

Fat stores in birds: an overlooked sink for carotenoid pigments?

J. J. NEGRO,*† J. FIGUEROLA,* J. GARRIDO‡ and A. J. GREEN*

*Department of Applied Biology, Estación Biológica de Doñana, CSIC, Avda. María Luisa s/n, 41013 Sevilla, Spain and ‡Department of Food Biotechnology, Instituto de la Grasa, CSIC, Avenida Padre García Tejero 4, 41012 Sevilla, Spain

Summary

1. Carotenoids are responsible for the most striking colours in birds, but also play an important role as enhancers of the immune system. Consequently, a trade-off between the ornamental and health functions of carotenoids in birds has been proposed.
2. Although it is well known that birds can store carotenoids in different organs and tissues, including the fat stores, until now all field studies of the regulation of carotenoid stores have focused on plasma carotenoids.
3. Carotenoids in the fat of 44 wild Greylag Geese (*Anser anser* L.) wintering in south-western Spain were identified and quantified. In addition, the relationships between carotenoids and the size of the fat stores, as well as the sex and age of the geese, were analysed.
4. The major carotenoid in goose fat was lutein. This and related carotenoids are also the most prevalent pigments in bird plasma and secondary sexual characters. We also detected β -cryptoxanthin, β -carotene, neochrome and neoxanthin. Total carotenoid concentration was negatively correlated with the size of the fat stores. Males had higher concentrations of carotenoids than females.
5. A possible explanation for these patterns is that male birds tend to have higher plasma carotenoids than females, a difference that could be transposed to fat if carotenoids diffused passively from the blood into adipose tissues. Carotenoids, however, may tend to remain in the fat stores. If this were true, fat would not be a reservoir of carotenoids, but a sink where a significant amount of these pigments would be sequestered, being no longer available for other functions.

Key-words: β -carotene, bird coloration, Greylag Goose, lutein, sexual selection

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Introduction

Carotenoids are currently the focus of intense research by evolutionary biologists because these pigments are responsible for some of the most striking colours in animal ornaments, which are likely to have evolved through sexual selection (Hill 1991; Negro *et al.* 1998). Behavioural ecologists are always looking for predictors of individual fitness, and the carotenoids contained in ornamental traits may possess this property (Hill 1990; Møller *et al.* 2000). The fittest individuals may concentrate more carotenoid in the ornaments (Hill 1996, 1999a; Olson & Owens 1999) because: (a) they ingest more carotenoids, which in vertebrates are always obtained from the diet (Goodwin 1950), (b) their genetic makeup for carotenoid absorption is superior, or (c) they are free of parasites or disease and

do not need to divert carotenoids to non-ornamental functions (as well as pigments, carotenoids are also antioxidants that can aid the immune system, Britton 1995; Surai & Speake 1998).

In birds, a trade-off between ornamental and health functions of carotenoids is widely supported (Bortolotti *et al.* 1996; Møller *et al.* 2000) but has recently been challenged (Hill 1999b). Citations about health benefits of carotenoids in recent ornithological literature are from medical studies with humans or other mammals, plus some studies with poultry (Allen 1992, 1993; Surai & Speake 1998). Most studies about wild birds have focused so far on variation of integumentary colour and its relation to pairing or breeding success (Hill 1991; Hill, Nolan & Stoeck 1999; Wolfenbarger 1999) or health status (Sundberg 1995; Figuerola *et al.* 1999; McGraw & Hill 2000). A few studies have also dealt with levels of plasma carotenoids and their relationship to external coloration (Hill *et al.* 1994; Bortolotti *et al.* 1996; Negro *et al.* 1998; Negro *et al.* 2000).

It has long been known that birds are able to store carotenoids in different organs, such as the liver or adipose tissue (Goodwin 1950; Fox 1953). High concentrations of the carotenoid astaxanthin have recently been measured in the fat of White Storks *Ciconia ciconia* (Negro & Garrido 2000). However, to our knowledge there are few studies evaluating how many carotenoids (and what types) end up in bird fat reserves (but see Surai, Royle & Sparks 2000). In order that carotenoid use by birds can be better understood, potential stores of carotenoids need to be investigated.

