Pheomelanin-Based Plumage Coloration Predicts Survival Rates in Birds

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ABSTRACT

Higher vertebrates synthesize two forms of melanin: eumelanin and pheomelanin. While the adaptive functions of eumelanin are diverse, those of pheomelanin, which is phototoxic and whose production consumes a key intracellular antioxidant (glutathione), are not clear apart from being involved in color patterns that confer concealment. The factors that have favored the evolution of pheomelanin thus remain a mystery, causing this pigment even to have been considered an "accident of nature." A recent hypothesis posits that pheomelanin has evolved because it represents an alternative mechanism to remove excess dietary cysteine, which can be toxic because of its oxidation. We tested for links between pheomelanin-based color and survival in both an intraspecific study of barn swallows *Hirundo rustica* and an interspecific study of 58 species of birds from North and Central America. As predicted on the basis that birds degrade excess dietary amino acids by transferring their amino group to uric acid synthesis, we found that under equal levels of uric acid in plasma, individuals or species with a higher intensity or greater proportion of plumage colored by pheomelanin (brown and chestnut coloration) had higher relative annual survival rates while controlling for the potentially confounding effects of age, sex, body size, and phylogenetic descent. Likewise, barn swallows with more intense pheomelanin-based coloration had higher prospects to survive the winter after controlling statistically for age, sex, body size, and level of uric acid. This supports the idea that pheomelanic traits evolve because of the removal of excess cysteine in nonstressful conditions, thus avoiding its toxic effects.

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Introduction

Melanins are the most common pigments in animals, where they are found mostly in the integument in two chemically distinct forms. Eumelanin is responsible for dark colors and is found in virtually all animal groups, while pheomelanin is responsible for reddish and chestnut (i.e., lighter) colorations and apparently is synthesized only by higher vertebrates (i.e., birds and mammals; Ito and Wakamatsu 2003). Vertebrates synthesize these pigments from dopaquinone, which produces eumelanin (a polymer of 5,6-dihydroxyindoles and 5,6-dihydroxyindole-2-carboxilic acid) when thiol groups from the amino acid cysteine are absent or in low levels in melanocytes, or pheomelanin (a polymer of benzothiazine derivatives) when cysteine/glutathione (GSH) concentration is higher than a threshold level (García Borrón and Olivares Sánchez 2011; Riley et al. 2011). The main physiological reservoir of cysteine is GSH, which is also the most important intracellular antioxidant (Wu et al. 2004). Therefore, pheomelanogenesis requires a constant supply of cysteine via GSH, which represents a consumption of this important antioxidant (Meyskens et al. 1999; Pavel et al. 2011). Furthermore, pheomelanin is phototoxic because it produces reactive oxygen species when exposed to UV radiation (Samokhvalov et al. 2005; Greco et al. 2009). However, the adaptive benefits of pheomelanin are obscure, which has led to this pigment being considered an "accident of nature" (Hill and Hill 2000).

Because pheomelanin-based color is necessary to produce skin, coat, and plumage patterns for camouflage or concealment, it has been suggested that the persistence of pheomelanin is mainly due to its camouflage and aesthetic properties (Hill and Hill 2000). These properties may favor the evolution of pheomelanin, but certainly not all pheomelanin-based color traits are involved in concealment patterns (Hoekstra et al. 2006; Bradley and Mundy 2008), and some concealment patterns can be achieved by eumelanin-based colors (Bortolotti 2006; Bradley and Mundy 2008). Thus, these properties can only partly, at best, explain the evolutionary origin and the maintenance of pheomelanin.

