Carotenoids in Eggs and Plasma of Red-Legged Partridges: Effects of Diet and Reproductive Output

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ABSTRACT

Carotenoids are important dietary constituents in birds. They serve as pigments and play numerous physiological roles in both the laying hen and developing embryo. However, factors determining the absorption of carotenoids and their allocation to different functions are numerous and complex, and causal relationships are generally poorly known. Our objective was to determine the degree to which carotenoid levels in egg yolks and the plasma of hens were influenced by differences in diet and reproductive output in captive red-legged partridges. Carotenoid concentrations were measured by high performance liquid chromatography in two feeds (high and low carotenoid content) and in yolks and plasma of hens near the start and end of laying. Early in the laying season, plasma and yolk carotenoids varied with diet and were correlated with one another. Late in the season, a dietary effect was evident only for yolks, and there was no relationship between plasma and egg levels of individual hens. However, plasma carotenoids at the end of laying were strongly correlated with the number of eggs that had been laid. Dietary availability, although important, could explain some variation in carotenoid levels in plasma and egg yolks only in the context of reproductive history.

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Introduction

Carotenoids are bioactive molecules synthesized mainly by plants. Their molecular structure, over 600 forms, and biological functions show remarkable diversity (Goodwin 1984; Krinsky et al. 1989; Britton 1995). In birds, carotenoids function as pigments in feathers and skin, antioxidants, precursors of vitamin A, and play various roles in the endocrine and immune systems (Brush 1990; Olson and Owens 1998; Møller et al. 2000; Surai et al. 2001*a*, 2001*b*). In eggs, they are involved in regulation of embryonic development by way of their antioxidant properties (Surai and Speake 1998; Surai et al. 2001*a*, 2001*b*).

Metabolism of carotenoids in animals, including birds, is poorly understood (Brush 1990). We know that, generally, after ingestion carotenoids are absorbed in the small intestine together with other fat-soluble nutrients and delivered with portomicrons to the liver (Surai et al. 2001*a*). We also know that the liver incorporates carotenoids into very low density lipoproteins (VLDL), where they are delivered to the egg yolk. As a result of exchange between different lipoprotein particles and metabolism of VLDL by lipoproteinlipase, carotenoids appear in low-density lipoproteins (LDL) and high-density lipoproteins (HDL) and are thus delivered to a variety of peripheral tissues. It is not known exactly to what extent carotenoids are metabolized in the liver; however, there is evidence indicating that carotenoid interconversion can take place there (Møller et al. 2000).

While the efficiency of absorption and assimilation of carotenoids are known to be affected by components in the diet and general health of the hen, the relationships are complex (Surai et al. 2001*b*; Surai 2002). For example, factors related to diet other than carotenoid content, genetics, hormonal status, parasites, sex, age, and season, to name a few, may act independently or interact with each other (e.g., Hudon 1994; Bortolotti et al. 1996; Surai et al. 2001*b*). While such relationships have been documented, it is not known to what degree they are causal in influencing carotenoid absorption or assimilation. Similarly, little is known as to what degree variation in carotenoids could be used to indicate current or past physiological states.

Most avian research on carotenoids has focused on applied issues in the rearing of captive or domesticated species: zookeepers must maintain the bright colors of display animals (Brush 1981), and the poultry industry must satisfy consumer demand for pigmented meat and egg yolks (Marusich and Bauernfeind 1981). It is well documented that supplementation

