

# How feather colour reflects its melanin content

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## Summary

1. Melanin coloration is the most common type of colour in animals. Many species use melanin-based black, brown, grey or earth-toned colours as intraspecific signals of quality.
2. Melanin comes in two main forms in vertebrates – eumelanin and pheomelanin – and these are said to create different colours, with eumelanin giving a darker black, brown or grey appearance and pheomelanin a lighter reddish or buff hue.
3. However, the few studies that have examined the melanin content of animal colours, namely from bird feathers, have shown that both pheomelanin and eumelanin are present in nearly all melanized patches. These pigment types also have different synthetic origins and may be differentially costly to display.
4. Thus, to fully understand the costs and benefits of melanic colours, we must first quantitatively determine the relationship between pigment composition and colour characteristics.
5. We studied melanin content and colour variation in the ventral chestnut-coloured plumage of male and female Barn Swallows (*Hirundo rustica erythrogaster*) from North America.
6. Total melanin concentration explained significant variation in the hue, saturation and brightness of male breast plumage and female breast plumage. Eumelanin is the predominant type in these feathers, constituting over 75% of total melanins. Eumelanin and pheomelanin concentration were significantly positively correlated in the feathers of females but not males.
7. In both sexes, levels of both eumelanin and pheomelanin concentrations were significantly and positively correlated with hue, saturation and brightness. In males but not females, however, the ratio of eumelanin-to-pheomelanin was also an important predictor of colour, with smaller ratios conferring browner hues. Males also deposit more pheomelanins, but not eumelanins or total melanins, in breast feathers than do females. These results suggest that pheomelanin plays a unique role in the colouring strategy of male Barn Swallows.

*Key-words:* Barn Swallow, eumelanin, *Hirundo rustica*, pheomelanin, plumage coloration, sexual selection

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## Introduction

Bird colours have emerged as a model system for understanding differences in the evolutionary significance and information content of ornamental traits in animals (Hill & McGraw 2006a,b). Different colours are produced by different mechanisms, and thus bring with them different costs and benefits of display. As such, it is important for each type of colour that we understand the pigmentary and structural nature of coloration and how the processes of colour production

are linked to selectively advantageous phenotypes and genotypes.

Melanin pigmentation is the most common form of colour in animals. Melanin gives black, brown, grey and earth-toned colours to bird plumages, and many such colours, like the throat patch of male House Sparrows (*Passer domesticus*), are used within species as social or sexual signals of quality (Hill 2006; Senar 2006). Despite its prevalence, however, we know much less about the honesty-reinforcing mechanisms underlying melanin colours than comparable pigment-based ornaments, such as the carotenoids (McGraw 2006). This is largely because of the historical difficulties biochemists have had with analysing large melanin

polymers. Several hypotheses have been proposed for how melanin colours may signal quality, involving hormonal (Buchanan *et al.* 2001), dietary (McGraw 2003) or social (McGraw, Dale & Mackillop 2003) mechanisms, but to date there are few experimental tests of or support for any of these.

We also lack a fundamental understanding of how melanin signals are best elaborated at the pigmentary level. How does a male Eastern Bluebird (*Sialia sialis*), for example, use melanin pigments to acquire the richest rufous breast colour possible? One obvious strategy would be to deposit high concentrations of melanin in feathers. However, because melanin comes in two main forms in vertebrates – eumelanin and phaeomelanin, which are thought to confer different colours (black/brown/grey *vs* reddish-brown/buff, respectively) and are both found in all melanized tissues analysed to date (McGraw 2006) – it is conceivable that more subtle variation in the types and amounts of melanins drives maximal colour expression. To date, no study has considered within a species how the relative or absolute amounts of eumelanin and phaeomelanin contribute to colour elaboration in birds.

