

# The liver but not the skin is the site for conversion of a red carotenoid in a passerine bird

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**Abstract** Carotenoids may provide numerous health benefits and are also responsible for the integumentary coloration of many bird species. Despite their importance, many aspects of their metabolism are still poorly known, and even basic issues such as the anatomical sites of conversion remain controversial. Recent studies suggest that the transformation of carotenoid pigments takes place directly in the follicles during feather growth, even though the liver has been previously recognised as a storing organ for these pigments with a certain potential for conversion. In this context, we analysed the carotenoid profile of plasma, liver, skin and feathers of male Common Crossbills (*Loxia curvirostra*). Interestingly, the derivative feather pigment 3-hydroxy-echinonone was detected in the liver and in the bloodstream (i.e. the necessary vehicle to transport metabolites to colourful peripheral tissues). Our results demonstrate for the first time with empirical data that the liver may act as the main site for the synthesis of integumentary carotenoids. This finding contradicts previous assumptions

and raises the question of possible inter-specific differences in the site of carotenoid conversion in birds.

**Keywords** Carotenoid conversion · Feather pigments · Follicles · Liver · *Loxia curvirostra*

## Introduction

Carotenoids have important functions in many physiological processes. They can work as antioxidants, immunomodulators and photoprotectants or take part in vitamin synthesis and intercellular communication (McGraw 2006). Carotenoid pigments are also used by many bird species as integumentary colorants, being responsible for most of their red, orange and yellow displays (Stradi 1998). Since birds cannot synthesise carotenoids *de novo*, they must acquire them directly from the diet. Once they have been consumed, dietary carotenoids are either directly incorporated into different body tissues (e.g. Negro et al. 2001) or metabolised before their deposition in the integument (Stradi 1998).

Current knowledge about species-specific metabolic processes involving carotenoids is still limited. For those species in which the carotenoids deposited in the plumage are not present in the diet, there are few data about the enzymes that catalyse the biochemical transformations, and the energetic costs of the conversions have not yet been assessed. Moreover, the anatomical site for carotenoid conversion remains controversial (McGraw 2006). Several authors have proposed the integument as the metabolically active site for processing of carotenoids that will be displayed in feathers (Stradi 1998; Inouye 1999; McGraw 2004), while others consider the liver as the most logical place (Schiedt et al. 1985; Torrissen et al. 1989;

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Brush 1990; Hill 2000). The possibility of hepatic conversion of certain carotenoids have been documented in birds and other vertebrates. The metabolism of pro-vitamin A carotenes, such as  $\beta$ -carotene, occurs in the liver and the small intestine (Wyss et al. 2001; Wyss 2004). Dietary xanthophyll modifications concerning anhydrolutein synthesis in Zebra Finches (*Taeniopygia guttata*) seem to take place at hepatic levels as well (McGraw et al. 2002). To date, however, no study has yet reported carotenoid derivatives colouring the avian integument in the liver, and recent publications support the idea that birds manufacture them directly at peripheral tissues (McGraw 2004, 2006).

The Common Crossbill (*Loxia curvirostra*) is a cardueline finch in which adult males display carotenoid-based ornamentation on throat, breast and rump. Colouration varies from dull yellow to bright red, although the majority of birds are reddish orange (Stradi 1998). This colouration is mainly attributed to 3-hydroxy-echinenone, a carotenoid that may derive from the oxidation of the non-xanthophyll dietary precursor  $\beta$ -cryptoxanthin (Stradi 1998). The relative proportion of this major red pigment in relation to other minor carotenoids determines the definitive hue of every individual (Hill 2000). These features make the Common Crossbill an appropriate model species for the study of feather pigmentation and carotenoid conversion in birds.

We examined the carotenoid content of liver, serum, skin and feathers of crossbill males. Our aim was to determine the anatomical origin of the metabolically derived ketocarotenoids that occur in the red plumage of most individuals but are not available in their diet. If these metabolites would be present in the liver and the bloodstream, the vehicle that circulates metabolites to colourful peripheral tissues (Hill 2000), hepatic conversion of dietary carotenoids ought to be assumed in this species.