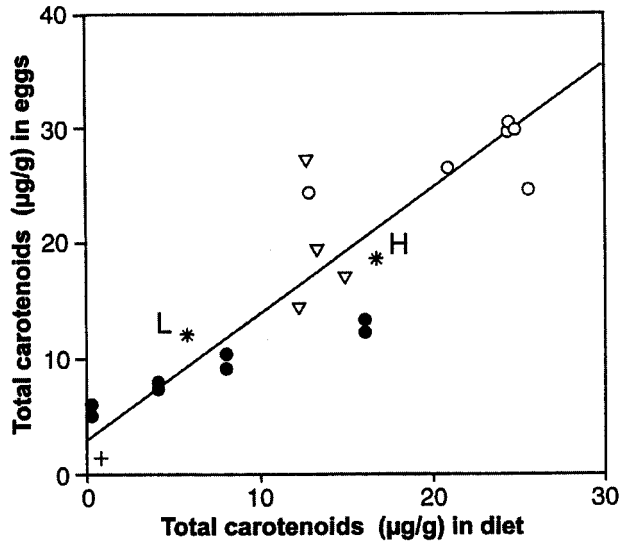

Figure 1. Scatterplot of total carotenoid concentration (μg/g) in diet versus in egg yolks in poultry (*filled circles*, Lai et al. 1996*a*, 1996*b*; *open triangles*, Surai et al. 1998; *open circles*, Gonzalez et al. 1999; *plus sign*, Strand et al. 1998), and red-legged partridges in this study (*asterisks*) on diets containing relatively high (*H*) and low (*L*) concentrations of carotenoids.

of the diet of chickens increases yolk carotenoids (Fig. 1); however, relatively little attention has been paid to factors determining plasma concentrations or to what degree plasma levels relate to yolk composition (Surai et al. 2001*b*). Our knowledge of such relationships in wild species is truly rudimentary (see Surai et al. 2001*a*, 2001*c*).

Poultry studies have focused primarily on population-level phenomena; i.e., there has been less attention to how individual birds may vary in response to availability of, or physiological demands for, carotenoids. Current studies in other fields use different approaches and require different information. For example, the focus on the individual is of considerable interest in more theoretical biology where potential adaptive strategies for optimizing carotenoid allocation, both within an individual as well as between the hen and her eggs, are being studied (Negro et al. 1998; Royle et al. 1999; Blount et al. 2000; Møller et al. 2000). The current proliferation of research is being stimulated by the appreciation of the diversity of potential functions of carotenoids and how carotenoids in plasma, or color, may be used as indicators of the health status of individuals; e.g., in behavioral ecology (Bortolotti et al. 1996; Shykoff and Widmer 1996; Olson and Owens 1998; Møller et al. 2000) and toxicology (Boily et al. 1994; Fernie and Bird 2001). What is needed is a better understanding of how the allocation of carotenoids to different functions varies among individuals and whether deposition levels in tissues (e.g., plasma and eggs) are indicative of variation in availability (i.e., diet) and physiological demands (i.e., reproduction).

Our model is the red-legged partridge (*Alectoris rufa*), a popular European game bird, bred in captivity to restock hunting states. This species shows coral-red coloration in the legs, bill, and lores (fleshy area anterior to the eye) due to carotenoid pigments. Previously, we showed that the plasma concentrations of carotenoids in captive partridges, even when kept on a constant diet, varied with age, sex, and season (Negro et al. 2001). In this study, our objective was to determine the degree to which hens deposited carotenoids in eggs, given differences in dietary availability and varying degrees of reproductive output.

Methods

This study was carried out at the Lugar Nuevo partridge breeding facility in southern Spain, near Andujar, in April–June 1999. We started with 90 laying females placed in separate breeding pens. Each female was paired to a male, and both birds shared a pen composed of a wire cage (120 × 80 × 50 cm) and an attached nest box for egg laying. All birds were exposed to natural photoperiod and temperature.

For several months before the experiment, the birds were fed a Purina maintenance diet for partridges. On February 1, 1999, more than 2 mo before the laying of eggs, the birds were switched to one of two diets formulated for breeding partridges. Half of the pairs ($n = 45$) were given a partridge diet manufactured by Nanta, while the remainder was given a different partridge formulation by Purina España. The main macronutrients varied slightly between diets, whereas carotenoid content was substantially different (Table 1); hereafter, the Nanta and Purina diets will be referred to as "high" and "low," respectively. Given that the carotenoid content of chicken eggs stabilizes 11 d after the hens are placed on a high-carotenoid diet (Surai and Speake 1998; see also Surai et al. 2001*b*), our feeding experiment was conducted with sufficient time to detect a dietary effect.

