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Carotenoid content and reflectance of yellow and red nuptial plumages in widowbirds (*Euplectes* spp.)

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Summary

1. Ornamental carotenoid coloration is commonly based on several different pigments with different nutritional and metabolic constraints. The identification and quantification of carotenoid pigments is therefore crucial to the understanding of signal content and signal evolution.
2. In male widowbirds (*Euplectes* spp.), the striking yellow and red carotenoid colours have been measured by reflectance spectrometry and studied with respect to sexual selection through male contest competition, but their biochemical mechanisms have not been analysed.
3. Here we use reflectance analysis and high performance liquid chromatography (HPLC) to describe the species-specific colours and plumage carotenoids in three widowbird species: yellow-mantled widowbird (YMW) *Euplectes macrourus*, red-shouldered widowbird (RSW) *E. axillaris* and red-collared widowbird (RCW) *E. ardens*.
4. YMW yellow ('hue' colorimetric $\lambda R50 = 522$ nm) derives from the two 'dietary yellow' xanthophylls lutein and zeaxanthin, together with small amounts of 'derived yellow' pigments (3′-dehydrolutein and canary xanthophylls).
5. RCW red ($\lambda R50 = 574$ nm) is achieved by the addition of low concentrations of 'derived red' 4-keto-carotenoids, notably α- and β-doradexanthin and canthaxanthin.
6. RSW red ($\lambda R50 = 589$ nm) is, in contrast, created by high concentrations of 'dietary yellow' pigments (lutein, zeaxanthin) and 'derived yellow' anhydrolutein, the latter only recently described in birds.
7. The two different mechanisms of producing red plumage are compared with other bird species and discussed with regard to costs and signal 'honesty'.

Key-words: carotenoid pigmentation, sexual dichromatism, sexual selection, status signalling, weaverbirds.

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Introduction

Bright yellow and red colours in birds and other animals are generally assumed to be caused by carotenoid pigments deposited in feathers, skin or scales (Fox & Vevers 1960), but this mechanism has only been confirmed in a few species, and analysed in detail in even fewer (McGraw 2006). However, thanks to modern analyses of plumage carotenoids in cardueline finches and several other species (e.g. Stradi *et al.* 1998; Inouye *et al.* 2001; McGraw *et al.* 2001; McGraw 2006), this situation is improving. Nevertheless, for most carotenoid plumages (see e.g. Gray 1996), pigment biochemistry and physiology remain to be explored, as illustrated by recent discoveries of a new avian carotenoid (anhydrolutein) in estrildid finches (McGraw, Adkins-Regan & Parker 2002; McGraw & Schuetz 2004) and the noncarotenoid psittacofulvins in parrots (Stradi, Pini & Celentano 2001; Hill & McGraw 2004). In addition to the value of comparative biochemistry of animal coloration, identification and quantification of carotenoid pigments are essential to the understanding of ecological and social selection pressures on carotenoid displays.

There are approximately 700 known naturally occurring carotenoids, most of which are produced by and restricted to photosynthetic plants and microorganisms (Britton 1991; Armstrong & Hearst 1996; Demmig-Adams & Adams 2002; Raila *et al.* 2002; McGraw 2006). The basic structure is a conjugated C40 hydrocarbon chain with or without cyclic end groups (Britton 1995; Armstrong & Hearst 1996). Carotenoids are broadly divided into two major classes: carotenes, which are pure hydrocarbons, and xanthophylls, which have more or less oxygenated end rings (Wolf *et al.* 2000). All carotenoids

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absorb strongly in the indigo-blue spectral region (400–500 nm) (Goodwin 1980), thereby creating striking yellow to red coloration of the reflective integument in which they are deposited (Andersson & Prager 2006).

In addition to being strong colorants, carotenoids have several features making them particularly interesting mechanisms of animal communication. First, as carotenoids cannot be synthesized *de novo* in animals, they must be obtained through the diet from carotenogenic plants or microorganisms (Brockmann & Völker 1934; Goodwin 1984; McGraw 2006). It has therefore been suggested that dietary constraints maintain condition-dependent ('honest') variation in carotenoid colour signals (Endler 1980; Hill 1990). As alternative or complementary processes, 'carotenoid honesty' has subsequently been suggested to be mediated by parasite interference with carotenoid uptake and expression (Hamilton & Zuk 1982; Milinski & Bakker 1990; Thompson *et al*. 1997; Zahn & Rothstein 1999) or direct allocation conflicts between integumentary pigmentation, and immunological or antioxidant functions (Lozano 1994; Olson 1999; von Schantz *et al*. 1999; Möller *et al*. 2000; Lozano 2001; Blount *et al*. 2003). The relative importance of the above mechanisms are debated (Olson & Owens 1998; Hill 1999; Lozano 2001; Hill, Inouye & Montgomerie 2002), and interpretations are complicated by the fact that carotenoid colours typically are made up of several pigments that can differ in all of the above aspects.

