



Melanin coloration in New World orioles I: carotenoid masking and pigment dichromatism in the orchard oriole complex

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Carotenoids produce the brilliant red, orange, and yellow colors of many animals. However, melanin pigments can also confer some of these same hues. Because carotenoid and melanin colors are produced in different ways and may serve different signaling functions, either within or between species, it is important to establish whether one or both types of pigment are responsible for coloration. We have discovered what appears to be an evolutionary switch from carotenoid- to melanin-based color in two sexually dichromatic New World orioles. Using a combination of reflectance spectrometry and chromatographic analyses of plumage pigments, we found that the chestnut plumage of adult male orchard orioles *Icterus spurius* is produced predominantly by phaeomelanins. Orchard oriole feathers also contain carotenoids, which appear to be masked by the high concentration of phaeomelanins. In contrast, both carotenoids and phaeomelanins appear to contribute to color in adult male Fuertes's orioles *I. fuertesi*. Moreover, yellow yearling male and female plumage in both species is produced by carotenoids alone. The masking of carotenoids with phaeomelanins in orchard orioles is interesting in light of the signaling roles that carotenoids are thought to play. In addition, these plumage differences produce a unique case of age and sexual pigment dimorphism in orchard and Fuertes's orioles.

Carotenoids and melanins are two of the most commonly studied color pigments used by animals. Traditionally, the bright red to yellow ornaments found in birds, fish, lizards, and frogs have been attributed to carotenoids and achromatic black or brown elements to melanins (reviewed in Fox 1976, Olson and Owens 1998, Hill 2002). Carotenoid and melanin colors are produced through different proximate mechanisms, which have played an important role in understanding their signaling function (McGraw and Hill 2000). In particular, carotenoids cannot be synthesized by vertebrates and must be obtained from the diet, although subsequent modifications may then occur (Brush 1990, Hill 2000). Many studies have claimed that these limited or costly carotenoids may reflect condition and can be used by females (or males) to evaluate the quality of a potential mate (e.g., Hill 1991, 2002, Grether 2000, Faivre et al. 2003). Melanins are synthesized from amino acid precursors (Jawor and Breitwisch 2003), and achromatic (black or brown)

melanin ornaments have been demonstrated to be independent of health (McGraw and Hill 2000). Several studies have demonstrated that these melanin ornaments often serve other functions, such as signal social status (Rohwer 1975, Senar and Camerino 1998, but see Thusius et al. 2001, Tarof et al. 2005).

These differences between carotenoids and melanins have played a role in developing theories of color signaling and in some cases may also play a role in how behavioral experiments are designed. However, several recent studies have found that melanins produce some of the chestnut, orange and yellow colors that were previously attributed in whole or part to carotenoids, including the orange cheeks of the zebra finch *Taeniopygia guttata* (McGraw et al. 2003a), the chestnut of European and American barn swallows *Hirundo rustica rustica* and *H. r. erythrogaster* (McGraw et al. 2004a, b), and the yellow portion of red-winged blackbird *Agelaius phoeniceus* epaulets (McGraw et al. 2004c). Although it has been known that melanins could produce yellow,

orange and rust colors for several decades (Fox 1976), within passerine birds it was often assumed that yellow, orange, and red colors were produced by carotenoids until recently (Johnson and Lanyon 2000, Omland and Lanyon 2000, also see discussions in Brush 1990, McGraw et al. 2004a, b, c). These brighter melanin colors are often produced by phaeomelanins, rather than eumelanins, and indicate that such generalizations about color may be overly simplified.