We selected six females in each diet that started laying at approximately the same time (first egg laid April 5–12), thereby controlling for any effect of laying date. We collected all eggs laid by those females 1–3 d after laying, thereby minimizing embryonic development. Two females in the high diet group

Table 1: Nutrient composition of the two diets of the red-legged partridge

Diet	Total Carotenoids[a] (mg/kg)	Protein (%)	Fat (%)	Ash (%)
High	16.60	17	2	10
Low	5.94	21	4	11

[a] Carotenoids were measured by our lab; other constituents were taken from manufacturers' labels.

and one in the low diet group terminated laying prematurely for unknown reasons and were removed from further analysis. Immediately upon collection, the whole eggs were numbered, frozen, and stored at −20°C. The yolk was later removed intact from the egg, taking advantage of the fact that albumin thaws more quickly than yolk. Frozen yolk samples were packed in dry ice and transported by air to the United Kingdom for analysis.

Blood was sampled from the 12 females from which eggs were analyzed on April 12 and June 16. Hereafter, the April and June periods are referred to as "early" and "late," respectively. While in the early period all females were similar in that they had laid between one and three eggs, the late sample exhibited somewhat more variability with respect to number of eggs laid. Clutch size by the time of the late sample was comparable for both diets, and varied among hens from 20 to 34 eggs. For each sample, 0.25 mL of blood was drawn from the brachial vein. Blood was extracted at 1000–1200 hours, transported in a cooler, and centrifuged within 24 h of collection. Resulting plasma was frozen at −20°C until analysis. Total plasma carotenoids were measured spectrophotometrically, using acetone as a solvent and a reading absorbance of 476 nm (see Bortolotti et al. 1996; Tella et al. 1998). Carotenoid concentration was estimated as μg/mL of plasma using the extinction coefficient of lutein in acetone (Mínguez-Mosquera 1997).

Carotenoid extraction from egg yolk was performed as previously described (Surai et al. 2000). In brief, egg yolk or tissues (0.2–0.5 g) were homogenized in 2 mL of 1 : 1 (v/v) mixture of 5% NaCl solution and ethanol, followed by the addition of 3 mL 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, and the residue was dissolved in 1 mL of methanol : dichloromethane (1 : 1, v/v) and centrifuged, and the supernatant was used for carotenoid determination.

Carotenoids were determined by high performance liquid chromatography as described previously (Surai et al. 2000), using a Spherisorb type S3ODS2, 5-μ C18, reverse-phase column, 25 cm × 4.6 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. Peaks were identified by comparison with the retention times of a range of carotenoid standards (variously obtained from Sigma, Poole, U.K.; Fluka, Gillingham, U.K.; Apin, Abingdon, U.K.; and Hoffman-La Roche, Basel, Switzerland), as well as using coevolution of individual carotenoids with known standards.

As egg and yolk sizes did not vary with diet or across the laying sequence (G. R. Bortolotti, J. J. Negro, and P. F. Surai, unpublished data), our findings were the same whether we presented absolute levels or concentrations, so we present only the latter. Statistical tests are two-tailed except in cases where only one direction was plausible, in which case it is noted as being one-tailed.

Results

There were no obvious effects of diet on reproduction. Considering all 12 hens, the body mass of birds on the low diet tended to be marginally heavier at the time of the early blood sample (i.e., shortly after laying began; high: mean = 402, SE = 17.7; low: mean = 438, SE = 7.69, Mann-Whitney *U*-test, $Z = -1.92$, $P = 0.054$). For the remaining analyses only birds that continued laying for the normal breeding season are compared. Partridges on the high diet on average produced 36 eggs (SE = 2.2), whereas those on the low diet averaged 34 eggs (SE = 4.1; $Z = -0.369$, $P = 0.73$). Egg characteristics were also similar between groups; we could not detect a difference in mass (g) of eggs in the early (high: mean = 18.48, SE = 0.718; low: mean = 16.96, SE = 0.482, $Z = -1.470$, $P = 0.19$) or late periods (high: mean = 17.93, SE = 0.398; low: mean = 17.40, *SE* = 0.220, $Z = -0.984$, $P = 0.41$). Similarly dietary effects on yolk mass (g) approached significance in the early (high: mean = 6.44, SE = 0.233; low: mean = 5.79, SE = 0.299, $Z = -1.960$, $P = 0.063$) but not late eggs (high: mean = 6.10, SE = 0.229; low: mean = 6.11, SE = 0.240, $Z = 0.00$, $P = 1.0$).

