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Differential ability of carotenoid C4-oxygenation in yellow and red bishop species (*Euplectes* spp.)

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ABSTRACT

Male breeding plumages of African widowbirds and bishops (*Euplectes* spp.) show striking variation in carotenoid-based plumage coloration, with saturated yellow or orange-red patches of different size. Yet, from observations and experiments, agonistic signaling appears to have been a generalized sexual selection pressure for redness in the genus. Recent results show that yellow and red widowbird colors derive from distinctly different pigment profiles, and suggest that species vary in their ability to metabolize ingested carotenoids. We used reflectance spectrometry and High Performance Liquid Chromatography (HPLC) to describe the species-specific colors and plumage carotenoids of the congeneric yellow-crowned bishop (*E. afer*) and southern red bishop (*E. orix*). Results show that the yellow rump color of *E. afer* primarily derives from direct-deposited, dietary yellow pigments, i.e. lutein and zeaxanthin. In the red breast of *E. orix*, these are complemented by smaller amounts of derived red C4-keto-carotenoids: mainly α-doradexanthin, but also β-doradexanthin, canthaxanthin, astaxanthin and adonirubin. We also performed a diet supplementation experiment to investigate the relative importance of nutritional and metabolic constraints in determining the differential occurrence of C4-keto-carotenoids, and thus red plumage color, in the two species. Our results indicate that *E. orix*, but not *E. afer*, can manufacture red C4-keto-carotenoids (α-doradexanthin and canthaxanthin) from yellow dietary precursors (lutein and β-carotene).

1. Introduction

Sexual selection for colorful bird plumages, through mate choice or male competition, has been well documented (Andersson, 1994; Hill, 2006b). Most of the bright red, orange and yellow plumage colors are caused by carotenoid pigments, including carotenes and their oxygenated derivatives xanthophylls (Fox and Vevers, 1960; Brush, 1978; Stradi, 1998). Carotenoid displays have been shown to correlate with male quality or vigor in birds (e.g. Hill, 1991, 1992; Gray, 1996; Hill, 1999; Griffith and Pryke, 2006) and other vertebrates (e.g. Kodric-Brown, 1989; Milinski and Bakker, 1990), and carotenoid coloration has become a textbook example of condition-dependent signaling (Andersson, 1994; Johnstone, 1997). In accordance with the 'handicap principle', reliability is ensured by disproportionately large costs for low-quality individuals to develop or maintain a conspicuous display (Zahavi, 1975; Grafen, 1990), but the mechanism of cost for carotenoid pigmentation is still contentious (see Olson and Owens, 1998; Hill, 2006a).

Since birds, like other vertebrates, lack the ability to de-novo synthesize carotenoids, these must be ingested (Völker, 1934; Goodwin, 1984). The main constraints of pigmentation may thus be the availability of dietary carotenoids (Endler, 1980; Hill, 1990), or the assimilation efficiency of the animal (Kodric-Brown and Brown, 1984, Hudon 1994). Other costs may derive from the fact that carotenoids are needed in cell differentiation, vision and vitamin A synthesis (Olson, 1999; Meyer, 2002), as well as in immune function and antioxidant defense (Lozano, 1994; von Schantz et al., 1999; Blount et al., 2003), all of which may conflict with their use in integumentary displays. Given that red pigments are less abundant than yellow in most avian diets (Goodwin, 1980, 1984), and that enzymatic conversion between the two is a costly process (Hudon, 1991; Hill, 1996), it has furthermore been suggested that red carotenoid displays generally are costlier to produce than yellow (Hill, 1996).

Carotenoids are highly unsaturated C40 hydrocarbons with or without cyclic end groups (Britton, 1995; Armstrong and Hearst, 1996). The striking yellow to red coloration caused by these pigments derives from selective absorption of indigo-blue (400–500 nm) light by the conjugated double-bond system (Goodwin, 1980; Britton, 1995). As the wavelength of maximum absorption (λ_{max}), and thus the spectral location (hue) of reflected light, depends on the number of conjugated double bonds (Fox and Vevers, 1960; Goodwin, 1980), carotenoid color is potentially affected by small structural re-arrangements of molecules. Enzymatic conversion of ingested pigments have been documented in several avian taxa (Brush, 1990; Latscha, 1990; Stradi et al., 1997; Schiedt, 1998). According to suggested metabolic pathways (Stradi et al., 1997, Fig. 3), red C4-keto-carotenoids (e.g., aDx, Cx) derive from enzymatic addition of

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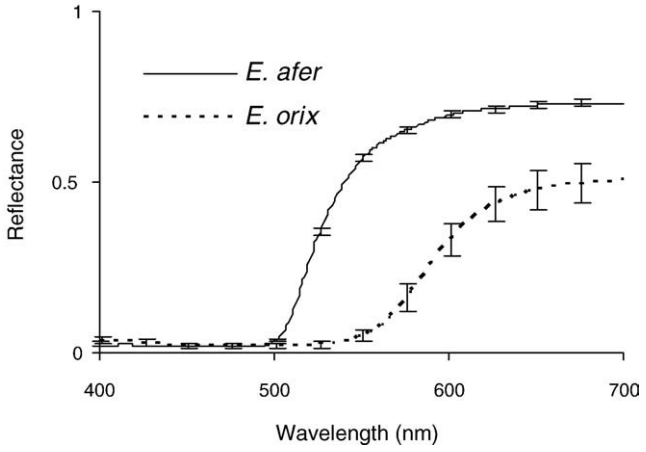

Fig. 1. Spectral reflectance (mean ± S.E.) for the yellow rump of wild *E. afer*, and the red breast of wild *E. orix*.

conjugated carbonyl groups to the C4 positions (C4-oxygenation) of dietary yellow precursors. Dehydrogenation of C3-carbonyls, in contrast, produces isolated carbonyl groups (e.g., in canary xanthophylls and 3-dehydrolutein), and furthermore converts affected end rings from β- to ε-configuration, thus shifting λ_{max} towards slightly shorter wavelengths. In addition, the hue of saturated carotenoid colors depends on the amount of deposited pigments (Hudon et al., 2003; Andersson and Prager, 2006), and the potential formation of carotenoid–keratin complexes (Stradi et al., 1995a).