A new hypothesis for the evolution of pheomelanin has recently been proposed (Galván et al. 2012*b*). This hypothesis posits that pheomelanin has evolved because of the benefits of removing cysteine during the incorporation of thiol groups into the structure of the pigment (Ito et al. 2011), because cysteine can cause toxicity if in excess as a result of the generation of

hydrogen peroxide during its autoxidation of the corresponding disulphide, which in turn can even decrease GSH levels (Viña et al. 1983; Munday 1989). The availability of cysteine depends mainly on the cysteine content of the diet (Lu 1999). In birds, excess cysteine, which occurs when its content in diet is higher than needed for protein synthesis or probably also as a reaction to constant oxidative stress, results in the production of sulfate, thus contributing to metabolic acidosis and a variety of associated problems such as thinning of egg shells and poor growth (Klasing 1998). Excess dietary amino acids in birds are degraded through deamination, by which the amino group is incorporated in the synthesis of uric acid (Klasing 1998). Indeed, very high protein levels result in increased uric acid levels in the blood (Klasing 1998). Therefore, excess dietary amino acids are partly removed through uric acid synthesis in birds, but pheomelanogenesis may represent a specific mechanism for removal of the excess of a particular amino acid, that is, cysteine. This function may explain the adaptive value of pheomelanin and the evolutionary origin and maintenance of this pigment.

The consumption of cysteine during pheomelanogenesis may thus be of adaptive value or represent a physiological cost depending on the needs of GSH for antioxidant protection. Therefore, the adaptive value of pheomelanin-based traits would depend on the levels of environmental oxidative stress, that is, stress generated by exogenous factors acting on all individuals of a population (Galván and Alonso-Alvarez 2009; Galván et al. 2012*b*). Under relatively low levels of environmental oxidative stress—for example, under conditions of low thermal stress or parasite prevalence—excess cysteine is more likely to occur, and the production of pheomelanin may be adaptive (Galván et al. 2012*b*). Under relatively high levels of environmental oxidative stress, by contrast, there would be more need for GSH, and the production of pheomelanin may thus reduce the availability of this compound for antioxidant protection and constitute a physiological cost (Galván et al. 2012*b*). Indirect support for these predictions may be provided by studies of birds that show that the expression of pheomelanin-based traits may limit the development of costly physiological processes or viability under adverse, stressful environmental conditions. Thus, a negative relationship between the extent of integument colored by pheomelanin and brain size, whose production requires high GSH levels for antioxidant protection, has been found (Galván and Møller 2011). Similarly, the extent of pheomelanic integument is negatively related to the capacity to resist the effects of ionizing radiation, a process that produces oxidative stress, among bird species (Galván et al. 2011). The extent of pheomelanic integument is also positively related to the prevalence of cataract in birds, which GSH critically contributes to prevent (Galván et al. 2012*a*). Western bluebirds *Sialia mexicana* with a greater expression of pheomelanic breast plumage coloration have been reported to be more likely to die of an epidemic (Keyser and Siefferman 2005). It has also been reported that more pheomelanic individual barn owls *Tyto alba* are more sensitive to physiological stress caused by corticosterone than less pheomelanic birds (Almasi et al. 2008) and that tawny owls *Strix aluco* belonging to the pheomelanic morph have lower viability during adverse environmental conditions than conspecifics belonging to the eumelanic morph (Karell et al. 2011). Some support is also provided by studies of mammals, because pheomelanin increases the photosensitization of cells to UV-induced oxidative damage in humans and mice (de Leeuw et al. 2001; Takeuchi et al. 2004; Samokhvalov et al. 2005), and pheomelanin content, together with other factors such as melanocortin 1 receptor genetic variance, is positively related to cancer risk in humans (Hill and Hill 2000; Simon and Peles 2010).