New World orioles *Icterus* also have colorful plumage that may be due to melanins. Most orioles have bright yellow or orange coloration. Biochemical analysis of the orange plumage of Baltimore orioles *Icterus galbula* revealed a mixture of several carotenoids (Hudon 1991), and other studies have considered orioles as having carotenoid plumage (Gray 1996, Omland and Lanyon 2000). However, adult male orchard orioles *I. spurius* have chestnut-colored plumage and adult male Fuertes's orioles *I. fuertesi*, their sister species (Clements 2000, Baker et al. 2003), have an ochre or tan color (Howell and Webb 1995, Jaramillo and Burke 1999, Baker et al. 2003), suggesting that color in these two orioles may at least in part be due to melanins rather than carotenoids. Orchard and Fuertes's orioles are sexually dichromatic, and females of both taxa have lemon-yellow plumage (see illustrations in Jaramillo and Burke 1999). Orchard and Fuertes's orioles also exhibit delayed plumage maturation, with the color of yearling males very similar to that of females (reviewed in Enstrom 1993). Even though their visual appearance suggests that female and yearling male colors are produced by carotenoids, it is possible that this plumage may also contain melanins.

The first goal of this study was to determine whether the colorful plumage of adult male orchard and Fuertes's orioles is produced by melanins, carotenoids, or both. If melanins produce the color, we wanted to determine whether they were simply replacing carotenoids or masking them. We also addressed whether females and yearling males maintain carotenoid-based plumage and represent a unique case of age and sexual pigment dimorphism.

Methods

Spectral analysis

Since several recent studies have shown that the visual appearance of plumage may be misleading (McGraw et al. 2003a, 2004a, b, c), we used both reflectance spectrometry and biochemical analysis to investigate the chemical basis of colorful plumage in orchard and Fuertes's orioles. An Ocean Optics USB 2000 portable

spectrometer was used to gather reflectance spectra from both museum and field specimens. Illumination was provided by a full spectrum light source (Ocean Optics PX-2) via a bifurcated fibre optic cable that allowed for illumination and detection within the same probe. A custom matte-black anodized aluminum cylinder with a 1.27 cm aperture was attached to the probe and prevented ambient light from entering during measurement. We measured three different colored body regions, the breast, belly, and rump. All measurements were taken perpendicular to the surface of the body, in replicates of three, with the probe completely removed between replicates. Whenever possible, measurements were taken from non-overlapping regions. All measurements were relative to a Spectralon diffuse white standard and corrected for dark noise. Raw spectral data were gathered into one nanometer bins, replicates were averaged, and representative spectra were generated from the five individuals measured.

Spectral measurements were taken from museum specimens at the Smithsonian National Museum of Natural History and the Delaware Museum of Natural History (Appendix 1). Five each of adult male, female, and yearling male orchard orioles were measured. Five adult male and five female Fuertes's orioles were also measured; however, we were limited to two yearling male specimens. In addition, we compared our measurements from the breast of adult male orchard oriole museum skins to measurements from living birds ($n = 11$) captured using mist nets (see below). Field measurements and museum specimens were compared using two values derived from reflectance spectra. First, we compared the average reflectance (over 300–700 nm) from museum specimens to field measurements. Then we generated composite reflectance spectra from museum specimens and field measurements and identified the wavelength at which these two composite spectra differed the most. We then compared the reflectance of museum specimens to field measurements at this wavelength. We used a Wilcoxon-Mann-Whitney test to examine these differences between museum specimens and living birds.

Biochemical analysis

Orchard orioles were captured using mist nets and feathers were removed from the ventral surface (breast and belly) while taking standard banding measurements. All birds were given USFWS bands prior to release, which ensured that the same bird was not measured more than once (Table 1). Feathers were stored at -20°C until analysis. Fuertes's oriole feathers were removed from the ventral region of

Table 1. Concentration of carotenoid and melanin pigments in orchard and Fuertes's oriole feathers.