Widowbird and bishop (*Euplectes spp*) males typically display saturated yellow or red carotenoid colors contrasting against black melanin pigmentation, and these color patterns are emphasized through posturing in agonistic and courtship interactions (Crook, 1964). In behavioral observations and experiments, the size and redness (hue) of color patches have been shown to function in male–male competition over territories (Pryke et al., 2001; Andersson et al., 2002; Pryke et al., 2002; Pryke and Andersson, 2003a,b). Yet, extant taxa show remarkable variation in carotenoid color expression, ranging from the small, yellow epaulettes of certain widowbirds, to the extensive orange-red patches of many bishop species. This diversity may reflect interspecific differences in fitness consequences of being colorful, e.g. in predation risk. Alternatively, the evolution of more elaborate displays may be constrained by dietary or physiological limitations in some lineages.

To begin to unravel the evolution of conspicuous color signals in the *Euplectes*, detailed knowledge of proximate mechanisms is crucial. A carotenoid basis of plumage coloration in bishops was first suggested by Kritzler (1943), and has subsequently been confirmed by High Performance Liquid Chromatography (HPLC) analyses of feather pigments in three species of the congeneric widowbirds (Andersson et al., 2007). The results indicate that the yellow xanthophylls lutein and zeaxanthin are abundant in all species, and the principal contributors to yellow colors of the genus. Red signals, on the contrary, are achieved either by addition of relatively small amounts of red C4-keto-carotenoids (mainly α-doradexanthin and canthaxanthin), or by deposition of high concentrations of both dietary and derived yellow pigments (Andersson et al., 2007).

Despite striking differences in coloration, *Euplectes* taxa show a high degree of ecological and behavioral similarity (Craig, 1980). All species primarily feed on grass seeds, which typically contain lutein and zeaxanthin, as well as smaller amounts of β-carotene and β-cryptoxanthin (Goodwin, 1980; McGraw et al., 2001). Interspecific differences in pigmentation thus seem more likely to reflect variation in physiological utilization, rather than in dietary access to carotenoids. In principle, any process responsible for absorption, transport or metabolism of carotenoids could be involved (Davies, 1991; Stradi et al., 1995b; Badyaev et al., 2001). The phylogenetic distribution of keto-carotenoid occurrence has, however, suggested to us that differences derive from a single gain of a C4-oxygenase enzyme (Schiedt, 1998) acting on dietary precursors in the red 'bishop-ardens clade' (see Prager et al., 2008) only (unpublished data, S. Andersson and M. Prager). In particular, the consistent absence of monoketo-carotenoids, e.g. echinenone, in *Euplectes* feathers makes direct synthesis of canthaxanthin from ß-carotene the most likely scenario.

Although general effects of carotenoid deprivation on plumage color has been described in several studies (e.g. Brockmann and Völker, 1934; Hill, 1992), surprisingly few diet manipulation experiments have been performed to test hypothesized metabolic pathways (Stradi et al., 1997) for specific derived carotenoids. Exceptions include studies on captive Flamingo (*Phoenicopterus ruber*; Fox et al., 1969), Bullfinch (*Pyrrhula pyrrhula*; Stradi et al., 2001) and Red Crossbill (*Loxia curvirostra*; Stradi et al., 2001), which suggest production of C4-keto-carotenoids adonirubin from ß-cryptoxanthin, and canthaxanthin from ß-carotene, respectively. Brush and Power (1976) also suggested ß-carotene as the precursor of canthaxanthin in the house finch, although recent studies find ß-cryptoxanthin to 3-hydroxy-echinenone conversion to be of greater importance for coloration in this species.

In this study, we explore metabolic constraints on plumage coloration in the *Euplectes*, using HPLC and reflectance spectrometry. We thus describe the identities and concentrations of the pigments associated with feather color in one yellow species, the yellow-crowned bishop *E. afer*, and one red species, the southern red bishop *E. orix*. We also perform a diet manipulation experiment to test for differences in enzymatic capacity between the two species, specifically regarding their ability to produce canthaxanthin from ß-carotene.

2. Materials and methods

2.1. Study species

The bishops and widowbirds, forming the genus *Euplectes* (Passeridae: Ploceinae), are sexually and seasonally dichromatic weaverbirds, breeding semi-colonially and polygynously in moist grasslands of Equatorial and Southern Africa. During the prenuptial molt, males replace their mottled brown non-breeding plumage (similar to that of females) with black body plumage and contrasting yellow or orange-red patches. While the male yellow-crowned bishop (*E. afer*) displays a bright yellow crown, back and rump, the orange-red nuptial coloration of the male southern red bishop (*E. orix*) extends also to the throat and breast regions. Unlike their widowbird congeners, which before breeding molt into more or less elongated black tails, both species have brown rectrices of normal length (3–4 cm), that are retained throughout the year.

2.2. Sampling protocol

Plumage reflectance and feather samples of wild *E. orix orix* (breast patch, $n = 7$) and *E. afer taha* (rump, $n = 13$) males were acquired in KwaZulu–Natal, South Africa, between November and December 1995, and in December 1997, respectively. Three wild-caught *E. afer* and two *E. orix*, of unknown geographic origin, were purchased from an authorized distributor for zoo dealers (Imazo AB, Vara, Sweden), for use in the diet manipulation experiment subsequently performed at the University of Gothenburg, Sweden. Based on plumage characters, e.g. the presence of a yellow and brown collar patch, the purchased *E. afer* were classified as the West-African subspecies *E. afer afer*. Based on plumage and mtDNA sequence comparison, the purchased *E. orix* males were identified as the West-African subspecies *E. orix nigrifrons*. In addition, one *E. orix orix* male was brought to Gothenburg from KwaZulu–Natal. Reflectance, feather and

plasma samples were taken from captive birds following 12 months on carotenoid-poor diet (cous–cous, with less than 0.6 μg/g lutein + carotenoid-free vitamin premix). A β-carotene supplement of 10% CWS beadlets (F. Hoffmann-La Roche, Basel, Switzerland) dispersed in water was then mixed into the diet (0.02 mg/g, i.e. ca. 0.1 mg/bird/day). Diet supplementation continued for 3–19 weeks until a new prenuptial molt took place, after which measurements were repeated.