The composition of carotenoids in bird plumages is determined both by the processes responsible for their absorption and transport, and by the metabolic capacities of the birds to modify the pigments obtained from the diet (Davies 1991; Stradi *et al*. 1995b; Badyaev *et al*. 2001). For most carotenoids, little is known about the availability of their precursors and, especially, the costs of metabolic derivation (Davies 1985; Brush 1990; Hill 1996; Olson & Owens 1998), both factors obviously pertinent for their potential function as 'honest signals'. One important observation by Hill (1996) is that in most birds, 'red' carotenoids (λ_{max} *c*. 470 nm) are much less common than 'yellow' carotenoids (λ_{max} *c*. 450 nm) (Goodwin 1980, 1984). 'Red' pigments are instead often metabolically derived from the yellow precursors, typically by oxygenation (also known as allylic oxidation) (Davies 1985; Britton 1995), adding double-bonded oxygen atoms ('keto-groups') to the end rings, thereby shifting the absorptance λ_{max} towards longer wavelengths. One suggestion from these observations is that red carotenoid displays generally carry higher acquisition or production costs (or both) than yellow displays (Hudon 1991; Hill 1996). However, as discussed below, if much smaller amounts of 'red' pigments are required to produce a given colour, derived red pigments may be more cost-effective (and thus less 'honest') colorants than large amounts of a 'yellow', albeit more common, pigment.

The extravagant breeding plumages of male widowbirds (*Euplectes* spp.), in addition to elongated tails of varying length, also contain striking yellow and red patches on wings or body. In the congeneric 'bishops', similar but more extensive plumage colours have been shown to be based on several carotenoids (Kritzler 1943), but these have not been analysed with modern methods. Moreover, some recent biochemical studies (McGraw *et al*. 2004a,b) of the melanin basis of what humans perceive as less chromatic ('brownish') red and yellow plumages (Fox & Vevers 1960), as well as the noncarotenoid but equally chromatic psittacofulvins in parrots (McGraw & Nogare 2004), have led to some confusion as to when carotenoid pigmentation can be assumed. With reflectance spectrometry (Andersson & Prager 2006) and some experience of carotenoid- vs. melanin-based reflectance shapes, the general distinction between these mechanisms is simple (sigmoid vs. straight slope), but when a mixture of the two colorants is suspected, a biochemical analysis is necessary to confirm and quantify the relative contribution of carotenoids and thereby the potential for carotenoid-mediated honest signalling.

Sexual selection on *Euplectes* nuptial plumages has been documented in five species, showing uniquely strong female preferences for elongated tails (M. Andersson 1982; S. Andersson 1989, 1992; Pryke, Andersson & Lawes 2001a; Pryke & Andersson 2002, 2005). In contrast, the striking carotenoid plumage patches function primarily in male contest competition (Pryke, Lawes & Andersson 2001b; Andersson *et al*. 2002; Pryke *et al*. 2002; Pryke & Andersson 2003a,b). In one species, however, the yellow-mantled widowbird *Euplectes macrourus* (Gmelin 1789), the yellow colour signal was not studied whereas tail manipulations (albeit on few males) seemed to affect male competition (territory retention) rather than the attraction of nesting females (Savalli 1994).

As regards the information content and 'honesty' of the long, graduated tails, the condition dependence through stressful growth and/or aerodynamic hindrance has been indicated in several studies (Andersson 1994; Andersson *et al*. 2002; Pryke & Andersson 2002, 2005). The mechanisms and costs of the carotenoid colour displays, however, are not known, and have become especially interesting to explore in the light of an apparent intra- and interspecific trade-off between the two signals (tail length and plumage colour) (Andersson *et al*. 2002).

In this study, we analyse the species-specific reflectance shapes and plumage carotenoid content in three widowbird species that have been studied with regard to sexual selection and signalling: yellow-mantled widow, red-shouldered widowbird *E. axillaris* (Smith 1838) and red-collared widowbird *E. ardens* (Boddaert 1783). By comparisons with the growing knowledge of dietary origins, uptake and metabolism of carotenoids in other seed-eating birds (Stradi *et al*. 1997; Inouye *et al*. 2001; McGraw & Hill 2001), we discuss the implications for 'carotenoid honesty' and evolution of the conspicuous colour signals in African widowbirds.