The aim of the current study was to identify and quantify carotenoid pigments in the fat reserves of Greylag Geese (*Anser anser* Linnaeus) wintering in Spain. We looked for possible sex- and age-related variation in carotenoids stored in fat. Such variation may be expected because male and adult birds typically show brighter carotenoid-dependent coloration than females and immatures (Bortolotti *et al.* 1996; Gray 1996). These trends have been also observed in plasma concentrations of carotenoids (Hill *et al.* 1994; Bortolotti *et al.* 1996), but it is unknown whether there are biases due to gender or age in fat stores.

Wintering geese have sizeable fat stores that show important seasonal and individual variation. This fat presents a yellowish colour due to carotenoid pigments. Greylag Geese have no carotenoid-dependent plumage coloration, but their beaks vary in colour from pink to orange. The orange hue is due to carotenoids, as with many other Anseriformes, including ducks (Johnson 1999) and swans. Bill colour in the Greylag Goose is brighter during the breeding season (A. D. Fox, personal communication), and brighter in adults than in immatures (Baker 1993), as expected of sexually selected traits (Andersson 1994). The carotenoids contained in fat might be available for ornamentation of the bill (or perhaps for other physiological functions), at the time of mate and territory acquisition. Nonetheless, whether carotenoids contained in adipose tissue can be remobilized via the blood transport system to be used for physiological functions remains unclear (Fox 1953).

Materials and methods

Geese that had been shot illegally and confiscated by the police were used. The geese were shot in the midwinter period (December 1998 to January 1999) in the Guadalquivir marshes of southwestern Spain. Their folded wing length, tarsus length and body mass were measured on receipt. The birds were surgically sexed and their age was assessed by plumage characteristics (Baker 1993).

ESTIMATION OF TOTAL FAT CONTENT AND SAMPLE COLLECTION

Two different methods were used to score fat content in each goose. First, fat was scored according to a 0–8 scale developed by Kaiser (1993) for passerines. Zero

denotes no fat, and 8 maximum fat deposition. Additionally, fat content was estimated by making an incision with a scalpel in the fat layer of the breast, and measuring the thickness of the fat layer with a metal ruler (mm). Two fat samples, from the belly and breast areas, were taken from each individual for carotenoid analysis. A few individuals only had fat in one of these areas, affecting sample size. The samples were stored at –20 °C until analysis.

CAROTENOID ANALYSES

Extraction and saponification procedure

The fat in which the carotenoid pigments were embedded made the extraction of carotenoids difficult. Therefore, a saponification was used and it was assumed that any esterified carotenoids that might be present in the fat would be obtained in a non-esterified form. Carotenoids were extracted and purified as follows: 0.5–2 g of fat was cut into small pieces with the help of scissors and placed in a glass flask with 20 ml of diethyl ether. The material was sonicated for 1 min and left under cover for 30 min. The solution with the carotenoids was filtered in a 500 ml decanting funnel. The residue was rinsed twice with 20 ml of diethyl ether that was added to the decanting funnel. Then 50 ml of KOH/MeOH 10% (w/v) were added to the ether solution and the decanting funnel was shaken gently. After 1 h with periodic shaking, distilled water was added. The aqueous phase was discarded and the ethereal phase was repeatedly rinsed with distilled water until neutral pH was reached. The ethereal phase was then filtered with anhydrous Na₂SO₄ (2%) and evaporated in a rotary evaporator until sample dryness. The resulting pigments were redissolved in an adequate volume of acetone and kept frozen at –20 °C until analysis by TLC (thin-layer chromatography) and HPLC (high-performance liquid chromatography).

TLC

Silica gel plates prepared in our laboratory with Kieselgel 60 GF₂₅₄ (Merck, Darmstadt, Germany) were used, with petroleum ether/acetone/diethylamine (10 : 4 : 1) as eluent.