2.3. Plumage reflectance

Reflectance was measured with a USB2000 spectroradiometer system (OceanOptics, Dunedin, USA), including a fibre-optic reflectance probe and a HL2000 tungsten-halogen light source, using CSpec software (Ancal, USA). The measuring configuration, using a home-made 'Probe holder' fitted on the probe ferrule, was 'Coincident Normal' (CN: coaxial illumination and reading beams, perpendicular to the plumage plane; Andersson and Prager, 2006), taking three to five scans and removing the probe between each. The objective colorimetric λ_{R50} (wavelength at which reflectance is halfway between its minimum and its maximum), was used as a measure of the spectral location, i.e. hue of plumage colors. For these and other details see Andersson and Prager (2006).

2.4. Carotenoid analysis

Colored feathers (5–7) were plucked with flat-tipped tweezers, and stored dark and dry in brown envelopes until analysis. Following a carotenoid extraction protocol modified from Stradi et al. (1995a), approximately 1 mg of colored barbs was trimmed off from feathers after washing these in hexane. The sample was homogenized in 3–4 ml methanol for 15 min at 27 Hz, in a Retsch MM2000 micronizer with ZrO containers (Hann, Germany). The white keratin residue was filtered off with a 0.2 μm syringe filter (GHP Acrodisc®). After evaporation under nitrogen in a speedvac (Savant DNA120, Holbrook, USA), the residue was resuspended in 150–200 μL acetone and placed at −78 °C overnight. The precipitate was filtered off with another 0.2 μm filter and the solvent evaporated in the speedvac for 5–10 min. The final residue was dissolved in 100 μL of the mobile phase (70:30 acetonitrile: methanol) and immediately analyzed by High Performance Liquid Chromatography (HPLC): 20–40 μL sample solution was injected into a RP-18 column (ODS-AL, 150 × 4.0 mm i.d., YMC Europe GmbH, Schermbeck, Germany), fitted on a ThermoFinnigan (San Jose, USA) HPLC system with PS4000 ternary pump, AS3000 autosampler, and UV6000 UV/VIS diode-array detector. Column temperature was maintained at 30 °C and the flow-rate at 0.6 mL/min.

To discriminate between potential constraints on carotenoid metabolism and deposition, respectively, we also analyzed circulating carotenoids in captive birds. Ca. 150 μL blood was thus drawn from the neck vein with a 1 mL syringe. Samples were centrifuged at 800 g for 10 min, and 100 μL plasma transferred to till 1 mL ice-cold acetone, then shaken and stored at −78 °C overnight. Thawed and vortexed samples were filtered through a 0.2 μm syringe filter (GHP Acrodisc 13 mm) and speedvac evaporated. Carotenoid residues were resuspended in 10 μL THF and 90 μL of the mobile phase, before HPLC analysis (see above).

For all samples, two-dimensional (peak height vs. time at 450 nm and 470 nm wavelength) and three-dimensional (peak height vs. time and position in 300–700 nm interval) chromatograms were obtained, inspected and analyzed in ChromQuest 4.0 software (ThermoFinnigan, San Jose, USA). Peaks were identified first by comparisons to published accounts of relative retention times and spectral absorptance characteristics using similar methods (e.g. Britton, 1995; Stradi et al., 1997; Stradi, 1998) and quantified by comparisons to standard runs of the same or closely related carotenoids: Lutein (β,ε-carotene-3, 3'-diol), zeaxanthin (β,β-carotene-3, 3'-diol), canthaxanthin (β,β-carotene-4, 4'-dione) and astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) were kindly provided by Roche Vitamins Inc. (Basel, Switzerland), and 2',3'-anhydrolutein (2',3'-didehydro-β,ε-caroten-3-ol) by F. Khachik. 3'-dehydrolutein (3-hydroxy-β,ε-caroten-3'-one), α-doradexanthin (3,3'-dihydroxy-β,ε-caroten-4-one), adonirubin (3-hydroxy-β,β-carotene-4, 4'-dione) and the canary xanthophylls A (3'-hydroxy-ε,ε-caroten-3-one) and B (ε,ε-carotene-3,3'-dione) were extracted from feather samples of species with known carotenoid profiles, i.e. Pekin Robin (*Leiothrix lutea*), Great Spotted Woodpecker (*Dendrocopos major*), Bullfinch (*Pyrrhula pyrrhula*), and Siskin (*Carduelis spinus*) (Stradi, 1998).

Due to isomerism around one of the double bonds in the central chain, many carotenoids can occur in different configurations, designated 'cis (Z)' or 'trans (E)' isomers (Britton, 1995), but the all-E-configuration appears to be the most common state of natural carotenoids (McGraw, 2006). Since cis-isomers are thus likely extraction artifacts (Schiedt and Liaaen-Jensen, 1995; Stradi et al., 1995a), they were pooled with the presumed parent carotenoids.

Based on the likely metabolic origins and spectral absorptance shape, identified carotenoid pigments were classified as 'dietary yellow', 'derived yellow' (both with multi-peaked curves with $\lambda_{max} < 455$ nm) or 'derived red' (smooth curves with $\lambda_{max} \geq 455$ nm). Species values of carotenoid concentration, as well as reflectance, are given as mean ± SE.