Taxon	Sex	Age	ID no.	Lutein (ug/g)	Average	SE	Eumelanin (ug/g)	Average	SE	Phaeomelanin (ug/g)	Average	SE	Phaeomelanin: carotenoid ratio
Orchard	M	Adult	1721-78641 ¹	138	125	36	785	770	15	8550	10173	2037	81
Orchard	M	Adult	1721-78639 ¹	181			740			7749			
Orchard	M	Adult	1721-78646 ¹	57			785			14220			
Orchard	M	Yearling	1721-78628 ¹	161	313	105	775	538	118	51	59	5.0	0.19
Orchard	M	Yearling	1721-78633 ¹	265			420			68			
Orchard	M	Yearling	1721-78640 ¹	513			420			59			
Orchard	F		1721-78635 ¹	40	113	37	190	345	131	42	38	4.4	0.33
Orchard	F		1721-78626 ¹	133			605			29			
Orchard	F		1721-78638 ¹	165			240			41			
Fuertes	M	Adult	DMNH44150 ²	342	283	48	1435	1753	199	623	800	116	2.8
Fuertes	M	Adult	DMNH44146 ²	189			2120			761			
Fuertes	M	Adult	DMNH40009 ²	319			1705			1017			
Fuertes	F		DMNH44143 ²	5	154	95	3295	2962	180	108	110	26	0.71
Fuertes	F		DMNH44151 ²	126			2675			66			
Fuertes	F		DMNH44145 ²	332			2915			155			

¹USFWS band number.

²Museum voucher number.

museum specimens at the Delaware Museum of Natural History (Table 1).

Carotenoids were extracted from 3–5 mg of feather material using the procedures of McGraw et al. (2003b). First, we separately washed feathers in 5 ml ethanol and 5 ml hexane for 30 min, blotted them dry, and trimmed off the carotenoid-pigmented barbules for analysis. Colored feather portions were weighed to the nearest 0.1 mg with an electronic balance. We then ground pigmented barbules for 15 min at 30 Hz using a Retsch MM200 mixer mill in the presence of 3 ml methanol. Resulting mixtures were centrifuged for 5 min at 3000 rpm, and the supernatant was transferred to a clean tube. We evaporated the solvent to dryness under a stream of nitrogen, and the pigment residue was resuspended in 200 μ l of HPLC mobile phase (methanol:acetonitrile:dichloromethane, 50:46:4) prior to analysis.

HPLC analysis was performed following the methods of McGraw et al. (2004b). We injected 50 μ l of each sample into a Waters Alliance 2695 HPLC system fitted with a Develosil RPAqueous RP-30 column (250 \times 4.6 mm) and a built-in column heater set at 30°C. We used an isocratic system at a constant flow rate of 1.2 ml min⁻¹ for 60 min to allow sufficient time for both xanthophylls and carotenes to elute if they were present. Data were collected from 250–600 nm using a Waters 2996 photodiode array detector. We identified pigments by comparing their respective retention times and absorbance maxima to those of authentic reference carotenoids run as external standards. Lutein (absorbance-max = 447 nm; retention time = 7.8 min) was the only carotenoid present in all feathers from both species. Lutein concentration was determined using Empower software (version 5.0) by comparing peak areas at max absorbance (447 nm) to an external standard curve.

We analyzed the eumelanin and phaeomelanin content of feathers following the methods of McGraw et al. (2004a). To determine eumelanin concentration, 3–5 mg of colored barbules were trimmed and homogenized in water (1:100, w/v), and 400 μ l of the homogenate were added to 800 μ l of 1 M H₂SO₄, oxidized with 3% KMnO₄. 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 μ l feather homogenate with 500 μ l 57% hydriodic acid at 130°C in the presence of H₃PO₂ 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).

Results

Spectral analysis

As in previous studies (Endler and Th  ry 1996, McGraw et al. 2003c), we found no significant difference in color between museum skins and living birds. Living birds (breast, $n = 11$) had an average reflectance of $10.24 \text{ SE} \pm 0.48$ percent; museum specimens (breast, $n = 5$) averaged $10.12 \text{ SE} \pm 0.23$ percent ($P > 0.9$). Although average reflectance would be likely to detect changes in brightness (i.e., due to fading), average reflectance might not be able to detect changes occurring at a localized region of the spectrum. To address this issue, we also compared field and museum specimens at 472 nm, the wavelength at which their averaged spectra differed the greatest. At 472 nm the reflectance from living birds was $29.53 \text{ SE} \pm 1.03$ percent; the reflectance from museum specimens was $28.90 \pm \text{SE } 1.02$ percent ($P > 0.6$).