The aim of this study is to indirectly test in birds the hypothesis that the expression of pheomelanic traits is adaptive under low levels of environmental oxidative stress because pheomelanogenesis helps to remove excess cysteine, which is more likely to occur under such conditions (Galván et al. 2012*b*). The identification of gradients of environmental oxidative stress is thus of key importance for testing the adaptive value of pheomelanic traits (Galván and Alonso-Alvarez 2009). We tested the hypothesis in a data set of 58 individual barn swallows *Hirundo rustica* and 58 other species of birds from the United States and Panama (there was no intention in equaling sample sizes for both studies, but they happened to coincide). As our data set comprised information collected under "average" conditions of stress (i.e., no evidence of particularly high levels of thermal stress, parasite prevalence, or food scarcity in some individuals or species), we assume that our information is related to low environmental oxidative stress levels. If pheomelanogenesis has evolved as a mechanism to remove excess cysteine parallel to the general excretory role of uric acid in birds (Klasing 1998), low environmental oxidative stress levels should render the expression of pheomelanic traits positively related to viability both at intra- and interspecific levels. Thus, two individuals or species with similar excretory capacity through uric acid (i.e., with similar uric acid levels) but differing in the amount of pheomelanin being produced (i.e., with different intensity or extent of plumage colored by pheomelanin) should enjoy different relative survival rates for a given age (in an intraspecific test) or body size (in an interspecific test). In particular, individuals or species with more pheomelanin should have a higher survival rate.

Material and Methods

Intraspecific Study

Study Area and Feather Samples. We studied barn swallows during April–August 1996 in a study area of 45 km^2 around the village of Kraghede, Denmark (57°12′N, 10°00′E), as part of a long-term study (Møller 1994). Barn swallows breed inside barns and other buildings while foraging indoors or in neighboring fields. Barn swallows were captured at least once a week with mist nets placed in front of doors or windows, allowing for a capture probability of more than 98% (Møller and Szép 2002). Barn swallows are ca. 20-g aerial insectivorous passerines. Males and females are similar in phenotype with the exception of the length of the outermost tail feathers, which are longer in males than in females (Møller 1994), and the more

deeply chestnut (i.e., melanin-based) coloration of throat and forehead in males (Ninni 2003). A total of 10–20 feathers from the throat of birds were collected and stored in the dark until measurements were made.

Color Measurements. The intensity of melanin-based color of the throat plumage patch of barn swallows was measured in the laboratory using an Avantes AvaSpec-2048 spectrometer (range 200–1100 nm) with ultraviolet (deuterium) and visible (halogen) lamps (AvaLight-DH-S light source, Avantes, Eerbek) and a bifurcated 400-μm fiber-optic probe (FCR-7UV400-2-ME, Avantes). The fiber-optic probe both provided illumination and obtained light reflected from the sample. For each bird, 10–20 feathers were mounted on a black cardboard background such that they resembled the natural appearance of the feather patch. Measurements were relative to a white reference tile (WS-2; Avantes) and were taken by orienting the probe perpendicular to the samples. An average spectrum of three readings on the same point of the distal part of feathers was obtained for each bird and plumage patch. The probe was removed after each measurement. Reflectance curves were determined by calculating the median of the percentage of reflectance in 10-nm intervals beginning at 300 nm and ending at 700 nm (using AvaSoft software, ver. 7.1, Avantes) to cover the full spectral range that can be detected by birds (Cuthill et al. 2000). Representative spectral curves are shown in figure 1*A*.

Spectral data were assumed to represent a measure of total brightness (i.e., the summed reflectance across the entire spectral range), as this is the best predictor of pheomelanin concentration in feathers of both sexes in barn swallows, with lower values (i.e., darker colors) denoting higher pheomelanin concentration (McGraw et al. 2005). Brightness measurements were repeatable ($r = 0.81$, $F_{57,116} = 14.37$, $P < 0.0001$). Although the chestnut throat feathers of barn swallows contain both pheomelanin and eumelanin and the content of both pigments is negatively related to brightness (McGraw et al. 2005), we made no predictions for eumelanin because the production of this pigment does not consume cysteine as pheomelanin does (see above). Therefore, although plumage brightness may serve as a proxy for both the pheomelanin content and the eumelanin content of feathers, our hypothesis is formulated only for pheomelanin, so any result in the predicted directions should be attributed to pheomelanin production.

