

## Ecological, morphological and phylogenetic correlates of interspecific variation in plasma carotenoid concentration in birds

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### Abstract

Carotenoids are important as pigments for bright coloration of animals, and as physiologically active compounds with a wide array of health-related benefits. However, the causes of variation in carotenoid acquisition and physiology among species are poorly known. We measured the concentration of carotenoids in the blood of 80 wild bird species differing in diet, body size and the extent of carotenoid-based traits. Preliminary analyses showed that diet significantly explains interspecific variability in plasma carotenoids. However, dietary influences were apparently overridden by phylogenetic relationships among species, which explained most (65%) of this variability. This phylogenetic effect could be due partly to its covariation with diet, but may also be caused by interspecific differences in carotenoid absorption from food to the blood stream, mediated, for example by endothelial carriers or gut parasites. Carotenoid concentrations also decreased with body size (which may be explained by the allometric relationship between ingestion rate and body mass), and correlated positively with the extent of carotenoid-dependent coloration of plumage and bare parts. Therefore, 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.

### Introduction

Carotenoids have recently attracted much attention among evolutionary ecologists (Olson & Owens, 1998). Carotenoids are responsible for the brightest animal coloration – red, orange, yellow and green hues (Britton, 1995) – and in addition to their pigmentary properties, they may also provide health benefits because of their antioxidant and immunostimulating properties (Britton, 1995; Surai *et al.*, 2001). Birds have been extensively used as models for studying the function of carotenoids in vertebrates. Several studies have demonstrated that individuals with the brightest carotenoid-dependent

coloration are preferred as mates (Burley & Coopersmith, 1987; Hill, 1991; Johnson *et al.*, 1993; Sundberg, 1995), suggesting that carotenoids play an important role in sexual selection (Shykoff & Widmer, 1996; Negro *et al.*, 1998; Olson & Owens, 1998). Other studies have shown a function of carotenoid-derived traits for offspring signalling to parents (Lyon *et al.*, 1994; Saino *et al.*, 2000). Additionally, the brightness of carotenoid-based traits and the concentration of carotenoids in plasma are correlated with several measures of health and immunocompetence of the individuals (Hill & Montgomerie, 1994; Dufva & Allander, 1995; Bortolotti *et al.*, 1996, 2000; Figuerola *et al.*, 1999; Saino *et al.*, 1999). These findings suggest a trade-off between ornamentation and health needs for carotenoids (Lozano, 1994; Shykoff & Widmer, 1996; Negro *et al.*, 1998; von Schantz *et al.*, 1999), which is the focus of current research and intense debate (Hill, 1999; Møller *et al.*, 2000; Lozano, 2001; Surai *et al.*, 2001).

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For carotenoid-based signals to be honest indicators of quality they have to be costly to produce. The traditional explanation for costliness of carotenoids is that they are difficult to obtain (Olson & Owens, 1998; Negro *et al.*, 2002). As carotenoids are produced only by bacteria, algae, fungi and plants, birds have to acquire carotenoids from their food, incorporate them into the blood stream, and allocate them to integument pigmentation or health functions (Olson & Owens, 1998). Recent work has shown that diet influences intraspecific variability in plasma carotenoid concentration of individuals (Bortolotti *et al.*, 2000; Negro *et al.*, 2000, 2002), and that such variability is reflected in their expression of colour in plumage and bare parts (Hill *et al.*, 1994; Bortolotti *et al.*, 1996; Saino *et al.*, 1999; Negro *et al.*, 2000; see also Grether *et al.*, 1999 for fish). However, the ability to use the carotenoids present in the food may be limited by the physiology (Hudon, 1994; Bortolotti *et al.*, 1996, 2000; Negro *et al.*, 1998; Saino *et al.*, 1999), nutrition (Hill, 2000), and genotype of the individual (Olson & Owens, 1998, but see Bortolotti *et al.*, 2000), as well as by environmental agents such as intestinal parasites interfering with the absorption of carotenoids in the intestine (Ruff *et al.*, 1974; Augustine & Ruff, 1983; Allen, 1987; McGraw & Hill, 2000).

