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Forum

European barn swallows use melanin pigments to color their feathers brown

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The barn swallow (*Hirundo rustica*) has figured largely in studies of sexual selection and exaggerated traits (Møller, 1994, 2001). In addition to the elongate tail streamers that confer a series of reproductive advantages to individuals in European populations, these birds develop a brown patch of feathers on the face and throat that has also been the focus of several empirical tests of honest advertisement theory (e.g. Camplani et al., 1999, Saino et al., 1999). Researchers studying this trait have assumed that these feathers contain carotenoids, and several conclusions have been drawn about the physiological and sexual functions of carotenoids in relation to this color ornament (Camplani et al., 1999, Saino et al., 1999). However, to date there is no biochemical evidence in the primary literature supporting the idea that brown feathers in barn swallows contain carotenoids. Here, we demonstrate that barn swallows from Europe (*Hirundo rustica rustica*) do not deposit carotenoids into brown feathers, but instead color this plumage with melanins.

To our knowledge, the first coverage in the primary literature of the pigmentary basis of brown plumage coloration in European barn swallows was in Saino et al. (1999). In this study of plumage ornamentation, blood-carotenoid status, and immune performance, the authors made the statement: "red-to-chestnut coloration of forehead, chin, and throat patch is mainly caused by melanin, although small amounts of lutein have also been found in these feathers (Stradi R, unpublished data)" (Saino et al., 1999: 442). However, in the previous year, a book published by R. Stradi (1998) had already used high-performance liquid chromatography to document a larger suite of carotenoids in barn swallow plumage that included lutein, zeaxanthin, and 3-hydroxy-echinenone. The most recent studies of brown plumage color in barn swallows have referred to this ornament only as 'carotenoid-based' or 'lutein-based' (Camplani et al., 1999, Møller and Mousseau, 2001, 2003), citing Saino et al. (1999) as evidence but without mention or consideration of melanins. These inconsistencies in the literature motivated our interest in exploring the biochemical basis of plumage color in this species.

We tested for the presence of carotenoids and melanins in brown feathers from wild-caught barn swallows in Scotland (*H. r. rustica*) (see Buchanan and Evans [2000] for details on this population). We plucked throat feathers from 27 males and 27 females during the breeding seasons of 2002 and 2003 and mounted them on index cards for color scoring (as in Keyser and Hill, 1999; Safran and McGraw, 2004). These cards were stored in the dark at room temperature for up to seven months prior to analysis. This method of storage is common procedure for plumage-pigment studies conducted by the first author (e.g., McGraw et al., 2003a), and even longer-term feather storage (e.g., as museum skins) does not change the pigment composition of carotenoid-colored bird feathers (e.g., Endler and Théry, 1996, McGraw et al., 2003b). Thus, we are not concerned that the pigment content of feathers was altered prior to analysis.

First, we used UV-VIS spectrophotometry to characterize the spectral-reflectance profile of brown feathers (following the methods of Siefferman and Hill [2003]). Based on their different molecular structures, and thus their different light-absorbance and -reflectance capabilities, carotenoids and melanins give distinct color signatures to animal tissues. As large, conjugated polymers, melanins absorb light throughout UV and visible wavelengths and characteristically impart black, brown, or gray colors that exhibit gradually increasing reflectance across the bird-visible spectrum (Prota, 1992; Riley, 1997). In contrast, colorful carotenoids strongly absorb blue-green wavelengths (430–500 nm; Britton et al., 1995), and carotenoid-colored bird feathers consequently have reflectance spectra that exhibit a discrete peak in the yellow, orange, or red wavelengths (see Keyser and Hill [1999] for an example in the house finch, *Carpodacus mexicanus*). We found that barn swallow feathers showed a melanin-typical spectrum that steadily increased in reflectance from short to long wavelengths (Figure 1).