Methods

STUDY SPECIES AND SAMPLE COLLECTION

The widowbirds (*Euplectes* spp.) are sexually and seasonally dichromatic weaverbirds (subfamily *Ploceinae*), breeding semicolonially and polygynously in moist grasslands of equatorial and southern Africa. During the prenuptial moult, males replace their mottled brown nonbreeding plumage (similar to that of females) with slightly to extremely elongated black tail feathers and black body plumage with contrasting yellow or orange–red patches. In South Africa, the red-collared widowbird *E. ardens ardens* (RCW hereafter) has a long tail (*c*. 22 cm) and a crescent-shaped red collar patch, while the red-shouldered widowbird *E. axillaris axillaris* (RSW) has a very slightly elongated tail (*c*. 8 cm) and an orange–red wing patch (lesser and wing coverts). From these two species, measurements and samples were collected as part of studies of their ecology and behaviour in Natal, South Africa (e.g. Pryke *et al*. 2001a; Pryke & Andersson 2003a). For comparison with the only yellow-coloured widowbird in which sexual signalling has been studied (Savalli 1994), we analysed yellow wing coverts from the yellow-mantled widowbird *E. macrourus macrocercus* (YMW), collected and kindly provided by Dr C.G. Wiklund outside Asmara, Eritrea. Coloured feathers (five to seven) were plucked with tweezers and stored in dark and dry conditions in brown envelopes, until analysis.

REFLECTANCE AND COLORIMETRICS

Plumage reflectance was measured, and colorimetric variables computed, for all RCW and RSW individuals from which feather samples were collected. In addition, we made sure that the reflectance curves were representative of the two study populations. For YMW, reflectance was measured on 10 specimens of the subspecies *E. macrourus macrocercu*s in the skin collection at British Museum of Natural History, Tring, UK (accession numbers 1912.10.15.1516–17, 23–26; 1923.8.7.1854; 1927.11.5.883–884, 889). Reflectance was measured with a USB2000 spectroradiometer system (OceanOptics, Dunedin, Florida, USA), including a fiber-optic reflectance probe, a HL2000 tungsten-halogen light source and using CSpec software (Ancal, Las Vegas, Nevada, USA). The measuring configuration, using a home-made 'Probe holder' (Andersson & Prager 2006) fitted on the probe ferrule, was 'Coincident Normal' (CN: coaxial illumination and reading beams, perpendicular to the plumage plane; Andersson & Prager 2006), taking three to five scans and removing the probe between each scan. Reflectance was analysed only in the 400–700 nm range, as the full range measurements of these and other saturated carotenoid colours consistently show negligible ('flat') reflectance in the ultraviolet range (320–400 nm; unpublished data). As detailed in Andersson & Prager (2006), the objective colorimetrics computed were 'Hue' (λR50; wavelength at which reflectance is halfway between its minimum and its maximum), 'Carotenoid chroma' (C_{CAR}; R_{700}–R_{450}/R_{700}) and 'Brightness' (R_{AV}; average reflectance 400–700 nm).

CAROTENOID ANALYSIS

Extraction and HPLC chemicals (methanol, n-hexane, acetonitrile and acetone) were obtained from VWR International (Stockholm, Sweden) and syringe filters from Pall Gelman Sciences Inc. (Ann Arbor, MI, USA). Following a carotenoid extraction protocol modified from Stradi, Celentano & Nava (1995), approximately 1 mg of coloured barbs was trimmed from feathers after washing 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, using a 0·2 µm syringe filter (GHP Acrodisc®). After evaporation under nitrogen in a speedvac (Savant DNA120, Holbrook, Arizona, USA), the residue was resuspended in 150–200 µL acetone and placed at –78 °C overnight. The precipitate was filtered off, using 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 analysed 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, CA, USA) HPLC system with PS4000 ternary pump, AS3000 autosampler, and UV6000 UV/VIS diode-array detector. The column temperature was maintained at 30 °C and the flow-rate at 0·6 mL min^{-1}. Two-dimensional (at 450 nm and 470 nm) and three-dimensional (300–700 nm) chromatograms were obtained, inspected and analysed in ChromQuest 4·0 software (ThermoFinnigan). Peaks were identified by comparing relative retention times and spectral absorptance characteristics with earlier analyses of plumage pigments (e.g. Britton 1995; Stradi *et al*. 1997; Stradi 1998). Pigment concentrations (per gram coloured feather barbs from which carotenoids were extracted) were calculated from known initial sample weight, injection volume and calibration coefficients obtained from identical analyses of standard solutions with known concentrations 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), kindly provided by Roche Vitamins Inc. (Basel, Switzerland), and 2′,3′-anhydrolutein (2′,3′-didehydro-β,ε-caroten-3-ol) provided by F. Khachik. For identification purposes, some standards, such as 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

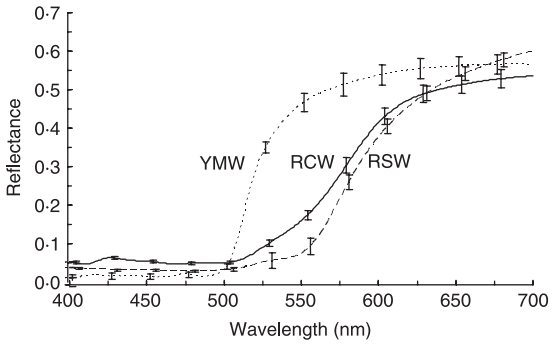

Fig. 1. Spectral reflectance curves (mean ± SE) with standard error bars for yellow-mantled widowbird (YMW, dotted line), red-collared widowbird (RCW, solid line), and red-shouldered widowbird (RSW, dashed line). Note sigmoidal reflectance shapes revealing carotenoids rather than melanin as the single (YMW) or predominant (RCW, RSW) source of absorptance (i.e. the inverse of reflectance).

