 27 mg/kg of β-carotene. The high β-carotene level was chosen to match the values usually utilized in Italian Grey Partridge breeding farms (near the highest value utilized in poultry, with a high safety margin with respect to the NRC recommendation, NRC, 1994; Villamide and Fraga, 1999). The low β-carotene level was one tenth the β+ amount, nearly equal to the minimum nutrient requirement reported by NRC (1994). The rearing food was a powdered mixture, commonly used by aviculturists to provide proper nutrition during egg laying (nutrition facts: protein 19.5%, fat 3.7%, ash 11.5%. Vitamin E addition per kg of food: 50 mg). Each pair had food and water available *ad libitum*.

2.2. Measurements on adults

We measured 8 body, blood and immune response variables before the beginning of the experimental treatment with β-carotene (January) and after the laying of the last egg (July). Body mass was measured with an electronic balance (±0.01 g accuracy) and tarsus length with a calliper (±0.1 mm accuracy).

2.2.1. Haematic and immune characteristics

At the beginning of the experimental period, blood from each parent was drawn from the brachial vein into 75 mm heparinized capillary tubes to measure the erythrosedimentation rate (ES rate) and haematocrit value. The ES rate is diagnostic of many acute and chronic diseases, including infections and rheumatic and inflammatory diseases (Merilä and Svensson, 1995). The ES rate was measured as the ratio between the length of the capillary tube not occupied by blood cells and the total length, after the capillaries stood vertically for 4 h in a refrigerator at 4 °C (Cucco et al., 2002). Haematocrit is an easily measured serological variable, diagnostic of acute and chronic

diseases, bacterial infections, anemia, dehydration or may reflect nutrition deficiencies of some minerals (Rupley, 1997). Blood samples were centrifuged in a portable apparatus for 4 min at 1200 *g*; the haematocrit was expressed as volume of the part of the capillary occupied by blood cells/blood volume in the capillary.

We used the phytohaemoagglutinin (PHA) test to estimate the cell-mediated immune response (Lochmiller et al., 1993). Subcutaneous injection with PHA produces a local inflammation, and its relative thickness (wing-web index) is directly related to the immune condition (Merino et al., 1999). This wing-web index is routinely applied in avian studies and is assumed to be proportional to the intensity of T-lymphocyte cell-mediated immunocompetence (Smits et al., 1999). In late January of each year, we measured the thickness of the wing-web area of the breeding individuals (32 females and 32 males) with a spessimeter (Alpha spa, Milan, Italy, accuracy ±0.01 mm); the birds were then injected with 0.25 mg of PHA (Sigma L-8754) diluted in 0.05 mL phosphate-buffered saline solution (PBS). After 24 h, we re-measured the web thickness at the injection point.

In July, at the end of the breeding season, we re-measured the individual body mass, ES rate, haematocrit and immune response to PHA injection.

2.2.2. Plasma carotenoid levels

To determine the concentration of β-carotene in Grey Partridge plasma, we drew blood from the alar vein of each individual into heparinized microcapillary tubes. We then centrifuged the tubes at 1200 *g* for 4 min, and stored the samples at −20 °C. To extract carotenoids, we thawed the samples to room temperature (light-protected) and used a Reagent kit for HPLC analysis of β-carotene in serum/plasma distributed by Chromsystems diagnostics and Microline sas (code. 32000). 50 μL of an internal standard (code. 32004) was added to 100 μL of plasma in a light-protected reaction vial, then mixed briefly. We added 50 μL precipitation reagent (code 32005), then 200 μL extraction buffer (code 32006), and centrifuged the mixture for 10 min at 12,000 ×*g*. The supernatant was transferred to a light-protected autosampler vial, and 50 μL were injected into the HPLC system (Waters Alliance 2695, Millipore Corp., Bedford, MA, USA) fitted with an RP-18 HPLC column (150 4.6 mm ID; Waters). An isocratic system, using the aforementioned mobile phase for 10 min, was used for analysis at a constant flow rate of 1.5/1.8 mL min^{-1}. The analyte (α-*cis*-β and all-*trans*-β-carotene) was quantified by the inclusion of an internal standard, which was a non-natural carotenoid-derivative, so that a single detection wavelength was required. We confirmed the identity of plasma pigments by comparing their retention times to those of authentic reference carotenoids provided by Roche Vitamins Inc. (*trans*-β-carotene, 8.3 min). Carotenoids were detected at max for each pigment (wavelength 453 nm) using a Waters 2996 photodiode array detector (Waters Chromatography, Milford, MA, USA). The concentration of β-carotene was determined by comparing peak areas (integrated with EMPOWER software) to those of an internal standard (code 32004 retention time, 3.8 min; 453 nm).