3. Results

3.1. Color and plumage carotenoids of wild E. afer and orix

Spectral reflectance curves for the yellow ($\lambda_{R50} = 527.8 \pm 0.5$ nm) rump of *E. afer* and the red ($\lambda_{R50} = 589.8 \pm 3.0$ nm) breast of *E. orix* both show distinct sigmoid shapes, typical of saturated carotenoid colors (Andersson and Prager, 2006; Andersson et al., 2007, Fig. 1). The carotenoid contribution to these plumage colors is further illustrated by representative three-dimensional HPLC chromatograms, derived from colored feather barbs of each of the two study species (Fig. 2). Full names, acronyms and chemical structures of all

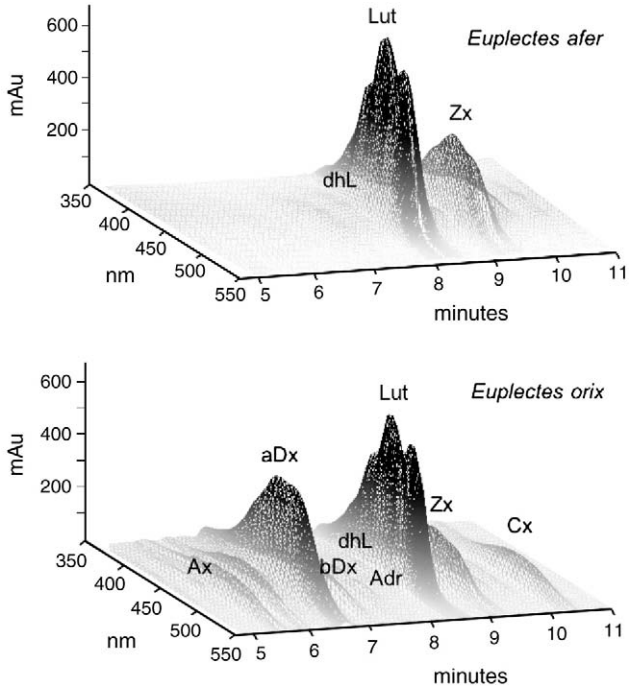

Fig. 2. Three-dimensional HPLC chromatograms representative of wild *E. afer* and *E. orix* feather carotenoid profiles. Peak height is given in milli-absorbance units, and carotenoid acronyms are explained in Fig. 3.

identified carotenoids are shown in Fig. 3. This figure also illustrates the likely metabolic pathways and precursor pigments, including β-carotene and β-cryptoxanthin, which were not detected in the feathers but are the likely precursors of canthaxanthin and adonirubin, respectively.

Although total pigment concentrations do not differ (Welsh $t_{2,7} = 1.02$, $p = 0.34$) between *E. afer* ($788.4 \pm 29.8\ \mu g\ g^{-1}$) and *E. orix* ($881.3 \pm 86.2\ \mu g\ g^{-1}$) feathers, species-specific pigment profiles (i.e. carotenoid composition) show several interesting variations that likely contribute to inter-specific differences in plumage hue (Fig. 4). The yellow color of *E. afer* rump feathers primarily derives from the direct-deposited dietary yellow pigments lutein (retention time, RT 8.5 min, wavelength of max. absorptance, λ_{max} 447 nm) and zeaxanthin (RT 9.1 min, λ_{max} 453 nm), together with smaller amounts of the derived yellow carotenoid 3'-dehydrolutein (RT 7.6 min, λ_{max} 447 nm) (Fig. 4). While the same yellow pigments also contribute to the red breast hue of *E. orix*, they are in this species complemented by several derived red keto-carotenoids: mainly α-doradexanthin (RT 6.5 min, λ_{max} 456 nm), but also β-doradexanthin (adonixanthin, RT 7.1 min, λ_{max} 466 nm), canthaxanthin (RT 10.3 min, λ_{max} 475 nm), astaxanthin (RT 5.5 min, λ_{max} 475 nm), and small amounts of adonirubin (RT 7.5 min, λmax 475 nm) (Fig. 4). The monoketo intermediates in the pathways to canthaxanthin and adonirubin, i.e. echinenone and 3-hydroxy-echinenone, respectively, were not detected in wild *E. orix* feathers, however.

Following zeaxanthin in both *E. afer* and *E. orix*, two small peaks with pronounced secondary UV-A peaks and slightly reduced λ_{max} values (typical of cis isomers; Britton, 1995) were identified as 9,9'Z- and 13,13'Z-zeaxanthin, respectively. Cis-isomers likewise followed directly after lutein (probably 13,13′Z-lutein) and β-doradexanthin.

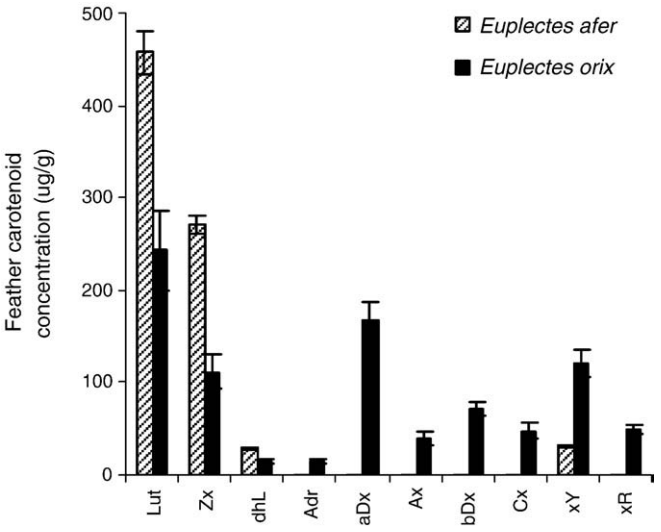

Fig. 4. Average (±SE) concentrations ($\mu g\ g^{-1}$) of detected carotenoid pigments in the nuptial plumages of wild *E. afer* and *E. orix*. Acronyms of identified carotenoids are explained in Fig. 3. Unidentified yellow and red pigments are labeled 'xY' and 'xR', respectively.