Uric Acid Levels of Plasma. We took two 75-μL capillaries of blood from the brachial vein of barn swallows. These were subsequently stored in a cooling box before being transported to the lab, where they were centrifuged with a hematocrit centrifuge for 5 min. Plasma and red blood cells were separated, and the plasma fraction was entered into the Kodak-Ektachem biochemical analysis equipment. We used the biochemical analyzer for uric acid as described in the instructions. The Kodak-Ektachem is known to provide highly accurate estimates of uric acid and other metabolites (Külpmann et al. 1990). The analyses were performed within 12 h after blood collection.

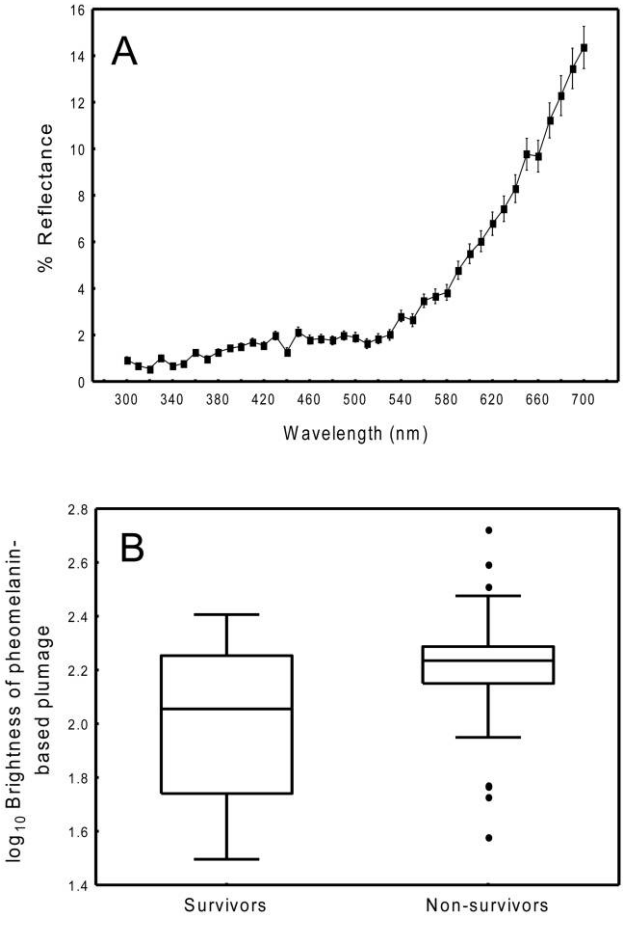

Figure 1. *A*, Spectral reflectance ($\pm$SE) of the melanin-based plumage of adult barn swallows during the breeding season. *B*, Color intensity (brightness) of melanin-based plumage of barn swallows that survived or did not survive the subsequent winter after capture, showing that survivors had higher pheomelanin (and eumelanin) concentrations in feathers than nonsurvivors ($b = -4.03$, $\chi^2_1 = 10.50$, $P = 0.001$). The horizontal bars represent the median, the boxes represent the interquartile range, and the vertical bars represent the nonoutlier range, while the dots represent outliers.

Interspecific Study

Survival Rate and Uric Acid Levels. Annual adult survival rates and uric acid levels of plasma (mg/dL) were obtained from the data provided by Cohen et al. (2009) for 58 species of birds captured in different locations of the United States and Panama. Detailed methods on how annual survival rates and uric acid levels were obtained are described in Cohen et al. (2009).

Melanin-Based Plumage Coloration. Using color plates, we scored the melanin-based plumage coloration of the 58 bird species. Several authors have used this method previously (e.g., Biard et al. 2009; Caro 2009; Galván and Møller 2011), and it has been shown to be a reliable method for quantifying different components of plumage color that is even correlated with the

avian perception of color (del Val et al. 2009; Seddon et al. 2010).

