

Red-winged blackbirds *Agelaius phoeniceus* use carotenoid and melanin pigments to color their epaulets

Kevin J. McGraw, Kazumasa Wakamatsu, Anne B. Clark and Ken Yasukawa

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Over the past three decades, the red-winged blackbird *Agelaius phoeniceus* has served as a model species for studies of sexual selection and the evolution of ornamental traits. Particular attention has been paid to the role of the colorful red-and-yellow epaulets that are striking in males but reduced in females and juveniles. It has been assumed that carotenoid pigments bestow the brilliant red and yellow colors on epaulet feathers, but this has never been tested biochemically. Here, we use high-performance liquid chromatography (HPLC) to describe the pigments present in these colorful feathers. Two red ketocarotenoids (astaxanthin and canthaxanthin) are responsible for the bright red hue of epaulets. Two yellow dietary precursor pigments (lutein and zeaxanthin) are also present in moderately high concentrations in red feathers. After extracting carotenoids, however, red feathers remained deep brown in color. HPLC tests show that melanin pigments (primarily eumelanin) are also found in the red-pigmented barbules of epaulet feathers, at an 'approximately equal concentration to carotenoids. This appears to be an uncommon feature of carotenoid-based ornamental plumage in birds, as was shown by comparable analyses of melanin in the yellow feathers of male American goldfinches *Carduelis tristis* and the red feathers of northern cardinals *Cardinalis cardinalis*, in which we detected virtually no melanins. Furthermore, the yellow bordering feathers of male epaulets are devoid of carotenoids (except when tinged with a carotenoid-derived pink coloration on occasion) and instead are comprised of a high concentration of primarily pheomelanin pigments. The dual pigment composition of red epaulet feathers and the melanin-only basis for yellow coloration may have important implications for the honesty-reinforcing mechanisms underlying ornamental epaulets in red-winged blackbirds, and shed light on the difficulties researchers have had to date in characterizing the signaling function of this trait. As in several other birds, the melanic nature of feathers may explain why epaulets are used largely to settle aggressive contests rather than to attract mates.

K. J. McGraw (correspondence), Department of Neurobiology and Behavior, Cornell University, Ithaca, NY 14853 USA. K. Wakamatsu, Fujita Health University, School of Health Sciences, Toyoake, Aichi, 470-1192 Japan. A. B. Clark, Department of Biological Sciences, Binghamton University, Binghamton, NY 13902 USA. K. Yasukawa, Department of Biology, Beloit College, Beloit, WI 53511 USA. Present address of K. J. McGraw: School of Life Sciences, Arizona State University, Tempe, AZ 85287-4501 USA. E-mail: Kevin.McGraw@asu.edu

The bright yellow, orange, and red colors of bird feathers are generally produced by carotenoid pigments (Fox and Vevers 1960, Brush 1978). Carotenoids are acquired from dietary sources and are often used as colorants in feathers and bare parts to attract mates (Hill 1999).

Because carotenoids appear to be difficult to obtain from the environment (Grether et al. 1999, Hill et al. 2002), and to accumulate physiologically (McGraw et al. 2003) in many species, there is a growing body of evidence demonstrating the condition-dependent nature

of carotenoid-based color signals (Olson and Owens 1998, Møller et al. 2000).