While intraspecific studies of birds are proliferating, interspecific variability in acquisition of carotenoids from food, their transport and allocation are much less well studied (Britton *et al.*, 1995; Olson & Owens, 1998; Surai *et al.*, 2001). Diet is considered to play a major role in interspecific variability in carotenoid acquisition because food varies much more between than within species. Herbivores are expected to obtain more carotenoids than insectivores, and the latter more than vertebrate-eating species, with omnivores falling somewhere in between (Gray, 1996; Olson & Owens, 1998). To our knowledge, however, information on the relationship between diet and carotenoids in blood is restricted to a study on 14 bird species artificially fed in captivity (Slifka *et al.*, 1999). On the other hand, other studies suggest that those species acquiring more carotenoids are able to better express external carotenoid-based coloration. Trams (1969) found higher levels of plasma carotenoids in the red-plumaged scarlet ibis (*Eudocimus ruber*) than in the related white ibis (*E. albus*). Likewise, Hill (1995a) reported that bird species with carotenoid-based plumage showed redder plasma hues (indicators of carotenoid concentration) than species without. These results suggest a link between concentration of plasma carotenoids and integumentary coloration. However, the results of interspecific studies dealing with the role of diet on the absorption of carotenoids (Slifka *et al.*, 1999) and its expression on plumage (Hill, 1995a) were limited by the reduced number of species considered and the lack of control for phylogeny-derived effects. Data from closely related species are not independent because they may share traits inherited from a common ancestor (Harvey &

Page, 1991). Thus, both the acquisition of carotenoids from the diet and their incorporation in integuments may be phylogenetically influenced.

We assessed the relative contribution of diet and the extent of carotenoid-dependent coloration on interspecific variability in blood carotenoid concentration in birds while controlling for phylogenetic relationships among them. Given that information on carotenoid concentration in other bird tissues is only available for a few species, we selected plasma concentration of total carotenoids as a measure of recent carotenoid acquisition (Surai *et al.*, 2001). In humans, one of the best-studied vertebrate species, the pattern and concentration of carotenoids in plasma reflect those measured in most tissues (Parker, 1989). We obtained data on plasma carotenoid concentrations from free-living birds belonging to 80 species in eight different orders, which differed widely in diet and the extent of carotenoid-based traits. We also considered the potential influence of body size because of its major implications in vertebrate ecology and physiology (Peters, 1983).

## Methods

### Study areas and sampling

Fieldwork was carried out in Baja California Sur (México) in November–December 1996, and in Spain between December 1996 and February 1997. We measured winter plasma carotenoids because species with carotenoid-based traits may show different maxima in plasma concentrations depending on whether carotenoids are displayed in the plumage (peak observed at the time of moult, at the end of the breeding period, Hill, 1995b), or in bare parts such as the bill or legs (peak observed at the start of the breeding season, Negro *et al.*, 1998, 2001). Thus, when comparing species with carotenoids in the feathers with those with carotenoids in the skin or with no external carotenoids, winter is the only time when basal levels of circulating carotenoids are expected for all of them. Another reason to choose winter for sampling was that no species were breeding at that time. Laying females, for example deposit large amounts of carotenoids in eggs (Surai *et al.*, 2001), and their plasma carotenoid levels decrease concurrently (Negro *et al.*, 1998). Therefore, we expect gender and age variations within species (Hill *et al.*, 1994; Figuerola & Gutiérrez, 1998; Negro *et al.*, 1998, 2001) to be reduced in winter compared with other times of the year (see below).