2.3. Measurements on eggs

In total, 1040 eggs laid by 32 Grey Partridge hens were collected from April to June 2002, and 1037 eggs laid by another 32 hens were collected in 2003. Eggs were collected the day of laying, individually marked and weighed with an electronic balance (±0.01 g precision). The eggs were also measured (length and breadth with a calliper, ±0.1 mm).

1877 of the 2077 laid eggs were incubated for 26 days in a commercial incubator at 37.5 °C and 60% humidity, while 200 eggs (100 each year) from 40 different females (5 eggs each, specifically the 5th, 7th, 10th, 13th and 16th in the laying sequence) were brought to the lab for chemical analyses. Half of the analysed eggs were from 20 randomly selected pairs of the low-carotene group, and half from 20 pairs of the high-carotene group.

In the lab, the eggs were separated into their constituent parts with a domestic egg separator sieve. Shell, albumen and yolk were carefully weighed with an electronic balance (±0.01 g). Yolk was then homogenized and stored at −20 °C until analysis, while albumen was frozen without centrifugation. Yolk was chemically analysed to assess lipids, proteins, lutein, β-carotene, and total carotenoids concentration. Lipids were extracted with a Soxhlet apparatus, and protein content was assessed with the Kjeldahl method.

Concentrations of yolk β-carotene were measured using high-performance liquid chromatography (HPLC). An aliquot of yolk (0.2–0.5 g) was homogenized in 2 mL of a 1:1 (v/v) mixture of 5% NaCl solution and ethanol, followed by the addition of 3 mL of hexane and further homogenization for 3 min. After centrifugation, hexane was collected and the extraction was repeated twice. Hexane extracts were combined and evaporated under N_2, the residue was dissolved in 1 mL of methanol:dichloromethane (1:1, v/v) and centrifuged, and the supernatant was used for carotenoid determination. β-Carotene was determined by HPLC with a WatersTM Alliance 2695 Modul System (Millipore Corp., Bedford, MA, USA), using a Spherisorb type S3ODS2, 5-m C18, reverse-phase column, 25 mm (Phase Separation, Clwyd, U.K.) with a mobile phase of acetonitrile–methanol (85:15) and acetonitrile–dichloromethane–methanol (70:20:10) in gradient elution using detection by absorbance at 445 nm (Waters 2996 photodiode array detector, Waters Chromatography). Peaks were identified by comparison with the retention times of carotenoid standards and integrated with EMPOWER software.

The concentration of total egg yolk carotenoids was measured spectrophotometrically. Egg yolk samples were mixed with hexane, acetone, toluene and ethanol (10/7/7/6), and centrifuged at 8000 ×*g* for 10 min. In the resulting supernatant, we determined the absorbance of the carotenoids peak at 450 nm using a Beckman Du-640 spectrophotometer. We calibrated carotenoid concentrations (μg mL^{-1}) using standard curves of β-carotene (Sigma).

Lysozyme activity was measured by the method of Osserman and Lawlor (1966): an agar gel with a dried strain of *Micrococcus lysodeikticus* (Sigma), which is particularly sensitive to lysozyme activity, was inoculated with 25 μL of albumen.