HPLC

A Waters 600E instrument equipped with a quaternary pump was used (Waters Cromatografía, Barcelona, Spain). Carotenoid analyses were carried out by using a reverse phase C₁₈ column (Spherisorb ODS-2250 × 4 mm I.D.) and a precolumn of the same material with a particle size of 5 µm. Samples were injected with a Rheodyne 7125 valve equipped with 20 µl loop (Rheodyne, Rohnent Park, CA, USA). The eluent system was a gradient described in Mínguez-Mosquera & Hornero-Méndez (1993): acetone–water (75/25) for 5 min,

linear increase up to (95/5) over 5 min (conditions were constant for 7 min). Then a convex gradient (100/0) over 5 min, and a return to initial conditions (75/5) for 5 min (linearly). Flow rate was 1.5 ml min^{-1} . Data were acquired between 350 nm and 550 nm with a Waters 996 diode-array detector (Waters Cromatografia, Barcelona, Spain).

Quantitative determination of carotenoids

Reference carotenoids were obtained from fresh green plants in our laboratory, according to Mínguez-Mosquera (1997). Known dilutions of reference lutein, the main carotenoid in goose fat (see below), were injected in the HPLC machine to build a calibration curve at 450 nm. Concentration of individual carotenoids was calculated from HPLC areas recorded at 450 nm. Total carotenoid concentrations were also calculated from the HPLC chromatogram as if they were lutein. The TLC and HPLC systems that were used are not able to separate lutein and zeaxanthin, so that our estimate of lutein concentration could contain some zeaxanthin. Nonetheless, additional tests (data not shown) suggested a dominance of lutein.

STATISTICAL ANALYSES

Residuals from regressions between body mass and morphometric measurements of birds are often used as a crude index of their 'condition' (Brown 1996; Green 2001). Although we had more direct measures of fat stores, various residual indices relating mass to wing and tarsus length were tried, using both traditional least squares and model II regression methods. In no case did these indices explain as much variation in carotenoid concentrations as fat score or fat thickness. Such residual indices were therefore not included in the final analyses.

To examine the relationship between carotenoids and different goose characteristics general linear modelling (GLM) was used. These models allow the error distribution of the dependent variable and the link function linking the independent to the dependent variables to be adjusted to the characteristics of the data. The distribution of carotenoid concentration data (both total and lutein) was greatly skewed, adjusting to a gamma distribution. GLMs with a gamma-distributed error function and a log link were thus used (see Crawley 1993). A forward stepwise procedure was followed until no additional variable entered significantly in the model. For variables not entering in the model, their partial effects were presented when added to the final model.

Results

IDENTIFICATION OF CAROTENOID PIGMENTS

A typical HPLC chromatogram of a saponified pigment extract of goose fat is presented in Fig. 1. No

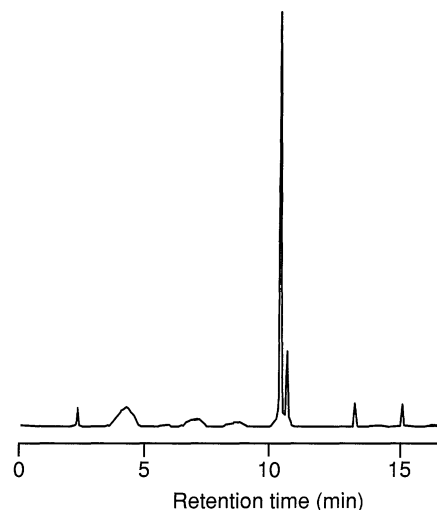


Fig. 1. Typical carotenoid pattern of Greylag Goose (*Anser anser*) fat by HPLC. Lutein and its *cis*-isomer are the major pigments, at about 10 min retention time.