Reflectance spectra from the chestnut-colored plumage of adult male orchard orioles showed a gradual increase from short to long wavelengths (Fig. 1a). This spectral shape is characteristic of the predominantly phaeomelanin-based chestnut colors found in other birds (McGraw et al. 2004a, Safran and McGraw 2004). Melanin-like reflectance spectra were found in all colored regions that we measured in adult male orchard orioles (Fig. 1a). Yellow female and yearling male plumage had spectral characteristics of carotenoids (Fig. 1b and c), including a peak in the ultraviolet region of the spectrum, a minor peak around 475 nm, a sharp increase in reflectance between 500–550 nm, and a plateau at longer wavelengths (Bleiweiss 2004, also see examples in MacDougall and Montgomerie 2003 Fig. 1, Mays et al. 2004 Fig. 1).

Reflectance spectra from adult male Fuertes's orioles contained spectral characteristics of carotenoids and melanins and varied slightly in shape across body regions. The reflectance spectra from the rump showed stronger characteristics of a melanin based color (Fig. 2a; McGraw et al. 2004a, Safran and McGraw 2004). In contrast, the reflectance spectra from the breast and belly had a minor peak around 475 nm (Fig. 2a). This peak is also found in female and yearling male Fuertes's orioles (Fig. 2b and c) and is characteristic of carotenoids such as lutein (MacDougall and Montgomerie 2003 Fig. 1, Mays et al. 2004 Fig. 1). Reflectance spectra from female and yearling male Fuertes's orioles (Fig. 2b and c) were very similar to the reflectance spectra of female and yearling male orchard orioles (Fig. 1b and c).

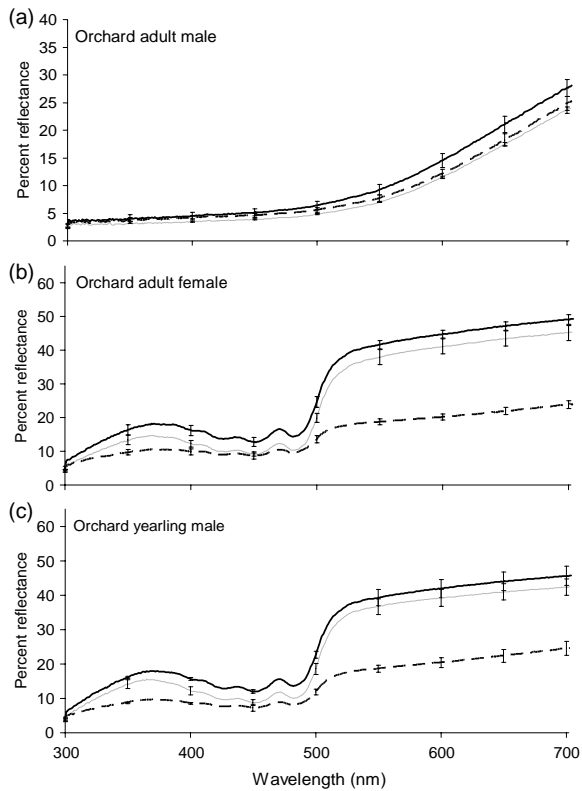


Fig. 1. Reflectance spectra of colored plumage from adult male (a), female (b), and yearling male (c) orchard orioles. Measurements were taken from belly (solid black line), breast (thin gray line), and rump (dotted black line). Spectra are averages from 5 individuals with \pm SE shown at 50 nm intervals.