A dietary effect was detectable in concentrations (μg/mL) of plasma carotenoids in the early blood sampling period (high: mean = 2.06, SE = 0.34; low: mean = 1.14, SE = 0.15, $Z = 2.24$, $P = 0.012$, one-tailed test) but not in the late sampling period (high: mean = 1.86, SE = 0.41; low: mean = 1.38, SE = 0.22, $Z = 0.88$, $P = 0.19$, one-tailed test). There was no significant correlation between plasma carotenoids in the early and late periods ($r_s = -0.357$, $P = 0.35$; Fig. 2, *top*).

Concentrations of carotenoids in egg yolks were strongly associated with diet and reproduction. Overall, the proportions of the major types of carotenoids in yolks were similar to those in feed (Table 2). Only data on total carotenoids are presented hereafter. To put the relationship between dietary and yolk concentrations of partridges into the perspective of a better known system, we examined data on poultry. We fitted a reduced major axis regression to feed and egg concentrations reported in the literature (yolk concentration = 1.264 + 1.195 × dietary concentration; Fig. 1). The levels observed in partridges closely match those predicted from the poultry data; early eggs on the high and low diets would be predicted to have 21.10 and 8.36 (g/g, respectively; Fig. 1), which are similar to observed values of 18.78 and 11.97 (Fig. 3).

Eggs from the high diet had significantly higher carotenoid concentrations in both the early (high: mean = 18.78, SE = 2.594; low: mean = 11.97, SE = 1.054, $Z = -1.960$, $P = 0.032$, one-tailed test) and late samples (high: mean = 10.61, SE = 0.614; low: mean = 7.00, SE = 0.667, $Z = -2.449$, $P = 0.008$, one-tailed test; Fig. 3). Yolk concentrations were not

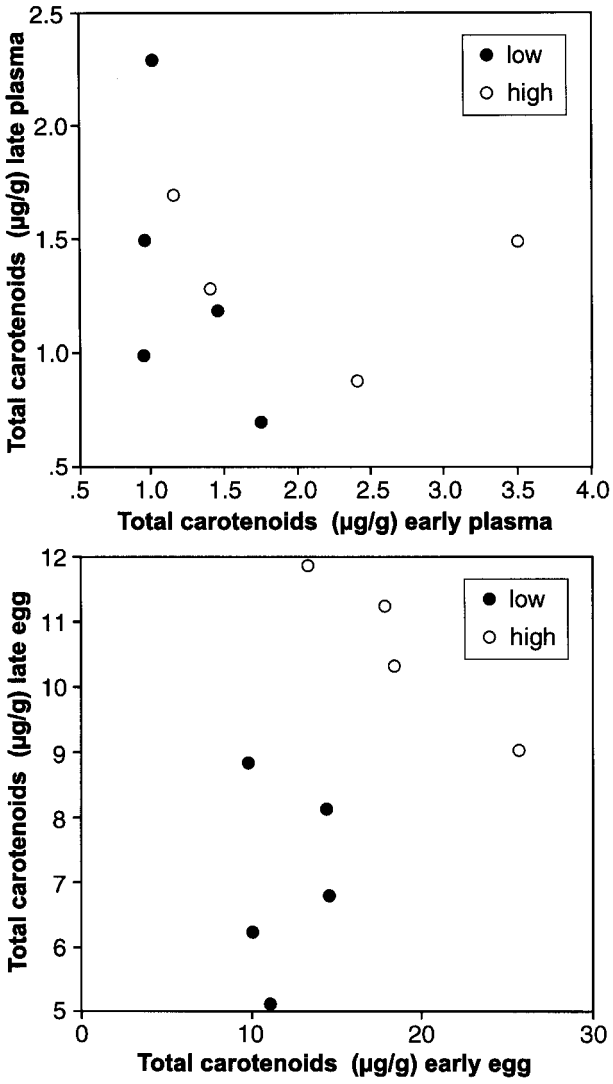

Figure 2. Relationship between carotenoid concentrations (μg/g) in the early and late sampling periods for plasma (*top*) and egg yolks (*bottom*) of individual red-legged partridges on high- (*open circles*) and low- (*filled circles*) carotenoid diets.

consistent for individual hens between the two time periods ($r_s = 0.450$, $P = 0.224$; Fig. 2, *bottom*).