Biochemical studies indicate, however, that not all red-, orange-, and yellow-colored bird feathers can be assumed to contain carotenoids. Parrots, for example, use a unique class of polyenal lipochromes to generate their spectrum of plumage colors (Stradi et al. 2001). McGraw et al. (2004) showed that the yellow downy plumage of domestic chicks *Gallus domesticus* and the yellow feathers of adult king *Aptenodytes patagonica* and macaroni *Eudyptes chrysolophus* penguins lack carotenoids and instead harbor as-of-yet uncharacterized fluorescent compounds. Still others have shown that melanin pigments are responsible for the buff-yellow feather colors in certain breeds of chicken (Frank 1939), turkey *Meleagris gallopavo* (Somes and Smyth 1965) and quail *Coturnix japonica* (Shiojiri et al. 1999). Thus, as in other animals (e.g. the similarly colored pterin and carotenoid pigments in fishes and lizards; Macedonia et al. 2000, Grether et al. 2001), it seems that detailed biochemical analyses are needed before researchers can be sure of the carotenoid basis of feather colors. This is particularly critical for studies of ornamental color signals, since different types of pigmentary and structural colors can be governed by different expression mechanisms and can serve unique sexual- or social-signaling functions (McGraw and Hill 2000a, McGraw et al. 2002a).

One of the best-studied color ornaments in birds is the red and yellow shoulder badges (or epaulets) of the red-winged blackbird *Agelaius phoeniceus* (Searcy and Yasukawa 1995, Beletsky 1996). The upper marginal coverts of males are brilliant red and bordered by yellow upper lesser coverts (Searcy and Yasukawa 1995). These epaulets are more exaggerated (e.g. larger in size, richer in color) in male blackbirds compared to females and juveniles, and are overtly displayed in sexual contexts during the breeding season (Yasukawa and Searcy 1995). Males erect epaulet feathers during flight and perch displays that occur in the presence of females or male competitors (Yasukawa and Searcy 1995). These ornaments are presumed by most to be carotenoid-based (Miskimen 1980, Irwin 1994, Johnson and Lanyon 2000, Hill 2002, Pryke and Andersson 2003), but this has never been confirmed biochemically.

The aim of this study was to describe the types of pigments that color the ornamental epaulet feathers of male red-winged blackbirds. We collected and analyzed the carotenoid content of yellow and red epaulet feathers from wild-caught male blackbirds using high-performance liquid chromatography (HPLC). We also tested for the presence of melanins in these feathers, with techniques that have been used to characterize the pheomelanin and eumelanin composition of yellow, red, brown and black hair and fur in mammals (Ito and

Fujita 1985) and the black to reddish-brown plumage of pigeons *Columba livia* (Haase et al. 1992).

Methods

Collection of samples

We collected 5 red and 5 yellow epaulet feathers from each of three adult males: one captured from Champaign-Urbana, Illinois in the autumn of 1995 and saved as a museum specimen, and the others plucked fresh from wild males netted on 7 September 2001 in Seneca Falls, NY and 14 August 2002 in Ithaca, NY. All feathers were stored in the dark at room temperature prior to analysis.

Carotenoid analyses

All procedures were performed in the laboratory of R. Parker from the Division of Nutritional Sciences at Cornell University. We used conventional thermochemical techniques (Hudon and Brush 1992, McGraw et al. 2002b) to extract carotenoid pigments from both red and yellow epaulet feathers. Feathers were first washed sequentially in ethanol and hexane for 15 min each to remove surface lipids. Three to five mg of pigmented barbules were then trimmed from feathers and placed in 2 ml acidified pyridine in a 9 ml glass tube. We filled the headspace of the tube with argon and held it at 95°C for 3 h. After heating, the tube was cooled to room temperature, and to the pyridine we added 2 ml water and 2 ml hexane:tert-butyl methyl ether (1:1). We shook the mixture for 2 min and centrifuged at 3000 RPM for 5 min. The upper, colored phase containing the carotenoids was removed, transferred to a fresh tube, and evaporated to dryness under a stream of nitrogen. We dissolved the residue in 200 µl of HPLC mobile phase (methanol:acetonitrile, 1:1, v/v) for analysis.

