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Effects of maternal carotenoid availability in relation to sex, parasite infection and health status of nestling kestrels (*Falco tinnunculus*)

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SUMMARY

Mothers can strongly influence the development of their offspring, and if maternal resources are limited, they may influence optimal reproductive strategies. In birds, maternally deposited carotenoids are a prominent component of egg yolk and are vital for the development of the embryo. However, results of long-lasting fitness consequences of this early nutritional environment have been scarce and inconsistent. In addition, sex-biased sensitivity to different egg components is one of the mechanisms postulated to account for sex-linked environmental vulnerability during early life. However, this important aspect is usually not accounted for when investigating maternal investment in carotenoids. In this study we gave carotenoid (lutein) supplements to female Eurasian kestrels (*Falco tinnunculus*) before and during egg laying. The experiment increased female plasma carotenoids, but this effect was not apparent in hatchling and fledgling plasma carotenoid concentration. Also, results showed that carotenoid supplementation increased the high density lipoprotein to low density lipoprotein ratio in adult females, suggesting that dietary carotenoids may influence lipid metabolism. Furthermore, the effect of the treatment was manifested in several nestling health state parameters. Nestlings of carotenoid-supplemented females were infested by less intestinal parasite groups, had higher lymphocyte concentrations in blood plasma, and were less stressed (heterophile to lymphocyte ratio) than control nestlings. In addition, an interaction between the experimental treatment and nestling sex was apparent for globulin concentrations, favouring the smaller male nestlings. Thereby, suggesting that males benefited more than females from an increase in maternal carotenoid investment. Our study shows that an increase in carotenoids in the maternal diet during egg laying favours nestling development in kestrels, and may also affect nestlings in a sex-specific way.

Key words: antioxidants, maternal effects, lipoproteins, globulins, heterophile, lymphocyte, nematodes, coccidia

INTRODUCTION

Offspring phenotype is not only determined by genetic inheritance, but can also be influenced by the mother affecting the environment in which genes will be expressed. Such maternal effects can have profound effects on an individual's fitness, and if maternal resources are costly to produce or acquire, they may influence optimal reproductive strategies (Lindström, 1999; Metcalfe and Monaghan, 2001; Mousseau and Fox, 1998; Naguib and Gil, 2005; Qvarnström and Price, 2001). In birds, the developing embryos rely completely on maternally derived nutrient deposited in the eggs. Egg yolks are typically rich in carotenoids that belong to the family of antioxidant pigments. There is a growing body of evidence to show that they play a fundamental role for avian development. Egg yolk largely consists of highly unsaturated fatty acids that nourish the developing embryo, but concurrently make embryo tissues vulnerable to free radical attack (Surai et al., 1998; Surai, 1999). Antioxidants, such as vitamins A and E, and carotenoids enhance the overall antioxidant capacity available to the embryo and hatchling (Blount et al., 2000; Edge et al., 1997; Karadas et al., 2005; Surai et al., 2003; Surai et al., 1999; Surai and Speake, 1998). Carotenoids are also directly involved in the development and activation of the immune system (Alonso-Álvarez et al., 2004; Blount et al., 2003a; Fitze et al., 2007; Koutsos et al., 2003; McGraw and Ardia, 2004; Møller et al., 2000) and are known to be involved in numerous other physiological processes in animal tissues (Krinsky, 1994; Møller et al., 2000; Olson and Owens, 1998; Rock, 1997). Moreover, recent experimental work showed that early-life exposure to carotenoids (either in yolk or the diet) has long-lasting effects on the assimilation and efficient use of carotenoids (Blount et al., 2003a; Karadas et al., 2005; Koutsos et al., 2003).

However, animals are unable to synthesize carotenoids *de novo*, and their availability in food is often limited (e.g. Hill, 1990; Hill, 1992). Therefore they make a good candidate for mediating life-history trade-offs. The availability of carotenoids in birds may be compromised by challenges to an individual's health, as is shown by associations with condition (Bortolotti et al., 1996; Hill and Montgomerie, 1994), exposure to parasites (Alonso-Álvarez et al., 2004; Figuerola et al., 1999; Hõrak et al., 2004a; Møller et al., 2000), environmental contamination (Camplani et al., 1999; Eeva et al., 1998) and carotenoid-based coloration (e.g. Arriero and Fargallo, 2006; Blount et al., 2003b; Fitze et al., 2003; Fitze et al., 2007; Martínez-Padilla et al., 2007; Møller et al., 2000). Birds fighting infections also often have reduced levels of carotenoids in plasma and in integument (Alonso-Álvarez et al., 2004; Hõrak et al., 2004b;

Hõrak et al., 2007; Martínez-Padilla et al., 2007; Saino et al., 1999). The transfer of carotenoids into yolk may therefore have to be traded against their allocation to maternal maintenance, what makes carotenoid availability of fundamental importance during reproduction for laying females (Blount, 2004). Actually, egg-laying capacity has been shown to be limited by carotenoid availability (Blount et al., 2004), and Bertrand et al. (Bertrand et al., 2006) recently demonstrated that carotenoid availability can modulate the trade-off between egg production and resistance to oxidative stress. Also, as expected, experimentally increased dietary intake of carotenoids before laying always increased the concentration in egg yolk, not only in captive birds (e.g. Bortolotti et al., 2003; Surai and Sparks, 2001; Surai and Speake, 1998), but also in several species of natural bird populations (Berthouly et al., 2007; Biard et al., 2005; Blount et al., 2002a; Blount et al., 2002b; Ewen et al., 2006; McGraw et al., 2005; Remeš et al., 2007; Royle et al., 2003), suggesting that carotenoid availability might be generally limited for breeding females.

Furthermore, prenatal developmental conditions may affect sons and daughters differentially, resulting in sex-biased mortality and/or sex-specific effects on offspring phenotype influencing offspring fitness (e.g. Müller et al., 2005). In general, the more susceptible sex will have the greater variability in reproductive success and thus provide higher fitness returns in favourable environments. Therefore, mothers may increase their own reproductive success by investing more into the sex that provides the greater fitness return under the given environmental conditions (e.g. Badyaev et al., 2002; Sheldon et al., 1998; Trivers and Willard, 1973). Sex-specific vulnerability has been attributed to different nutritional requirements and thereby different vulnerability to poor nutritional conditions (Gorman and Nager, 2004; Griffiths, 1992; Torres and Drummond, 1997) (reviewed by Fargallo et al., 2006), but is also affected by sexual differences in gonadal hormone production, and the sex-specific organization of physiology, endocrinology, immunity and postnatal behaviour (Balthazart and Adkins-Regan, 2003). In fact, different vulnerability to yolk concentrations of, for example, testosterone and/or corticosterone have recently been detected in different species (Love et al., 2005; Müller et al., 2005; Naguib et al., 2006). However, to our knowledge, only one study on a natural bird population evaluated possible sexual differences in the impact of maternally transmitted carotenoids on offspring phenotype, which found that great tit (*Parus major*) male hatchlings from carotenoid-supplemented mothers were heavier than their sisters (Berthouly et al., 2008).