Table 1
Adult Grey Partridges

Parameter	Females				Males			
	mean±SD		Statistic		mean±SD		Statistic	
	β+	β−	$F_{1,62}$	P	β+	β−	$F_{1,62}$	P
Beginning of the experimental period								
Mass (g)	379.1±28.1	389.7±26.8	2.42	0.13	380.5±20.6	391.9±27.7	3.36	0.07
Tarsus length (mm)	43.3±1.52	43.8±1.45	2.02	0.16	44.7±1.5	45.3±1.1	3.34	0.07
Haematocrit	37.0±8.4	36.9±12.5	0.01	0.99	38.8±9.3	41.8±5.8	1.45	0.24
ES rate	0.41±0.23	0.46±0.19	3.81	0.06	0.44±0.26	0.44±0.18	0.01	0.97
Immune response (mm)	0.39±0.32	0.38±0.25	0.03	0.87	0.38±0.34	0.37±0.24	0.11	0.74
β-carotene (μg mL^{-1})	20.7±11.4	19.7±15.3	0.04	0.84	13.8±7.7	24.6±27.8	1.54	0.23
End of experimental period								
Mass variation (g)	10.9±41.5	2.6±38.3	0.49	0.49	−10.2±22.6	−12.9±20.6	0.58	0.45
Haematocrit	41.0±5.6	40.4±8.8	0.45	0.50	40.9±5.1	40.3±4.7	0.18	0.67
ES rate	0.46±0.15	0.51±0.12	8.10	0.006**	0.48±0.13	0.48±0.12	0.87	0.35
Immune response (mm)	0.47±0.18	0.27±0.17	16.5	0.001***	0.36±0.23	0.32±0.18	0.32	0.57
β-carotene (μg mL^{-1})	26.5±18.6	36.3±28.5	0.70	0.42	15.4±9.65	28.9±30.6	1.59	0.23

Comparison of mean values of mass and haematological parameters in two groups fed with different quantities. $N=64$ pairs, mean±SD for homogeneous samples are reported. Statistically significant after sequential Bonferroni correction.
** $=P<0.01$.
*** $=P<0.001$.

Standard dilutions of crystalline hen egg white lysozyme (Sigma) (25, 100, 500 and 1000 μg/mL) were run with each group of test samples. The plates were incubated at room temperature (24–26 °C) for 18 h, during which bacterial growth was inhibited in the area of the gel surrounding the albumen inoculation site. The diameters of the cleared zones are proportional to the log of the lysozyme concentration. This area was measured using an *ad hoc* ruler, and converted on a semilogarithmic plot into hen egg lysozyme equivalents (HEL equivalents, expressed in mg mL^{-1}) according to the standard curve.

2.4. Chick survival

The 1877 eggs not utilized for biochemical analyses were incubated in a commercial incubator. On the day of hatching, chicks were individually marked by numbered plastic rings on their legs to allow subsequent identification. The chicks received a standard chicks Partridge diet of cereal pellet, and were raised together for 6 weeks in two heated pens in a nursery room. Growth, immunocompetence and behavioural data were analyzed elsewhere as part of a complementary study on chick behaviour (Cucco et al. 2006a). Here we utilized chick's survival records, assessed by tracking the presence of each chick in the enclosure at least every second day.

2.5. Statistical analysis

The effects of β-carotene treatment on adult characteristics were determined by analysis of variance. The effects of treatment on egg characteristics were ascertained by nested analysis of variance, because eggs laid by the same female may not be independent of each other. Indeed, our experimental design had a nested structure in that all eggs in a clutch pertained to one or the other of two treatments, *i.e.* low or high β-carotene in the parental diet. In the nested models, the effect of clutch was nested within treatment, thereafter indicated as clutch (treatment), and we also included egg position in the laying order as covariate. Statistics were computed by Systat 8.0 (Wilkinson, 1992).

3. Results

3.1. Effect of β-carotene supplementation on adult health

At the beginning of the experimental period, there were no differences in body mass, size, blood ES rate and haematocrit, immune response or plasma carotenoid concentrations between either the females or males assigned to the two groups fed with different concentrations of β-carotene (Table 1).

At the end of the experimental period, there were significant differences in ES rate and immune response between females fed with the different diets, while males did not differ in any variable (Table 1). Females fed with more β-carotene showed a better immune response and lower ES rate than the females of the other group.

3.2. Effect of diet on egg quality

Eggs laid by females assigned to the two groups fed with different β-carotene concentrations did not differ in mass, length, breadth, yolk and albumen weight, and concentration of proteins, lipids, β-carotene and total carotenoids, but were significantly different in lysozyme content (Table 2). Eggs laid by β+ females had a higher concentration of lysozyme (+28.4% difference; β+=3.68±0.17 mg/mL *vs* β−=2.87±0.17 mg/mL).