Fifty µl of each sample was injected into a Waters™ 717plus Autosampler HPLC (Millipore Corp., Bedford, MA) fitted with a Develosil RPAqueous RP-30 column (250 × 4.6 mm ID; Nomura Chemical Co. Ltd., Japan) and an Eppendorf TC-50 column heater (set at 32°C; *sensu* McGraw et al. 2002c). We used an isocratic system (HP 1050 Series Isocratic Pump) at a constant flow rate of 1 ml/min for 60 min to allow sufficient time for both xanthophylls and carotenes to elute. Data were collected from 250–600 nm using a Waters™ 996 photodiode array detector (Waters Chromatography, Milford, MA). The minimum detection limit of this PDA detector is 0.0001 AU (absorbance units), which amounts to approximately 0.005 mg of carotenoid per gram of pigmented feather portion using this protocol. Total carotenoid concentration (mg/g) was determined using

an absorbance spectrophotometer (Bausch and Lomb Spectronic 1001), and by applying the following formula (*sensu* Hudon and Brush 1992):

$$\frac{A \times \text{volume of extract (ml)} \times 10}{E \times \text{feather mass (g)}}$$

where A is the absorbance of the sample at λ_{max} (475 for our red pigment extracts) and E is the extinction coefficient of the relevant carotenoids (2350 in ethanol, which is the approximate average value of the main carotenoids present; Bauernfeind 1981).

Melanin analyses

All procedures were performed in the laboratory of S. Ito and K. Wakamatsu at the Fujita School of Health Science in Japan. Methods of analyzing both phaeomelanins and eumelanins in bird feathers follow those in Haase et al. (1992). To determine eumelanin content, colored feather barbules were homogenized in water (1:100, w/v) and 400 μl of the homogenate were added to 800 μl 1 M H_2SO_4 , oxidized with 3% KMnO_4 . The resulting oxidation product (pyrrole-2,3,5-tricarboxylic acid; PTCA) was analyzed via HPLC (Ito and Fujita 1985, Ito and Wakamatsu 1994). Phaeomelanins were examined by hydrolyzing 200 μl feather homogenate with 500 μl 57% hydriodic acid at 130°C in the presence of H_3PO_2 for 24 h, and subsequently analyzing the product (4-amino-3-hydroxyphenylalanine; 4-AHP) using HPLC with electrochemical detection (Wakamatsu et al. 2002). Amounts of eumelanin and phaeomelanin were obtained by multiplying the amount of PTCA and 4-AHP by conversion factors of 50 and 9, respectively (Ito and Fujita 1985, Wakamatsu and Ito 2002). Analyses of all samples were performed in duplicate, and we report averages of these values here.

Results

Pigments in red epaulet feathers

In our HPLC analyses of lipid fractions from red epaulet feathers, we identified five major peaks that were present in all three of the males studied (Fig. 1). By comparing the retention times (t_R) and absorbance properties (λ_{max}) of each peak to authentic reference carotenoids donated by Roche Vitamins Inc. (Parsippany, NJ) and Dr. Riccardo Stradi (University of Milan, Italy), we matched the pigments present in red feathers to the following five carotenoids (in all-*trans* form): canary xanthophyll A, astaxanthin, lutein, zeaxanthin, and canthaxanthin (Fig. 1). Two *cis* isomers of lutein were also present and are believed to be artifacts that are

formed during the thermochemical extraction process (Mays et al. 2004). Astaxanthin was the most common carotenoid (35% of total) in all three males, followed by lutein (28%), canthaxanthin (23%), zeaxanthin (12%), and canary xanthophyll A (3%) (Table 1). Overall, these carotenoids occurred at an average concentration of 1.87 mg pigment/g feather (Table 1).

After carotenoid extraction, however, the once-red epaulet feathers retained a deep brown hue. Subjecting these feathers to further acidified-pyridine treatment yielded no additional carotenoids. HPLC tests for melanins, however, identified a substantial amount of melanin pigments in these same carotenoid-pigmented feather portions (Table 1). Both eumelanins and phaeomelanins were present in red epaulet feathers, with eumelanins comprising 83% of total. The overall melanin concentration was roughly equivalent to that of overall carotenoid concentration (Table 1).