### Field and laboratory procedures

Passerines were mist-netted, whereas larger species were trapped using a variety of devices, such as bal-chatri traps, rocket nets, clap nets and Wain Wright traps. All birds were released at the site of capture immediately after blood collection. Blood ( $\geq 30 \mu\text{L}$ ) was extracted from the

brachial or jugular veins in syringes or microcapillary tubes and then transferred to vials. Blood samples were transported in a cooler to our laboratories within a day of collection. Upon arrival, samples were centrifuged at 3000 r.p.m. for 10 min, to separate plasma from cells. The plasma was frozen at  $-20^{\circ}\text{C}$  until analysis. The concentration of total plasma carotenoids was measured spectrophotometrically (e.g. Allen, 1987; Tella *et al.*, 1998). The plasma was mixed with pure acetone (1 : 9) and centrifuged at 10 000 r.p.m. for 10 min. In the resulting supernatant, we determined the absorbance of the carotenoid peak at 476 nm using a Beckman Du-70 (La Paz, Mexico) or a Pharmacia Ultrospec 2000 (Sevilla, Spain) spectrophotometer. We calibrated carotenoid concentrations ( $\mu\text{g mL}^{-1}$ ) using standard curves of lutein (alpha-carotene-3,3'-diol, Sigma, Madrid, Spain) obtained independently for the two spectrophotometers used, thus allowing the comparison of results (Wellburn, 1994).

### Morphological and dietary variables

Relative importance of carotenoids in the pigmentation of plumage and bare parts (i.e. the presence of yellow, orange, red and noniridescent bright green colours, see Gray, 1996) was scored blindly by the same person (JJN) in seven body regions following Yezerinac & Weatherhead (1995), thus weighting each one according to the proportion of the body surface area covered, and using colour plates from field guides (Peterson & Chalif, 1973; Peterson *et al.*, 1987). The chestnut- or rufous-coloured feathers in some passerines that we studied, including the breast in the robin (*Erithacus rubecula*), and the stonechat (*Saxicola torquata*), the breast and tail in the bluethroat (*Luscinia svecica*), as well as the tail in black redstart (*Phoenicurus ochruros*) were scored as having zero carotenoids (see Appendix) because the pigments responsible for those colours are phaeomelanins (Brush, 1978, and authors' unpublished data based on high-performance liquid chromatography determinations).

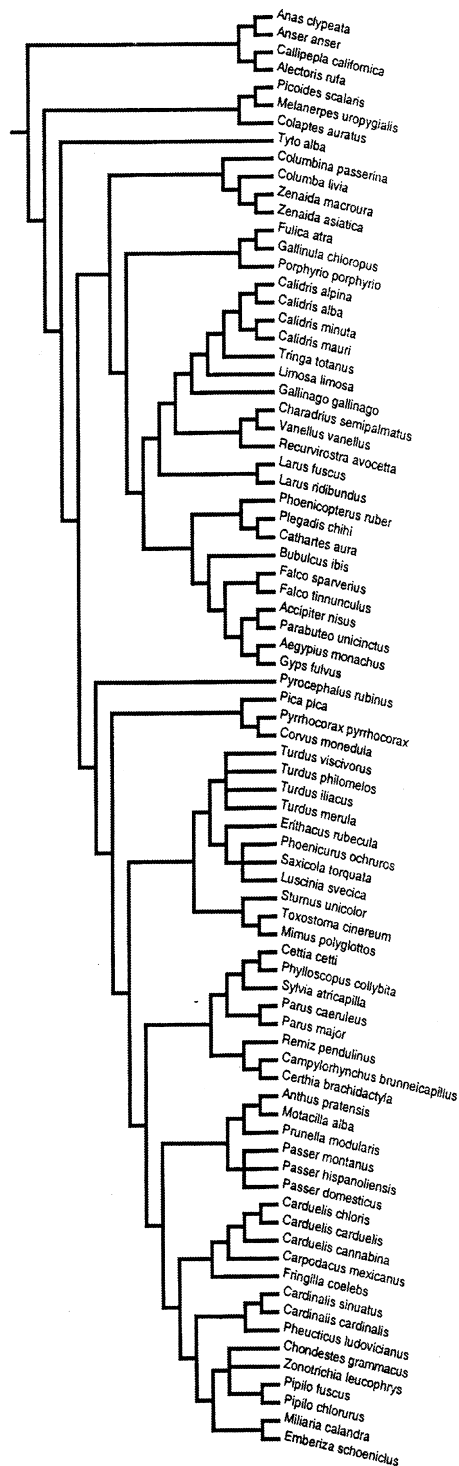
For dichromatic species, we scored the coloration of the male, which was always the most colourful sex. Winter diet was assigned to one of six categories ranked from lower to higher potential concentrations of carotenoids in food: (i) species strictly feeding on vertebrates, (ii) feeding on both vertebrates and invertebrates, (iii) feeding only on invertebrates, (iv) omnivorous, (v) feeding on invertebrates and plants, and (vi) feeding exclusively on plants. Dietary information and mean body mass of each species were obtained from several sources (Cramp, 1985–1992; Cramp & Perrins, 1993–1994; Rodríguez-Estrella, 1997).