In *E. orix* feathers, there were also several minor yellow peaks (RT 4.8–6.7 min, λ_{max} 423–450 nm) that did not match any of our reference pigments. These were classified as 'unidentified yellow' pigments (labeled 'xY' in Figs. 4, 6 and 7). Additionally, *E. orix* feathers included moderate amounts of an unidentified pigment (RT 5.9 min) with λ_{max} around 455 nm, and without signs of multipeaked

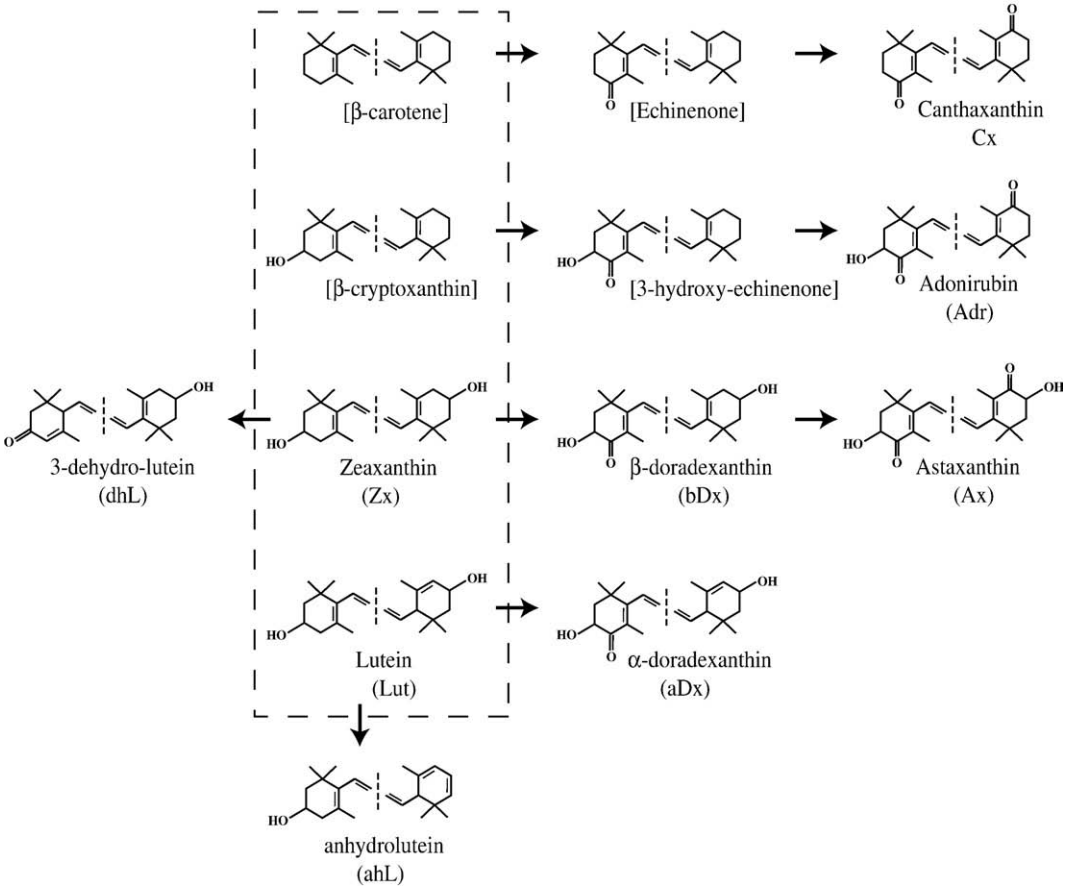

Fig. 3. Chemical structure and hypothesized dietary (inside box) or metabolic (outside box) origin of identified plumage carotenoids. Arrows pointing left, downwards and right indicate enzymatic dehydrogenation, dehydration and C4-oxygenation, respectively. Note that β-carotene and β-cryptoxanthin are assumed precursors, and echinenone and 3-hydroxy-echinenone are assumed intermediate derivatives, although none of these has been detected in the feather analyses.

structure. We classify this pigment as 'unidentified red' (labeled 'xR' in Figs. 4, 6 and 7).

3.2. Pigment and color responses to diet manipulation

After carotenoid deprivation, all *E. afer* and *E. orix* males molted to paler, less chromatic nuptial plumage colors, with more short-wave (yellow-shifted) hues than in wild populations. The transition was particularly evident in *E. orix*, whose orange-red plumage was replaced by dull yellow ($\lambda_{R50} = 526.0 \pm 7.1$ nm) feathers. In contrast, *E. afer* males maintained an almost wildtype yellow color ($\lambda_{R50} = 511.3 \pm 2.3$ nm). Change in spectral location due to low carotenoid-access is seen as a displacement of reflectance curves to the left, in Fig. 5. Reflectances have been normalized to equal maximum values, to illustrate differences in plumage hue independent of variation in brightness ($R_{normal} = R/R_{max}$).

HPLC analyses indicated that feathers molted under carotenoid deprivation had lower total pigment content (*E. afer*: 113.2 ± 14.9 vs. 788.4 ± 29.8 μg g^{-1}, *E. orix*: 179.0 ± 38.5 vs. 881.3 ± 86.2 μg g^{-1}), than feathers sampled from wild populations. In both species, the dominant feather carotenoid was still lutein, either from not depleted tissue storage (e.g. in the liver) or direct deposition from the small amounts (ca 0.6 μg/g) available in the cous–cous diet. Consequently, the yellow feathers of carotenoid-deprived *E. orix* also contained small amounts of α-doradexanthin, the monoketo-derivative of lutein, although in lower ratio to lutein than in the wild (0.2 vs. 0.7). All other C4-keto-carotenoids detected in wild *E. orix* plumages were absent in experimental birds, as was the major feather pigment zeaxanthin. As in wild birds, experimental feathers also contained minor amounts of 3'-dehydrolutein and a few unidentified derived yellow pigments.