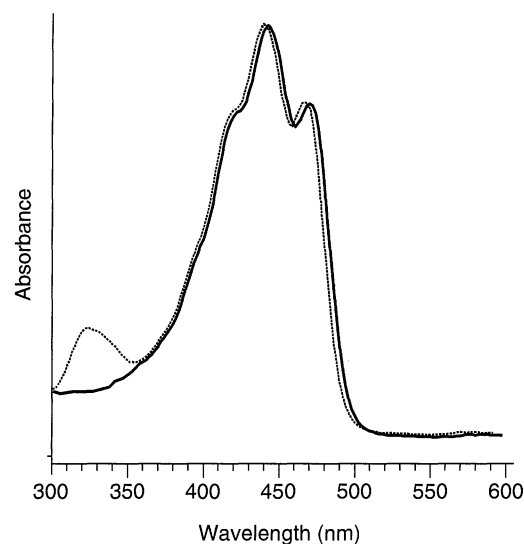


Fig. 2. Electronic absorption spectra in the HPLC eluent system (see Methods) of lutein (solid line) and *cis*-lutein (dashed line) from fat of free-living Greylag Goose (*Anser anser*).

qualitative differences were observed between breast and belly samples. The chromatograms showed a well-defined peak at about 10.5 min together with a lower peak at 11 min. Comparing retention time and the shape of the absorption spectra with those of the available standards in the same eluent system, the first peak was consistent with lutein. The pigment responsible for the second peak presented an absorption spectrum very similar to that of the previous pigment but showed an additional peak at 340 nm, characteristic of *cis*-isomerization of lutein (Fig. 2). Additionally, TLC analyses were consistent with the previous identification, although in this case it was not possible to separate the *cis*-isomer from its corresponding *trans*-, which is

the most abundant in fat (see also Mínguez-Mosquera 1997). In addition, two small peaks usually emerged at 13.5 and 15.6 min (Fig. 1) that, according to their chromatographic and spectroscopic characteristics, were tentatively identified as β -cryptoxanthin (426, 450 and 480 nm) and β -carotene (428, 454 and 482), respectively (Mínguez-Mosquera 1997).

In the chromatograms, a wide band was usually present (with a retention time of about 4 min) composed of a mixture of highly polar pigments. For the samples in which the signal of the wide band was more intense, we obtained absorption spectra with the same eluent system as for HPLC, and at different times. In some cases there were two clearly different components, with absorption maxima at 401, 424 and 450 nm, and 416, 440 and 468 nm, respectively. It is hypothesized that they correspond to the presence of the carotenoids neochrome and neoxanthin (Mínguez-Mosquera 1997).

In most HPLC chromatograms of the individual samples, diffused absorption areas were also observed between 4 and 10 min of retention time. These areas were also considered when estimating total carotenoid concentration, as they possibly corresponded to mixtures of degraded pigments.

When performing TLC of the pigment extracts a yellow band with a $R_f = 0.40$ was always obtained, identical to reference lutein. The absorption spectra in benzene (429, 456 and 488 nm) and chloroform (430, 452 and 482 nm) were also identical to the ones for reference lutein. Depending on the samples, other bands were sometimes obtained that correspond to the different carotenoid pigments detected by HPLC. Owing to this intersample variability regarding presence/absence of the minority pigments, an aliquot of all extracts for TLC analysis was pooled. The pooled sample yielded five yellow bands, with R_f values of 1.0, 0.56, 0.40, 0.18 and 0.15. When the plate was sprayed with diluted HCl, the first two bands ($R_f = 1.0$ and $R_f = 0.56$) did not change their colour, thus indicating that those carotenoids had no functional groups, as is the case with β -carotene and β -cryptoxanthin. The third band, with $R_f = 0.40$, was brown in colour at the centre with a green rim, indicative of the presence of two hydroxylic groups, as is the case with lutein. The last two bands turned blue, pointing to the presence of an epoxy group, such as those of neoxanthin and neochrome. Overall, these results confirm the tentative identification of carotenoid

Table 1. Fat score (Kaiser 1993) and fat thickness (mm) in wintering Greylag Geese according to age and sex

	Fat score	Fat thickness	n
Juvenile males	2.09 \pm 2.01	4.73 \pm 1.74	11
Adult males	3.00 \pm 2.67	5.20 \pm 2.20	10
Juvenile females	2.50 \pm 1.51	5.25 \pm 1.67	8
Adult females	3.17 \pm 2.37	5.13 \pm 2.56	15

pigments in the goose samples previously made using HPLC.