canary xanthophylls A (3′-hydroxy-ε,ε-caroten-3-one) and B (ε,ε-carotene-3,3′-dione) were extracted from feather samples from previously analysed species, i.e. pekin robin *Leiothrix lutea*, great spotted woodpecker *Dendrocopus major*, bullfinch *Pyrrhula pyrrhula* and siskin *Carduelis spinus* (Stradi 1998).

Owing 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 all-E-configuration appears to be the most common state of natural carotenoids (McGraw 2006). All identified isomers were combined with the parent carotenoid.

Results

PLUMAGE REFLECTANCE

The species-specific plumage reflectance curves are shown in Fig. 1, with associated colorimetrics in the top section of Table 1. The difference in spectral location or 'hue' (λR50) is large (*c*. 50 nm) between YSW and the two red species. Less obvious to the human eye, RSW has an approximately 14 nm 'redder' (more long-wave) hue than RCW (Table 1, $F_{1,32} = 11{\cdot}2$, $P = 0{\cdot}002$). With regard to the proximate mechanisms (discussed below), there are also interesting interspecific differences in spectral shape that are not captured by the above colorimetrics. In particular, the deviation in RCW and RSW, but not in YMW, from the sigmoid reflectance shape typical of pure carotenoid pigmentation (Andersson & Prager 2006) suggests some contribution of brown melanin, especially in RSW (see Discussion).

IDENTIFIED CAROTENOIDS

Figure 2 shows representative HPLC three-dimensional chromatograms derived from coloured feather barbs of each of the three study species. Full names, acronyms

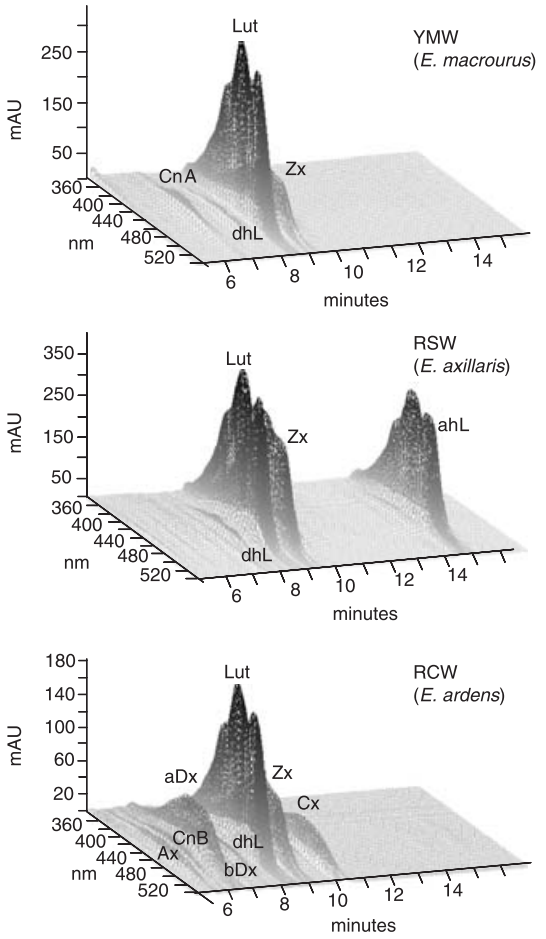

Fig. 2. Three-dimensional HPLC chromatograms representative of the three species-specific carotenoid profiles, YMW (top), RSW (middle) and RCW (bottom). Pigment acronyms (see also Fig. 3) are explained here, in order of appearance (retention time): Ax, astaxanthin; CnB, canary xanthophyll B; aDx, α-doradexanthin; CnA, canary xanthophyll A; bDx, β-doradexanthin (adonixanthin); Adr, adonirubin; dhL, 3′-dehydrolutein; Lut, lutein; Zx, zeaxanthin; Cx, canthaxanthin; and ahL, anhydrolutein. Identified carotenoids are also listed together with their concentrations in Table 1.

and chemical structures of all identified carotenoids are shown in Fig. 3. This figure also illustrates the likely metabolic pathways (see Discussion) and precursor pigments ('substrates'), including β-carotene and β-cryptoxanthin, which were not detected in the feathers but are the likely precursors of canthaxanthin and adonirubin, respectively. In contrast to cardueline finches (Stradi *et al*. 1995b; Inouye *et al*. 2001), the one-keto intermediates echinenone and 3-hydroxy-echinenone (Fig. 3) were not detected in RCW, suggesting efficient and/or more or less simultaneous C4-oxygenation of both end rings of the precursor molecules (β-carotene and β-cryptoxanthin, respectively). However, β-doradexanthin (adonixanthin), the one-keto product of zeaxanthin, was present in larger concentrations than the end-product astaxanthin (Table 1), which probably is due to a slower conversion of hydroxylated end groups. The large amounts of α-doradexanthin, derived from