### Statistical analyses

We first assessed whether there were sex and/or age effects on plasma carotenoids during winter in 11 species from five different orders (*Anas clypeata*, *Carduelis*

*cannabina*, *Columbina passerina*, *Emberiza schoeniclus*, *Falco sparverius*, *Fringilla coelebs*, *Fulica atra*, *Passer domesticus*, *Passer hispaniolensis*, *Pyrrhocorax pyrrhocorax* and *Zonotrichia leucophrys*) for which age and/or sex were identifiable in winter and more than 20 individuals were sampled (range: 23–141 individuals, see Appendix). One- or two-way ANOVAS revealed that neither age nor sex explained variability within species in carotenoid plasma concentration (all  $P = \text{n.s.}$ ). Therefore, potential interspecific biases in bird sampling related to differences in age and sex of individuals are unlikely to affect our comparative analyses. Another potential source of bias is sample size, i.e. a small number of birds may not adequately represent the plasma concentration values for a species. Thus we tested whether variability in plasma carotenoids was larger among than within species through an analysis of repeatability (Zar, 1996), using each individual as an independent measure for a given species. As repeatability of plasma carotenoid concentrations within species was high (intra-class correlation coefficient,  $r_i = 0.76$ ,  $F_{60,780} = 42.82$ ,  $P < 0.001$ ), we concluded that single individuals provide repeatable values for the species. Nonetheless, multivariate analyses were repeated excluding species for which only one individual was sampled to ensure our results were not biased by these species. For species from which more than one individual was sampled ( $n = 61$ ), we used the median of plasma carotenoid concentrations (see values in Appendix) for further analyses, as the median is less influenced than the mean by variability in sample sizes (Zar, 1996). Nonetheless, statistical analyses were repeated using the mean (also reported in Appendix) and both results (not shown) and conclusions remained the same.

Statistical analyses exploring the relationships between plasma carotenoids, diet and morphology included Spearman correlations and multivariable Generalized Linear Models (GLM) using GENMOD procedure (SAS, 1997), with a gamma error distribution, a log link function and the untransformed values of carotenoid concentration. Additionally, we used two approaches to deal with the phylogenetic relationships among species. We first used a nested ANOVA (Bell, 1989; Harvey & Pagel, 1991) to examine how plasma carotenoid concentration varied between and within different taxonomic levels (order, family and genera). We then included order and families nested within orders in a GLM to estimate the percentage of variation in plasma carotenoids explained by phylogeny (estimated from taxonomic relationships), diet and morphological traits. Genera were not controlled for in GLMs because most (86%) were represented by only one species, making it difficult to obtain accurate regression estimates. Secondly, a phylogenetic regression (Grafen, 1989) was calculated with program PHYLO.GLM for GLIM (Crawley, 1993), and used to investigate the relationships between plasma carotenoid concentration and the dietary and morphological variables in more detail. For this



**Fig. 1** Phylogenetic tree for the 80 bird species sampled for concentration of total plasma carotenoids.

analysis, a working phylogeny (see Fig. 1) was constructed using Sibley & Ahlquist (1990) and Sibley & Monroe (1990). Branch lengths were calculated according to

