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Astaxanthin is the major carotenoid in tissues of white storks (*Ciconia ciconia*) feeding on introduced crayfish (*Procambarus clarkii*)

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Abstract

We studied the carotenoid pigments in plasma, skin and body fat of white storks (*Ciconia ciconia*) from a colony in Spain feeding mainly on the recently introduced red swamp crayfish (*Procambarus clarkii*). In control colonies, where crayfish was absent, plasma was collected for comparison. Our objective was to determine whether the astaxanthin contained in the crayfish reached the blood, accumulated in fat, and finally was deposited in the red-colored bill and legs. If that was true, the visual cues provided by those tegumentary areas would be altered, with potential behavioral consequences. Plasma carotenoids were directly extracted with acetone, whereas skin and fat samples needed harsher conditions, i.e. grinding, sonication and extraction with diethyl ether. The extracts were analyzed by thin-layer chromatography (TLC) and UV/Vis spectroscopy. In crayfish-eating storks, astaxanthin was confirmed to be the dominant pigment in all analyzed tissues. This red pigment was absorbed unchanged in the gut, and was responsible for the red color of plasma and the abnormal orange pigmentation of the feather-covered skin. It was also present in large quantities in the exposed bill and tarsi, which are typically red-colored in the stork. Control storks with no crayfish in the diet only presented lutein in their plasma.

Keywords: White stork; Crayfish; Bird color; Astaxanthin; Lutein; Carotenoids; TLC analysis; UV/Vis spectroscopy

1. Introduction

Carotenoids are responsible for the brightest colors in the integument of birds (Brush, 1990). Many potential sexual signals are pigmented by carotenoids, and thus these substances have attracted the attention of evolutionary biologists dealing with animal communication. Apart from their well known pigmentary properties, there is growing evidence that carotenoids also play an essential role in vertebrates as physiological modulators (Britton, 1995). However, the research on the physiology and metabolism of carotenoids in wild animals is still in its infancy (Bortolotti et al., 1996).

Most studies about carotenoids in wild birds have related the brightness of carotenoid-dependent plumage to different fitness traits, such as mating or breeding success (Hill, 1993). The iden-

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tification and quantification of carotenoids in bird tissues other than feathers (Brush, 1981; Hudon and Brush, 1992; Stradi et al., 1995, 1996, 1997) has rarely been performed, even though carotenoids, that are necessarily obtained from the diet, can be stored in different organs, the skin and body fat (Fox, 1962; Czeczuga, 1979; Brush, 1981).

In a previous paper (Negro et al., in press), we reported the probable presence of astaxanthin, the typical crustacean carotenoid, in the plasma and skin of Spanish white storks (*Ciconia ciconia*) eating an introduced crustacean, the red swamp crayfish (*Procambarus clarkii*) from North America. White storks are large wading birds with bright red bills and tarsi due to carotenoids. Although no studies are currently available on the significance of the red coloration in this species, the bill and tarsus brightness could be indicative of the condition of the bird and be used for mate acquisition, as it is presumed in other bird species with similar displays (Lozano, 1994; Shykoff, 1997; Negro et al., 1998).

Here we expand our previous work with detailed comparative studies of carotenoid composition in plasma, skin (including bill and tarsi), and fat of white storks from the crayfish-eating colony. We have included for comparative purposes pigments extracted from plasma of storks with normal diets (no crayfish), and pigments directly extracted from crayfish. Standard canthaxanthin and astaxanthin, two ketocarotenoids typical of crustaceans (Castillo et al., 1982), have been used as well. Those are the principal pigments found in the skin and feathers of flamingos (*Phoenicopterus* spp.), possibly the most thoroughly studied bird species in relation to carotenoid metabolism (Fox, 1962; Fox and Hopkins, 1966; Fox and McBeth, 1970; Brush, 1981).

Our aims were first to determine whether astaxanthin, the novel pigment in the diet of storks, was transported as such in the blood, accumulated in fat stores, and finally was deposited in the integument of bill and legs, in which case it could interfere with the presumed visual signals provided by those areas. Second, we wished to introduce field biologist to relatively simple and straightforward chemical protocols, such as UV/Vis spectroscopy and thin-layer chromatography, for the qualitative analysis of carotenoids in different bird tissues.

2. Materials and methods

2.1. Biological materials

In 1998 and 1999, we collected blood samples from nestling storks: (a) at a breeding colony near Doñana National Park in the province of Sevilla (southern Spain), where crayfish captured in the nearby marshes and rice fields were the main food item; and (b) from control colonies in the province of Madrid (central Spain) where crayfish was never recorded in the diet. Blood was transported to the laboratory in coolers and centrifuged within 24 h of collection to obtain the plasma, that was stored at -20°C (for more details about collecting dates and sample sizes see Negro et al., in press). From storks found freshly dead in the Doñana area, we also collected skin (about 2 g/sample) from the wing area, neck, bill and the legs, as well as body fat (about 2 g). The latter tissues, that were intensely red or orange colored, were frozen at -20°C until analysis. Finally, we collected and froze at -20°C several red swamp crayfish from the Doñana marshes. Reference lutein was freshly extracted from green plants in our laboratory. Hoffman–La Roche (Roche, Madrid) kindly supplied standards of canthaxanthin and astaxanthin.