Evidence of the effect of an increase in maternally transmitted carotenoids on offspring performance in wild bird populations is very scarce and inconsistent up to now. Although a positive effect of yolk carotenoids on nestling tarsus length was found in blue tits (*Cyanistes caeruleus*) (Biard et al., 2005), this was found not to be the case in the closely related great tits (Remeš et al., 2007), norin barn swallows (*Hirundo rustica*) (Saino et al., 2003a). A beneficial effect of maternally transmitted carotenoids on cellular immunity was only detected in barn swallows (Saino et al., 2003a), whereas results with great tits are confusing (Berthouly et al., 2007; Remeš et al., 2007). Effects on hatching and fledgling success were only studied on zebra finch (*Taeniopygia guttata*) populations in captivity, one finding a positive effect (McGraw et al., 2005), but in the other no such effect was apparent (Bertrand et al., 2006). These minor effects of an increase in yolk carotenoids on offspring performance might be explained by high natural abundance of carotenoids or other antioxidants in the adult and nestling diet. Additionally, conflicting results of different studies may be explained by species-specific features of their life-histories. Therefore, it might be interesting to study carotenoid supplementation in species where carotenoid availability is typically limited in the diet. In this sense, raptors are a good model because as carnivores they are predicted to have difficulty in acquiring carotenoids (Olson, 2006; Olson and Owens, 1998; Tella et al., 2004).

In this study, we explore sex-related effects of an increase in carotenoid availability before egg laying on nestling health status in Eurasian kestrels (*Falco tinnunculus* Linnaeus 1758), a sexually dimorphic raptor in which adult females are 20% heavier than adult males (Massemin et al., 2000). In this small raptor, there may be a potential trade-off between diet quality and quantity, since carotenoid pigment coloration was associated with the consumption of invertebrates but not voles in adult Eurasian kestrels (Casagrande et al., 2006), and in the closely related adult American kestrels (*Falco sparverius*) carotenoid levels in birds nesting in territories with more voles were lower (Bortolotti et al., 2000). We supplemented a group of females with dietary carotenoids (xanthophylls) 3 weeks before the start of egg laying until clutch completion, maintaining another group as control. We evaluated the effect of a pre-laying supplementation with carotenoids on female, hatchling and fledgling circulating carotenoids. Furthermore, we evaluated the effect on body condition and health status in females and fledglings by means of a series of haematological condition parameters [albumin and globulin concentrations, albumin to globulin ratio (nutritional status), lymphocyte and heterophile concentrations, heterophile to lymphocyte ratio (stress indicator), high-density lipoprotein (HDL – good cholesterol lipoprotein) to low-density lipoprotein (LDL – bad cholesterol lipoprotein) ratio]. In addition, we evaluated the intestinal parasite burden diversity in nestling faeces and the nestling cell-mediated immunity (CMI) using the phytohaemagglutinin (PHA) skin test, which has become a standard method of assessing cell-mediated components of the immune response in birds (Smits et al., 1999; Tella et al., 2002).

As was stated above, according to the role that carotenoids play during embryonic development, we expect that the carotenoid treatment of the mothers will benefit the development of the different physiological systems of their chicks, over those of the control group.

With respect to a potential sex-related effect, previous studies showed that especially under conditions of food scarcity, male kestrel nestlings, the smaller sex, have lower competitive ability (Fargallo et al., 2003) and thereby produce a lower cell-mediated immune response than their female nest-mates (Fargallo et al., 2002; Fargallo et al., 2003; Fargallo et al., 2007). For that reason, here we predict male nestlings to be more stressed compared to female nestlings [higher heterophile to lymphocyte (H:L) ratio (e.g. Laaksonen et al., 2004; Moreno et al., 2002; Ots et al., 1998)]. In addition, Berthouly et al. (Berthouly et al., 2007) found that during increased stress situations early in life, maternally transmitted carotenoids seem to be more important for the development of a competent cellular immunity in nestlings than their overall nutritional status. It can be expected that male kestrels, facing a more stressed situation during the nestling phase, may be more susceptible to a change in the micro-nutritional developmental environment than females; and thereby could benefit more from an increased maternal carotenoid investment. In that case, these benefits should be reflected in better health state parameters in male nestlings enjoying an increase in maternally transmitted carotenoids compared to control males.

MATERIALS AND METHODS

Study area and studied species

The study was conducted during the breeding season of 2005 in the Campo Azálvaro region (40°40′N, 4°20′W), a homogeneous mountain grassland area in central Spain (1300 m above sea level). Nest boxes have been installed in the area progressively since 1994, and the breeding population has been followed up since then (Fargallo et al., 2001). During the breeding season of 2005, the study area had 65 nest boxes and 45 kestrel pairs started breeding. Laying date differs among years, but usually starts in the second fortnight of April. Kestrel females lay one egg every 2 days (Aparicio, 1994), and mean clutch size in the population is five (range three to six eggs) (Fargallo et al., 2001). Females are responsible for incubation and the feeding of newly hatched young, while males are the main food providers to females and chicks from pre-laying to fledgling independence (Village, 1990; Tolonen and Korpimäki, 1994) (P. Vergara and J. A. Fargallo, in press).

Data collection and experimental protocol

We performed a food supplementation experiment prior to laying, starting on the 4th April, which was 25 days before the first egg was found in the population, thereby covering more than enough the period required for egg formation in this species (9 days) (see Meijer et al., 1989). During the same breeding season of 2005, simultaneous observational studies were performed during the pre-laying period in relation to agonistic behaviour (Vergara and Fargallo, 2007; Vergara et al., 2007). Those observations started before the carotenoid supplementation experiment, and the day the experiment started, 41 of the finally 45 (91%) breeding pairs were established. To each nest box we assigned an experimental treatment (carotenoid *vs* control) in an alternating way, so that treatments were randomly distributed over the study area. We used 1/8 of farmed Japanese quail (*Coturnix c. japonica*; 15 g) to administer the carotenoids, and the same amount of quail without carotenoids was supplied to the control group. It is known from previous studies that kestrels absorb predominantly lutein, which is an oxygenated carotenoid, i.e. a xanthophyll (Casagrande et al., 2006). These carotenoids were kindly supplied by Kemin Foods (Herentals, Belgium; OroGlo Layer Dry 20) in the form of a dietary supplement made of crystalline lutein derived from marigold flowers. Xanthophyll concentration in the product was 1.8% lutein (0.2% isomers) in free alcohol forms readily available for absorption (Biard et al., 2005; Blount et al., 2002b). We treated each piece of quail with a mix of 0.2 ml of vegetable oil and 5 mg of carotenoids, and control pieces were treated with 0.2 ml of oil only. Nest boxes were visited every 2 days, and the quail piece was left in the nest box according to the given treatment. All pieces disappeared every two days. In kestrels, once the pairs are established, females usually remain in the territory while males go out hunting to feed them (Village, 1990), so that food supplements placed in the nest boxes were most likely consumed by the females (Martínez-Padilla and Fargallo, 2007).

At the beginning of the food supplementation, it is likely that some control pairs ate some carotenoid supplemented quails. We did not eliminate carotenoids from the diet of control pairs, which access these components also through their natural diet; but we increased carotenoid availability for females in the carotenoid group because as the pre-laying period advances, females stay more and more time in the nest surroundings and they are very aggressive against other females in nest proximities (Vergara and Fargallo, 2007; Vergara et al., 2007).

Food supplementation continued until 4 days after clutch completion, and the subsequent day we captured females at the nest box while incubating. In that way we could compare female parameters at the same stage of their breeding period. We measured body mass and structural size (wing, tail and tarsus length) and took a blood sample (1 ml) from the brachial vein. The blood was placed in a 1.5 ml Eppendorf tube containing EDTA buffer. Two smears were made immediately after extraction and were fixed with absolute ethanol within 12 h. All blood samples were stored in a cooling bag in the field, and when back in the lab, plasma was separated from blood cells by centrifugation and stored at –20°C upon analysis. Blood cells were conserved in absolute ethanol for analysis of nestling sex.