SEX- AND AGE-RELATED DIFFERENCES IN FAT CONTENT

No differences in fat score were found according to age or sex (Kruskal Wallis test, $\chi^2_3 = 2.00$, $P = 0.57$, $n = 44$, Table 1). Likewise, fat thickness did not differ between males and females ($F_{1,40} = 0.12$, $P = 0.73$) or juveniles and adults ($F_{1,40} = 0.07$, $P = 0.79$), and the interaction between these two factors was not statistically significant ($F_{1,40} = 0.20$, $P = 0.66$, Table 1).

CAROTENOID CONCENTRATION IN GOOSE FAT

Mean carotenoid content in the fat of male and female Greylag Geese is given in Table 2. Total carotenoid concentration in the breast and belly were significantly correlated (Spearman correlation, $\rho = 0.53$, $P = 0.0004$, Table 3). Lutein and its isomers represented a major fraction of total carotenoids deposited in both the belly and in the breast (breast = $41.10\% \pm 13.60$, belly = $55.21\% \pm 12.83$, means calculated using individuals with samples analysed for both body areas, Wilcoxon paired-rank tests = -362.5 , $n = 41$, $P < 0.001$).

Concentration of total carotenoids in the breast was negatively correlated with fat score (Table 3 and Fig. 3). In the belly, carotenoid concentration was also negatively correlated with estimated fat content but in this case with fat layer thickness. Carotenoid concentration in both locations was higher in males than in females.

The thickness of the fat layer was negatively correlated with absolute lutein concentration in the breast and belly (Table 4). Males presented higher concentrations of lutein in the belly than females. The same pattern was detected in the breast, but differences were not statistically significant ($\chi^2 = 2.84$, 1 df, $P = 0.09$).

CAROTENOID CONCENTRATION IN FAT COMPARED WITH PLASMA

In the absence of detailed studies on Greylag Geese, to estimate the total fat content of our birds we used Johnson *et al.*'s (1985; p. 572) formula for the Greater White-Fronted Goose *Anser albifrons*. According to this formula, the mean size of the fat stores in the analysed Greylag individuals should be of ≈ 445 g of fat in

Table 2. Carotenoid content ($\mu\text{g g}^{-1}$ wet mass) in fat of male and female Greylag Geese wintering in Spain (two samples, from breast and belly, per individual; see Materials and methods)

Gender	Total carotenoids	Lutein + isomer	Other carotenoids	Sample size
Females	1.79 \pm 2.18	0.82 \pm 1.01	0.96 \pm 1.22	38
Males	3.17 \pm 4.39	1.71 \pm 3.15	1.45 \pm 1.45	39
Population	2.49 \pm 3.52	1.27 \pm 2.38	1.21 \pm 1.35	77

Table 3. GLM models testing the relationship of absolute carotenoid concentration ($\mu\text{g g}^{-1}$) in the breast and belly with different goose characteristics. Error distribution = gamma, link = log

	Breast estimate	χ^2	P	Belly estimate	χ^2	P
Sex	0.453 ± 0.189	5.35	0.02	0.647 ± 0.216	8.08	0.005
Age		0.00	0.95		2.29	0.13
Fat score	-0.186 ± 0.043	15.02	<0.0001		0.18	0.67
Fat layer		1.09	0.30	-0.420 ± 0.054	39.85	<0.0001
Initial deviance	21.14			54.81		
Final deviance	13.51			20.80		