In *E. afer*, diet supplementation with ß-carotene did not seem to have any substantial effect on the carotenoid profile, or plumage color (Figs. 5–6). In particular, there was no sign of keto-carotenoids in the subsequently molted feathers. In *E. orix*, however, ß-carotene supplementation obviously caused canthaxanthin to reappear in feathers (Fig. 6), largely restoring plumage reflectance to that in the wild ($\lambda_{R50} = 565.9 \pm 3.3$ nm, Fig. 5).

Plasma pigment profiles for *E. afer* and *E. orix* showed considerably lower concentrations, but qualitatively corresponded well with feather data (Fig. 7). Importantly, plasma samples of carotenoid-deprived and ß-carotene supplemented *E. afer* invariably lacked keto-carotenoids, whereas the plasma of deprived *E. orix* contained small amounts of α-doradexanthin, which was complemented by relatively large amounts of canthaxanthin after supplementation.

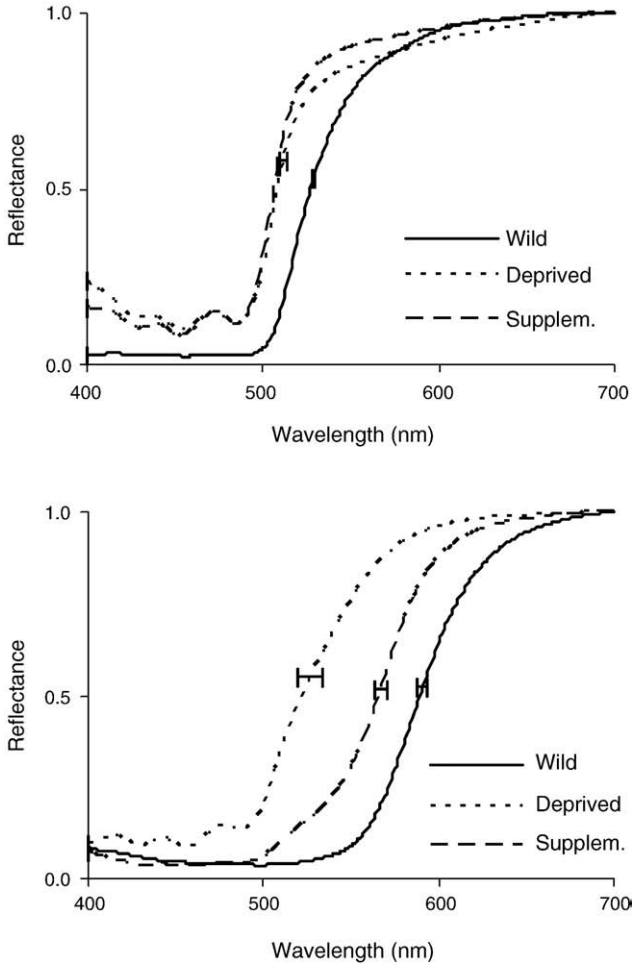

Fig. 5. Mean spectral reflectance, with standard error of hue (λR50), for wild, carotenoid deprived and β-carotene supplemented *E. afer* (top panel) and *E. orix* (bottom panel). To illustrate differences in spectral position, independent of variation in brightness, reflectance spectra have been normalized to equal maxima.

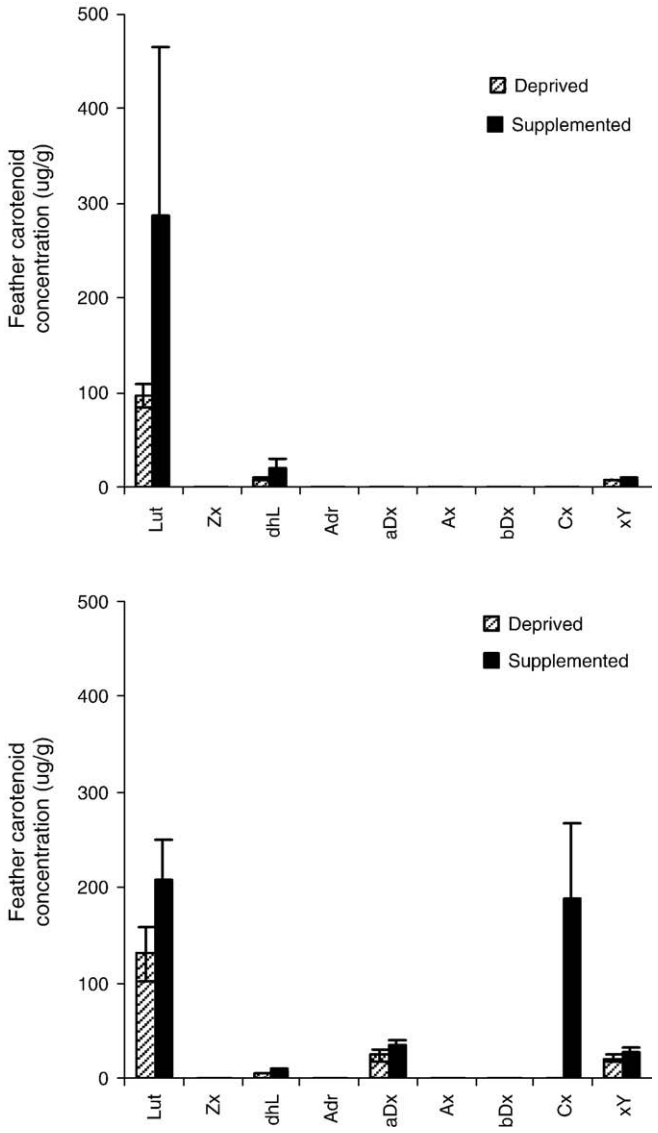

Fig. 6. Average (±SE) concentrations (μg g^{-1}) of detected carotenoid pigments in nuptial feathers of carotenoid deprived and β-carotene supplemented *E. afer* (top panel) and *E. orix* (bottom panel). Acronyms of identified carotenoids are explained in Fig. 3. Unidentified yellow and red pigments are labeled 'xY' and 'xR', respectively.