Carotenoid-containing songbird feathers that are depigmented with acidified pyridine are often nearly white in color (K. McGraw pers. obs.), which led us to suspect that the co-occurrence of carotenoids and melanins in colorful feathers is an uncommon phenomenon. To test this idea, we investigated the melanin content of carotenoid-pigmented feather barbules in two other songbird species: the American goldfinch *Carduelis tristis* and the northern cardinal *Cardinalis cardinalis*. Distal ends of colorful feathers in male goldfinches and cardinals contained low melanin concentrations (Table 1). The ratio of carotenoids-to-melanins in feathers was 7–10 times greater than that found in the red epaulets of *A. phoeniceus* (ca. 1:1).

Pigments in yellow epaulet feathers

The yellow bordering feathers from the epaulets of two males completely lacked carotenoids (Table 1). The third male displayed a tinge of pink in his yellow feathers, and this wash of color could be attributed to a dilute concentration of carotenoids (Table 1) that was removed after treatment with acidified pyridine. In all cases, these feathers retained a deep yellow hue after carotenoid extraction. We isolated an even higher concentration of melanin pigments (ca. 13–16 mg/g) from yellow epaulet feathers than was found in red feathers (Table 1). Here, phaeomelanins comprised the majority of melanins (82% of total).

Discussion

Carotenoids

We describe the pigmentary basis of epaulet coloration in male red-winged blackbirds. Red feathers contained not just one, but a suite of carotenoid pigments (as in

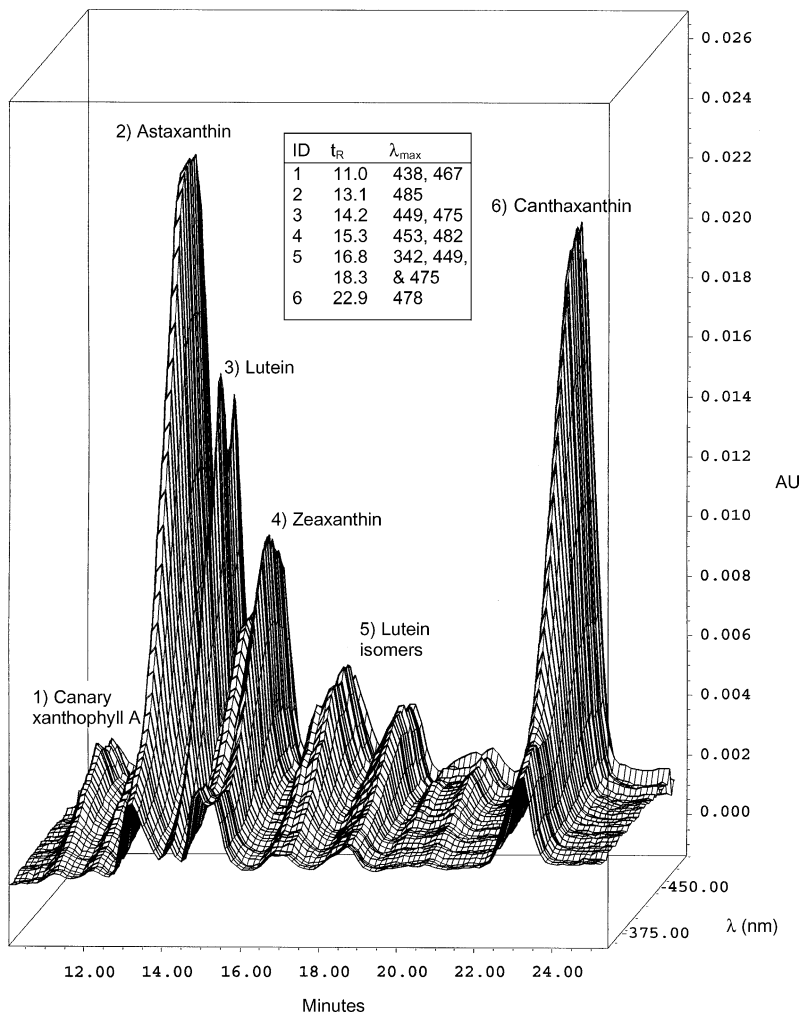


Fig. 1. Three-dimensional HPLC chromatogram depicting the carotenoid profile of red epaulet feathers in male red-winged blackbirds (see text for methods). Pigment names are given above their respective peaks; retention times and absorbance characteristics are provided in the key. AU = absorbance units.