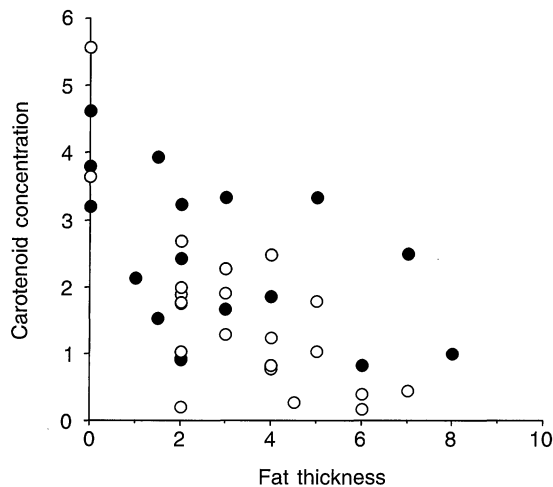


Fig. 3. Relationship between fat thickness (mm) and absolute carotenoid concentration in the breast ($\mu\text{g g}^{-1}$) for males (●) and females (○).

males and 490 g in females. These estimates suggest that, in females, means of 0.405 mg of lutein and 0.877 mg of total carotenoids are stored in fat. The equivalent figures for males are 0.764 and 1.413 mg, respectively. The volume of blood was calculated from the formulas of Hanwell, Linzell & Peaker (1971). From a volume of blood of 191.4 ml in males and 170.3 ml in females, a mean concentration of carotenoids of $3.33 \mu\text{g ml}^{-1}$ of plasma (authors' unpublished data), and a haematocrit of 46% (mean for Anseriformes, Prinzinger & Misovic 1994), the quantity of carotenoids circulating in the blood will approach 0.306–0.344 mg, i.e. less than half the amount of carotenoids in fat.

Discussion

CAROTENOID COMPOSITION

The major carotenoids in goose fat were lutein and its *cis*-isomer, which represented about 50% of total carotenoids in each sample. At far lower concentrations β -cryptoxanthin, β -carotene, neochrome and neoxanthin were also detected. Recently, Surai *et al.* (2000) have reported a similar array of carotenoids in abdominal fat of free-living Lesser Black-Backed Gulls *Larus fuscus*.

Birds are able to absorb xanthophylls intact from the diet in the gut (Schiedt 1998), and the fact that lutein and its *cis*-isomer were the major carotenoids in our samples is no surprise. Free lutein in the geese that were analysed possibly came from green plants, as these birds are herbivorous. However, the presence of the lutein isomer could be due to our carotenoid extraction protocol. As explained above, saponification is a rather lengthy process and, given the low stability of carotenoids, part of the free lutein could have suffered isomerizations. Lutein is the most prevalent carotenoid in bird plasma (Brush 1990; Tella *et al.* 1998), and the blood transport system distributes this pigment to the soft tissues, including skin, liver (Schiedt 1998) and, as shown by our results, also to the adipose tissue.

β -carotene and β -cryptoxanthin are poorly absorbed, at least in the domestic chicken (Schiedt 1998), but they have been detected in the skin of several wild and domestic bird species (Czeczuga 1979). Curiously enough, these two carotenes, with recognized provitamin A activity (Schiedt 1998), have been detected in large amounts (29% and 13% of total carotenoids, respectively) in the skin of domestic geese *Anser anser*.

Table 4. GLM models examining the relationship of absolute lutein concentration ($\mu\text{g g}^{-1}$) in the breast or belly with different goose characteristics. Error distribution = gamma, link = log

	Breast estimate	χ^2	P	Belly estimate	χ^2	P
Sex		2.84	0.09	0.727 ± 0.256	7.29	0.007
Age		0.77	0.38		0.27	0.60
Fat score		0.94	0.33		0.55	0.50
Fat thickness	-0.286 ± 0.073	13.98	0.0002	-0.421 ± 0.064	32.36	<0.0001
Initial deviance	25.85			64.25		
Final deviance	18.26			27.56		

domesticus (Czeczuga 1979: no analyses were performed on fat). Neochrome and neoxanthin are two carotenoids found in most photosynthetic organisms and they may have been absorbed unspecifically when the geese fed on green plants.