Clutches laid by β+ or β− females did not differ in laying date (Julian date of the first egg: β+=115.7±11.3 days, $N=32$ pairs;

Table 2
Comparison of eggs laid by Grey Partridges in two groups fed with different β-carotene quantities

	MSS	*df*	*F*	*P*
Mass [a]				
Treatment	3.39	1	0.18	0.68
Clutch (treatment)	19.20	61	62.28	<0.001
Laying order	0.06	1	0.18	0.67
Error	0.31	2007		
Length [b]				
Treatment	63.17	1	1.63	0.21
Clutch (treatment)	38.83	61	36.82	<0.001
Laying order	1.69	1	1.60	0.21
Error	1.06	2009		
Breadth [c]				
Treatment	0.26	1	0.03	0.86
Clutch (treatment)	8.14	61	10.40	<0.001
Laying order	5.38	1	6.86	0.009
Error	0.78	2008		
Yolk weight [d]				
Treatment	1.11	1	2.36	0.14
Clutch (treatment)	0.47	18	3.62	<0.001
Laying order	2.85	1	21.82	<0.001
Error	0.13	79		
Albumen weight [e]				
Treatment	0.38	1	0.44	0.52
Clutch (treatment)	0.87	18	3.09	<0.001
Laying order	4.92	1	17.41	<0.001
Error	0.28	79		
Protein concentration [f]				
Treatment	1.94	1	0.29	0.60
Clutch (treatment)	6.79	20	2.38	0.004
Laying order	29.18	1	10.23	0.002
Error	2.85	76		
Lipids concentration [g]				
Treatment	8.79	1	0.99	0.33
Clutch (treatment)	8.91	20	2.44	0.003
Laying order	157.90	1	43.29	<0.001
Error	3.65	76		
β-carotene concentration [h]				
Treatment	50.39	1	0.57	0.46
Clutch (treatment)	88.90	40	0.94	0.57
Laying order	97.92	1	1.04	0.31
Error	94.27	156		
Total carotenoids concentration [i]				
Treatment	3.23	1	0.59	0.45
Clutch (treatment)	5.48	18	1.57	0.09
Laying order	8.27	1	2.37	0.13
Error	3.49	79		
Lysozyme concentration				
Treatment	0.276	1	5.02	0.03
Clutch (treatment)	0.055	18	2.36	0.005
Laying order	0.530	1	22.89	<0.001
Error	0.023	79		

β−=111.8±11.6 days, N=32 pairs; $F_{1,61}$=1.82, P=0.18 n.s.), but β+ clutches showed higher hatchability (β+=0.60±0.04 rate *vs* β−=0.46±0.04 rate; $F_{1,61}$=5.31, P=0.025). The hatching rate of each clutch was positively related to the mean lysozyme concentration measured in the 5 sampled eggs pertaining to the same clutch (Spearman r_s=0.46, P<0.05; Fig. 1).

After hatching, percent survival of chicks hatched from clutches laid by β+ females did not differ from that of chicks hatched from clutches laid by β- females, both at 10 days of age (Chi-square=0.158, P=0.69 n.s.) and at 21 days of age (Chi-square=0.05, P=0.94 n.s.).

The position in the laying order did not significantly influence egg mass, egg length, β-carotene and total carotenoid concentration, but was significantly related (Table 2) to a decrease of yolk weight, protein and lysozyme concentrations, to an increase of albumen weight and lipid concentration (Fig. 2), and to a small (but still significant) increase of egg breadth (from 27.43±0.10 mm in the first eggs, to 27.89±0.11 mm in the last eggs, a difference of about 1.69% of the mean value). The decrease in lysozyme concentration with laying order was balanced with the observed increase in albumen volume, while such a compensation was not detected for carotenoids, as the concentration was constant while the yolk volume decreased with laying order.

4. Discussion

Our results show that in the Grey Partridge (a typical precocial bird with a large clutch size), carotenoid supplementation improves the health of laying females and also affects the composition of their eggs (lysozyme content) which is correlated to the number of hatchings.