We studied how variation in melanin-pigment content translates into variation in ornamental plumage-colour expression in male and female Barn Swallows from North America (*Hirundo rustica erythrogaster*). Ventral plumage in this subspecies: (a) varies in colour from deep to pale brown, (b) is rich in eumelanin and phaeomelanin pigments (McGraw *et al.* 2004a), (c) is sexually dichromatic, with males being more intensely coloured than females (Safran & McGraw 2004), and (d) is a sexually selected trait, with more colourful birds mating assortatively and raising more offspring annually (Safran & McGraw 2004). We scored plumage colour using reflectance spectrophotometry and analysed the eumelanin and phaeomelanin concentrations of feathers with liquid chromatography to address the following questions about the relationship between colour characteristics and pigment content:

1. How do eumelanin and phaeomelanin concentrations covary within a colour patch for males and females?
2. How well does eumelanin concentration, phaeomelanin concentration and total melanin concentration explain variation in tristimulus colour scores (hue, saturation and brightness) of male and female plumage?
3. Does the ratio of eumelanin-to-phaeomelanin account for significant colour differences among individuals?
4. What aspect of melanin-pigment variation best explains observed sex differences in ventral coloration?

## Methods

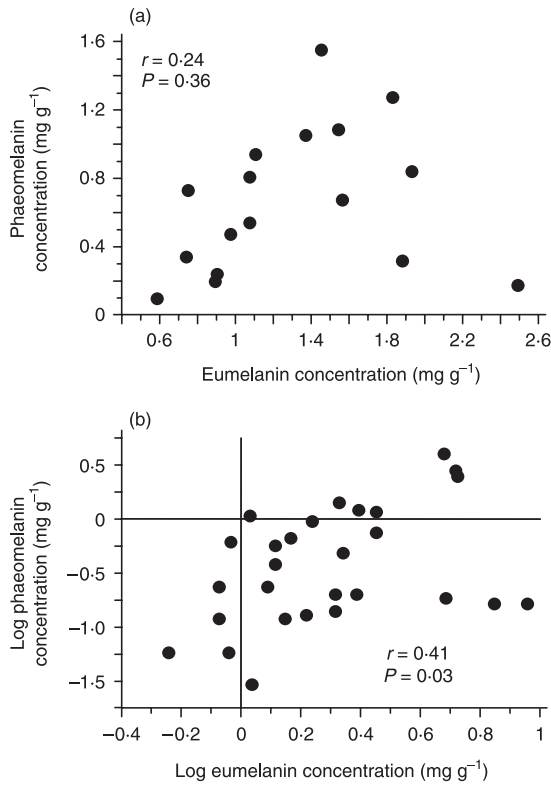
We studied Barn Swallows in Tompkins County, New York, USA, during the spring of 2001 (Safran & McGraw 2004; Safran 2004). Birds were captured with

mistnets within the first 2 weeks of the breeding season and were marked with enamel paints and aluminium rings for individual identification. At capture, we plucked patches of 3–10 feathers from the breast of each bird and mounted them on an index card, such that we recreated the natural appearance of the feather patch. These cards were stored in the dark and saved for later analysis.