4. Discussion

Investigating the proximate mechanisms of plumage coloration in a yellow and a red *Euplectes* species, we found that the yellow rump color of nuptial *E. afer* males depends on dietary yellow pigments, whereas the red breast of *E. orix* males is produced using derived red keto-carotenoids. Results from a diet manipulation experiment furthermore suggest that *E. orix*, but not *E. afer*, can manufacture red keto-carotenoids from yellow dietary precursors, presumably using a C4-oxygenating enzyme. This shows that the observed interspecific differences in pigment profiles and color expression can be explained by different capacities to metabolize ingested carotenoids.

4.1. Color and plumage carotenoids of wild E. afer and E. orix

The presented plumage pigment profile of wild *E. afer* samples is qualitatively similar to that of the yellow-mantled widowbird (*E. macrourus*; Andersson et al., 2007), suggesting that yellow *Euplectes* colors in general result from direct deposition of the dietary yellow carotenoids lutein and zeaxanthin. As these are the most abundant carotenoids in plants (Goodwin, 1980), including grass seeds, the main diet of *Euplectes* (Craig, 1980), it is not surprising to find them also in *E. orix*, as well as in several other birds (Goodwin, 1984; Stradi, 1998; McGraw, 2006). Like other *Euplectes* (Andersson et al., 2007), both *E. afer* and *E. orix* samples moreover contained varying amounts of 3'-dehydrolutein, presumably derived from dehydrogenation of zeaxanthin (Stradi, 1998). Since *E. afer* represents a branch basal to the main subclades of bishops and widowbirds (Prager et al., 2008), this supports our previous suggestion of a common and ancestral enzymatic dehydrogenation mechanism for the genus (Andersson et al., 2007).

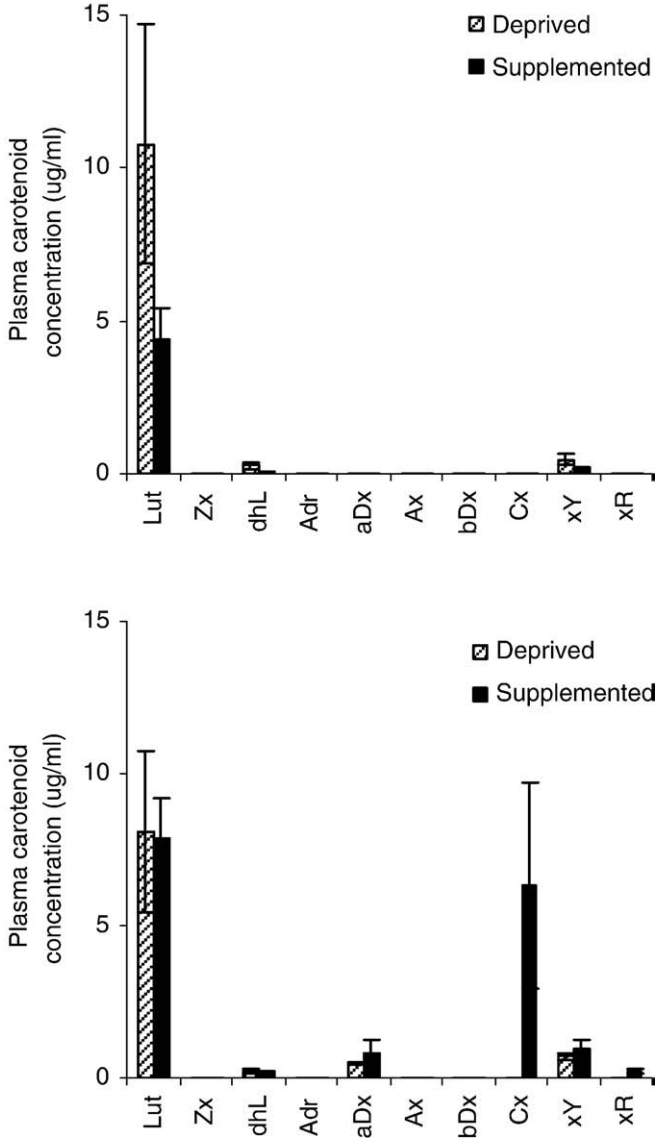

Fig. 7. Average (±SE) concentrations (μg g^{-1}) of detected carotenoid pigments in the plasma of carotenoid deprived and β-carotene supplemented *E. afer* (top panel) and *E. orix* (bottom panel). Acronyms of identified carotenoids are explained in Fig. 3. Unidentified yellow and red pigments are labeled 'xY' and 'xR', respectively.

Wild *E. orix* males complement yellow pigments with co-deposition of red keto-carotenoids (α-doradexanthin, β-doradexanthin, canthaxanthin, astaxanthin and adonirubin). These most certainly derived orange or red pigments have previously been identified in numerous bird species (Goodwin, 1984; Stradi, 1998; McGraw, 2006), including the red-collared widowbird (*E. ardens*; Andersson et al., 2007) which belongs to the same *Euplectes* subclade as *E. orix* (Prager et al., 2008). In contrast, the fan-tailed widowbird (*E. axillaris*), representing the widowbird sister clade, lacks keto-carotenoids altogether, and achieves a red plumage hue by deposition of large amounts of yellow pigments. The restriction of keto-carotenoids to the 'bishop-ardens' clade suggests a single gain (or re-expression) of a C4-oxygenase in the genus.

Due to low concentrations and poor chromatogram resolution, we were unable to determine whether any of the minor yellow peaks (RT 4.8–6.7 min, λ_{max} 423–450 nm) detected in *E. orix* represented canary xanthophylls A or B, previously identified in congeneric taxa (Andersson et al., 2007).