## Material and methods

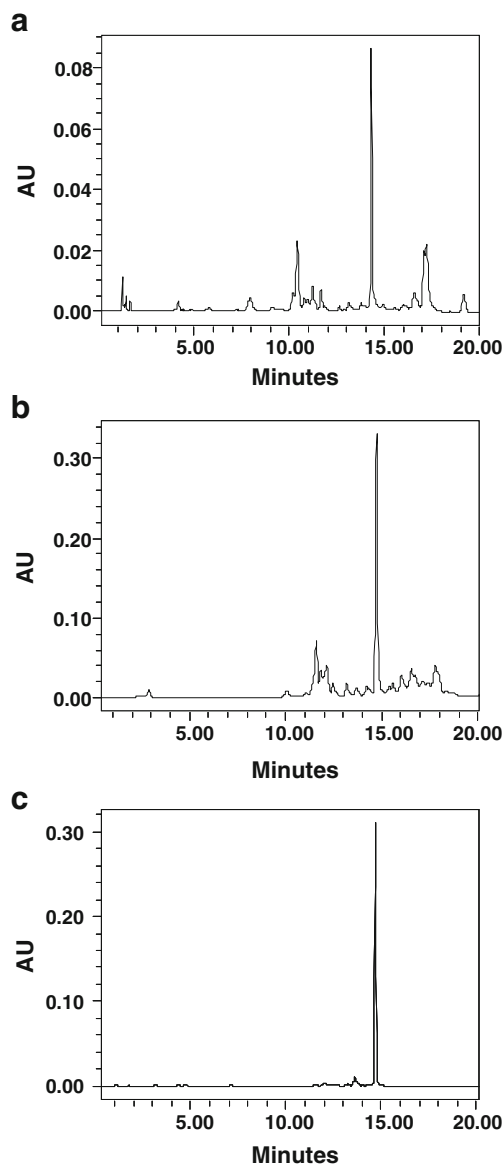
Liver, follicle-containing skin and feather samples were obtained from seven adult crossbill males that died accidentally during ringing sessions in the Catalanian Pyrenees between 2004 and 2006. All carcasses were immediately frozen at  $-20^{\circ}\text{C}$  until analysis. Once the birds were defrosted and dried, we scored the general plumage colour pattern of the individuals along a visual scale, which ranged from yellow to orange and red (for details see Del Val et al. 2009). We collected the feathers of their entire body and stored them in plastic bags under dark conditions at room temperature. We also removed the skin with the feather follicles and the liver. All samples were frozen at  $-80^{\circ}\text{C}$  before carotenoid extraction.

Breast feathers, skin and liver were subjected to an extraction procedure of carotenoids as follows: 0.01 g of feathers, 0.2 g of skin and 0.2 g of liver were introduced separately into 2-ml test tubes for subsequent extraction. We added 1 ml of *N,N*-dimethylformamide and placed the tubes in a  $60^{\circ}\text{C}$  water bath for 3 h, including sonication for 15 min every hour. We then centrifuged the samples at 12,000 rpm for 5 min and stored an aliquot of the supernatant at  $-30^{\circ}\text{C}$  until analysis by high-performance liquid chromatography (HPLC). Identification of 3-hydroxy-echinenone was conducted by separation and isolation of the pigment by thin layer chromatography and acquisition of UV-visible spectra with a PhotoDiode Array Spectrophotometer model HP 8452A in different solvents. Chemical derivatization microscale tests were also performed for the examination of 5,6-epoxide, hydroxyl and carbonyl groups (Eugster 1995). The chromatographic, spectroscopic and chemical properties of the pigment were compared with data in the literature (Foppen 1971; Davies and Köst 1988; Britton 1995). After this tentative identification, samples were analysed in a Waters 600E instrument equipped with a reverse-phase C18 column (Kromasil 5  $\mu\text{m}$ ,  $250 \times 4.6$  mm I.D.) and a precolumn with the same material. We used the chromatographic method described by Mínguez-Mosquera and Hornero-Méndez (1993), with a binary solvent gradient acetone–water at a flow rate of  $1.5 \text{ ml min}^{-1}$ . The diode array detector wavelength was set at 450 nm and the UV-visible spectra of each peak were recorded and stored online in the 350–600 nm wavelength range. Spectra and retention time of 3-hydroxy-echinenone were compared with those obtained using a pure standard. Quantification was performed using an external standard calibration curve at 450 nm from injection of progressive concentrations of the reference pigment.

Additionally, we analysed blood carotenoids from 14 moulting males captured in September 2004 and 2005. Blood samples (maximum 400  $\mu\text{l}$ /bird) were collected from the brachial vein into heparinized microhematocrit tubes. We kept the samples on coolers and centrifuged them at 11,000 rpm for 10 min in the next 8 h. Plasma was removed, transferred to Eppendorf tubes and frozen at  $-20^{\circ}\text{C}$  until analysis by HPLC. Carotenoids were extracted from thawed plasma by adding three parts of acetone (3:1, v/v). The mixtures were introduced in a room temperature bath and sonicated for 5 min in order to accelerate the extraction process. We subsequently centrifuged the samples at 13,000 rpm for 10 min, obtaining a supernatant with the carotenoids in solution. HPLC was carried out following the procedure described in Senar et al. (2008), and the quantitative determination of 3-hydroxy-echinenone was performed as described above for feathers and tissues.