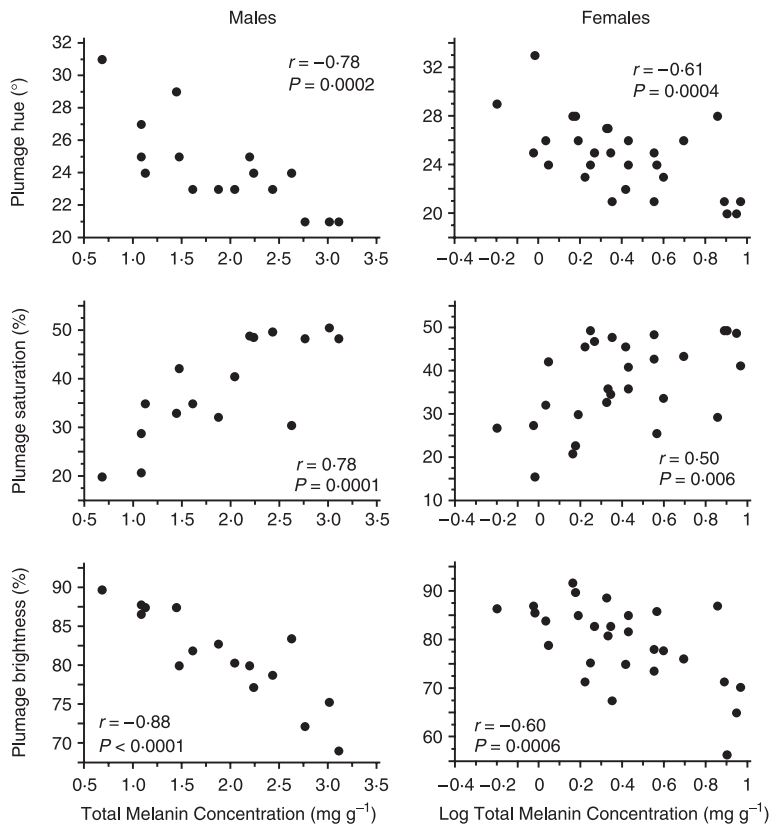
The colour of feathers was scored along three traditional axes of colour (hue, saturation and brightness) with a Colortron™ II reflectance spectrophotometer (Light Source Inc., San Rafael, CA). This unit quantifies visible light only, which is sufficient for this species because ventral plumage in Barn Swallows does not exhibit a unique ultraviolet reflectance peak (Safran & McGraw 2004). Each patch of breast feathers was scored three times, and we averaged these scores to determine mean hue, saturation and brightness for each bird. In a previous study, we then collapsed these scores into a single measure (PC1) using principal components analysis (Safran & McGraw 2004); however, for this study, we were interested in understanding how different types and amounts of pigments could explain variation in colour parameters, so we used tristimulus colour scores directly in analyses here.

We then analysed the melanin content of these feathers, following the protocol of McGraw *et al.* (2004b). To determine eumelanin concentration, brown barbules were trimmed and homogenized in water (1:100, w/v) and 400 µl of the homogenate were added to 800 µl 1 M H<sub>2</sub>SO<sub>4</sub>, oxidized with 3% KMnO<sub>4</sub>. The resulting oxidation product (pyrrole-2,3,5-tricarboxylic acid; PTCA) was analysed via high-performance liquid chromatography (HPLC) (Ito & Fujita 1985; Ito & Wakamatsu 1994). Phaeomelanins were examined by hydrolysing 200 µl feather homogenate with 500 µl 57% hydriodic acid at 130 °C in the presence of H<sub>3</sub>PO<sub>2</sub> for 24 h, and subsequently analysing the product (4-amino-3-hydroxyphenylalanine; 4-AHP) using HPLC with electrochemical detection (Wakamatsu, Ito & Rees 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 & Fujita 1985; Wakamatsu & Ito 2002).

Some of our colour and pigment variables were not normally distributed, so we log-transformed them to meet assumptions of parametric statistics. We used Pearson's correlational analyses to examine relationships between colour characteristics and pigment content. Similar results were found when data were not transformed and were analysed equally in the two sexes and for different colour and pigment measures, using non-parametric Spearman rank correlations. We also used two-sample *t*-tests (or Mann–Whitney *U*-tests when data were not normally distributed) to examine sex differences in non-transformed colour and pigment variables. Throughout the study, *n* = 17 for comparisons involving males and *n* = 28 for comparisons involving females.



**Fig. 1.** Correlation between levels of phaeomelanin and eumelanin in the breast feathers of (a) male and (b) female Barn Swallows.



**Fig. 2.** Relationship between total melanin content and tristimulus colour scores in the breast feathers of male and female Barn Swallows. Note here and elsewhere that lower hue scores correspond to browner plumage.