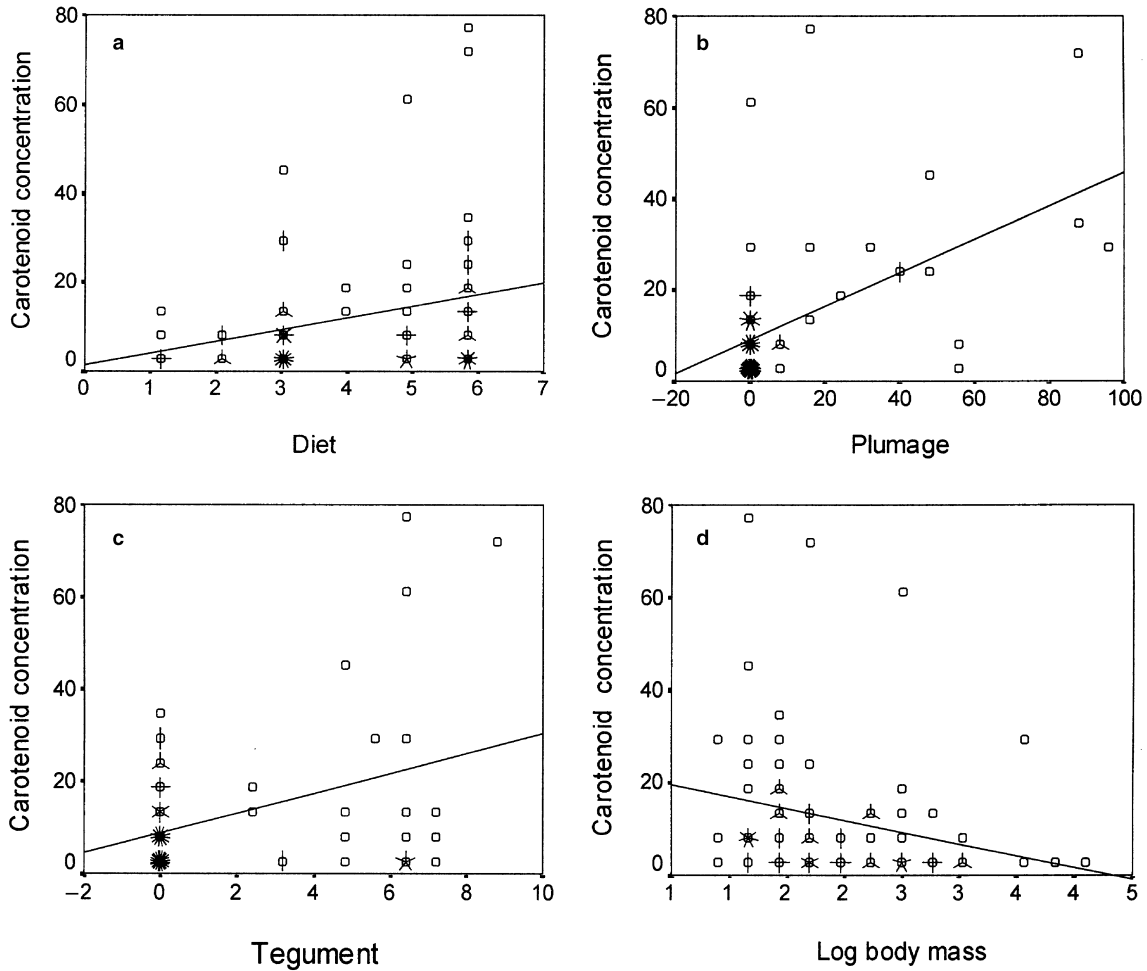
Grafen's (1989) method, because no detailed information on time of divergence was available. Phylogenetic regression was preferred over the more traditional Felsenstein's independent contrast method because of the limitations of the latter method to deal with most of the categorical variables used in the present study (see Harvey & Pagel, 1991). Plasma carotenoid concentration and body mass were log transformed to fit a normal distribution. All models were constructed following a forward stepwise procedure.