Biochemical analysis

Biochemical analysis revealed that the carotenoid lutein was present in adult male, yearling male, and female orchard and Fuertes's orioles (Fig. 3, Table 1). High concentrations of melanins were found in adult male orchard and Fuertes's orioles (Fig. 3, Table 1). The chestnut colored feathers from all three adult male orchard orioles had phaeomelanin concentrations that were several orders of magnitude higher than those of yellow female and yearling male feathers (Fig. 3, Table 1). The tan feathers from adult male Fuertes's orioles had a phaeomelanin concentration that was, on average, an order of magnitude lower than that of orchard orioles. This average was still almost an order of magnitude greater than the phaeomelanin concentration of female Fuertes's orioles (Fig. 3, Table 1). Although the sample size did not permit statistical treatment of the data, the extreme differences in phaeomelanin concentration provide strong support for these differences between sex, age, and species.

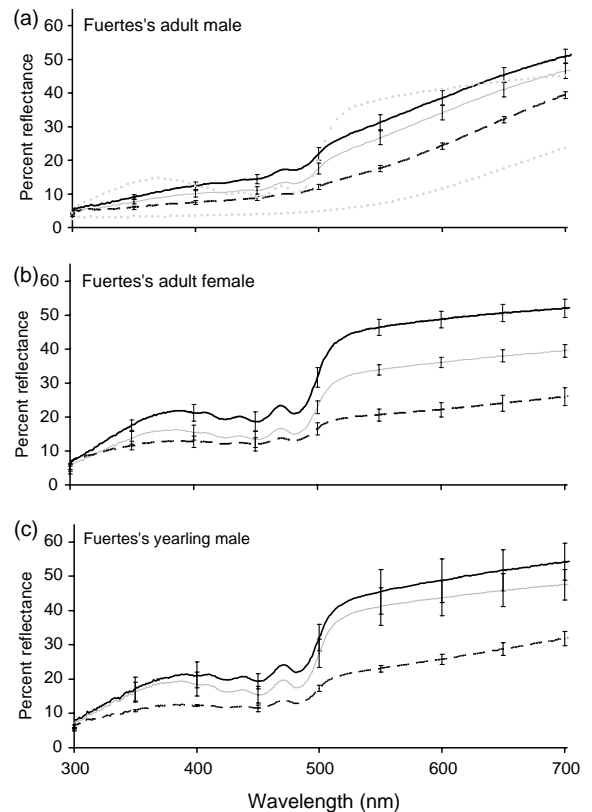


Fig. 2. Reflectance spectra of colored plumage from adult male (a), female (b), and yearling male (c) Fuertes's orioles. As in the orchard oriole (Fig. 1), measurements were taken from the belly (solid black line), breast (thin gray line), and rump (dotted black line). For comparison the male and female orchard oriole spectra are included with the adult male (dotted gray lines). Male and female spectra are averages from 5 individuals; yearling male spectra are from 2 individuals with \pm SE shown at 50 nm intervals.

Discussion

Melanins mask carotenoids

Our results indicate that in the chestnut-colored plumage of adult male orchard orioles, the presence of carotenoids is masked by a high concentration of melanins. These feathers do contain the carotenoid lutein; however, the high concentration of phaeomelanins effectively blocks any spectral or visual signature of carotenoids. This masking of carotenoids with a melanin-based color in adult males makes the orchard oriole unique to date. Carotenoid pigments are not found in the colorful melanin-based plumage of European and American barn swallows or eastern bluebirds *Sialia sialis*, which also contain high levels of phaeomelanins (McGraw et al. 2004a, b).