Earlier we showed that carotenoid levels in blood were associated with diet only in the early period. The same appears to be true for the relationship between blood levels and egg concentrations: plasma and yolk carotenoids were positively correlated in the early period ($r_s = 0.577$, $P = 0.026$, one-tailed test; Fig. 4, *top*); however, no such correlation existed in the late period ($r_s = -0.008$, $P = 0.49$, one-tailed test; Fig. 4, *bottom*). The lack of correlation in the late period may be a consequence of the variation in the clutch size among hens; i.e., variability in the demands made on her stores of carotenoids. That appears to be the case because there was a highly significant correlation between the hen's plasma carotenoid concentrations and the number of eggs that she laid up to the date the birds were bled ($r_s = -0.764$, $P = 0.017$) with no apparent influence of diet (Fig. 5, *top*). However, clutch size was not always complete by the time of the late sample. The percentage of eggs laid by the late sample varied among hens from 75% to 100% (mean = 85.5, SE = 2.88; all birds were combined because there was no effect of diet on clutch size). Even though laying had not ceased, it is likely that at the time of the late sample, the hens had already committed carotenoids to the remaining (i.e., final) few yolks because the ova would have been partially developed. This appears to be the case because when carotenoid concentrations in late-sample plasma were compared with total number of eggs laid, an even stronger negative correlation resulted ($r_s = -0.899$, $P = 0.001$; Fig. 5, *bottom*) than when only eggs up to the late blood sample were considered (Fig. 5, *top*). We could not detect any relationship between the late period yolk concentrations and the number of eggs laid either at the time of the late sample ($r_s = 0.370$, $P = 0.33$) or in total ($r_s = 0.276$, $P = 0.47$).

Discussion

While sample sizes were small, there were no substantial differences in the breeding performance of partridges on the two feeds. The general lack of response of reproduction to diet suggests that both feeds were adequate, or at least similar, with respect to satisfying nutritional requirements, as would be expected of commercial products. Our study of allowing captive birds to lay many eggs is relevant to this species in the wild. The ultimate clutch size laid by birds in this study (about 35 eggs) is within the range expected of red-legged partridges in the wild. Natural clutch sizes are typically 10–16 eggs (maximum 20) and often two clutches (one for each member of the pair to incubate concurrently) are laid per season; hens will also lay a second clutch if the first is destroyed (Cramp and Simmons 1980).

Plasma concentrations provided a limited amount of information regarding dietary input in this study. It is clear from studies of poultry that diet can influence plasma concentrations of carotenoids, but such information must be put in perspective. Variation among species no doubt plays some role (Surai et al. 2000; see also the diversity of carotenoid concentrations reported by Hill [1995*a*] and Tella et al. [1998]). To some degree our failure to detect other diet effects in partridges may stem from small sample sizes; however, it is clear that by the late period other factors must have come into play. In this study, there was a relatively modest difference between carotenoid concentrations in the high and low diets. Studies reporting that plasma concentrations respond to diet have a bigger difference between experimental feeds: in poultry, high/low feed ratios of 6.5 resulted in a plasma ratio of only 4.3 (Surai et al. 1999).