2.2. Pigment extraction

2.2.1. Plasma

Acetone was added at a ratio of 3:1 (v/v) to the plasma samples. The mixture was centrifuged at 13 000 $\times$ *g* during 10 min to precipitate the flocculant proteins. The supernatant, with the carotenoids in solution, was analyzed by spectrophotometry and thin-layer chromatography (TLC).

2.2.2. Skin and fat

The samples were gently ground by hand in a glass flask. About 40 ml of diethyl ether were added, the material was sonicated for about 1 min and left under cover for 2 h. The diethyl ether with the carotenoids in solution was recovered and evaporated in a rotary evaporator until sample dryness. The resulting pigments were re-dissolved in an adequate volume of acetone. This solution was analyzed by UV/Vis spectroscopy and TLC analysis.

2.2.3. Crayfish

Before thawing of the frozen specimen was completed, it was finely ground in a mortar, and then treated as previously described for skin and fat.

2.3. Pigment separation and analysis

2.3.1. Thin layer chromatography

Commercial plates of Kieselgel 60 F254 (Sharlau Cf 330) and other prepared in our laboratory with Kieselgel 60 GF254 (Merck, art. 7730) were used for chromatography with the following developers: petroleum ether/acetone/diethylamine 10:4:1 (eluant A); hexane/acetone 3:1 (eluant B), and benzene/ethyl acetate 1:1 (eluant C) (Mínguez-Mosquera and Garrido-Fernández, 1989; Muriana et al., 1993).

2.3.2. UV/Vis spectroscopy

It was carried out for the carotenoid extracts and the standards, and also after (a) reduction with $NaBH_4$, and (b) saponification with KOH/MeOH 10% (w/v). The apparatus used was a Hewlett–Packard UV/Vis spectrophotometer, model 8452A. Astaxanthin and lutein in the different tissues were quantified by determining the optical density of the carotenoid peak at 476 nm. Quantification of total carotenoids was performed by using a standard calibration curve for lutein in acetone at 476 nm. We deliberately used a lutein curve, instead of one for astaxanthin, because lutein has been proposed as a reference for estimating total carotenoids in birds (Tella et al., 1998), and was also used in a previous study about carotenoids in crayfish-eating storks (Negro et al., in press).

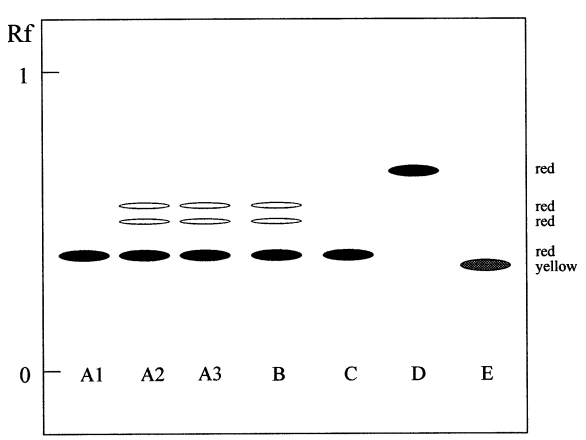

Fig. 1. Chromatography in silicagel plates 60 GF_{254} of pigment extracts from plasma, skin and fat, (A1, A2, A3) of crayfish-eating storks; red-swamp crayfish (B); standard astaxanthin (C); standard canthaxanthin (D); plasma from control stork (E). Solvent system: petroleum ether/acetone/diethylamine (10:4:1). 'Skin' included samples from four different locations (i.e. bill, tarsus, neck and wing) that yielded identical results.

3. Results

Developing the pigment extract from plasma of crayfish-eating storks (Fig. 1) with TLC, it showed a single red band with $Rf = 0.41$. This band was identical in color and Rf value to the ones of reference astaxanthin, and the main red band of the skin, fat and crayfish extracts, respectively. Two red-colored secondary bands also appeared in skin, fat and crayfish samples. These bands may be artifacts due to isomerization of astaxanthin.

We initially thought that those extra bands could have been esterified carotenoids, but we rejected this hypothesis because assays with saponified and unsaponified pigment extracts yielded identical chromatograms. Due to the low concentration of the two secondary bands, they were pooled to obtain their absorption spectrum, which resulted to be identical to the one of standard astaxanthin, except that there was a new absorption band at about 340 nm. This is characteristic of the presence of *cis*-isomers (Davies, 1976). Pigment extraction for skin, fat and crayfish took place under harsher conditions than in the case of plasma, and given the low stability of carotenoids, part of the free astaxanthin could have easily suffered isomerizations.