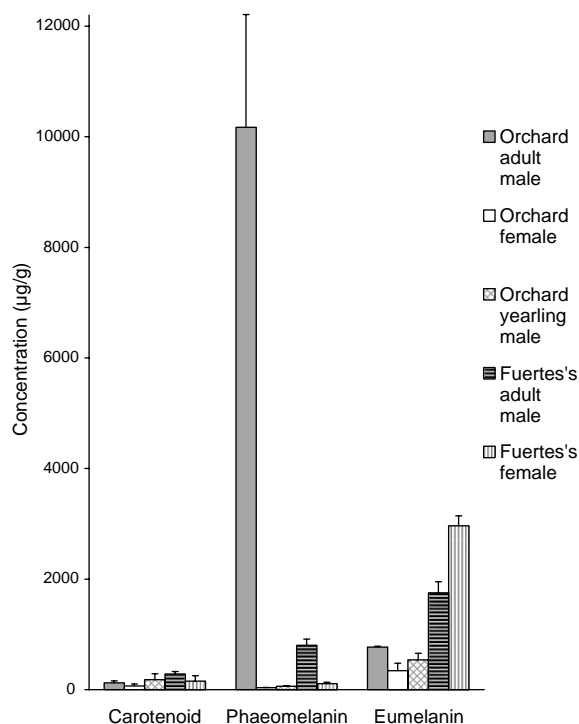


Fig. 3. The average concentration ($\mu\text{g/g}$) of carotenoids, eumelanins, and phaeomelanins in adult male, female, and yearling male orchard orioles, as well as adult male and female Fuertes's orioles. Only one type of carotenoid (lutein) is found, and it is present across all groups. Each bar is the average of 3 individuals \pm SE.

In addition, the same carotenoid pigment is present in the colorful plumage of yearling male, female, and adult male orchard and Fuertes's orioles. When yearling males molt into their full adult plumage (after second year), the observed pigment changes occur across all colored body regions. Thus orchard and Fuertes's orioles also represent a unique case of age and sexual dichromatism in which adult males have color that is produced by a different proximate mechanism.

Although we found considerable variation in carotenoid (as well as eumelanin and phaeomelanin) concentration within each age and sex class (Table 1), our results suggest that adult male orchard orioles contain levels of carotenoid pigments that are comparable to those found in females. This suggests that the concentration of pigments found in adult males would be high enough to produce carotenoid colored plumage had they not been masked. Further studies with larger sample sizes are necessary answer additional questions, such as whether yearling male orchard and Fuertes's orioles have a significantly greater concentration of lutein than females or adult males.

Why trade a carotenoid signal for a melanin one?

The masking of carotenoid-based plumage is interesting in light of the signaling roles that carotenoid colors play across a wide range of organisms. While we recognize that experimental studies demonstrating the condition-dependent and sexually selected nature of carotenoid-based plumage have yet to be performed in orioles, extending the signaling implications from numerous other passerines to orioles raises many exciting questions. If carotenoids are such honest and/or costly signals in all animals, then why would they be masked in orchard orioles? Why does this masking only occur in adult males, who are likely to be under the strongest sexual selection? Also, why do females and yearling males continue to express this putatively costly honest signal? Finally, why do Fuertes's orioles use a combination of both carotenoid and melanin pigments to produce a unique color? More research is needed in this genus and other taxa to understand the causes of such interspecific differences in elaborate colors and patterns.

Relationship between pigment concentration and spectral shape

In the adult male orchard oriole all spectral and visual characteristics of carotenoids appeared to be masked by melanins. These results suggest that carotenoids are not contributing to the visual signal in adult male orchard orioles. The only way to confirm that carotenoids were still present was through biochemical analysis. This masking appeared to be due to the high concentration of phaeomelanins found in orchard oriole plumage. In the Fuertes's oriole a lower concentration of phaeomelanins, combined with carotenoids, produced plumage with an ochre or tan color and a distinct spectral shape with characteristics of both pigments (Fig. 2a). Thus, the masking of carotenoids only occurs when high concentrations of phaeomelanins are present, and as the concentration decreases the spectral signature of carotenoids begins to reappear. Surprisingly, a high concentration of eumelanins was found in female Fuertes's orioles with little change in the reflectance spectra (Figs. 3 and 2b). Therefore, both plumage color and spectral shape appear to be largely dependent upon the concentration of phaeomelanins and the phaeomelanin:carotenoid ratio (Table 1). Future experiments investigating the effect that the selective removal of carotenoid and melanin pigments (from both adult male Fuertes's and orchard orioles) has on the reflectance spectrum presents and intriguing avenue for future research and will provide further insight into how each pigment contributes to the color signal.