Table 1. Reflectance colorimetrics of plumage colour and average concentration ± standard deviation of identified carotenoids in feathers. Concentrations are given in μg g^{-1}

	E. macrourus Mean ± SD	*E. axillaris* Mean ± SD	*E. ardens* Mean ± SD
Reflectance colorimetrics	(*n* = 10)	(*n* = 22)	(*n* = 17)
'Hue' (λR50)	521·7 ± 1·39	589·2 ± 1·67	573·5 ± 3·65
'Carotenoid chroma' (C_{CAR})	0·98 ± 0·00	0·95 ± 0·00	0·89 ± 0·01
'Brightness' (R_{AV})	0·31 ± 0·01	0·22 ± 0·01	0·26 ± 0·02
Plumage pigment concentrations (μg g^{-1})	(*n* = 8)	(*n* = 20)	(*n* = 14)
Dietary carotenoids			
Lutein	215·0 ± 85·5	518·9 ± 207·5	243·4 ± 92·8
Zeaxanthin	90·1 ± 62·7	416·7 ± 129·1	197·5 ± 129·1
Subtotal	305·1 ± 146·2	935·6 ± 325·1	440·9 ± 172·6
Derived 'yellow' carotenoids			
2′,3′-anhydrolutein	–	642·5 ± 198·3	–
3′-dehydrolutein	27·3 ± 19·9	65·3 ± 27·7	19·5 ± 12·7
Canary xanthophyll A	8·6 ± 13·4	25·7 ± 14·3	15·9 ± 5·4
Canary xanthophyll B	–	24·2 ± 14·8	37·7 ± 19·6
Unidentified 'yellow'*	–	21·0 ± 6·8	11·6 ± 6·8
Subtotal	35·9 ± 19·3	757·7 ± 242·9	73·1 ± 35·2
Derived 'red' carotenoids			
α-doradexanthin	–	–	113·3 ± 52·7
β-doradexanthin	–	–	31·5 ± 17·0
Adonirubin	–	–	6·0 ± 10·3
Canthaxanthin	–	–	20·9 ± 19·2
Astaxanthin	–	–	7·2 ± 11·8
Subtotal:	–	–	178·8 ± 68·1
Total carotenoid concentration:	341·0 ± 157·0	1715·3 ± 569·7	704·4 ± 227·6

*Unidentified yellow pigments were not included in the subtotal for derived yellow carotenoids, but were included in the measure of total carotenoid concentration.