When laying hens were fed on a sorghum-soybean diet, carotenoids were not detected in their blood serum; however, inclusion of lutein at the level of 200 mg/kg in such a diet was associated with lutein concentration in serum of 18.25 μg/mL (Haq et al. 1995). When the level of xanthophylls (as marigold meal) intubated to 46-d-old broilers was increased from 4.0 to 16.0 mg/kg body weight, serum xanthophyll concentration increased from 2.17 up to 3.13 and from 1.86 to 3.31, μg/mL after 18 and 28 h, respectively (Middendorf et al. 1980). When the level of lutein diester supplementation in a chicken diet was increased from 5 up to 80 mg/kg, lutein concentration in serum increased only from 1.4 up to 9.5 (g/mL; Tyczkowski and Hamilton 1986). The partridge diets in our study contained a carotenoid ratio (high/low) of only 2.8 (Table 1), and the hens had high/low plasma ratios of 1.7 and 1.0 for the early and late periods, respectively. Therefore, plasma responses to diet may be more readily observed under more extreme conditions or perhaps during a physiologically less demanding time. The two diets we used were not identical with respect to macronutrients (Table 1), and the differences in fat in particular may have played a role in reducing the dietary effect (Surai 2002). The lack of an obvious effect of diet in all aspects of this study emphasizes the need for considering the multitude of factors attributable to individual hens that may influence carotenoid availability, absorption, and, ultimately, allocation (Surai et al. 2001*a*; Surai 2002).

To what degree birds in the wild would vary in diet to the degree of experimental manipulation in poultry is unknown, but the smaller degree of feed differences in this study may be analogous to variation that might be experienced among hens within a wild population. A number of studies of wild species have correlated plasma concentrations of carotenoids with attributes of diet: variation in types of foods eaten among species (Hill 1995*a*) or within a species, according to food availability (Bortolotti et al. 2000) and season (Hill 1995*b*). Given the multitude of demands on carotenoids in the body, plasma levels may not always be a good indicator of dietary intake as these nutrients may be mobilized to meet various physiological needs.

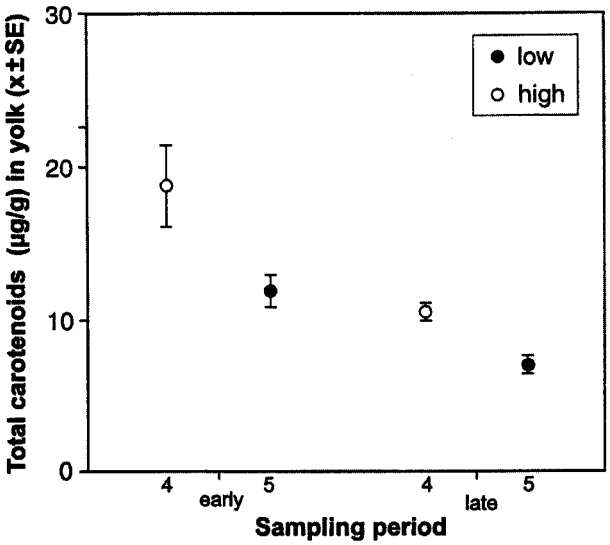

Figure 3. Mean ± SE total carotenoid concentrations (μg/g) in yolks from eggs in the early and late sampling periods for red-legged partridges on high- (*open circles*) and low- (*filled circles*) carotenoid diets.

One of the biggest demands on a hen's carotenoid stores is for yolks.

The correlation between carotenoids in plasma with those in yolks of the early period (Fig. 4, *top*) suggests that plasma levels were indicative of the hens' stores available for their eggs. Comparable data for other species, even poultry, are lacking. We suspect that the association we found would have been even stronger if plasma could have been evaluated just before the first egg was laid. Because it was not possible to predict clutch initiation date, and sampling birds all on the same day may be preferable to control other factors, we had to contend with the fact that blood sampling occurred within a few days of laying.