other songbirds; Stradi 1998). Two ketocarotenoids—astaxanthin and canthaxanthin—comprised over half (55–65%) of all feather carotenoids and were responsible for the red color of epaulets. These pigments are generally believed to be absent from songbird diets and instead are manufactured from basic dietary-carotenoid precursors (Goodwin 1984, Brush 1990, Stradi 1998, McGraw et al. 2001). Such metabolic precursors include lutein and zeaxanthin, which are common yellow pigments in avian diets (Goodwin 1984, McGraw et al. 2001) and were also found in the red epaulets of blackbirds. Zeaxanthin in particular is thought to serve as the substrate for astaxanthin synthesis in birds (Stradi 1998). Non-polar carotenoids such as β -carotene were not present in feathers (which is a common occurrence in birds; Stradi 1998), but may exist in the diverse diet of blackbirds and serve as a precursor for the formation of canthaxanthin, for example (e.g. as has been hypothesized in northern cardinals; McGraw et al. 2001).

Such a balanced mixture of both dietary precursors and metabolic derivatives in colorful feathers is quite

rare, not just within a species but within individual birds and feathers. Most carotenoid-pigmented species color their feathers with dietary compounds only (e.g. as in the lutein-based yellow plumage of yellow-breasted chats *Icteria virens*; Mays et al. 2004), or metabolic derivatives only (as in the canary xanthophylls and 4-oxo-carotenoids of many cardueline finches; Stradi 1998). Exceptions include the house finch *Carpodacus mexicanus*, where yellow males typically display only diet-derived products (e.g. lutein), while red males sequester purported metabolic derivatives in feathers (e.g. 3-hydroxy-echinenone; Inouye et al. 2001), and northern orioles *Icterus galbula* (Hudon 1991). It has been argued that one of the important costs of producing colorful carotenoid-based traits is the energetic demand of synthesizing these metabolically derived pigments (Hudon 1991, Hill 1996, 2000, McGraw et al. 2002b). The red-winged blackbird may be an ideal species in which to test this idea, since it is possible that only certain males can fully transform dietary carotenes and xanthophylls into the red ketocarotenoids

Table 1. Total complement of pigments found in the red and yellow plumage of males from three songbird species. Using HPLC, we analyzed the carotenoid and melanin content of red epaulet and yellow bordering feathers in adult male red-winged blackbirds, yellow breast feathers in adult male American goldfinches, and red breast feathers in adult male northern cardinals. All concentrations are given in mg of pigment per g of pigmented feather. For reference, black body feathers in adult blackbirds contain nearly pure eumelanin (20.95 mg/g eumelanin, 0.03 mg/g phaeomelanin). We show total carotenoid concentration (and not % composition) for goldfinches and cardinals since the proportions are not relevant to this study (and because individual pigment types do not match up with those of blackbirds).

	Red-winged blackbird						Northern cardinal	American goldfinch
	Male #1		Male #2		Male #3 ^a			
	red	yellow	red	yellow	red	yellow		
Carotenoid pigments								
Total concentration (mg/g)	2.0	none	1.7	none	1.9	0.01	1.5 ^b	1.2 ^c
% canary	3		1		5	4		
xanthophyll A								
% astaxanthin	37		33		35	37		
% lutein	26		27		30	29		
% zeaxanthin	17		10		8	9		
% canthaxanthin	17		29		22	21		
Melanin pigments								
Total concentration (mg/g)	2.0	15.6	1.6	13.3	1.7	16.1	0.20	0.13
% eumelanin	82	17	86	19	81	17	69	78
% phaeomelanin	18	83	14	81	19	83	31	22

^a = unlike the other two males, this blackbird displayed a tinge of pink color (due to the presence of carotenoids) on the yellow portion of his epaulet.