4.2. Pigment and color responses to diet manipulation

Carotenoid deprivation greatly affected the carotenoid profiles of both *E. afer* and *E. orix*. Feathers molted in this condition were largely devoid of all carotenoids except small amounts of lutein (a quarter and a half of the wildtype amounts in *E. afer* and *E. orix*, respectively), most likely absorbed from the cous–cous diet. In *E. orix* males, this resulted in a considerable drop in plumage hue. Similar effects were recently reported for captive cardueline finches, in which ß-cryptoxanthin deprivation caused red feathers pigmented with 3-hydroxy-echinenone to be replaced by yellow ones containing lutein only (Stradi et al., 2001). Atypical yellow hue of a wild-caught mutant cardinal (*Cardinalis cardinalis*) was likewise shown to result from lack of keto-carotenoids (McGraw et al., 2003). The pale orange feathers of carotenoid-deprived *E. orix* males were not completely depleted of keto-carotenoids, but contained traces of α-doradexanthin. As in Stradi et al. (1997), this was most likely produced by C4-oxygenation of ingested lutein. Despite a substantial decrease in lutein and 3'-dehydrolutein, and a complete loss of zeaxanthin, the color effect in carotenoid-deprived *E. afer* individuals was slight, in comparison to that in *E. orix*.

Dietary supplementation with ß-carotene caused a considerable red-shift in newly molted *E. orix* feathers, primarily from the reappearance of canthaxanthin in the plumage, most likely produced from C4-oxygenation of the supplied ß-carotene (Stradi et al., 1997). Wildtype color was not fully achieved, however, as the observed increase of canthaxanthin was insufficient to compensate for comparatively low amounts of α-doradexanthin, and complete lack of β-doradexanthin, astaxanthin and adonirubin, presumably due to the absence of the dietary precursors, zeaxanthin and ß-cryptoxanthin (Stradi et al., 1997). Neither ß-cryptoxanthin nor ß-carotene was detected in any wild or captive bird, even after *ad-lib* supplementation. We still believe that these are important precursor carotenoids, since both are typically present in seeds (Goodwin, 1980), but may be efficiently metabolized to vitamin A (Wyss et al., 2001; McGraw, 2006) or keto-carotenoids (Brush, 1990) in the small intestine or liver. We are

also confident that we would have detected these pigments if they had been present. For example, we have detected modest amounts of ß-carotene in plasma from other *Euplectes* after prolonged ß-carotene supplementation (unpublished data, S. Andersson and M. Prager).

The plumage hue of captive *E. afer* did not change much after ß-carotene supplementation, and no traces of keto-carotenoids were found in analyzed feathers. There is, however, an indication of increased lutein concentrations in the plumage of supplemented *E. afer*, as well as *E. orix*, specimens. If this holds for a larger sample, it may indicate that ß-carotene access augments, rather than suppresses or competes for, uptake or transport mechanisms of other dietary carotenoids. This needs further study with better controlled or labeled supplements.

A central result in our study is the absence of canthaxanthin in feathers of carotenoid-deprived individuals, and its subsequent reappearance in *E. orix*, but not *E. afer*, after ß-carotene supplementation. This verifies the occurrence of C4-oxygenation of dietary precursors in *E. orix*, and that this mechanism is lacking in *E. afer*. The complete absence of keto-carotenoids also in *E. afer* plasma, further points to differences in carotenoid metabolism rather than uptake, or transport, between the two species.

Provided that C3 hydroxyl groups do not cause insurmountable steric hindrance, a single enzyme may in principle catalyze C4-oxygenation of both carotenes and xanthophylls, thus producing all keto-carotenoids observed in *Euplectes* feathers. Lower conformational flexibility around C6–C7 bonds (John Landrum, pers. comm.), and/or the need for double bond displacement when adding a C4-keto-group, may explain why the putative enzyme seems to affect ß- but not ε-end rings. This has implications for the cost, and thus honesty, of red plumage signals in these species. An ability to convert the abundantly available seed carotenoid lutein (ß, ε; Goodwin, 1980), into the diketo-carotenoid astaxanthin (ß,ß) would presumably constitute a cheaper redness mechanism than either being restricted to the substrate α-doradexanthin, or using the less abundant zeaxanthin as astaxanthin precursor. Enzyme specificity for β-end rings is, however, supported by the apparent inability of captive *E. orix* to derive astaxanthin from dietary lutein.

As in the congeneric *E. ardens* (Andersson et al., 2007), and in contrast to finches (Stradi et al., 1995b; Inouye et al., 2001), the monoketo intermediates in the pathway to canthaxanthin and adonirubin, i.e. echinenone and 3-hydroxy-echinenone, respectively (see Fig. 3), were undetected in *E. orix*. This suggests efficient or more or less simultaneous C4-oxygenation of both end rings of the precursor molecules (β-carotene and β-cryptoxanthin, respectively). However, β-doradexanthin, the monoketo-derivative of zeaxanthin, was present in concentrations comparable to that of the end product astaxanthin, possibly due to a slower conversion of hydroxylated end groups. The large amounts of α-doradexanthin, derived from the likewise hydroxylated β-ring on lutein, may seem to contradict this, but this is probably a reflection of the higher concentration of the precursor (lutein) and that α-doradexanthin is not further modified. Isolated observations of echinenone have likewise been made in birds subjected to prolonged ß-carotene supplementation (unpublished data, S. Andersson and M. Prager), suggesting that monoketo compounds only persist at higher substrate-to-enzyme ratios.

5. Conclusions

Our results suggest that *E. orix*, but not *E. afer*, can manufacture red C4-keto-carotenoids from yellow dietary precursors. The occurrence of yellow-pigmented species, in a genus with strong sexual selection for redness, may thus be explained by physiological constraints on plumage pigmentation, rather than ecology. This illustrates how knowledge of proximate mechanisms is crucial for understanding the evolution and diversification of color signals in nature.

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