Nests were visited daily when hatching date was reached. Blood samples (50–100 μl) were taken from hatchlings (0–1 days old) by means of brachial vein puncture with a heparinized capillary, and the birds were then weighed and marked with indelible ink. Nestlings were then remarked every 5 days until banding. Nestling kestrels fledge when they are about 31 days old (range 25–36 days, N=15) (Bustamante, 1994). When nestlings reached the age of 25 days, we took fledgling body measurements and a blood sample (1 ml extracted from the brachial vein with a syringe). Blood samples of hatchlings and fledglings were handled in the same way as previously described for females. In addition, we used a phytohaemagglutinin-P (PHA) injection assay to evaluate *in vivo* the combined responses of T-cells, cytokines and inflammatory cells (cell-mediated immunity, hereafter CMI). Kestrel chicks were injected intradermally in the wing web with 0.3 mg of PHA dissolved in 0.1 ml of phosphate-buffered saline (see Fargallo et al., 2002). During the handling of nestlings, a faecal sample was taken from each one.

Laboratory analyses

Sex determination

The sex of chicks was determined from blood samples with molecular methods as described previously (Fridolfsson and Ellegren, 1999) applied on kestrels (Fargallo et al., 2002).

Intestinal parasite burdens

Fresh faecal samples from each fledgling were examined by the direct flotation method using a zinc sulphate solution to identify Nematoda (genera *Espiurida*, *Capillaria* and *Porrocaecum*) and Cestoda (Clyde and Patton, 2001; Greiner and Ritchie, 1994). For the coccidian genera, we sporulated the oocysts with 2.5% potassium dichromate for 14 days for proper identification of *Eimeria*, *Caryospora* and *Isospora* (Forbes and Fox, 2005).

Leukocyte count

White blood cell count (WBC) was determined with an improved Neubauer hemocytometer and Natt & Herrick solution in white cell dilution pipettes. Blood smears were stained with Diff-Quick stain, and 400 leukocytes were differentiated on each smear (Campbell, 1995; Wernery et al., 2004). The total count of heterophiles and lymphocytes was determined as the percentage of heterophiles (or lymphocytes, respectively) of the total leukocyte count of the blood smear multiplied by the WBC.

Lymphocytes are immune cells that assist in the recognition and destruction of many types of pathogens and a decreased lymphocyte number likely signals stress-induced immunosuppression and higher susceptibility to viral infections (Hõrak et al., 1998; Ots et al., 1998). By contrast, heterophiles are phagocytosing cells and their concentration increases during inflammatory processes, stress and

infections [Coles in Kilgas et al. (Coles, 1997; Kilgas et al., 2006a)]. The heterophile to lymphocyte (H:L) ratio is often used as a stress estimator in domestic and wild birds (Maxwell and Robertson, 1998; Moreno et al., 2002; Ots et al., 1998; Ots and Hõrak, 1996), including kestrels (Laaksonen et al., 2004; Martínez-Padilla et al., 2004), and was also recently shown to be related to survival probability in adult great tits (Kilgas et al., 2006b).

Plasma analyses

Plasma carotenoid concentration

The system employed is based on that used by Negro and Garrido-Fernández (Negro and Garrido-Fernández, 2000). In each plasma sample, the carotenoid pigment content was analyzed in duplicate. For each replicate, 20 μl of plasma was taken and placed into an Eppendorf tube containing 80 μl of acetone (HPLC grade). The closed tube containing the mixture of sample and acetone was vortexed for 1 min and later sonicated in an ultrasonic bath for 1 min. Finally the sample was centrifuged for 5 min at 17500 ***g***. The system of separation used was that described by Mínguez-Mosquera and Hornero-Méndez (Mínguez-Mosquera and Hornero-Méndez, 1993) for carotenoid pigments in fruits and lately used for carotenoid pigments separation from plasma (Negro et al., 2002). The method uses a reverse-phase column (Spherisorb ODS2) of 25 cm in length, 0.46 cm internal diameter, and with a particle size of 5 μm. Separation was performed using an acetone-water binary gradient with a flow of 1.5 ml min^{-1}. The volume of sample injected was 20 μl, and detection was performed at 450 nm using a fixed-wave UV–visible detector. Carotenoid pigments were identified by comparing their retention time and spectral data under the elution conditions with those obtained using pure standards (Goodwin, 1976; Mínguez-Mosquera and Hornero-Méndez, 1993). The individual concentrations of the carotenoid pigments in plasma samples were determined by comparing the area of each peak in the chromatogram with the areas of the calibration curve obtained using pure standards (Fig. 1). Results (in mg l^{-1}) are presented as the average of two measurements.

Plasma protein and lipoprotein electrophoresis

Plasma protein and lipoprotein electrophoresis fractions were determined on commercial agarose gels [Hydragel Protein (E), Sebia Hispania S.A., Barcelona, Spain] using a semi-automated Hydrasys System (Sebia Hispania S.A., Barcelona, Spain) with manufacturer's reagents to determine the concentration of albumin and globulins (α-, β- and γ-globulins) of the kestrels (Vergara et al., 2008) and the percentage of high density lipoprotein (HDL) and low density lipoprotein (LDL). Total plasma proteins were determined by the Biuret method (Lumeij and McLean, 1996), and plasma protein concentrations (g dl^{-1}), which were used in the analyses, were calculated by multiplying each protein fraction by the total protein value. From these values, the albumin:globulin ratio was computed as an index of general health (Cray and Tatum, 1998).

Plasma proteins provide important transport, immune and energy functions (Hõrak et al., 1998; Hõrak et al., 2002; Ots et al., 1998). Albumin is the largest single fraction of protein in the blood, and is generally considered a robust indicator of nutritional condition (Hõrak et al., 2002; Jenni-Eiermann and Jenni, 1998). A decrease in albumin concentration in the blood plasma accompanies almost any diseases and malnutrition (Jenni-Eiermann and Jenni, 1998; Ots et al., 1998). Furthermore, an increase in globulin concentration is expected after chronic or acute infections with inflammatory processes, and therefore, healthier individuals have a higher albumin to globulin ratio (Ots et al., 1998), which was also found to be related to survival probability in a natural population of adult great tits (Kilgas et al., 2006b).

Lipoproteins in the blood, a water-based medium, carry triglycerides, cholesterol and carotenoids around the body, and generally, in healthy individuals the LDL fraction is low and HDL fraction high (Krinsky, 1994; Rock, 1997).

Statistical analyses

The effect of the treatment on female plasma carotenoid composition and concentrations was investigated using a MANOVA with the general linear model (GLM). The initial models concerning females always included laying date and clutch size as covariates in order to account for possible seasonal variation and differences in reproductive investment. Other reproductive and female variables were analysed with ANOVA (GLM procedure).

General mixed linear models (GLMM) were used with the proc mixed (SAS 1989-96 Institute Inc., Cary, NC, USA) procedure to analyse nestling characteristics and generalized linear mixed models (GLIMMIX procedure) to analyse the presence or absence (binomial error, link=logit) of intestinal parasites (Littell et al., 1996). The initial models testing the effect of treatment (fixed effect) on nestling characteristics always included the fixed factor sex and the covariates brood size and hatching date, in order to account for seasonal variation, sibling competition and sexual differences and/or dimorphism. In addition, the interaction sex*treatment tested if the effect of the treatment was similar in both sexes. Nest-identity (nested within treatment) was included as a random factor in order to account for random variation due to the nest. Furthermore, the interaction nest-identity*sex was included as a second random factor. In this way pseudo replication was avoided since in each nest two blocks according to each sex were considered as independent points and not each individual nestling (see degrees of freedom from analyses). However, in some cases the interaction did not account for any variation, and the model then used nestlings as independent cases.