**Results**

**PRESENCE OF AND CORRELATIONS BETWEEN PIGMENT TYPES IN FEATHERS**

Total melanin concentrations averaged  $3.05 \pm 0.32$  (mean  $\pm$  SE)  $\text{mg g}^{-1}$  in swallow breast feathers and ranged from 0.6 to  $9.2 \text{ mg g}^{-1}$ . Eumelanin and phaeomelanin were detected in all feathers, with eumelanin being the predominant type (constituting  $78.2 \pm 2.2\%$  of total). Eumelanin and phaeomelanin concentrations were significantly positively correlated in the feathers of females but not those of males (Fig. 1).

**WITHIN-SEX CORRELATIONS BETWEEN COLOUR CHARACTERISTICS AND PIGMENT TYPES**

Hue, saturation and brightness levels were all positively intercorrelated within feather patches for both males and females (Table 1; also see Safran & McGraw 2004).

In males and in females, total melanin concentration was significantly correlated with plumage hue, saturation and brightness (Fig. 2). Respectively, in males and females, total melanin concentration explained 61% and 37% of the variation in plumage hue, 61% and 25% of the variation in plumage saturation, and 78% and 36% of the variation in plumage brightness. Broken down by individual pigment type, individual amounts of eumelanin and of phaeomelanin also were significantly predictive of our three colour measures (Table 2). When considering the relative amounts of the two melanins (the eumelanin-to-phaeomelanin ratio, E:P), no measure of colour in female breast plumage was correlated with the E:P ratio (all  $P > 0.5$ ). In contrast, male plumage hue and brightness were both significantly associated with E:P ratio, with lower hue and brightness scores (browner, darker plumage) corresponding to lower E:P values (proportionally more phaeomelanin present) (Fig. 3;  $r = -0.35$  and  $P = 0.18$  for saturation).

**Table 1.** Relationship between tristimulus colour measures for breast plumage of male and female Barn Swallows. Comparisons in the upper right corner of the table (in bold) are for males; those in the bottom left are for females. In all cases, birds with lower hue scores (browner) have more saturated (purer) and less bright (darker) colours

	Hue	Saturation	Brightness
Hue	–	$r = -0.74$ $P = 0.0004$	$r = 0.81$ $P < 0.0001$
Saturation	$r = -0.82$ $P < 0.0001$	–	$r = -0.85$ $P < 0.0001$
Brightness	$r = 0.81$ $P < 0.0001$	$r = -0.81$ $P < 0.0001$	–

**Table 2.** Correlations between colour parameters and the two types of melanin present in ventral Barn Swallow feathers. Results presented at the top of each cell (in bold) are for males; those below are for females

	Eumelanin concentration (mg g <sup>-1</sup> )	Phaeomelanin concentration (mg/g)
Hue	$r = -0.65$ , $P = 0.005$ $r = -0.58$ , $P = 0.001$	$r = -0.72$ , $P = 0.001$ $r = -0.45$ , $P = 0.02$
Saturation	$r = 0.76$ , $P = 0.0003$ $r = 0.47$ , $P = 0.01$	$r = 0.61$ , $P = 0.01$ $r = 0.37$ , $P = 0.05$
Brightness	$r = -0.80$ , $P < 0.0001$ $r = -0.57$ , $P = 0.001$	$r = -0.75$ , $P = 0.0005$ $r = -0.42$ , $P = 0.03$

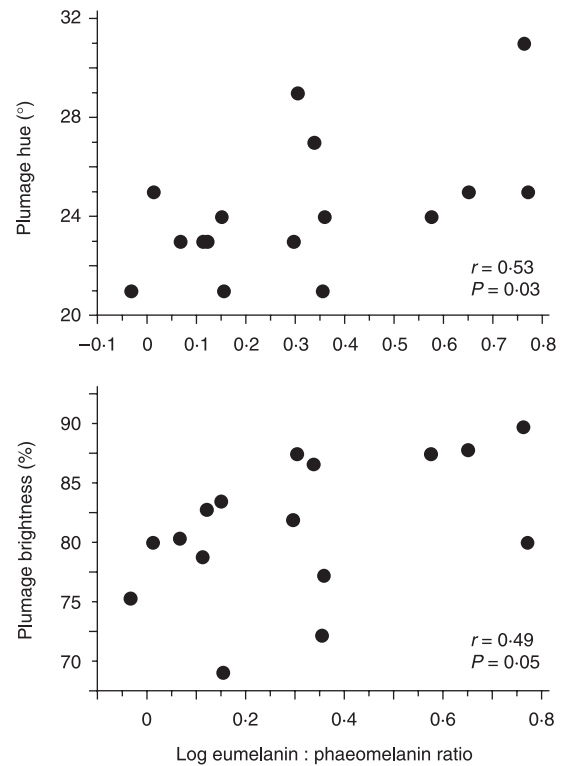
#### SEX DIFFERENCES IN COLOURS AND PIGMENTS