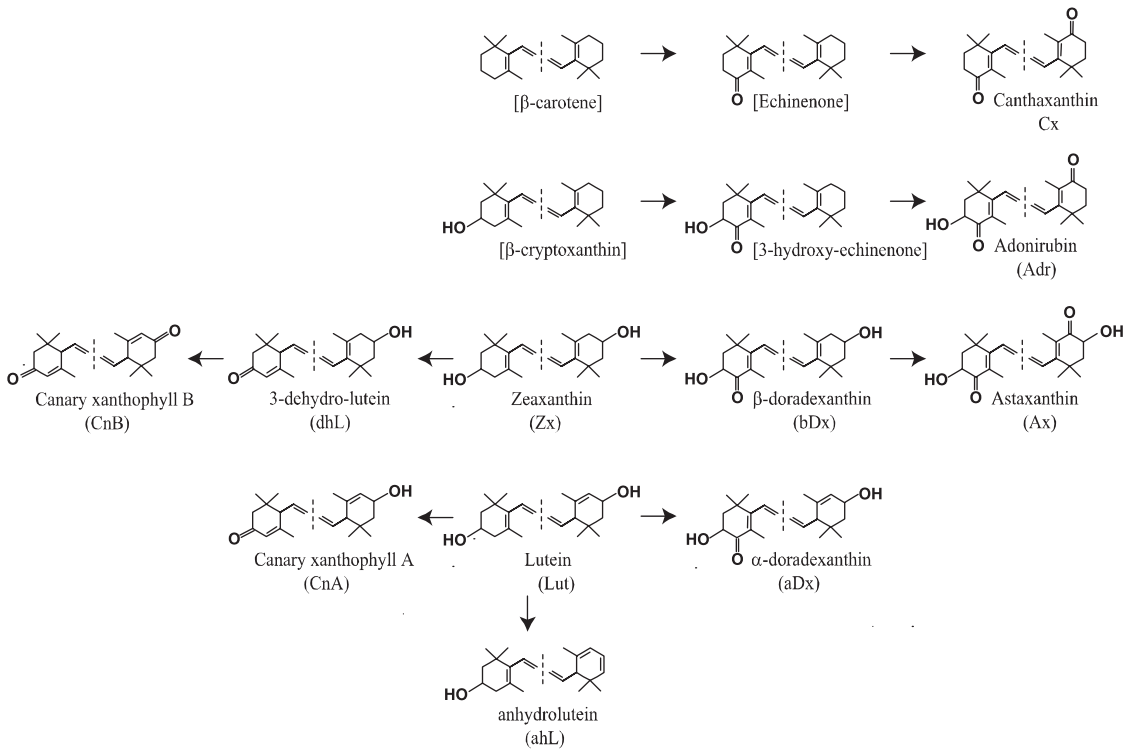

Fig. 3. Chemical structure and hypothesized dietary or metabolic origin of identified plumage carotenoids. Arrows pointing left, downwards and right indicate enzymatic dehydrogenation, dehydration and C4 oxygenation, respectively. Note that β-carotene and β-cryptoxanthin are assumed precursors, and echinenone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

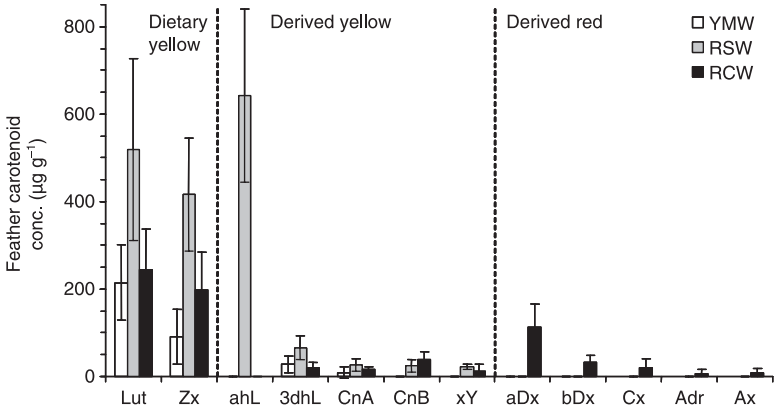

Fig. 4. Bar chart showing average (± SE) concentrations (μg g^{-1}) of identified carotenoid pigments (classified as Dietary, Derived yellow, and Derived red) in the nuptial plumages of three species of widowbird: yellow-mantled widowbird (YMW, open bars), red-shouldered widowbird (RSW, grey bars) and red-collared widowbird (RCW, black bars). Carotenoid acronyms are explained in Fig. 3.

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.

SPECIES-SPECIFIC CAROTENOID PROFILES

Table 1 and Fig. 4 summarize the average carotenoid concentrations detected in nuptially coloured feathers from the three species. Based on the likely metabolic origins (Fig. 3) and spectral absorptance shape, the pigments were classified as 'dietary yellow', 'derived yellow' (both with multipeaked curves with $\lambda_{max} < 460$ nm) or 'derived red' (smooth curves with $\lambda_{max} > 460$ nm). The main components in all species were the two common 'dietary yellow' xanthophylls lutein (RT 0, λ_{max} 447 nm) and zeaxanthin (Retention time, RT 0·6 min, λ_{max} 453 nm). However, apart from these typically direct-deposited plumage pigments (McGraw 2006), there were several interesting qualitative and quantitative interspecific differences as regards metabolically derived carotenoids. First, in the yellow feathers of YMW we identified canary xanthophyll A (RT –1·5 min, λ_{max} 443 nm) and 3′-dehydrolutein (RT –0·7, λ_{max} 447 nm). Both of these are also present in the red RSW feathers, which in addition contain canary xanthophyll B (RT –2·1 min, λ_{max} 440 nm), as well as large amounts of another derived yellow pigment, 2′,3′-anhydrolutein (RT 6·0 min, λ_{max} *c.* 448 nm), recently discovered also in the subfamily Estrildinae and supposedly derived by dehydration from lutein (McGraw *et al*. 2002). In addition, compared with YMW, RSW has more than twice as much lutein and 3′-dehydrolutein, and almost five times as much zeaxanthin (Table 1). In the absence of 'red' pigments (i.e. 4-keto-carotenoids), the red hue of RSW (Fig. 1) results from these very high concentrations of both derived and precursory 'yellow' pigments (see Discussion, and Andersson & Prager 2006).

In RCW, lutein and zeaxanthin were again the main fractions, together with smaller quantities of 3′-dehydrolutein and canary xanthophylls A and B. Only in this species, however, did we find 'derived red' carotenoids; primarily the keto-carotenoids α-doradexanthin (RT –1·8 min, λ_{max} 456 nm), β-doradexanthin (adonixanthin, RT –1·3 min, λ_{max} 466 nm), and canthaxanthin (RT 1·9 RT –1·3 min, λ_{max} 474 nm), but also astaxanthin and adonirubin (RT –2·9 and RT –1·3 min, respectively, λ_{max} 475 nm) (Table 1), in some individuals.