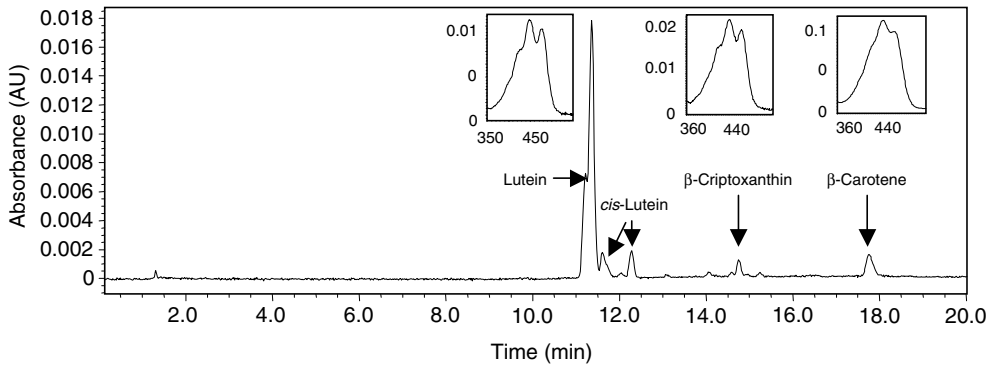

Fig. 1. Chromatogram of carotenoid profile in plasma of Eurasian kestrels. Insets show the UV–vis spectra (absorbance *vs* wavelength nm) of the eluted carotenoids. AU, absorbance units.

Table 1. Reproductive variables in carotenoid-supplemented and control kestrel breeding pairs

	Laying date (1=1 April)	Clutch size	% Hatching success	% Fledgling success
ANOVA	$F_{1,40}$=1.40, P=0.08	$F_{1,40}$=0.38, P=0.54	$F_{1,36}$=0.05, P=0.82	$F_{1,34}$=0.64, P=0.43
Control	9±1 May (22)	4.8±0.2 (22)	92.0±3.1 (19)	95.9±3.1 (17)
Carotenoid	6±1 May (20)	5.0±0.1 (20)	92.8±2.2 (19)	92.5±2.8 (19)

Values are mean ± s.e.m. (*N*).

Interactions and main effects were sequentially dropped from the model when not significant. Models were compared with the Akaike's information criterion (AIC), and the most parsimonious was retained (lowest AIC) (Burnham and Anderson, 1998). Degrees of freedom for type 3 tests of fixed effects were calculated using the Satterthwaite method. Tests of the residuals for normality and homoscedasticity were used to check the validity of the model, and dependent variables were transformed to meet these constraints if needed. The means of least square means between treatments or sexes were compared with adjusted P values using the Scheffé method.

Values are reported as means ± s.e.m.

RESULTS

Of the 45 kestrel pairs that started breeding, 38 produced hatching (19 control and 19 carotenoid). In total, 155 eggs hatched, and we were able to blood sample 150 hatchlings between 0 and 1 day of age. Fledging was reached in 36 nests (17 control and 19 carotenoid, 149 fledglings). We have information on carotenoid analysis in 103 fledglings, of protein electrophoresis and HDL and LDL cholesterol in 147 fledglings, of leucocyte counts in 149 fledglings, and of intestinal parasite burden in 131 fledglings. The loss of some chicks was due to predation on breeding adults by an eagle owl *Bubo bubo* (observation in the field by J.A.F., P.V. and L.D.N.).

We were able to capture and obtain all measurements from 34 females, 5 days after clutch completion (18 control and 16 carotenoid).

The breeding pairs of the carotenoid group tended to start breeding earlier, but clutch size, egg size, hatching success (% eggs hatched) and fledging success (% hatchlings fledged) did not differ between the two groups (Table 1).

Breeding females

Females did not differ in body mass (GLM $F_{1,30}$=0.32, P=0.57; carotenoid, 244.1±4.2 g; control, 246.1±3.9 g), tarsus length (GLM $F_{1,30}$=0.04, P=0.84; carotenoid, 49.63±0.43 mm; control, 49.75±0.37 mm) or wing length (GLM $F_{1,30}$=0.78, P=0.38; carotenoid, 25.39±0.17 cm; control, 25.17±0.16 cm) between treatments. The covariables laying date and clutch size were not significant in any case (all P>0.19).

Female plasma carotenoids consisted mainly of lutein, with small proportions of β-carotene, *cis*-lutein and β-criptoxanthin (Table 2). The pre-laying carotenoid supplementation significantly affected female plasma carotenoid concentrations (MANOVA: Wilk's lamda=0.69, $F_{4,27}$=3.08, P=0.032, N=34), whereas laying date and clutch size were not significant (P>0.8). Univariate results showed that the carotenoid supplementation mainly increased the concentration of female plasma lutein ($F_{1,30}$=12.51, P=0.001), but also β-carotene ($F_{1,30}$=5.20, P=0.029), *cis*-lutein ($F_{1,30}$=6.89, P=0.013) and β-criptoxanthin ($F_{1,30}$=4.66, P=0.034) were significantly increased (Fig. 2).

The supplement also significantly affected circulating lipoproteins. The HDL:LDL ratio was higher in carotenoid-supplemented females compared to control ones ($F_{1,30}$=10.69, P=0.0026; carotenoid, 2.12±0.12; control, 1.54±0.14). The covariables laying date and clutch size were not significant (all P>0.12).

The carotenoid supplement did not significantly affect plasma protein titres (all P>0.18) or white blood cell counts (all P>0.32). Laying date and clutch size did not significantly affect these variables (all P>0.38).

Hatchlings

Hatchling mass was negatively affected by laying date (GLMM $F_{1,35.9}$=9.99, P=0.0032) and marginally negatively by clutch size (GLMM $F_{1,36}$=3.55, P=0.067). Controlling for sex, no significant between-group differences were observed in hatchling body mass, or the interaction treatment↔sex (P>0.78).

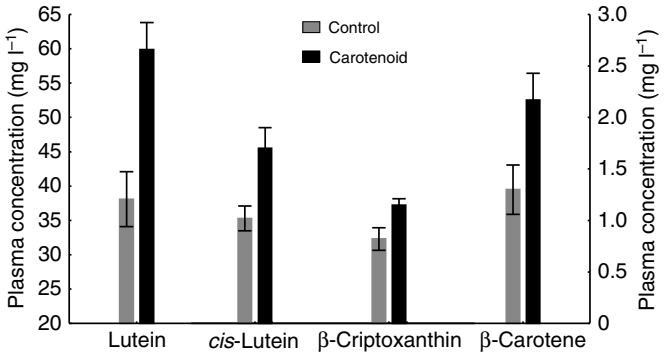

Fig. 2. Plasma carotenoid concentrations in females supplemented with carotenoids and in control females. Results of GLM statistical analysis are given in the text. The left-hand *y*-axis shows lutein concentration and the right-hand *y*-axis shows the concentration of the other carotenoids.