In previous work, we showed that a principal components analysis, which combined hue, saturation and brightness into a single colour parameter (PC1), revealed a significant difference in ventral coloration between the sexes (Safran & McGraw 2004). In the present subsample of birds, the breast plumage of males had lower hue scores than that of females (two-sample *t*-test,  $t = 2.1$ ,  $P = 0.04$ ), but there were no sex differences in plumage saturation or brightness (both  $P > 0.4$ ). We also found in these birds that the sexes differed in the phaeomelanin content of breast feathers (Fig. 4), but not in eumelanin (Mann–Whitney *U*-test,  $U = 240$ ,  $P = 0.78$ ) or total concentration ( $U = 207$ ,  $P = 0.31$ ).

#### Discussion

We tested the basic assumption that colour characteristics directly reveal pigment content for a melanin-based colour ornament in birds. We found that the total concentration of melanins was a strong predictor of the three traditional axes of colour in the ventral feathers of male and female Barn Swallows, explaining 25–78% of the variation in plumage hue, saturation and brightness. Thus, all tristimulus colour scores accurately reflect their melanin content, and this is perhaps no surprise given the strong intercorrelations that exist among the colour parameters (Safran & McGraw 2004; this study). This provides support for using a single colour measure, such as those derived from principal components analysis (e.g. Safran & McGraw 2004), to capture sexually and behaviourally relevant variation in Barn Swallow colour in future studies.

The range of colour variation explained by total melanin content varied substantially, however, being lowest in both sexes for plumage saturation. This presumably reflects the fact that saturation measures how relatively important certain wavelengths are to the perceived colour (also known as spectral curve shape) and that melanin colours are quite unsaturated, having only steadily increasing spectral curves with no sharp peaks (e.g. in Barn Swallows; Safran & McGraw 2004;

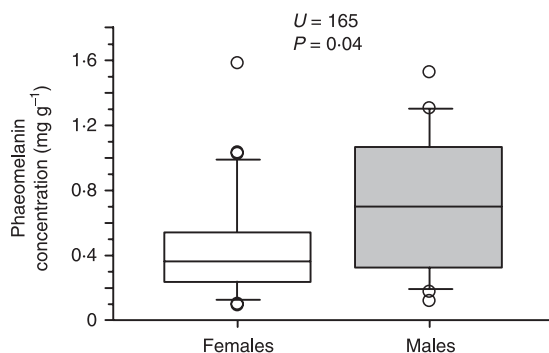


**Fig. 3.** Correlation between the eumelanin-to-phaeomelanin (E:P) ratio and two measures of plumage colour – hue and brightness – in the breast feathers of male Barn Swallows.

McGraw *et al.* 2004b). Colour variables were also better at capturing total pigment variation in males than in females, and this could reflect sampling differences (e.g. we analysed feather colours and pigments for more females than males), measurement differences (e.g. if colour and melanin can be determined more easily from male feathers, owing to a larger size), or perhaps pigmentation (e.g. emphasis on phaeomelanin) or structural differences in plumage between the sexes (see below).