The concentrations of carotenoids in the blood of adults and in the eggs were within the ranges reported in other species (Surai, 2002). Carotenoid concentrations span two orders of magnitude in birds, and may be influenced by sex, age and season (Negro et al., 2001a). The most abundant carotenoid found in our species' eggs was β-carotene, representing about 37% of total carotenoids, the remnant was mainly represented by lutein and zeaxanthin (unpublished data). Remarkably, in our

Notes to Table 2

General linear models with a nested design of mass, length, breadth, yolk weight, albumen weight, protein, lipids, β-carotene, total carotenoids and lysozyme concentration in relation to clutch treatment and position in the laying sequence. The effect of treatment is tested against the error term of clutch (treatment), Zar (1999).

[a] Least squares means±s.e.: β+=14.72±0.02; β−=14.63±0.02 (g).
[b] Least squares means±s.e.: β+=36.58±0.04; β−=36.16±0.04 (mm).
[c] Least squares means±s.e.: β+=27.79±0.03; β−=27.77±0.03 (mm).
[d] Least squares means±s.e.: β+=5.56±0.05; β−=5.77±0.05 (g).
[e] Least squares means±s.e.: β+=6.38±0.08; β−=6.26±0.08 (g).
[f] Least squares means±s.e.: β+=10.24±0.28; β−=9.93±0.26 (%).
[g] Least squares means±s.e.: β+=10.71±0.32; β−=10.04±0.29 (%).
[h] Least squares means±s.e.: β+=7.28±1.08; β−=8.36±1.01 (μg mL^{-1}).
[i] Least squares means±s.e.: β+=10.29±0.26; β−=10.65±0.26 (μg mL^{-1}).

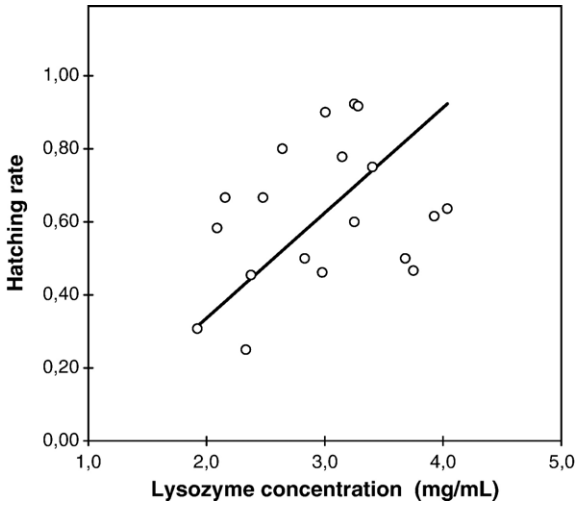

Fig. 1. Mean lysozyme concentration (mg mL^{-1}) and hatching rate of clutches laid by Grey Partridges (regression line calculated by reduced major axis procedure: $Y=0.288\ X-0.239$, $N=19$).

experiment the larger amount of β-carotene in the food did not translate into increased levels in the plasma of adults or in the egg yolk. Several studies reported that carotenoids in the plasma do not accurately reflect their abundance in the diet (Wolf et al., 2000; Negro et al., 2001a; McGraw et al., 2002; Bortolotti et al., 2003). Moreover, selective carotenoid absorption, storage (fat stores: Negro et al., 2001b) or utilization (feather coloration: McGraw et al., 2003) in different body compartments can occur. Since we did not sacrifice the breeders, our study does not clarify whether, or to what extent, the extra carotenoids provided to the high-carotene group were stored in the body.

Concerning the transmission of these substances from mother to eggs, it has been shown that dietary supplementation can increase the yolk carotenoid contents (Surai and Speake, 1998; Bortolotti et al., 2003; Surai et al., 2003; Karadas et al., 2005a,b). In Black-backed Gulls, Blount et al. (2002) showed that dietary supplementation of carotenoids resulted in eggs with increased yolk concentrations, but they also showed (Blount et al., 2001) that yolk enrichment involves physiological discrimination among carotenoids, indicating metabolic transformations and differential transfer from maternal diet to yolk. In the Red-legged Partridge, carotenoid concentrations in yolk vary with the diet only in the early reproductive season (Bortolotti et al., 2003). Nevertheless, it is clear from these studies that the dietary variation in carotenoids is not the only factor that produces individual differences: an important role of sex, age and season was demonstrated (Negro et al., 2001a). Moreover, the acquisition of carotenoids from the diet and their use for both health and display functions seem to be constrained by ecological and physiological aspects linked to the phylogeny and size of the species (Tella et al., 2004).