Table 2. Concentrations of detected carotenoids in plasma of hatchlings, fledglings and adult females

	Concentration (mg l^{-1})				
	Lutein	*cis*-Lutein	β-Criptoxanthin	β-Carotene	% Lutein
Hatchlings	6.5±1.9	0.1±0.09	0.008±0.05	0.008±0.1	99.8±0.3
Fledglings	22.2±2.1	0.6±0.1	0.5±0.05	0.4±0.1	94.1±0.4
Adult females	47.8±3.3	1.3±0.1	1.0±0.1	1.7±0.2	92.6±0.4

Hatchlings: *N*=38 nests, 150 hatchlings; fledglings: *N*=36 nests, 103 fledglings; adult females: *N*=35.

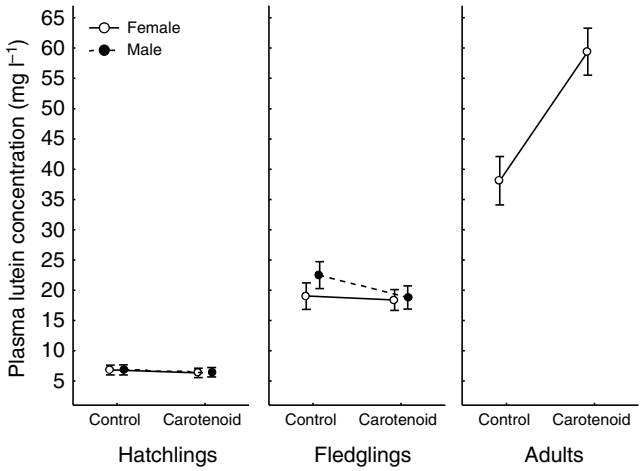

Fig. 3. Plasma lutein concentrations (mean ± s.e.m.) in hatchlings, fledglings and breeding females in control and carotenoid-supplemented groups. Filled circles, males; open circles, females.

Overall hatchling plasma carotenoids consisted of 99.8±0.3% lutein (Table 2), and in 95% of the hatchlings, it was 100% lutein. Therefore, we explored the possible effect of the food supplementation treatment on lutein titres only. None of the variables included in the initial model explained significant variation in hatchling lutein concentration (GLMM all $P>0.36$, treatment $F_{1,35.9}=0.21$, $P=0.65$; see Fig. 3).

Fledglings

Body size measurements and cell-mediated immunity

The treatment did not affect fledgling body mass (GLMM $F_{1,32.6}=0.02$, $P=0.89$; Fig. 4A), tarsus length (GLMM $F_{1,32.3}=0.02$, $P=0.88$, Fig. 4B) or wing length (GLMM $F_{1,32.3}=0.02$, $P=0.88$; Fig. 4C). In the model with CMI as a dependent variable, we also included nestling body mass as covariate (Fargallo et al., 2002). Only nestling body mass was retained in the model ($F_{1,142}=13.44$, $P=0.0003$). Treatment, sex, or their interaction did not significantly affect nestling CMI (GLMM all $P>0.14$; Fig. 4D).

Plasma carotenoids

Fledgling plasma carotenoids consisted of 94.1±0.4% lutein (Table 2) and laying date correlated negatively with lutein plasma concentration (GLMM $F_{1,30.3}=6.49$, $P=0.016$), and showed a positive non-significant correlation with tarsus length (GLMM $F_{1,98.7}=3.68$, $P=0.058$). The experimental treatment did not explain significant variation in plasma lutein concentration in fledglings (GLMM $F_{1,31.3}=0.71$, $P=0.40$; see Fig. 3) and there were no differences between the sexes (GLMM $F_{1,86.3}=0.94$, $P=0.33$; males, 20.45±1.45; females, 19.08±1.38; Fig. 3).

Furthermore, *cis*-lutein was negatively explained by laying date (GLMM $F_{1,30.6}=10.48$, $P=0.003$), but no variables included in the initial model explained significant variation in β-criptoxantin or β-carotene concentrations in fledglings (all $P>0.23$).

Plasma proteins

The total globulin concentrations were negatively correlated with fledgling body mass (GLMM $F_{1,80.1}=13.22$, $P=0.0005$) and the interaction treatment↔sex also explained significant variation (GLMM $F_{3,47.4}=5.14$, $P=0.0037$; Fig. 5A). In the control group, males and females did not differ in globulin levels (*post-hoc* Scheffé test: $P=0.98$), whereas in the carotenoid group, males showed lower globulin levels than females (*post-hoc* Scheffé test: $P=0.004$; Fig. 5A). Males did not significantly differ in globulin levels between the carotenoid and control group (*post-hoc* Scheffé test: $P=0.17$).

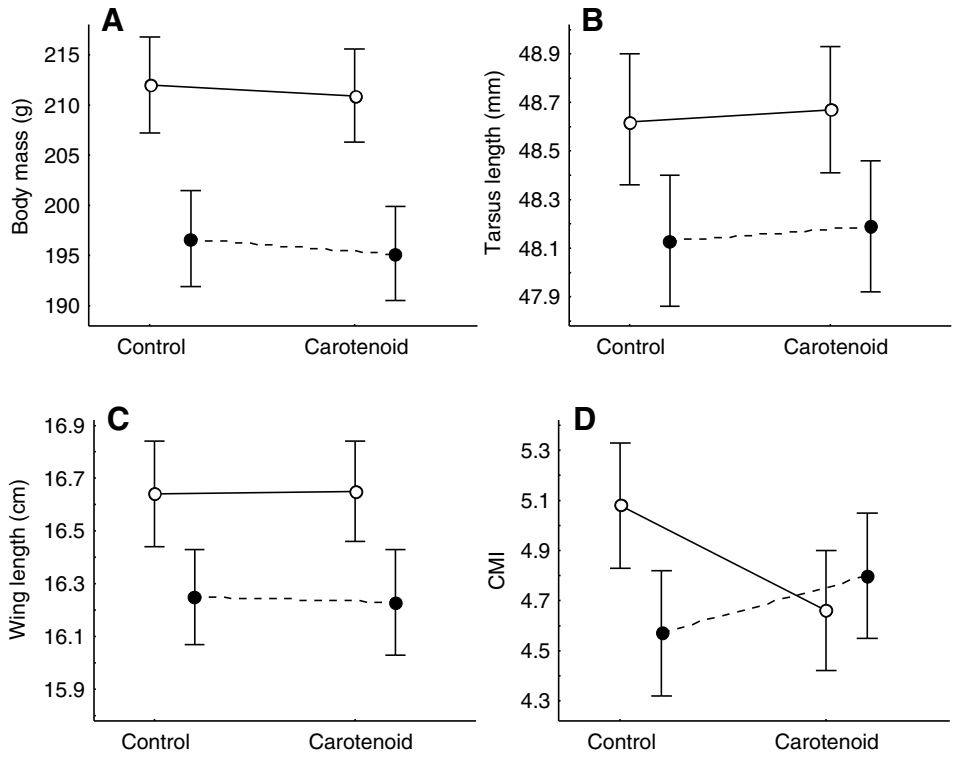

Fig. 4. Least square means (± s.e.m.) of nestling body mass (A), tarsus length (B), wing length (C) and cell-mediated immunity (CMI; D) in control and carotenoid-supplemented groups. Filled circles, males; open circles, females.