In the plasma from control storks we observed a single yellow band with a $Rf = 0.39$ (Fig. 1). This band co-eluted with the one due to lutein obtained from green plants. We further confirmed the plasmatic yellow band was due to lutein because its absorption maxima in two different solvents — i.e. light petroleum (418, 442, 470) and chloroform (430, 452, 482) were identical to the ones of the lutein extracted form plants. Given that lutein is a normal component of bird plasma (Brush, 1981; Tella et al., 1998), we will focus on the discriminatory red pigment. Standard canthaxanthin, on the other hand, had a faster elution and a single band of $Rf = 0.67$, much higher than any other band in the co-cromatographies, indicating that this pigment was absent in both the stork and crayfish samples.

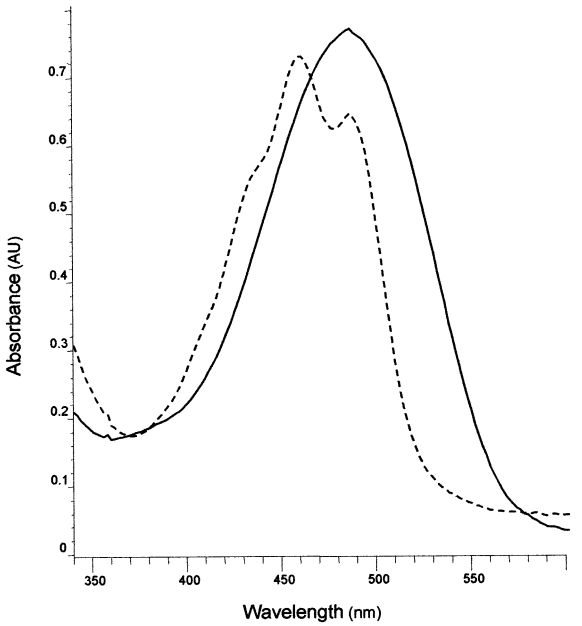

Fig. 2. Electronic absorption spectrum of astaxanthin in chloroform, before (—) and after (- - -) reduction with $NaBH_4$.

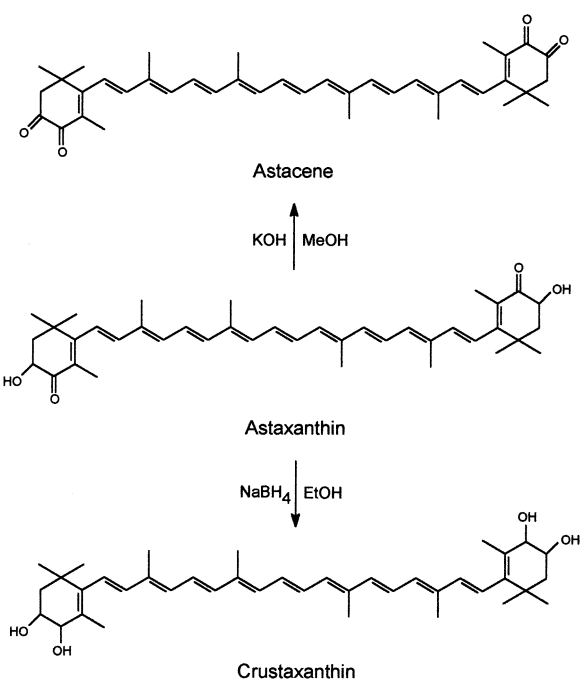

Fig. 3. Structural changes of astaxanthin after reduction (crustaxanthin) or after saponification (astacene) (Davies, 1976).

When TLC was carried out with eluants B and C, the carotenoid extract from plasma of crayfish-eating storks yielded single red bands with Rf values of 0.48 and 0.77, respectively. Those bands were again identical to the one of reference astaxanthin and the main band from crayfish extract.

The absorption spectra were identical for samples of crayfish-eating storks and the reference pigment, with the very wide single absorption band typical of astaxanthin, showing maxima at 472 nm in acetone, 476 nm in ethanol, 468 nm in hexane, 486 nm in chloroform, and 484 nm in benzene (as in: Foppen, 1971; Goodwin, 1976; Britton, 1995).

3.1. Chemical test — reduction and saponification

The red pigment reduced with $NaBH_4$ was subjected to absorption spectroscopy in different solvents, yielding the following λ_{max}: (424), 448, 474 nm in hexane, (434), 460, 486 nm in chloroform (Fig. 2), (426), 452, 476 nm in acetone, and (424), 450, 476 nm in ethanol. All these values — with slight variations — correspond to published values for crustaxanthin (Fig. 3), the reduced tetrahydroxilated derivative of astaxanthin (Foppen, 1971; Goodwin, 1976; Britton, 1995).