Following zeaxanthin in all three species, two small peaks with pronounced secondary UVA humps and slightly reduced λ_{max} values (typical of *cis*-isomers, Britton 1995) were identified as 9,9′*Z*- and 13,13′*Z*-zeaxanthin, respectively. Likewise, directly following lutein, was a lutein *cis*-isomer (probably 13,13′*Z*-lutein), whereas several early small peaks (RT –4 to –2 min and λ_{max} 430–445 nm) indicated a variety of canary xanthophyll isomers. As *cis*-isomers are likely to be extraction artefacts (Schiedt & Liaaen-Jensen 1995; Stradi *et al*. 1995a), all were pooled with the most likely parent carotenoid.

In RSW, there were small amounts of two unidentified 'yellow' peaks (xY in Fig. 4) that did not appear to be isomers of any identified pigments: The first was just before and mostly concealed by the higher 3′-dehydrolutein peak, why the spectral shape and λ_{max} was difficult to determine. The retention time corresponded to adonirubin (in RCW), but judged from the multipeaked spectral absorptance shape, this was more likely a 'yellow' pigment. The other unidentified RSW peak was a late but like the former a typically 'yellow' pigment (RT 11·8 min, λ_{max} 447 nm). Finally, in two RCW individuals, there was yet another unidentified yellow pigment (RT –2·5, λ_{max} 451 nm), closely following astaxanthin (RT –2·9 min).

Discussion

Long before this study, Kritzler (1943) used paper chromatography to separate the major carotenoid fractions of three other *Euplectes* species ('bishops'). Together with the present analysis of three 'widowbirds' in the same genus, the carotenoid contribution to ornamental coloration and sexual dichromatism in this group is thus firmly established, and of particular relevance to the agonistic signal function demonstrated in RCW and RSW (Pryke *et al*. 2002; Pryke & Andersson 2003a). Furthermore, as regards a recent classification of widowbird colours as 'carotenoid + melanin' (Hill 2006), it may be pointed out that RCW feathers (0·704 mg g^{-1}) and RSW feathers (1·71 mg g^{-1}) (Table 1) contain two and 10 times, respectively, as high carotenoid concentrations as in the 'carotenoid' plumages listed in the same table. Any impression that widowbird colour signals are less carotenoid-dependent than others is thus misleading, as they actually have among the highest recorded plumage carotenoid concentrations.

The dominance of lutein and zeaxanthin in *Euplectes* feathers is not surprising as these, together with carotenes and cryptoxanthins, are the most abundant carotenoids in plants (Goodwin 1980), including grass seeds, which is the main diet of widowbirds and bishops (Craig 1980). As in a wide range of other birds (Goodwin 1984; Stradi 1998; McGraw 2006), lutein and zeaxanthin are common, readily absorbed and direct-deposited pigments, with few obvious costs of acquisition or uptake. Enzymatic dehydrogenation of these common precursors produces 3′-dehydrolutein, and canary xanthophylls A and B (Fig. 3) (see also Stradi 1998; McGraw 2006). The first two of these 'derived yellow' pigments (3dhL and CnB) were found in all three species and appear to be present in most other *Euplectes* species as well (A. Johansson and S. Andersson, unpublished data), suggesting that a common (homologous) enzymatic dehydrogenation mechanism produces all the 'derived yellow' pigments to the left in Fig. 3. The adaptive function of this modification is not known (McGraw 2006), and seems unlikely to concern signalling, as derived yellow carotenoids have absorptance maxima (and thus the 'hue' of the resulting reflectance) at either the same or shorter wavelengths than their precursors. For example, absorptance λ_{max} of canary xanthophylls are 440–442 nm, compared with 447–453 nm in their precursors lutein and zeaxanthin.

In addition to lutein, zeaxanthin and their dehydrogenated derivatives, RSW also incorporates large amounts of 2′,3′-anhydrolutein. This carotenoid has recently been found in the plasma and/or liver of five estrildid finch species, two of which also use it in yellow plumage pigmentation (McGraw *et al.* 2002; McGraw & Schuetz 2004). Like in humans, 2′,3′-anhydrolutein presumably derives from dehydration of dietary lutein (Khachik *et al.* 1992; McGraw *et al.* 2002). As the estrildid finches also are grassland-dwelling granivores (Fry & Keith 2004), we might assume that a convergent enzymatic mechanism is responsible for the presence of 2′,3′-anhydrolutein in the feathers of RSW (Fig. 3).