Albumin levels were not significantly affected by any of the variables in the initial model (all $P>0.11$; GLMM treatment: $F_{1,31.2}=0.69$, $P=0.41$; sex↔treatment $F_{2,121}=0.16$, $P=0.85$; Fig. 5B). Owing to the significant effect on globulin concentrations, also the interaction treatment↔sex significantly explained variations in albumin to globulin ratio (GLMM $F_{3,79.4}=3.93$, $P=0.011$; Fig. 5C), other variables were excluded from the model (all $P>0.09$). In the control group, males and females did not differ in albumin to globulin ratios (*post-hoc* Scheffé test: $P=0.93$), but in the carotenoid group, males showed higher albumin:globulin ratios compared to females (*post-hoc* Scheffé test: $P=0.018$; Fig. 5C). Although male nestlings from the carotenoid group tended to have higher albumin to globulin ratios compared with male nestlings from the control group, this difference was not significant.

Leucocytes

Male fledglings had less lymphocytes in plasma than female fledglings (GLMM $F_{1,145}=6.10$, $P=0.015$; Fig. 6A), and fledglings from the carotenoid group had higher lymphocyte counts than fledglings from the control group (GLMM $F_{1,33.9}=5.24$, $P=0.028$; Fig. 6A). Heterophiles were negatively correlated with CMI (GLMM $F_{1,62}=4.82$, $P=0.031$, $N=142$), but their variation was not explained by the treatment or nestling sex (GLMM treatment: $F_{1,30}=0.67$, $P=0.42$; sex $F_{1,26.8}=0.71$, $P=041$; Fig. 6B). The heterophile to lymphocyte ratio was higher in males compared to female nestlings (GLMM $F_{1,22.4}=6.22$, $P=0.02$, $N=148$; Fig. 6C), but the treatment only showed a non-significant effect on the heterophile to lymphocyte ratio, with lower ratios in the carotenoid group (GLMM $F_{1,30.4}=2.05$, $P=0.16$; Fig. 6C).

Intestinal parasite prevalence

Fledgling kestrels had a mean of 1.9±0.9 (range: 0–5) different intestinal parasite groups. The percentage of fledglings infected with the studied parasite groups is given in Fig. 7. The carotenoid treatment significantly affected the number of studied intestinal parasite groups that were found in each kestrel fledgling (GLMM $F_{1,26.6}=5.20$, $P=0.03$, $N=131$; carotenoid, 1.7±0.1; control, 2.2±0.1). From the seven different parasite groups studied, only the prevalence of the nematode genus *Capillaria* was significantly lower in the carotenoid compared to the control group (GLIMMIX $F_{1,28.5}=8.36$, $P=0.0073$, $N=131$; carotenoid, 41%; control, 68%). Also the prevalence of the nematode genus *Porrocaecum* tended to be lower in the carotenoid compared to the control group (GLIMMIX

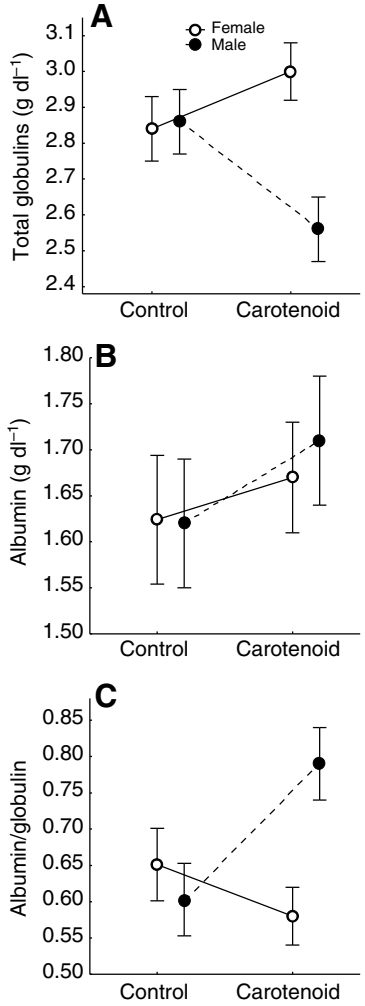

Fig. 5. Least square means (± s.e.m.) of plasma protein concentrations (A,B) and the albumin to globulin ratio (C) of male and female kestrel nestlings in control and carotenoid-supplemented groups.

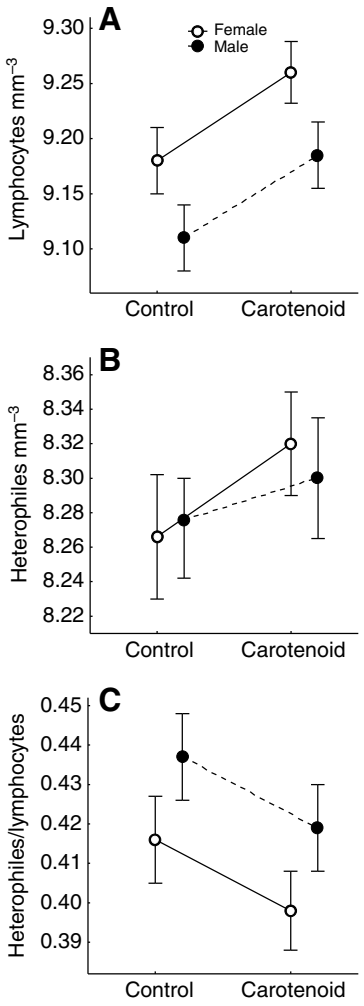

Fig. 6. Least square means (± s.e.m.) of lymphocyte (A) and heterophile (B) plasma concentrations, and heterophile to lymphocyte ratio (C) of male and female kestrel nestlings in control and carotenoid-supplemented groups.

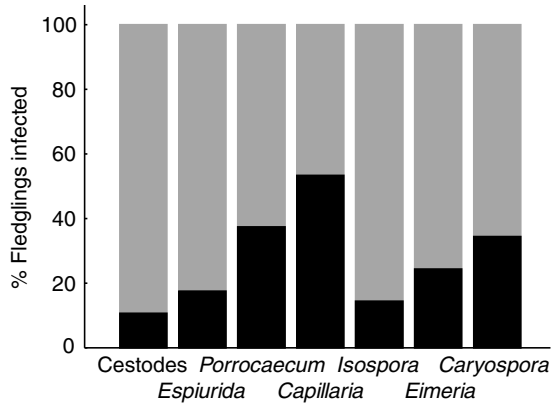

Fig. 7. The percentage of fledglings infected with different intestinal parasite groups: cestodes, nematodes (*Espiurida*, *Porrocaecum*, *Capillaria*) and coccidia (*Isospora*, *Eimeria*, *Caryospora*).

$F_{1,129}$=3.12, P=0.08, N=131; carotenoid, 30%; control, 46%). No significant differences in the prevalence of the nematode genus *Espiurida*, and the coccidian genera *Eimeria*, *Caryospora* and *Isospora* were found between treatments (all P>0.23). The other explanatory variables: sex, the interaction sex*treatment, laying date and brood size, did not affect significantly the number or presence of parasite groups (all P>0.23).