Breast plumage in Barn Swallows contained both eumelanin and phaeomelanin, with eumelanin constituting more than three-quarters of total melanins. This is comparable in ratio to the rufous breast plumage of male and female Eastern Bluebirds (McGraw *et al.* 2004a). When considered separately, eumelanin and phaeomelanin concentrations were both significant predictors of all of our colour variables. This suggests that they play equally strong roles in creating colourful plumage in swallows. Such a conclusion is supported by the fact that, in females, phaeomelanin and eumelanin concentrations are positively intercorrelated in feathers. In males, however, there is no such statistically significant correlation, and instead the eumelanin-to-phaeomelanin ratio is associated with plumage appearance in males (but not females), with feathers comparatively rich in phaeomelanin being a deeper, darker brown colour.

This hints at a unique, sex-limited role for phaeomelanin in the coloration strategy of male Barn Swallows.



**Fig. 4.** Sex difference in phaeomelanin content of breast feathers in Barn Swallows. Males deposit significantly more phaeomelanin into plumage than do females.

Consistent with this, males exhibit a deeper brown hue, and accordingly deposit significantly more phaeomelanin in their ventral plumage than do females. Phaeomelanin differs from eumelanin not only in appearance but also in its synthetic pathway, with the incorporation of cysteine (a sulphur-containing amino acid) driving phaeomelanogenesis over eumelanogenesis (Land & Riley 2000). Experimental studies of Mallards (*Anas platyrhynchos*) showed that the relative amount of the two melanin types in plumage was sensitive to androgen levels (Haase, Ito & Wakamatsu 1995); testosterone treatment stimulated proportionally increased phaeomelanin synthesis in the nuptial plumage of Mallard drakes. Since melanin colours are widely seen as testosterone-dependent (Evans, Goldsmith & Norris 2000) badges of status in avian societies (Senar 2006), it is tempting to link these studies and suggest a common mechanistic link between pigment accumulation and signal function. We await future studies of the control mechanisms and behavioural role (e.g. male–male competition *vs* female choice) of ventral plumage colour in Barn Swallows.

The idea for testing how pigment variation predicts colour variation was previously intimated for carotenoid-based signals in birds (e.g. in House Finches, *Carpodacus mexicanus*, Inouye *et al.* 2001; Greenfinches, *Carduelis chloris*, Saks, McGraw & Horak 2003; American Goldfinches, *Carduelis tristis*, McGraw & Gregory 2004), and there are a few interesting contrasts between these studies and ours that deserve attention. First, not all tristimulus scores accurately reflected their carotenoid content in finches, contrary to what we found for melanins in swallows. Second, the extent to which pigment variation explained colour characteristics was much higher for melanin colour in swallows than for carotenoid colour in finches. This was true both for the number of colour parameters predicted by pigment content and for the tightness of each relationship. It is possible that these differences between studies are to some degree an artefact of the methods used to analyse the different colours and pigment types, but it is also likely that they are due to a biologically relevant difference in the absorbance properties of carotenoids and melanins. Melanins absorb light more strongly than carotenoids,

and the melanin-containing feathers of swallows are inherently less reflective (darker) than carotenoid-containing plumage in finches. Shawkey & Hill (2005) have shown that sexually selected carotenoid-based plumage in male goldfinches requires a structurally based reflective layer to derive its colour; thus, structurally based components of the feather may contribute more to total spectral output and variation from carotenoid-containing feathers than from melanin-based plumage. More work on additional species, particularly for melanin colours, is needed to test this hypothesis.

A common thread among these studies on colour and pigment content for carotenoids and melanins, however, is that there can be specialized biochemical strategies for ornamental coloration that have gone previously unrecognized. In House Finches, for example, a particular dietary carotenoid precursor and metabolic pathway is thought to be responsible for brilliant red (*vs* orange and yellow) colour (Inouye *et al.* 2001), and this knowledge has been important for targeting how the honesty of these signals is reinforced (Hill 2000). Here, we show for the first time that subtle variations in melanin content can generate differences in ornamental colour within and between the sexes. Our aim should now be to test the commonality of this colouring strategy and how various environmental, physiological and genetic challenges stand in the way of following such a strategy.

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