## Results

A total of 862 birds from 80 species were sampled, representing eight orders, 25 families and 64 genera. The median concentration of total plasma carotenoids varied from 0.38 to 53.63  $\mu\text{g mL}^{-1}$  among species (see Appendix). Univariate analyses showed that concentration of plasma carotenoids correlated positively with diet ( $r_s = 0.31$ ,  $P < 0.01$ ; Fig. 2a) and extent of carotenoid-based plumage ( $r_s = 0.50$ ,  $P < 0.001$ ; Fig. 2b) and negatively with body mass ( $r_s = -0.41$ ,  $P < 0.001$ ; Fig. 2d), but did not correlate significantly with the extent of carotenoid-based bare parts ( $r_s = 0.16$ ,  $P = 0.14$ ; Fig. 2c). However, all four variables were significant when analysed multivariately through a GLM (see model 1 in Table 1). This model explained 46% of the original deviance (final deviance 42.70, d.f. = 75). The variable explaining more deviance was plumage (10.6%), followed by bare parts (7.9%), body mass (6.7%) and diet (5.1%).

The above results might be biased by phylogenetic relationships between species, as phylogeny strongly influenced interspecific variability in plasma carotenoid concentration: a nested ANOVA ( $F_{64,15} = 2.71$ ,  $P < 0.05$ ) showed that taxonomic levels explained 65% of the variance. Most taxon-related variation was explained by order (38% of the variance), followed by family (14%) and genera (13%). We therefore controlled for taxonomy in the GLM regression, obtaining a more robust model which explained 81% of the original deviance for interspecific variability in plasma carotenoids (model 2 in Table 1). The concentration of plasma carotenoids was positively correlated with the extent of carotenoid-based coloration both in bare parts and in plumage, explaining 4.8 and 1.9% of the original deviance, respectively. Body mass of the species was negatively related to their levels of circulating carotenoids (3.3% of the original deviance). Diet, however, resulted only marginally significant once phylogenetic relationships among species were controlled for (model 2 in Table 1). These results were nearly identical when excluding those species from which only one individual was sampled (order,  $P < 0.001$ ; family,  $P < 0.001$ ; body mass,  $P < 0.01$ ; bare parts,  $P < 0.001$ ; plumage,  $P < 0.05$ ; diet,  $P = 0.12$ ).

The more robust phylogenetic regression, using the working phylogeny shown in Fig. 1, confirmed the



**Fig. 2** Relationships between concentration of plasma carotenoids ( $\mu\text{g mL}^{-1}$ ) in 80 species of birds and their (a) diet, (b) carotenoid-based plumage coloration, (c) carotenoid-based coloration of teguments (bare parts), and (d) body mass.

Parameter	d.f.	Estimate	SE	$\chi^2$	P
<b>Model 1</b>					
Intercept	1	1.0638	0.2533		
Body mass	1	-0.0002	0.0001	10.26	0.0014
Bare parts	1	0.1081	0.0317	11.98	0.0005
Plumage	1	0.0154	0.0042	15.77	0.0001
Diet	1	0.1574	0.0546	7.89	0.0050
<b>Model 2</b>					
Intercept	1	-0.0767	0.5524		
Order	7	0.0000-2.3530	0.0000-0.6490	53.01	0.0001
Family	17	-1.4768-0.1077	0.0000-0.7380	37.49	0.0029
Body mass	1	-0.0002	0.0001	13.21	0.0003
Bare parts	1	0.1274	0.0291	18.46	0.0001
Plumage	1	0.0101	0.0037	7.96	0.0048
Diet	1	0.1540	0.0820	3.41	0.0648

**Table 1** The results of GLMs explaining variability in the concentration of plasma carotenoids among 80 species of birds. Model 1, without controlling for taxonomy; Model 2, controlling for taxonomy. Different estimates were obtained for each family and order included in model 2, and the reported values correspond to the extremes of the range of estimates.

**Table 2** The results of a phylogenetic regression explaining variability in the concentration of plasma carotenoids among 80 species of birds.

Parameter	d.f.	Estimate	F	P
Log body mass	1,70	-0.1957	5.27	0.02
Bare parts	1,70	0.0614	17.28	<0.0001
Plumage	1,70	0.0055	9.02	0.004
Diet	1,69		1.77	0.19

positive relationships between plasma carotenoids and the relative presence of carotenoids in the plumage and bare parts, a negative relationship with body mass, and no significant effects of diet when phylogeny was accurately controlled for Table 2.