Eumelanin and pheomelanin normally occur simultaneously in tissues (Ozeki et al. 1997), but the darker colors conferred by eumelanin (Toral et al. 2008) make evident the lower content of this pigment in chestnut and brown colors compared with black and gray colors (Galván and Alonso-Alvarez 2009). Furthermore, many bird species have feather melanin contents of high purity (>90% of either eumelanin or pheomelanin; McGraw and Wakamatsu 2004; J. J. Negro, personal communication). Therefore, similar to Galván and Møller (2011), Galván and Rey Benayas (2011), and Galván et al. (2011), we considered black and gray plumage colors to be predominantly generated by eumelanin and chestnut and brown colors to be predominantly generated by pheomelanin. We did not consider blue, green, yellow, or red colorations assumed to be generated by other pigments (e.g., carotenoids, porphyrins, or psittacofulvins) or by feather structures unless chemically identified as being melanin based by Toral et al. (2008).

Thus, I. Galván quantified the proportion of melanic plumage parts by examining illustrations in del Hoyo et al. (1992–2002, 2003–2007). Illustrations of both resting and flying adults in breeding plumage were examined. The method used by Beauchamp and Heeb (2001) and Galván (2008) was followed to obtain estimates of the proportion of eu- and pheomelanic color present in the plumage of each species by assigning scores that ranged from 0 (total lack of melanic color) to 5 (all melanic). It must be noted that eu- and pheomelanic color patches can coexist in the same feathers, and thus the sum of the two color scores in a species that presents both color types is not always necessarily 5; higher values are also possible. The mean scores for males and females were used. The scoring was made blindly with respect to uric acid and survival data. The scores assigned to the 58 species of birds are shown in table A1 in the online edition of *Physiological and Biochemical Zoology*. Pheomelanic color scores obtained by this method are positively related to the concentration of pheomelanin in the plumage of birds (Galván et al. 2012*a*).

Data Analyses

Intraspecific Analyses. By means of a generalized linear model (GLM) with a binomial response distribution and a logit link function, we tested whether the capacity of barn swallows to survive from the breeding season when they were captured (1996) to the next season (1997), with the scores of survivors being equal to 1 and nonsurvivors being equal to 0, is predicted by the intensity of pheomelanin-based coloration ($\log_{10}$ transformed), controlling for uric acid levels of plasma ($\log_{10}$ transformed). Because the survival probability obviously depends on age and this trait is also related to the pheomelanin-based color intensity of barn swallows (Galván and Møller 2009), the age of birds was included as a covariate in the model. Sex was also included as a covariate in the model because male and female barn swallows have different survival prospects (Møller and Szép 2002), and barn swallows are sexually dichromatic regarding throat melanin-based coloration (Galván and Møller 2009). The length of outer tail feathers, a quality indicator in barn swallows, has also been found to be related to survival prospects of this species (Møller and Szép 2002), although we did not find any relationship between tail length and brightness of pheomelanin-based plumage (GLM with $\log_{10}$ brightness as response variable and $\log_{10}$ tail length, sex, and their interaction as predictors: tail length: $F_{1,54} = 0.47$, $P = 0.494$, tail length × sex: $F_{1,54} = 0.00$, $P = 0.943$), so tail length was not included in the model. Nonsignificant terms were not removed from the model because our hypothesis explicitly requires controlling for these terms.