In our study, the blood and yolk carotenoid levels were unrelated to the diet, but we observed an influence of the carotenoid-rich diet on Grey Partridge females. The reaction to PHA injection and the erythrosedimentation rate indicate an improved immune condition and health state. Recent studies in birds have generally confirmed the positive effects of carotenoids on immune conditions (Olson, 1989; McGraw and Ardia, 2003, 2004; Tanvez, 2004; but see Smith et al. 2006). In our study, a similar beneficial effect was not found in males. A possible

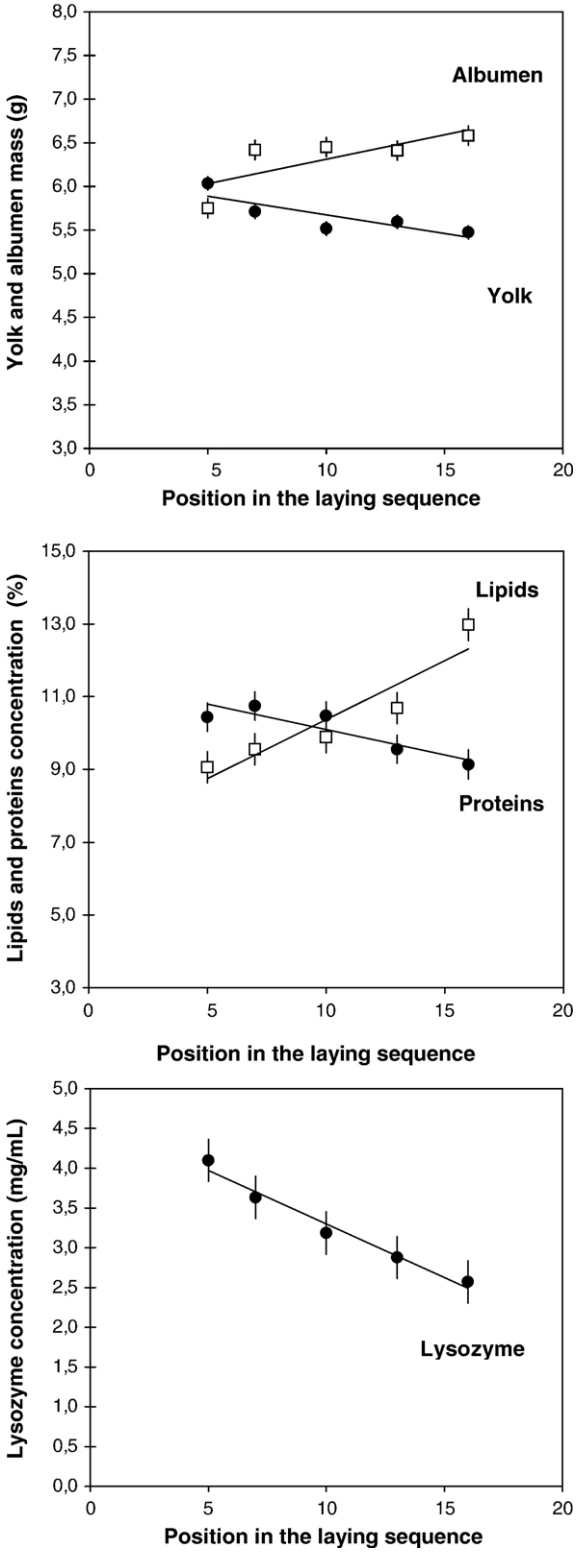

Fig. 2. Yolk and albumen mass (g), proteins and lipids concentration (%), and lysozyme concentration (mg mL^{-1}) in relation to the position in the laying sequence.

explanation could be a less intense metabolic expenditure of males, which are not engaged in egg production. Furthermore, according to Müller et al. (2003), the gender difference is possibly linked to higher plasma levels of testosterone in males, which might have an immunosuppressive effect. However, it has been recently reported that testosterone can increase bioavailability of carotenoids, thus buffering the immunosuppressive effect (Blas et al., 2006). Hence, more studies are needed to better understand male–female differences in immune response (Forbes, 2006) and their possible links to diet.