## Discussion

Diet has traditionally been thought of as a major constraint for the acquisition of carotenoids by bird species. Herbivores, feeding on primary producers, would have more ready access to carotenoids than invertebrate-eaters, and these would have an advantage over species preying upon vertebrates (Olson & Owens, 1998). However, this intuitively appealing hypothesis lacked empirical support. Given that accurate information on carotenoid content of food is only available for some captive species (Surai *et al.*, 2001), we used a broad categorization of diets covering species from strict vertebrate-eaters to strict herbivores (Olson & Owens, 1998). Both univariate and multivariate analyses showed that interspecific variation in circulating carotenoids is partially explained by variability in potential dietary access to them. However, the role of diet could be overridden by a stronger phylogenetic effect, which accounted for most (65%) of the variance in plasma carotenoids among species.

Our latter result suggests that phylogeny may strongly constrain the acquisition of carotenoids in birds. However, as Sheldon & Whittingham (1997) emphasized, although a trait may be correlated with phylogeny, such a correlation is not a demonstration of a phylogenetic constraint and may merely reflect a concomitant ecological constraint. In our case, the most evident ecological constraint covarying with phylogeny is diet. Obviously, ecological, morphological and physiological traits shared by phylogenetically related species mean that, for example dietary differences within raptors are much lower than between raptors and passerines, and thus a substantial amount of the variance explained by phylogeny could reflect dietary affinities among related species. In fact, a nested ANOVA shows that all the variability in diet of our sampled species may be explained by taxonomic levels (order: 62%, family: 20%, genera: 18%). This is in part due to the fact that diet was evaluated qualitatively, resulting, for example in

all species within the same genera being scored equally. Therefore, it is very difficult to disentangle the roles of diet and phylogeny, and further comparative analyses would be desirable when the actual concentration of carotenoids in the food of a number of bird species is available. However, the fact that our model 2 including phylogenetic effects explained much more variance in plasma carotenoids than model 1 where phylogeny was not considered (83% vs. 46% of the original deviance), suggests that in addition to diet, phylogenetic-related factors may also cause interspecific variability in carotenoid concentration.

To our knowledge, the issue of whether phylogeny can affect the acquisition of carotenoids independently of their availability in diet has not previously been studied. We suggest that there are several possible mechanisms requiring further investigation. First, a carotenoid-rich diet does not necessarily mean that the animal has access to those carotenoids. Interspecific differences in plasma carotenoid concentrations may be related to differences in the selective absorption processes in the small intestine (Yang & Tume, 1993). Carotenoids are thought to move into the enterocyte by passive diffusion (Furr & Clark, 1997). If so, species differences in absorption may arise due to differences in gut luminal events such as pH, gut motility and liposome and micelle formation, as well as variation in the type and amount of dietary fat (Furr & Clark, 1997). In addition, for a carotenoid to be incorporated into the bloodstream, the appropriate lipoproteins must be present in endothelial cells in the intestine (Rock *et al.*, 1996; Furr & Clark, 1997), and this physiological aspect could be genetically fixed (Olson & Owens, 1998). In fact, the low levels of carotenoids found in the plasma of the white ibis when compared with its congener the scarlet ibis, despite similar diets, was attributed to the lack of the appropriate lipoproteins needed to transport carotenoids from the gut to the blood stream in the former species (Trams, 1969). Therefore, the efficiency of carotenoid absorption may vary among species (Yang & Tume, 1993; Rock *et al.*, 1996; Surai *et al.*, 1998), and this variability, and other physiological traits (Garland & Carter, 1994), could be phylogenetically determined. Nonetheless, phylogenetic constraints may sometimes be overcome, as suggested by the differential carotenoid absorption showed by the two closely related species of ibis (Trams, 1969). Secondly, parasites may also play a role. It is well known that Coccidia (protozoan gut parasites) inhibit the absorption of carotenoids from the gut to the blood both in poultry and some wild species (Ruff *et al.*, 1974; Augustine & Ruff, 1983; Allen, 1987; Tyczkowski & Hamilton, 1991; McGraw & Hill, 2000). Moreover, the prevalence of coccidiosis varies among the groups of birds studied so far, such as cranes, partridges, ducks, doves and birds of prey (e.g. Kutzler *et al.*, 1982; Carpenter *et al.*, 1984; Boch & Schneidawind, 1988). Therefore, the risk of infection by Coccidia could shape some hosts' behavioural and ecological traits, which in

turn would be phylogenetically correlated, thereby partially explaining the phylogenetic effect we found. Unfortunately, the paucity of information available on prevalence of *Coccidia* in wild bird species prevented us from including it in our analyses so as to test this hypothesis.