DISCUSSION

Female physiological status and reproductive parameters

Lutein supplementation before and during egg-laying caused a significant increase in all female plasma carotenoids, i.e. xanthophylls but also β-carotene. A significant correlation between carotenoid intake and plasma levels has previously been found in several bird species (Alonso-Álvarez et al., 2004; Bertrand et al., 2006; Blount et al., 2002b; Blount et al., 2003b; Hõrak et al., 2006c). This result indicates that supplemented females assimilated more carotenoids than controls, suggesting that carotenoids may be a limiting resource for laying female kestrels. However, carotenoid-supplemented females also showed a significant increase in the HDL:LDL ratio. Lipoproteins in the blood, a water medium, carry triglycerides, cholesterol and carotenoids around the body. The distribution of carotenoids among lipoproteins depends much on the physical properties of individual carotenoids and the composition of the lipoproteins (Parker, 1996; Trams, 1969). The hydrocarbon carotenes (e.g. β-carotene) are predominantly associated with LDL (low-density lipoprotein, sometimes referred to as bad cholesterol lipoprotein), whereas the more polar carotenoids (e.g. lutein) are distributed more evenly between LDL and HDL (high-density lipoprotein, sometimes referred to as good cholesterol lipoprotein) (Parker, 1996). Generally, in healthy individuals the LDL fraction is low and the HDL fraction high (Krinsky, 1994; Rock, 1997). Therefore, the higher HDL:LDL ratio in supplemented females probably allowed for a better distribution of carotenoids (lutein) from the liver to other body tissues. Recent work showed that a diet rich in lettuce, which is high in a number of antioxidants, affected lipid metabolism (cholesterol HDL:LDL ratio) and antioxidant status (vitamins C, E and carotenoids) in the rat (Nicolle et al., 2004). In addition, supplementation with lycopene, a strong antioxidant predominantly present in tomatoes, affected the HDL:LDL ratio in Japanese quail (*Coturnix coturnix japonica*) in a similar way as in the present study (Sahin et al., 2006). Our study indicates that carotenoid supplementation in kestrels can cause a shift in the HDL:LDL ratio, suggesting that dietary carotenoids may influence lipid metabolism. Unfortunately, we did not measure the concentration of lipoproteins, but it is also possible that carotenoid supplementation increased HDL and/or LDL plasma concentrations. In a recent study, lutein supplementation increased plasma triglyceride levels in greenfinches (*Carduelis chloris*) (Hõrak et al., 2006a). Since lipoproteins constitute the major part of plasma triglycerides, Hõrak et al. (Hõrak et al., 2006c) suggest that lutein supplementation eventually leads to increased lipoprotein assembly in the liver. Maternal lipoprotein metabolism was affected by carotenoid supplementation, so it is also possible that the experiment not only increased the maternal transfer of carotenoids, but also additionally increased or modified the transfer of other egg compounds. Because carotenoids were not injected directly into eggs, we cannot exclude the possibility that it was not the carotenoids *per se*, but carotenoids together with other compounds that had an effect on several physiological processes during embryonic development, which affected several health state parameters later in life, days before fledging.

By contrast, carotenoid supplementation did not affect female body mass, as it did, for example, in blue tits (Biard et al., 2005) and zebra finches (Alonso-Álvarez et al., 2004). We also did not find significant differences between experimental groups in laying date, clutch size, hatching and fledging success. As mentioned before (see Introduction), discrepancies in the effects of pre-laying carotenoid supplementation on reproductive parameters probably indicate interactions with other environmental parameters that differed between studies and species.

Hatchlings

The transfer of carotenoids to eggs has been repeatedly shown to be determined by their availability in the mothers' diet, and experiments involving carotenoid provisioning always produced a significant increase in egg yolk carotenes compared with the control group (e.g. Biard et al., 2005; Bortolotti et al., 2003; Karadas et al., 2005; Koutsos et al., 2003; Remeš et al., 2007; Schaeffer et al., 1998; Surai et al., 2003; Surai et al., 1998). In addition, maternal circulating carotenoids showed a significant correlation with the mean clutch concentration of carotenoids in poultry (Schaeffer et al., 1998) and gulls (Blount et al., 2002b). We allowed all eggs to hatch and young to be raised with the objective of exploring the effects of pre-laying carotenoid supplementation on nestling performance without altering nest composition or brood size. Our experiment largely increased kestrel female plasma carotenoid status compared with control females, but we did not find this effect reflected in hatchling plasma lutein concentrations. During embryonic development, carotenes are transferred from the egg yolk mainly to the liver, and at hatching, the concentration of carotenoids in the liver far exceeds that of any other tissue (Surai et al., 2001b; Surai and Speake, 1998). Post-hatch, the liver then undergoes a dramatic depletion in carotenoids that are redistributed among other tissues, including the plasma. In addition, the liver carotenoid content is very sensitive to changes in yolk carotenoid levels, whereas the other tissues of the embryo are much less responsive (Surai and Speake, 1998). Even though a significant effect of pre-laying carotenoid supplementation was previously detected in the plasma of hatchling chicks (Surai and Speake, 1998), the fact that it in our experiment there was no difference in the hatchling plasma lutein concentrations was probably due to the fact that carotenoids were still mainly stored in the liver at that age, with very low concentrations of circulating plasma carotenoids (see Table 2). If blood samples had been taken form older hatchlings differences in

blood plasma carotenoids might have been apparent. We opted for taking blood samples from hatchlings as early as possible with the aim of detecting the transfer of maternal carotenoid availability, excluding as much as possible an environmental component. This is because cross-fostering experiments in American kestrels showed that plasma carotenoids of nestlings at 22 days were mainly environmentally determined (Bortolotti et al., 2000).

Fledglings

The prelaying carotenoid supplement given to females affected their fledgling leucocytes and plasma protein concentrations, and also resistance to intestinal parasites. However, we did not detect significant effects of the treatment on fledgling circulating carotenoids, body mass, body size and CMI. As stated before, plasma carotenoids of the closely related American kestrel fledglings were mainly environmentally determined (Bortolotti et al., 2000), as was also the case for yellow-legged gulls (*Larus michahellis*) (Rubolini et al., 2006). In addition, in nestling kestrels, plasma carotenoid concentration has been shown to vary between different bird families (Costantini et al., 2007), and were increased when nestlings were supplemented with carotenoids (Casagrande et al., 2007; Costantini et al., 2007). However, this effect disappeared 1 week after the supplementation (Costantini et al., 2007) or once it became apparent in skin colouration, indicating a quick reduction of the pigments after involvement in physiological processes (Casagrande et al., 2007). Therefore, plasma carotenoid concentrations of fledglings most likely reflected their actual diet and since experimental nests were randomly distributed in the study area, no differences in diet between the experimental groups are expected. Moreover, plasma carotenoid concentrations were negatively related to laying date and positively to tarsus length, suggesting a major influence of parental quality. The prelaying carotenoid supplementation did not affect fledgling body size or mass. Previous findings, as mentioned in the Introduction, together with the present study, suggest that the effect of yolk carotenoids on post-hatch growth is not ubiquitous, and is most likely dependent on other environmental interactions.