We then used biochemical techniques to identify the types and amounts of pigments in brown feathers. First, we used high-performance liquid chromatography (e.g., Stradi et al., 1995a) to determine carotenoid content. Although feathers reflected light in an overall pattern consistent with the presence of melanin, it was possible that carotenoids were also present in dilute quantities, since birds can co-pigment feathers with both carotenoids and melanins (Lucas and Stettenheim, 1972). We employed two different methods to extract carotenoids from feathers—a thermochemical method (sensu Hudon and Brush, 1992; McGraw et al., 2002, 2003a,b) and a mechanical method (used by Stradi et al. [1995a,b, 1996, 1997, 1998, 2001] in all of their studies of plumage carotenoids). For each method, 3–5 mg of brown-pigmented barbules were trimmed from the throat feathers of 15 males and 15 females, washed sequentially in ethanol and hexane, and blotted dry. As in other studies, solvent washes yielded no carotenoids. For the thermochemical procedure, we added 1 ml acidified pyridine to the barbules in a 9-ml glass tube, filled the headspace of the tube with argon (to prevent pigment oxidation), and held the tube at 95°C for 4 h. After this time, we cooled the tube to room temperature, added 1 ml water, inverted the tube a few times by hand, and then added 3 ml hexane:*tert*-butyl methyl ether (1:1, v/v; to recover both polar and non-polar lipids). We shook the tube vigorously for 2 min. For the mechanical procedure, we ground barbules for 15 min at 30 Hz in a Retsch® MM200 mixer mill (Retsch Inc., Newtown, PA) using a zirconia grinding jar (fitted with a Teflon O-ring) and balls and in the presence of 3 ml methanol. After grinding, methanol was removed and added to a 9-ml glass tube. At this point in both procedures, we

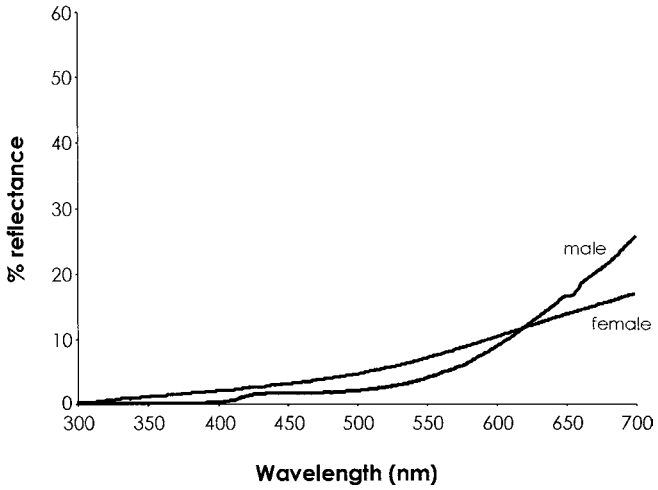

Figure 1
Representative UV-VIS reflectance spectra for breeding male and female European barn swallows (*Hirundo rustica rustica*) from Scotland.

centrifuged the mixtures for 5 min at 3000 RPM, transferred the supernatant to a fresh tube, and evaporated the solvent to dryness under a stream of nitrogen. For both procedures, we ran a positive control (yellow, carotenoid-pigmented contour feathers from American goldfinches, *Carduelis tristis*; McGraw et al. 2001, 2002) along with our samples.

HPLC analyses follow those in McGraw et al. (2003a,b). Residues were redissolved in 200 μl HPLC mobile phase (see below) and 50 μl was injected into a Waters™ 717plus Autosampler HPLC (Millipore Corp., Bedford, MA) fitted with a Develosil RPAqueous RP-30 column (250 × 4.6 mm; Nomura Chemical Co. Ltd., Aichi, Japan) and an Eppendorf TC-50 column heater (Hamburg, Germany) set at 27°C. We used two different isocratic systems (Hewlett-Packard 1050 Series Isocratic Pump), both at a constant flow rate of 1.2 ml/min, to analyze xanthophylls and carotenes separately, if they were present: (1) for xanthophylls, we used acetonitrile:methanol:chloroform (46:46:8, v/v/v) as the mobile phase; (2) for carotenes, we used methanol:dichloromethane (50:50, v/v) as the mobile phase. Data were collected from 250–600 nm using a Waters™ 996 photodiode array detector (Waters Corporation, Milford, MA).

HPLC analyses yielded no detectable levels of carotenoids in brown barn swallow feathers. Solvent extracts were colorless. Extracted feathers retained their deep brown hue. It is important to note that the minimum detection limit of our PDA detector is 0.0001 AU (absorbance units), which amounts to approximately 1 ng of carotenoid per 50 ml injection (or 5 μg of carotenoid per gram of pigmented feather portion). Equal portions of feather barbules from yellow- or red-colored plumage in other songbirds, however, yield 20–400× more carotenoids than this (0.1–2 mg/g; McGraw et al., 2002; Stradi et al., 1996, 1997). We proceeded to analyze a much larger sample of feather barbules (~40 mg) pooled from 10 individuals, which lowered our detection limit to 0.5 μg/g, but we still found no carotenoids.