Once controlling phylogeny, body mass still significantly explained variance in plasma carotenoids among species, with larger species having lower carotenoid concentrations. This result could be related to the allometric relationships between body mass and physiology in vertebrates. Both metabolic and ingestion rates increase with body mass of a species to the power 0.75. However, the total volume of blood is directly proportional (slope close to 1) to the body mass of the species (Peters, 1983). Therefore, large-bodied species ingest proportionally less food than small ones and may thus incorporate relatively fewer carotenoids to a proportionally equal volume of blood, resulting in lower concentrations of carotenoids.

Until recently almost nothing was known about the relationship between concentration of plasma carotenoids and bird colours. Hill (1995a) showed that species with carotenoid-based plumage had redder plasma than species lacking carotenoid pigmentation during moult. In this study, we confirm Hill's observations with a much larger set of species from both the Old and the New World, while controlling for phylogenetic relationships and measuring the actual concentration of carotenoids in blood. Moreover, we are the first to find a relationship between plasma carotenoids and the pigmentation of bare parts (see also Negro *et al.*, 2002). However, a fundamental difference between Hill's and our study is that we conducted ours in winter. Thus, species with external display of carotenoids have higher carotenoid levels in the plasma not only at times of the year when they are required for external pigmentation. Had we measured plasma carotenoids at the seasonal peaks for each species (moulting or mating seasons, Hill, 1995b; Negro *et al.*, 1998), we would have found ever stronger relationships. Nonetheless, our results do not demonstrate the causal basis underlying the evolution of carotenoid-dependent avian coloration. Although nothing is yet known about the amount of carotenoids required for health functions in birds (Hill, 1999), perhaps only species with carotenoid levels exceeding their specific health necessities are able to divert extra amounts to coloration (Gray, 1996). This possibility has also been suggested recently after analysing the relationship between diet and carotenoid pigment expression in a comparative study of doves (Mahler *et al.*, 2003). Alternatively, carotenoid-pigmented species could have evolved more efficient mechanisms for carotenoid absorption as a result of sexual selection. Although Mahler *et al.* (2003) did not find support for this hypothesis it is worthy of further examination.

Finally, we admit that the results of our comparative study, as others where accurate information for the independent variables is not available, need to be interpreted cautiously. More than 600 different carotenoids have been isolated from natural sources, and these carotenoids differ widely in their functional properties (Britton, 1995). However, no detailed information is available on the carotenoid composition of the diet for most free-living avian species. Moreover, the carotenoids responsible for bright coloration in birds vary among species, while few of them have been yet studied (Stradi, 1998), and health-related functions may also vary among types of carotenoids (Surai *et al.*, 2001). Nonetheless, we obtained a model that explained most (83%) of the interspecific variability in the concentration of total plasma carotenoids that was corroborated by phylogenetic regression. Although diet seems to play a role, we also propose the existence of previously overlooked physiological constraints to the acquisition of carotenoids, linked to phylogeny and body size, which may also limit their expression in external ornaments. We encourage researchers to conduct further comparative and experimental work to improve our understanding of the ecological, physiological and biochemical mechanisms behind these limiting factors.

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## Supplementary material

The following material is available at <http://www.blackwellpublishing.com/products/journals/suppmat/JEB/JEB634/JEB634sm.htm>

**Supplementary Appendix A1:** Mean (SD) and median concentration of total plasma carotenoids ( $\mu\text{g mL}^{-1}$ ), sample size ( $N$ ), average body mass (in g), and values of dietary and phenotypic variables (see Methods) for the 80 species of birds sampled belonging to different orders (O) and families (F).

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