A role of carotenoids in the development of the bird embryo has repeatedly been shown: domestic hen chicks with high tissue concentrations of maternally derived carotenoids have enhanced antioxidant protection (Surai et al., 2001a,b) and lymphocyte synthesis (Haq et al., 1996). A positive effect of dietary carotenoids on immunity was also demonstrated in recently hatched chicks of the Barn Swallow (Saino et al., 2002a), Moorhen (Fenoglio et al., 2002) and Grey Partridge (Cucco et al., 2006a).

In our study, we observed a substantial increase of lysozyme concentration in the eggs laid by females fed with the enriched diet. The other measured variables (mass, breadth, length, albumen and yolk volume, lipid and protein concentrations), as well as the previously discussed β-carotene and total carotenoid concentrations, did not vary. The lack of difference for these variables in response to dietary differences is similar to what has been reported in several studies (Ricklefs, 1977; Carey, 1996; Blount et al., 2004), and probably indicates that a proximate factor like food abundance or food quality only has an influence within the confines of a large heritable component of egg size (Ricklefs, 1977; Christians, 2002).

To our knowledge, this is the first report of a beneficial effect of maternal carotenoids on an antibacterial activity transmitted from mother to egg, *i.e.* lysozyme. Lysozyme is an antibacterial immune enzyme that digests bacterial cell walls (Sato and Watanabe, 1976), and is considered a reliable indicator of maternal beneficial effects for chick prospects of survival (Saino et al., 2002b). This substance affects egg hatchability and anti-parasite defence and viability of the offspring. At present, we do not know the metabolic link that could lead to enhanced accumulation of lysozyme in eggs when mothers are fed more carotenoids. One possible explanation is that the improved health condition of the females is translated into enhanced egg quality *via* the specific antibacterial activity the mother transfers early in egg formation. However, data on this point are very scanty, the only study available being those of Amar et al. (2004), who showed that lysozyme plasma concentration increased in β-carotene supplemented fishes. Since lysozyme is secreted by leukocytes, Amar et al. (2004) suggested that the augmentation of lysozyme by β-carotene could be *via* its stimulation of phagocytic cells.

Blount et al. (2004) questioned whether diet carotenoids are beneficial for egg production directly, by stimulating the synthesis and allocation of pigments in the egg, or indirectly *via* effects on maternal health. In our study, a beneficial effect of maternal health was found, even though mothers did not increase carotenoid abundance in their eggs. However, their eggs appear to be of better quality since they have higher lysozyme content, a factor that covaries with hatching rate.

Detailed investigations of intraclutch variation of egg components in different species have reported a variety of relationships between egg size or components and laying sequence (Kennamer et al. 1997; Lessells et al., 2002). Generally, egg characteristics vary with laying order according to species-specific patterns, *e.g.* increasing, decreasing, or increasing up to the middle egg and decreasing thereafter (Aparicio, 1999). In the Grey Partridge, we found an increase of albumen and lipids and a decrease of yolk, proteins and lysozyme with laying sequence. The adaptive value of this pattern is largely an open question, which deserves long-term studies and experimental designs. Our data indicate that there is a sort of compensation between yolk decrease and albumen increase with laying sequence, so that the total weight of eggs is constant through the entire clutch. As suggested by Kennamer et al. (1997), in precocial species a larger amount of yolk may be necessary in the first laid eggs to allow the development of embryos for the longer period before clutch completion and the final synchronous hatching of all eggs. Finally, the negative relationship between position in the laying order and lysozyme concentration is in line with the suggestion of Saino et al. (2002b) that females have limited lysozyme production and the decline simply reflects exhaustion of maternal lysozyme; however, it also agrees with the adaptive interpretation that first laid eggs are more vulnerable to infections because they remain in the nest longer than last laid ones.

In conclusion, the results of our study on β-carotene supplementation enlarge the known range of beneficial effects of carotenoids (Surai, 2002), stressing their indirect role on female health and antibacterial activity of the eggs, and suggest the need of comparative studies and more detailed information on proximate causes and ultimate effects.

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