## Results

In all sample types (i.e. liver, blood, follicles and feathers), we detected a carotenoid with no fine structure that eluted at 14.80 min (Fig. 1a, b) and whose UV-visible spectrum showed only one maximum at 466 nm. This was consistent with a chromophore of nine conjugated double bonds plus two b-rings and the possibility of end groups containing ketonic functions. The maximum of the carotenoid pigment absorption spectra in different solvents were compared with those in the literature. All chromatographic, spectroscopic and chemical properties were consistent with the structure of 3-hydroxy-echinenone. In addition, the detected carot-



**Fig. 1** HPLC chromatograms for **a** a liver extract of an adult male crossbill *Loxia curvirostra*, **b** follicle-containing skin sample for a same sex and age individual and **c** 3-hydroxy-echinenone standard

enoid presented identical spectra and elution patterns compared to standard 3-hydroxy-echinenone analysed in our HPLC system (see Fig. 1c).

The liver extracts showed a strong yellow colouration. However, in the chromatogram resulting from the HPLC analysis, the signal was very weak in the visible range, with absorbance peaks in this region of the spectrum practically overridden by a strong signal below 400 nm and extending all along the chromatogram (data not shown). The strong absorbance of the liver sample in the UV region must have been responsible for most of the yellow colouration, but this was due to non-carotenoid pigments, possibly including bilirubin and biliverdin. When injecting the sample in the HPLC system, the tri-dimensional chromatogram afforded by the controlling software did not permit to observe the carotenoid pigments in the visible range because absorbance maxima were automatically set for the wavelengths of the more abundant non-carotenoid pigments co-eluting with them. However, when the chromatogram was set at 450 nm, and after zooming up about five times over the normal measuring level (or about 0.08 UA), it was possible to detect several peaks that we identified as potential carotenoids. The largest peak in liver samples (Fig. 1a), also detected in skin samples (Fig. 1b), was later shown to be 3-hydroxy-echinenone by comparison to the spectra and elution pattern of the standard pigment (Fig. 1c).

Further confirmatory analyses were carried out on the red carotenoid detected by HPLC. An acetylation test confirmed the presence of only one hydroxy group. A keto group reduction test confirmed the presence of this functional group. The chromatography of the pigment obtained after the reduction gave a single peak with a retention time very similar to that of the original non-reduced pigment and to the one of cryptoxanthin. This fact suggests that both the hydroxy and the keto group are placed in the same ionone ring, as it is expected from 3-hydroxy-echinenone. If both functional groups were placed in different rings, the reduction would have conducted to a pigment very similar to lutein, whose polarity and retention time is different from that of both cryptoxanthin, 3-hydroxy-echinenone and the reduced pigment obtained from 3-hydroxy-echinenone.

Identification was confirmed by HPLC, comparing the spectrum and the retention time of the extracted pigment and the pure standard.

Concentrations of 3-hydroxy-echinenone found in the liver, follicles and breast feathers of the seven dead crossbills analysed are shown in Table 1. The relative proportion of this pigment in different tissues was calculated as the percentage of its concentration in relation to the total amount of carotenoids. 3-Hydroxy-echinenone also appeared in eight plasma samples of the 14 moulting males we captured

**Table 1** Concentration (mg/kg) and relative proportion of 3-hydroxy-echinenone (in parenthesis and expressed as percent) in liver, follicles and breast feathers of Common Crossbill males (*Loxia curvirostra*)

Colour	Age	Month	Liver	Follicles	Feathers
Red	Yearling	October	0.35 (69%)	6.09 (70%)	141.73 (89%)
	Adult	October	1.22 (61%)	26.37 (65%)	No data
	Adult	October	0.01 (14%)	0.15 (100%)	No data
Orange	Yearling	October	0.12 (19%)	1.56 (36%)	41.58 (91%)
	Adult	July	0.19 (40%)	0.12 (100%)	24.56 (78%)
	Adult	July	0.11 (5%)	0.00 (0%)	1.92 (41%)
Yellow	Yearling	October	0.05 (35%)	0.55 (56%)	3.12 (14%)

Colour represents the overall plumage hue of the birds determined by visual assessment and month indicates the date when individuals were captured

additionally, with a mean concentration of  $1.35 \pm 0.45$   $\mu\text{g/ml}$  (mean  $\pm$  SE; range, 3.773–0.263  $\mu\text{g/ml}$ ).