Cell-mediated response was not significantly affected by the treatment or by sex or the interaction sex↔treatment. In natural and experimental situations of food shortage, nestling male kestrels have generally shown a lower cell-mediated immune response than their female nest-mates (Fargallo et al., 2002; Fargallo et al., 2003; Fargallo et al., 2007). In accordance with these previous findings, Fig. 4D shows the trend of males having a lower CMI than females in the control group, however, this difference did not reach significance using a Scheffé *post-hoc* adjustment. Up to now, very few studies have evaluated possible effects of maternal carotenoids on nestling cell-mediated immunity measured by PHA injection (Berthouly et al., 2007; Remeš et al., 2007; Saino et al., 2003a), and a significant result has only been found in a study where lutein was directly injected into the eggs of barn swallows (Saino et al., 2003a). Here we did not find an effect on cell-mediated immunity; however, the carotenoid treatment significantly lowered the number of intestinal parasite groups that were found in each kestrel fledgling. However, the treatment did not help nestlings avoid infection by coccidian parasites; but it did lower the prevalence of nematodes. Both nematode and coccidian infections may have detrimental effects on an individual's health and fitness (Brawner et al., 2000; Calvete, 2003; Draycott et al., 2006; Forbes and Simpson, 1997; Georgieva et al., 2006; Hill and Brawner, 1998; Hõrak et al., 2004a). Recently, Dalloul et al. (Dalloul et al., 2006) found that the injection of a lectin extracted from a mushroom into chicken embryo's had an immunoenhancing effect on cell-mediated immunity and coccidiosis. To our knowledge this is the first study showing a lowered intestinal parasite infestation due to different environmental conditions experienced during ontogeny in a wild bird population, thereby supporting the idea that maternal effects may exert considerable effects on an individual's capability to respond to immune challenges later in life. Unfortunately, we do not have information on the severity of infestation by intestinal parasites. A recent experimental study (Hõrak et al., 2006b) demonstrated individual variation in resistance to coccidiosis in greenfinches, but it was not determined if this was caused by genetic or ontogenetic differences in immune function. If nestlings of carotenoid supplemented females enjoyed a more optimal development, which allowed them to better resist parasitic infections later in life, it is possible that these nestlings maintained lower levels of infection compared to control nestlings (Hõrak et al., 2006b).

Furthermore, nestlings of carotenoid-supplemented females showed higher lymphocyte concentrations. Carotenoids decrease immunosuppressive peroxides, and enhance the production of lymphocytes and the phagocytic ability of heterophiles and macrophages (reviewed by Lozano, 1994; Møller et al., 2000; Surai et al., 2001a). The higher concentration of lymphocytes in kestrel nestlings of carotenoid-supplemented females may indicate that these nestlings were able to develop their immune system more rapidly, as was previously also suggested for blue tit nestlings from carotenoid enriched eggs (Biard et al., 2005). Because of the higher lymphocyte concentration in nestlings from the carotenoid group, these also tended to have a lower H:L ratio, indicating lower stress compared to the control group. This result supports the recent suggestion in a study on great tits that the beneficial effects of maternally transmitted carotenoids may be mediated by their positive direct or indirect influence on physiological and behavioural processes involved in stress resistance and competitiveness (Berthouly et al., 2007).

Aside from this effect, in both experimental groups, male nestlings showed lower lymphocyte numbers and a higher H:L ratio compared to female nestlings. Lymphocyte number in the peripheral blood is known to decrease in response to different stressors, and particularly after exhausting physical activities [e.g. Hoffman-Goetz and Pedersen in Hõrak et al. (Hoffman-Goetz and Pedersen, 1994; Hõrak et al., 1998)]. In raptors, male nestlings are smaller than females, suggesting that males are in competitive disadvantage under conditions of food scarcity (Anderson et al., 1993; Fargallo et al., 2003). Therefore, males are most likely suffering from more intensive sibling competition compared to females, provoking their more stressed situation (less lymphocytes, higher H:L ratio).

Furthermore, the experiment also affected nestling plasma protein concentrations. Based on albumin concentrations, kestrel nestlings did not differ in nutritional condition between experimental groups (Hõrak et al., 2002; Jenni-Eiermann and Jenni, 1998). But the experimental treatment did affect globulin concentrations, although only in the male nestlings (Fig. 5). Since an increase in globulin concentration is expected after chronic or acute infections with inflammatory processes, healthier individuals have a higher albumin to globulin ratio (Ots et al., 1998; Kilgas et al., 2006b).

In the present study, male nestlings of carotenoid-supplemented females showed the highest albumin to globulin ratio, being healthier than males from the control group. Female nestlings did not differ in albumin to globulin ratio between experimental groups, and sexual differences were only manifested in the carotenoid supplemented group.

Sexual differences in kestrel nestling condition and/or CMI have been interpreted as a reduced competitive capacity of male nestlings, the smaller sex (Fargallo et al., 2003), or also as a result of different hormonal configuration of both sexes (Fargallo et al., 2002). However, this last possibility was refuted in a recent experimental study (Fargallo et al., 2007). Another alternative could be that prenatal environmental conditions drive sexual differences in physiological processes because the sexes may differ in their vulnerability and/or sensitivity to specific egg components for their normal development (Gorman and Nager, 2004; Love et al., 2005; Naguib et al., 2006; Naguib and Gil, 2005). In this sense, our results of globulin levels indicate that an increased maternal investment in lutein benefited males but had no effect on females.

Although we find benefits of maternal carotenoids for both sexes in fighting off intestinal parasites and the development of the immune system (leucocytes), we cannot know if increased pre-laying carotenoid availability caused a sex-biased maternal investment (Saino et al., 2003b; Verboven et al., 2005) or if a general increase in the investment of carotenoids in the total clutch benefited more males than females post hatch with respect to globulin levels.

We are aware that with the presented experimental design, effects of maternal investment *per se* and post hatch parental care on fledgling health status cannot be distinguished since we did not perform the usually employed cross-fostering experiments to address these questions (e.g. Biard et al., 2005). We believe that parental quality was randomly distributed among experimental groups, since the experiment was randomly distributed along the study area, 90% of the territories were established before the experiment started and no significant differences in reproductive parameters were found between treatments. However, carotenoid availability may have influenced egg investment and thereby nestling performance, but it could have also influenced parental care; our manipulation, however, was mainly directed towards females before and during egg-laying. Prey-provisioning during incubation and the first two weeks post-hatch is exclusively performed by the male, and even afterwards, the contribution of males in nestling provisioning largely outweighs that of females (Tolonen and Korpimäki, 1994) (Vergara and Fargallo, in press). Then, during the manipulation period, males spent most of their time hunting to feed breeding females. Unless the carotenoid treatment had some effect on egg colouration or on female behaviour, which could potentially influence male behaviour, it is not expected that there would be any effect of the experiment on male parental care, and thereby on nestling rearing conditions. We did not monitor male feeding frequency during the nestling period, but during the post-fledging period, parents did not differ in feeding behaviour between treatments (Vergara and Fargallo, in press). For these reasons, we believe that the results of our study most likely indicate that maternal investment in carotenoids at least altered in some way post-hatch nestling physiological health state parameters. In addition, a recent study on great tits found that the effect of maternally transmitted carotenoids on nestling immune response depended on whether nestlings had been cross-fostered or not, and the study concluded that carotenoid supplementation compensated for the immunosuppressive consequence of nestling translocation (Berthouly et al., 2007). This indicates that although cross-fostering is a good tool for distinguishing between pre-and post-natal environmental effects, it is not free from additional complications. In fact, Remeš et al. (Remeš et al., 2007) did not mention the lack of cross-fostering experiments in their study in order to evaluate maternal carotenoid supplementation effects on nestling performance.

In conclusion, prelaying carotenoid supplementation to kestrel females increased adult female plasma carotenoid concentrations and altered the HDL:LDL ratio. The effect of the treatment of prelaying female was manifested in several nestling haematological health state parameters and in nestling intestinal parasite burden. Nestlings of carotenoid-supplemented females had a lower number of intestinal parasite groups, higher lymphocyte concentrations, and were less stressed than control nestlings. In addition, interactions between treatment and sex were apparent for globulin concentrations, favouring male nestlings, suggesting sex-specific benefits from carotenoid maternal investment in kestrels.

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