Carotenoid concentration in yolks varied quantitatively with diet (Fig. 3), but qualitatively, the similarity in types of carotenoids between feed and eggs (Table 2) suggests little metabolic conversion took place (see also Partali et al. 1987). Carotenoid

Table 2: Mean ± SE concentration and percent composition for carotenoids of two diets and in the early-laid eggs of the red-legged partridge

	Lutein		Zeaxanthin		β-Cryptoxanthin		Other	
	μg/g	%	μg/g	%	μg/g	%	μg/g	%
High								
Feed[a]	6.49	39.1	6.09	36.7	1.25	7.5	2.77	16.7
Eggs ($n = 4$)	7.81 ± 1.37	41.0	6.91 ± 1.10	36.4	.92 ± .16	5.1	3.15 ± .28	17.5
Low								
Feed	2.44	41.1	2.08	35.0	1.12	18.8	.30	5.1
Eggs ($n = 5$)	5.46 ± .41	46.0	3.60 ± .52	29.5	.62 ± .06	5.2	2.29 ± .35	19.2

[a] Feeds are represented by one sample each.

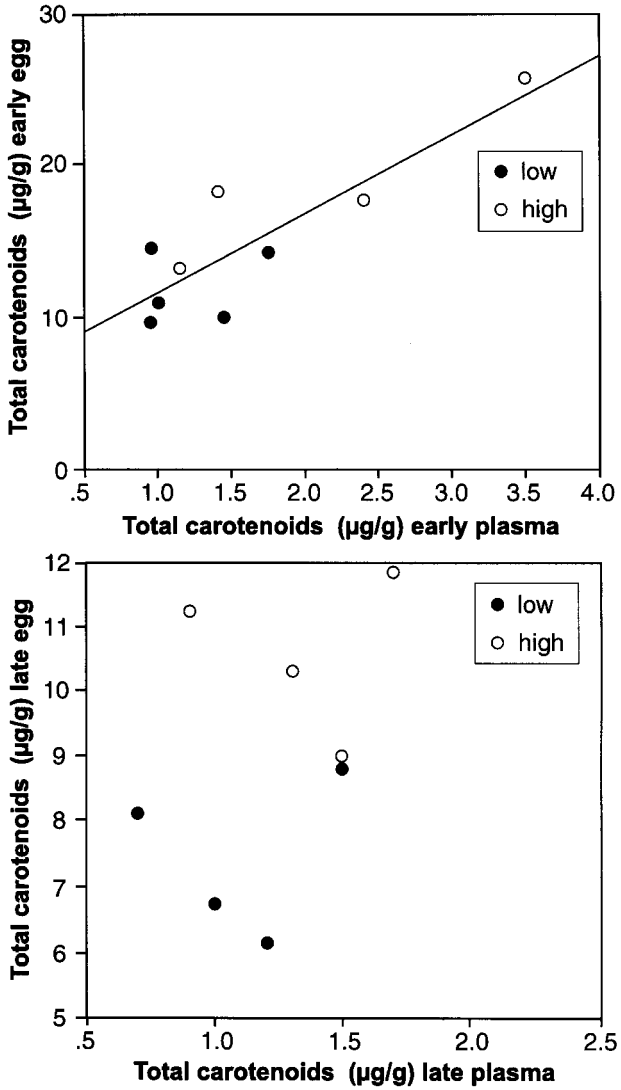

Figure 4. Relationship between carotenoid concentrations (μg/g) in the plasma versus egg yolks of individual red-legged partridges on high- (*open circles*) and low- (*filled circles*) carotenoid diets in the early (*top*) and late (*bottom*) sampling periods.

concentrations were well predicted by the chicken feed/egg relationship (Fig. 1). While levels declined from early to late in the season (see Fig. 3), individual hens were not consistent in the decline. The latter is expected as so many factors influence the ability of hens to absorb carotenoids from their feed, and subsequently the physiological requirements of the hen for carotenoids are many (Surai et al. 2001*a*). However, a major demand is the deposition of carotenoids in yolk, as suggested by the exceptionally strong correlation between plasma concentrations and number of eggs laid by each hen (Fig. 5). In poultry, a single yolk may contain about 40%–45% of the total carotenoids found in the liver (Surai and Speake 1998; Surai et al. 1999), which is the main site of carotenoid accumulation (Surai et al. 2000). If one takes into account the fact that in the ovary of the hen there are yolks of different size, that there could be 5–10 yolks, some of which are almost fully formed, then it is clear that more than 50% of total carotenoid reserves in the body are in ovary (P. F. Surai, unpublished data; see Nys 2000).