Next, we analyzed the melanic content of feathers, using methods previously employed to quantify melanins in the feathers of domestic pigeons (*Columba livia*; Haase et al., 1992). Melanins exist in two main forms in nature—eumelanin and phaeomelanin (Prota, 1992)—that bestow different colors on animals (black/gray vs. brown/chestnut, respectively), and this procedure allowed us to distinctly quantify both eumelanins and phaeomelanins in swallow feathers. To determine eumelanin concentration, brown barbules were trimmed from 12 males and 12 females and 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-hydroxyphenyalanine; 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).

These analyses yielded appreciable amounts of eumelanin and phaeomelanin in all throat feather samples (Table 1). Brown feathers ranged in total melanin concentration from 3–37 mg/g and contained an average ± SEM of 13.0 ± 1.8 mg/g. Phaeomelanin comprised a greater proportion of feather pigments than eumelanin (77 ± 2.7 % vs. 23 ± 2.7%, respectively), which may explain why these feathers are sometimes described as having a 'chestnut' or 'rufous' appearance (Saino et al., 1999). Overall, these melanin levels exceed all prior accounts of carotenoid concentration in bird plumage (e.g., McGraw et al., 2002, 2003b; Stradi et al., 1996, 1997), suggesting its potent pigmenting action in barn swallow feathers. Even if we assume that carotenoids do exist in these feathers at levels undetectable to us, melanins still would be greater than four orders of magnitude more concentrated in feathers than carotenoids, comprising more than 99.99% of feather pigments.

We realize that we did not examine plumage pigments for the exact populations or individuals used in prior studies of plumage color in *H. r. rustica* (e.g., in Italy, Denmark, the Ukraine). However, our evidence for the absence of carotenoids, but presence of melanins, in brown feathers matches that found in a previous study of ventral plumage color in barn swallows from North America (*Hirundo rustica erythrogaster*)

Table 1
Eumelanin and phaeomelanin concentrations of throat feathers in male and female European barn swallows (*Hirundo rustica rustica*), reported as mg of pigment per g of pigmented feather. Analyses of all samples were performed in duplicate, and we report averages of these values below.

	EUMELANIN (mg/g)		PHAEOMELANIN (mg/g)		TOTAL (mg/g)		PHAEO:EU RATIO	
Sex	Mean ± SE	95% CL	Mean ± SE	95% CL	Mean ± SE	95% CL	Mean ± SE	95% CL
Male	2.28 ± 0.28	1.67–2.89	12.09 ± 2.34	6.95–17.23	14.37 ± 2.49	8.89–19.85	6.02 ± 1.33	3.10–8.94
Female	2.59 ± 0.52	1.45–3.72	9.02 ± 2.42	3.70–14.34	11.61 ± 2.63	5.83–17.39	4.20 ± 1.02	1.94–6.45

(McGraw et al., 2004). Given the consistency of melanin-based plumage coloring across these barn swallow subspecies, it is hard to imagine that other *H. r. rustica* populations would adopt a wholly different, carotenoid-based pigmentation system.

Ultimately, our results indicate that conclusions drawn in previous studies about 'carotenoid-based' coloration in barn swallows (e.g., Camplani et al., 1999; Møller and Mousseau, 2001; Saino et al., 1999) are unjustified. Carotenoids are touted as potent diet-derived antioxidants in birds and other animals (reviewed in Lozano, 1994; Møller et al., 2000), and thus it has been argued that barn swallows that accumulate more carotenoids in their body or feathers are healthier and better able to endure severe environmental stress (e.g., radioactive contamination from Chernobyl; Camplani et al., 1999; Saino et al., 1999). From our work, however, it is clear that future studies of this plumage ornament should recast these arguments in light of the role of melanins as feather colorants. Unlike carotenoids, melanin pigments are not acquired from the diet or circulated through the body; instead, they are synthesized by animals from amino acids and done so locally in maturing feather follicles (Ralph, 1969). There may still be very good mechanistic reasons why melanic plumage color reveals individual quality in European barn swallows, but they should have nothing to do with carotenoid signaling.

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