FAT SCORES AND CAROTENOID CONCENTRATION

No detailed studies of the fat dynamics of Greylag Geese wintering at southern latitudes have been made, but studies in other species suggest that fat stores are generally at intermediate levels during this midwinter period (Gauthier, Giroux & Bédard 1992).

Negative correlations were found between total carotenoid concentration and fat content. These results could be explained by at least three hypotheses: (a) the assimilation of lipids is easier than that of carotenoids, which get 'diluted' as fat stores get large, (b) when fat is mobilized because of increased energetic demands, carotenoids tend to stay in the remaining fat stores, and (c) birds in better condition, as indicated by fat stores, are more efficient in their use of ingested carotenoids. According to the latter hypothesis (G. Hill, personal communication), birds could have mechanisms to prevent carotenoids from entering the fat, where they may become unavailable for other uses. Birds in better condition could have more efficient mechanisms. Currently we have no data to support any of these hypotheses. Nonetheless, the return of carotenoids from adipose tissue to the blood has not been established (Fox 1953), suggesting that carotenoids in fat may not be readily available for other functions mediated by the blood transport system.

MALE-BIASED CONCENTRATION OF CAROTENOIDS

The most puzzling result in our study is the significant male-biased carotenoid concentration in fat, even though neither fat score nor fat thickness differed among sexes. As the possible effect of fat content was controlled in the analyses, this result could be explained if carotenoids diffused passively to the adipose tissue, in relation to its concentration in the plasma, as demonstrated in experiments with domestic chicken (Schiedt 1998 and references therein). Male birds tend to have higher plasma concentration of carotenoids than females (see Introduction). For Greylag Geese, no sex differences have been reported for bill colour, but there could be a subtle and yet unrecognized dichromatism, like the one recently detected in the Red-Legged Partridge *Alectoris rufa* (Villafuerte & Negro 1998). However, sexual dichromatism is not the only explanation for sexual differences in carotenoid concentrations, which have also been reported in the loggerhead shrike *Lanius ludovicianus*, a species lacking external display of carotenoids (Bortolotti et al. 1996). The sex bias in carotenoid absorption and

accumulation could be related to hormones, which are in turn involved in lipid synthesis (P. Surai, personal communication). Nonetheless, our study was conducted in mid-winter when hormonal asymmetries are at a minimum.

ABSOLUTE AMOUNTS OF CAROTENOIDS DEPOSITED IN FAT STORES

Total carotenoid concentrations in the fat of Greylag Geese, of about 2–3 µg g⁻¹ fresh mass, were very similar to the mean value provided by Surai et al. (2000) for fat carotenoids of free-living gulls (2.54 ± 0.23 µg g⁻¹), and slightly lower than the values reported for the red or orange skin of the legs of several wild birds (Czeczuga 1979). On the other hand, the absolute amount of total carotenoids retained in goose fat averaged ≈1 mg, which is more than twice the amount that can be found in the whole blood volume in winter. The pigmentation potential of 1 mg of carotenoids is considerable, particularly if it had to be applied to the small surface of the beak.

Our study demonstrates that carotenoid content in goose fat is relatively large. These carotenoids were absorbed from the gut to the blood transport system, and later diffused to the adipose tissue. The pathways for this transfer in wild birds have not been investigated, but carotenoids are lipid-soluble substances (Schiedt 1998) that are transported by lipoproteins (Parker 1996). Further research is needed, however, to determine whether carotenoids stored in fat can be re-used later for ornamental or other functions (e.g. carotenoid provisioning of the egg yolk in the case of breeding females) when the fat is mobilized. In addition, more work is needed to determine how levels of circulating carotenoids are related to the quantity of carotenoids stored in organs and tissues, in order to assess if the levels of plasma carotenoids are a reliable predictor of the much larger quantities that can be stored in different body parts.

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