A particularly interesting question is how the 'red' hue of RSW is produced from only 'yellow' pigments. We consider two (not mutually exclusive) possibilities: (1) that the exceptionally high concentration (1·71 mg per g) of 'yellow' pigments by itself is responsible for the shift to an 'orange–red' hue, and (2) that the pigment absorptance spectra are altered by their binding to proteins. Regarding the first alternative, Andersson & Prager (2006) describe how hue and other colorimetrics change with the concentration (saturation) of carotenoids and other pigments (also see Hudon *et al.* 2003). This is because the spectral absorptance function of carotenoids does not drop abruptly to zero, but decreases gradually (although steeply) into longer wavelengths. This means that even after saturation (100% absorptance in the spectral interval of the peak), subsequent increases in pigment concentration will keep shifting the hue towards the orange–red (see Andersson & Prager 2006). The alternative (or additional) mechanism is that attachment to the feather keratin causes a change in the electronic structure, and thus a shift to longer wavelength absorptance of one of the carotenoids (supposedly anhydrolutein, which is unique to RSW). This was suggested for the red face patch of the European goldfinch *Carduelis carduelis*, which also contains only 'yellow' pigments, and this idea was supported by resonance Raman spectroscopy (Stradi *et al.* 1995a). Similar analyses of RSW feathers, combined with more detailed studies of reflectance variation in relation to pigment concentration, will indicate which of the above mechanisms is most likely in this case.

Instead of relying on either large quantity or binding biophysics of yellow pigments, RCW acquire its orange–red hue using moderate amounts of lutein and zeaxanthin in combination with lower concentrations of 'red' keto-carotenoids (λ_{max} 465–473 nm). As the latter generally are scarce or absent in granivorous diets (Hill 1996) their presence in these plumages depends on metabolic modification, more precisely C4-oxygenation of yellow, dietary precursors (Stradi *et al.* 1997; Fig. 3). Accordingly, the main keto-carotenoids found in RCW, i.e. α-doradexanthin, astaxanthin, adonirubin and canthaxanthin, probably derives from C4-oxygenation of lutein, zeaxanthin, β-cryptoxanthin (β,β-caroten-3-ol) and β-carotene (β,β-carotene), respectively (see, e.g. Stradi *et al.* 1997; Fig. 3). All four precursors above are known to be present (in low concentrations) in some grass seeds, but only lutein and zeaxanthin, are with certainty, found in avian plasma and feathers (McGraw *et al.* 2001). The absence of β-cryptoxanthin and β-carotene in plasma may suggest that these ingested carotenoids were completely consumed by either C4-oxygenation (to Adr or Cx, respectively) or cleavage to retinol (Wyss *et al.* 2001) or, most likely, a combination of these.

From a signal evolution and diversification perspective, it is particularly interesting with the occurrence of two distinctly different mechanisms of red carotenoid coloration; either large quantities of dietary yellow pigments (RSW) or smaller amounts of derived red pigments (RCW). This implies convergent evolution in response to similar selection pressures on coloration. In both species, the red colour patches function as agonistic signals in male contest competition ('intrasexual selection') over breeding territories (Pryke *et al.* 2001b, 2002; Pryke & Andersson 2003a,b). Our results thus confirm a carotenoid basis of 'redness' in both RSW and RCW, but the two separate mechanisms suggest that different kinds of costs (and thus different information content) apply to colour production in each species. Whereas RSW presumably invests more in terms of acquisition, transport and deposition of dietary pigments, RCW has additional costs relating to enzymatic conversion of dietary pigments into red keto-carotenoids.

In YMW, with yellow ornamental coloration, the signalling function is unknown, as well as the intended receiver (i.e. females or rivals). It would be particularly interesting to know if there is stabilizing signal selection on 'yellowness', or if there is a pre-existing bias for red coloration also in this species, which appears to represent a more ancestral branch than the red-coloured widowbirds (Andersson & Prager, unpubl. data). If the latter, it would imply that YMW either has not yet been endowed with any of the two 'redness' mechanisms in RSW and RCW, respectively, or that there are balancing costs from, e.g. nutrition or predation. Given the very minor ecological differences (diet, habitat, etc.) within the *Euplectes*, ecological constraints seem unlikely and we rather believe that there are physiological adaptations in both RSW and RCW that have not evolved (or been lost) in YMW. In RCW, this would of course be the keto-carotenoid producing C4 oxygenase (see above), and in RSW it might be mechanisms of more efficient uptake, transportation or deposition of the yellow carotenoids. The latter may also be related (functionally or genetically) to the production of anhydrolutein: This is supported by two observations: (1) preliminary data showing anhydrolutein in another widowbird species with a red patch, the long-tailed widowbird *E. progne*, and (2) well resolved phylogenetic evidence (Andersson *et al*. unpublished data) suggesting that YMW is basal to both RCW and RSW, in which the two different 'redness' mechanisms thus appears to be derived. Further work on the phylogeny of carotenoid pigmentation in *Euplectes* may confirm this picture and allow us to comparatively and experimentally test the selection pressures behind the divergence in ornamental coloration.

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