After saponification with KOH-MeOH, both the storks' red pigment and standard astaxanthin yielded absorption spectra very similar to the one for the untransformed pigments, but showing a bathochromic shift of two nanometers toward higher wavelengths. When chromatographing the saponified pigments with eluant A, both the storks' sample and the saponified reference astaxanthin yielded identical bands with $Rf = 0.07$, as expected of a more polar pigment (i.e. astacene) than astaxanthin (Fig. 3) (Davies, 1976).

Concentrations of total carotenoids in the different tissues that we analyzed are given in Table

Table 1
Concentration of total carotenoids in different tissues of crayfish-eating storks[a]

Samples		Total carotenoids
Skin[b]	Neck	176.70
	Wing	28.83
	Tarsus	341.86
	Bill	403.33
Fat[b]		181.04
Plasma[c]		8.19
Plasma[c](control)		1.09

[a] Plasma carotenoid concentration of control storks ($n = 10$) are included for comparative purposes.
[b] mg pigment/kg tissue (four samples)
[c] mg pigment/l plasma (ten samples)

1. Data are given separately for each skin area, and it can be noted that carotenoid concentrations are much higher in tarsi and bill than in neck and wing. This result was anticipated as bill and legs are typically red-pigmented, whereas the skin in neck and wing is hidden under the feathers and normally unpigmented. Regarding plasma, there is a clear difference in the concentration of carotenoids depending on the diet. Concentration is much higher in storks with a rich source of dietary carotenoids (i.e. crayfish), which are indeed the ones with unusual integumentary pigmentation.

4. Discussion

TLC analyses showed that plasma was the easiest and most reliable material for the comparative study of stork pigments. Astaxanthin and its isomers seemed to be the dominant carotenoids in crayfish-eating storks' tissues, while lutein dominated in the plasma of control storks, that showed no traces of astaxanthin. The latter was not surprising, as lutein is the most common carotenoid in bird plasma (Brush, 1981; Tella et al., 1998). The fact that lutein was not observed in samples from crayfish-eating storks does not mean that it was completely absent. However, the dominant pigment (i.e. astaxanthin) would mask a very low concentration of lutein, which would thus play little role in pigmentation. On the other hand, canthaxanthin, the most prominent pigment in the plasma and tarsal skin of the flamingoes (Fox and Hopkins, 1966), was not detected in any of the samples that we analyzed. Diet of the two species is however very different. Flamingoes are highly specialized birds that mainly feed on micro-algae and microcrustaceans. Storks prey upon a wide variety of macro-invertebrates and vertebrates, including human refuse (see, e.g. Negro et al., in press)

The fact that astaxanthin was the predominant carotenoid in all colored tissues of crayfish-eating storks supports the hypothesis that the birds ingested the pigment from the crayfish, and that it was transported unchanged from the gut to the blood, and then deposited as such in the fat, the exposed skin of bill and legs, and also in body skin, that is normally unpigmented in this species. This is the more plausible hypothesis, even though biosynthesis of astaxanthin from β-carotene has also been suggested (Fox and McBeth, 1970; Rodríguez et al., 1973). In the flamingoes, for instance, astaxanthin was found in feathers and skin but it was not detected in the plasma or in any internal organs or tissues (Fox, 1962). Even when the flamingoes were given a diet rich in astaxanthin, there was little transmission from the intestine to the blood (Fox and McBeth, 1970).

Astaxanthin has been reported in the plumage and skin of several bird species, other than flamingoes, such as pheasants, partridges and domestic fowl (Czeczuga, 1979; Brush, 1981; Schiedt, 1998). The case of the crayfish-eating storks is exceptional, however, in that astaxanthin has been made available to storks in large quantities only very recently. Crayfish were first released in the Doñana marshes in 1973 for human consumption, and storks quickly made them the bulk of their diet (Negro et al., in press). An immediate result of the easy assimilation of astaxanthin by the storks has been the possibility for changing the color — and thus the behavioral message — of bill and legs. High levels of intraspecific variation in bird colors are likely to have major fitness consequences (Zahn and Rothstein, 1999), although we have no proof that this is happening to storks. The color shift was particularly evident in nestlings, whose bill and legs were bright red-colored in the crayfish-eating colony, and mostly black in nestlings elsewhere. As for the adults, we have not compared colors, but it is a possibility that those eating mainly crayfish presented a deeper red hue. The actual concentration of astaxanthin in bill and legs of the adults that we analysed was, in fact, much higher in the integument of those locations than in any other tissue.

Two questions remain as how much crayfish — or dietary astaxanthin — is needed to alter skin pigmentation in storks, and for how long they keep it once they turn into a different diet (as they would in their African winter quarters). We have recently started feeding experiments using captive storks, that may provide some insight into those and related questions.

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