It is also necessary to take into account that carotenoids are delivered to egg yolk as a part of VLDL accumulating in the yolk. At the time of laying, VLDL concentration increases more than 200 times (Yu et al. 1976) as a result of their increased

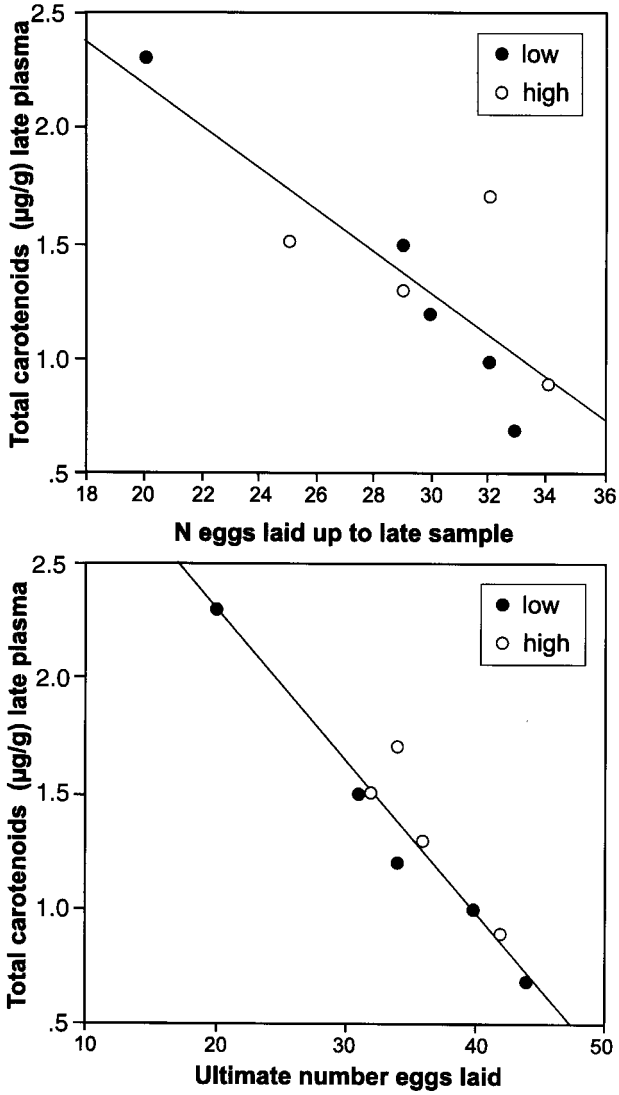

Figure 5. Relationship between carotenoid concentrations (μg/g) in the plasma of individual red-legged partridges in the late sampling period on high- (*open circles*) and low- (*filled circles*) carotenoid diets, and the number of eggs that were laid at the time of the sample (*top*) and the ultimate number of eggs laid (*bottom*).

synthesis in the liver in response to estrogen stimulation. Similarly, the administration of estrogens to domestic fowl stimulates redistribution of lipoproteins such that production of VLDL increased by 400 times, the LDL level rose by 70 times, and the HDL level significantly decreased (Kudzma et al. 1979). VLDL is the main delivery system for carotenoids into the developing oocyte. There are some specific unique features of VLDL in laying hens. First, the diameter of VLDL particles synthesized in the liver of laying hens is about 30 nm, significantly smaller than that in immature hens Second, these so-called yolk-targeted VLDLy are resistant to hydrolysis by LPL (Walzem 1996). The receptor-mediated uptake of intact VLDLy by binding to the follicular apoB receptor for endocytosis (Walzem 1996) means that carotenoids present in VLDLy will be transported in to the developing follicle. Since the concentration of VLDL is strongly dependent on reproductive stage, one could expect more uniform data at the beginning of the reproductive period, when most birds are laying eggs, in comparison to the end, when some have already finished reproduction.

Overall, our results help to clarify how reproduction and carotenoid content of diets influence allocation of these important micronutrients to eggs and plasma. Before one can effectively interpret variation in carotenoid levels in these tissues, one must have a context of reproductive history.

Acknowledgments

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