^b = obtained from triplicate analyses of feather samples donated by G. Hill, Auburn University.

^c = data from McGraw et al. 2002b.

that are critical for red epaulet coloration, while others leave many of the food pigments unconverted (which show up directly in feathers). Follow-up studies relating pigment concentration and composition to plumage color in this species are now needed to begin to address how variable and important these dietary and synthetic carotenoid products are.

Melanins

Carotenoids were not the only colorful pigments present in epaulet feathers, however. Red epaulet feathers retained a brown color after carotenoid extraction, and yellow bordering feathers were essentially devoid of carotenoids, except for the occasional wash of red on the feathers that was due to a small amount of carotenoid pigment. To our knowledge, this is the first documentation of a yellow color in songbirds that is not generated by carotenoids. Stradi (1999) reviewed over 100 species of songbirds that use carotenoids to color their feathers yellow, orange, and red. We are currently studying another apparent case of non-carotenoid-based yellow plumage in male red-billed queleas (*Quelea quelea*; K. McGraw and J. Dale, unpubl. data).

Both the brown color of red feathers and the yellow of bordering feathers can be attributed to the presence of melanin pigments. Melanins occur in two primary forms in nature: 1) eumelanin, which confers dark black, brown, or gray hues, and 2) phaeomelanin, which gives a

yellow and reddish-brown appearance (Prota 1992). Consistent with this notion, red epaulets that appeared brown after removing carotenoids contained predominantly eumelanins, while the yellow coverts contained predominantly phaeomelanins. The unusually high concentration of melanins in yellow feathers is similar to the profile of blonde hair in humans (total melanin ca. 17 mg/g, 96% phaeomelanin; Ito and Fujita 1985). The concentration of melanins detected in red feathers was similar to that found in the brown, ventral plumage of North American barn swallows *Hirundo rustica erythro-gaster* (McGraw et al. 2004), and closely approximated the concentration of carotenoids found in the same epaulet feathers. Still, it is difficult to calculate the relative contributions of carotenoids and melanins to the color of red blackbird feathers, because it requires information on the extinction coefficients of the different compounds, which has been challenging to estimate for large melanin polymers whose full biochemical structure has proven difficult to characterize (Prota 1992). Future studies addressing this question should first be aimed at quantifying feather coloration in relation to the relative abundance of carotenoids versus melanins for a large number of blackbirds.

Carotenoids and melanins are known to occur regularly in the same feather in birds, most often being deposited in different sections of the feather to generate unusual plumage colors (e.g. the olive-green appearance of greenfinches *Carduelis chloris*; Lucas and Stettenheim 1972). However, when carotenoids are confined to distal

regions of the feather, as is the case in the ornamental red, orange, and yellow plumage of other songbirds, these areas are thought to be otherwise unpigmented or sparsely populated with melanins (to produce a gray color; Lucas and Stettenheim 1972). We suspect that intense co-pigmentation of ornamental epaulets with red and yellow carotenoids and brown and yellow melanins in red-winged blackbirds may be an uncommon phenomenon and one that warrants more detailed feather-microstructural investigations as well as renewed theoretical considerations for the sexual-signaling function of this colorful trait.