Fuertes's orioles differ from orchard orioles (and most other birds studied to date) because they use a combination of carotenoids and phaeomelanins to generate an intermediate spectral shape. Fuertes's oriole plumage differs from the olive plumage found in other birds, including adult male orioles (e.g., *I. graduacauda*), which appears to be due to mixture of carotenoids and eumelanins (Lucas and Stettenheim 1972). Plumage perceived as olive by the human eye is measured spectrally as an unsaturated yellow. The reflectance plateau of olive plumage occurs at much lower values than yellow carotenoid plumage; however, unlike the intermediate spectral shape of the Fuertes's oriole, no changes in the overall shape or location of the reflectance spectrum occur (C. Hofmann pers. obs.). Thus, orchard and Fuertes's orioles provide interesting insight into how various concentrations of carotenoids and phaeomelanins may be combined to produce unique colors with distinct spectral shapes. The co-deposition of carotenoids and melanins found in orchard and Fuertes's orioles also suggests that the signaling functions of carotenoid and melanin colors may be more complex than previously thought (i.e., a carotenoid/melanin dichotomy may be overly simplified) and both pigments may be used to produce unique colors, potentially with novel or joint signaling functions. In addition to the fact that melanins may produce bright, colorful plumage, McGraw et al. (2004c) found co-deposition of carotenoids and melanins (without the masking of carotenoids) in red-winged blackbird epaulets. Co-deposition also appears to occur convergently in Widowbird (*Euplectes*) epaulets and collars (K. McGraw unpubl. data). Further biochemical studies are necessary to determine whether examples of carotenoid and melanin co-deposition are widespread or only occur in a handful of taxa.

In summary, using a combination of spectral and biochemical analyses, we found that in adult male orchard orioles a carotenoid-based color is masked by a melanin-based color. In the Fuertes's oriole a combination of the two pigments produces a unique type of plumage coloration. Sexual and age dichromatism is also unique in these two orioles since both female and yearling male orchard and Fuertes's orioles have carotenoid-based coloration. The masking of carotenoids with a melanin-based color in orchard orioles is interesting in light of the very different signaling roles that carotenoids and melanins are thought to play in many other organisms as well as the supposed costs associated with producing carotenoid based colors.

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Appendix 1. Museum voucher numbers of orioles measured in this study.

Species	Sex	Age	Voucher no.
Orchard oriole	M	Adult	SMNH593366
Orchard oriole	M	Adult	SMNH593363
Orchard oriole	M	Adult	SMNH565182
Orchard oriole	M	Adult	SMNH422083
Orchard oriole	M	Adult	SMNH394401
Orchard oriole	M	Yearling	SMNH609478
Orchard oriole	M	Yearling	SMNH362719
Orchard oriole	M	Yearling	SMNH593365
Orchard oriole	M	Yearling	SMNH564227
Orchard oriole	M	Yearling	SMNH597265
Orchard oriole	F		SMNH341292
Orchard oriole	F		SMNH593367
Orchard oriole	F		SMNH597587
Orchard oriole	F		SMNH564228
Orchard oriole	F		SMNH597549
Fuertes's oriole	M	Adult	DMNH44149
Fuertes's oriole	M	Adult	DMNH44150*
Fuertes's oriole	M	Adult	DMNH44147
Fuertes's oriole	M	Adult	DMNH44146*
Fuertes's oriole	M	Adult	DMNH40009*
Fuertes's oriole	M	Yearling	DMNH44138
Fuertes's oriole	M	Yearling	DMNH360293
Fuertes's oriole	F		DMNH44145*
Fuertes's oriole	F		DMNH44151*
Fuertes's oriole	F		DMNH44143*
Fuertes's oriole	F		DMNH44138
Fuertes's oriole	F		DMNH360293

*Indicates specimen from which feathers were analyzed.