## Discussion

Carotenoid-derived red colouration in birds has been shown to act as an ornament, signalling the nutritional and health status of the individual and its ability to locate high-quality resources (reviewed in McGraw 2006). Moreover, colouration based on red carotenoids may entail more metabolic costs in comparison to yellow and orange pigmentation due to the biochemical expenditure involved in their oxidation (Hill 1996). This might explain the intra-specific variation in the expression of this type of colouration (Hill 2000), and several studies have demonstrated that differences in plumage pigmentation are used by females to choose high-quality males (Hill 2006).

However, and despite their importance (a recent theoretical study suggests that red carotenoids are more efficient antioxidants than the yellow xanthophylls, Martínez et al. 2008), most of the physiological and biochemical mechanisms concerning red colour displays remain poorly understood (see also Toral et al. 2008). One of the most controversial points is the identification of the anatomical site of feather carotenoid synthesis (McGraw 2006).

To date, the most widespread idea is that integumentary carotenoids are directly processed at colourful tissues (Stradi 1998; Inouye 1999; McGraw 2004) since analyses of plasma and liver pigments in several songbird species never revealed the existence of those carotenoid derivatives that were ultimately described in their feathers (Brush 1990; Inouye 1999; McGraw et al. 2006). Nevertheless, feather follicles contained a mixture of dietary and synthetic carotenoids, suggesting that they could act directly as metabolically active sites of pigment production (Inouye 1999; McGraw 2004).

However, our finding of the primary red feather pigment of male crossbills, 3-hydroxy-echinenone, in the liver and plasma of the individuals we examined suggests for the first time a different metabolic route. 3-Hydroxy-echinenone does not appear in the diet of birds and has to be metabolically transformed from ingested pigments (Stradi et al. 1996). Based on parsimonious chemical modifications, previous studies have proposed  $\beta$ -cryptoxanthin as the most likely non-xanthophyll precursor of this derived ketocarotenoid (Stradi et al. 1996). Since carotenoid pigments are delivered to peripheral body tissues through the bloodstream from sites of conversion and storage (Hill 2000), the presence of 3-hydroxy-echinenone in liver and serum can be explained only by a hepatic conversion of dietary  $\beta$ -cryptoxanthin and subsequent delivery of resultant 3-hydroxy-echinenone to follicular cells via plasma. Maturing follicles would just accumulate this red derivative until the pigment would be incorporated finally into growing feathers during moult (Fox 1962; Brush 1990; Hill et al. 1994; Hill 2000). Therefore, it is logical (and expected) to find the highest concentrations of 3-hydroxy-echinenone in germs and feathers.

The fact that levels of 3-hydroxy-echinenone in the liver showed great variation among samples also suggests that this pigment is converted there from a precursor. If the latter is available, 3-hydroxy-echinenone can be readily detected, but it may not be so when it has been diverted to target tissues (including final deposition in follicles and feathers). In the skin sample, the concentration of 3-hydroxy-echinenone was much higher, but the carotenoid profile was different to that of the liver. The minority carotenoids did not show up in the skin in the same proportion, suggesting that it was a specific accumulation site for 3-hydroxy-echinenone.

This surprising divergence with previous studies raises the question whether there are inter-specific differences in anatomical sites for conversion of carotenoids. Future studies need to determine how liver versus feather follicle

metabolism is distributed in the avian class (McGraw 2006). Understanding inter-specific variation in mechanisms of colour production may be the key to comprehend the different evolutionary pathways involved in colour signalling. The elucidation of the site of conversion of common yellow carotenoids into specific red ones is essential to consider possible physiological constraints and to focus analytical efforts. Research on avian circulating carotenoids has mainly been based on lutein and its isomers (see, e.g., Tella et al. 2004). So far, overlooked pigments in the blood should be searched there, as the hypothesised trade-off of carotenoids for ornamentation or as physiologically active molecules (McGraw 2006) is only possible if they actually circulated through the body but not so if they were produced at the feather follicles. Moreover, this new perspective on avian carotenoid metabolism represents a challenging topic for evolutionary biologists and should encourage researchers to conduct further comparative studies to improve our knowledge on the physiological mechanisms of carotenoid-based ornamental colouration in birds.

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