Implications

Several decades of research have been devoted to understanding the fitness consequences of variation in male epaulet size and coloration in *A. phoeniceus* (reviewed in Searcy and Yasukawa 1995, Beletsky 1996). Biologists have generally had a difficult time diagnosing the social or sexual significance of this trait. Although carotenoid-based color ornaments traditionally function in mate attraction (Hill 1999), female red-winged blackbirds do not seem to consistently use variability in epaulet size or coloration to make mating decisions (see Table 7.4 in Searcy and Yasukawa 1995). Moreover, carotenoid-containing ornaments traditionally are hampered by parasitic infections or other threats to an individual's health (Hill 2000, McGraw and Hill 2000a), but in male red-winged blackbirds there seems to be no link between epaulet characteristics and either parasitism (Weatherhead et al. 1993) or immunocompetence (Westneat et al. 2003). Instead, it appears from experimental manipulations of the epaulet that it functions more as a signal of aggressiveness and social status to rival males (Peek 1972, Smith 1972, Røskaft and Rohwer 1987), which is not a common feature of carotenoid ornaments (Wolfenbarger 1999, McGraw and Hill 2000b). Overall, redwing epaulets stand as an obvious, unexplained exception to the general pattern of carotenoid signaling in birds (Hill 2002).

With this new information about the biochemical properties of blackbird epaulets, however, we must reclassify the ornament as both carotenoid- and melanin-based and equally consider the mechanisms and functions of melanin displays in birds. In fact, several studies of melanin ornaments in birds reveal that they characteristically: 1) vary in size more than color (much like redwing epaulets, but not always like the carotenoid colors of finches, for example), and 2) function as status badges in avian societies (reviewed in Senar 1999, McGraw and Hill 2000a, McGraw et al. 2002a). Thus, under the existing framework, the epaulets of *A. phoeniceus* seem to function more like melanin badges

and less like carotenoid colors. It should be pointed out that most of the work on avian melanins have been focused on patches of eumelanin-based black plumage (e.g. the throat 'bib' of the house sparrow *Passer domesticus*), and that we are just now beginning to understand the control agents and signaling function of phaeomelanin-containing traits (e.g. in barn swallows and eastern bluebirds *Sialia sialis*; McGraw et al. 2004, Siefferman and Hill 2004).

Some may argue that the designations we have applied to the carotenoid- versus melanin-based signals of birds are overly simplistic, and that there are at least a few exceptions that complicate the patterns we report, particularly for carotenoid colors. In fact, we believe that detailed biochemical information for these exceptions bear critically on the functional significance of epaulets in red-winged blackbirds, and may even add support to the arguments we have built about the two types of color signals in general. The first exception is the African widowbirds, which show uncanny ecological, behavioral, and morphological convergences with *A. phoeniceus* from North America. Certain widowbird species develop virtually identical plumage colors and patterns to red-winged blackbirds, including jet-black body feathers and red-and-yellow epaulets (e.g. red-shouldered widowbird *Euplectes axillaris*). In the context of ornamental coloration, the two best-studied members of this group are the red-collared widowbird *Euplectes ardens* and the red-shouldered widowbird. In these species, as in red-winged blackbirds, females do not show a mating preference for males with redder plumage (Pryke et al. 2001a, Pryke and Andersson 2003), but instead colorful plumage functions as a social status signal (Pryke et al. 2001b, Pryke and Andersson 2003). Furthermore, the carotenoid profile has been described for these color ornaments and they too show extraordinary biochemical convergence with redwings, containing both dietary (lutein, zeaxanthin) and metabolically derived (canthaxanthin) forms (Pryke et al. 2002). Additional biochemical analyses of colorful widowbird feathers may in fact show a high concentration of melanins as well. The second and last exception is the red-billed quelea. Like other carotenoid signals, carotenoid-based beak color in this species functions as a condition-dependent indicator of quality, however, head and chest plumage that ranges in color from yellow to red is not condition-dependent (Dale 2000). As mentioned above, we have confirmed the absence of carotenoid pigments in these yellow feathers and continue to investigate the pigmentary basis of colorful plumage in queleas in light of its unique social function as a signal of individual identity (Dale et al. 2001). In the end, we encourage all behavioral ecologists to carefully consider the biochemical properties of their color signals of study so that we can accurately assess how they may be produced and function.

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