Interspecific Analyses. Bird species are not independent sample units because of common phylogenetic descent (Felsenstein 1985; Harvey and Purvis 1991). Thus, the effect of common ancestry among taxa can lead to an overestimation of degrees of freedom if phylogenetic relationships are not taken into account. We used phylogenetic eigenvector regression (PVR; Diniz-Filho et al. 1998; Desdevises et al. 2003) to correct for phylogenetic effects in the analyses. PVR is a comparative method that is widely used for a diversity of taxa and ecological questions (e.g., Giannini 2003; Kriloff et al. 2008; Montoya et al. 2008; Bisson et al. 2009; Galván and Møller 2011; Galván et al. 2011) because it has similar statistical performance even under evolutionary processes distinct from Brownian motion and provides results similar to those of other methods (Diniz-Filho and Torres 2002; Martins et al. 2002; Galván and Møller 2011; Galván et al. 2011). We first performed a principal coordinates analysis on the matrix of pairwise phylogenetic distances between the 58 bird species (after a double-center transformation). Then we selected the first six eigenvectors obtained by the broken-stick rule to account for the phylogenetic signal parsimoniously (Diniz-Filho et al. 2012). Eigenvectors extracted from double-centered phylogenetic distance matrices are able to detect the main topological features of the cladogram under different sample sizes or number of taxa used in the analyses (Diniz-Filho et al. 1998). The original matrix of phylogenetic distances between the 58 bird species and the reproduced matrix of distances estimated based on the first six eigenvectors were very similar (Mantel test with 999 randomized matrices to estimate significance: $r = 0.875$, $P < 0.0001$; test carried out using PopTools 3.2.3; Hood 2010). These eigenvectors were used as additional predictor variables in GLMs (see below) in order to control for phylogenetic effects. The phylogenetic hypothesis (see fig. A1 in the online edition of *Physiological and Biochemical Zoology*) was taken from the species-level supertree constructed by Davis (2008) to resolve the polytomies found in the phylogeny constructed by Cohen et al. (2009). We set all branch lengths equal to unity in our compiled phylogeny, thus assuming a speciational model of evolution.

By means of GLMs we investigated whether annual adult survival rate (arcsine transformed) could be explained by the pheomelanic color score of birds after controlling for uric acid level, which was added to the models ($\log_{10}$ transformed) as a covariate. The first six phylogenetic eigenvectors (hereafter

EV1–EV6) were entered as covariates. The same analysis was made including the eumelanic color score in the models instead of the pheomelanic score (both color scores were not included in the same models because they were strongly negatively correlated; effect of eumelanic score in GLM with pheomelanic score as response variable and eumelanic score and EV1–EV6 as covariates: $b = -0.89$, $F_{1,50} = 84.18$, $P < 0.0001$). Because there was a high variability among species in the number of individual birds that were sampled for uric acid levels (see Cohen et al. 2009) and this could lead to violations of the assumptions of phylogenetic comparative analyses (Freckleton 2009; Garamszegi and Møller 2010), we weighted the models by number of individuals examined per species, thus giving more importance to determinations of uric acid levels in species with information for many individuals. The distribution of the residuals of the models was examined to determine that the normality assumption was fulfilled. Again, nonsignificant terms were not removed from the model because our hypothesis explicitly requires controlling for these terms.

We also controlled for potential confounding variables in the models. In particular, and given the tight dependence of life history traits such as survival rate on body size (Blueweiss et al. 1978), we searched for possible effects of body size (as provided in Cohen et al. 2009) on pheo- and eumelanic color scores by means of GLMs. We also tested for possible effects of diet on both survival rate and pheo- and eumelanic color scores using the information provided by Cohen et al. (2009), summarized as the result of a principal components analysis (PC1 explaining invertebrate consumption and PC2 explaining the relative balance of fruits and seeds, with higher scores indicating more seed in the diet; Cohen et al. 2009). We considered diet a potential confounding variable because it may reflect interspecific differences in levels of excess protein. Latitude may also represent a confounding variable because survival rate is related to this variable in birds (Møller 2007). However, latitude was not associated with pheo- or eumelanic plumage color scores in a larger data set of species (Galván and Møller 2011), and in our data set pheo- and eumelanic plumage color scores did not differ between temperate and tropical species (GLM with climate zone [temperate vs. tropical] and EV1–EV6 as covariates: zone effect for model for pheomelanic score: $F_{1,50} = 1.69$, $P = 0.199$; zone effect for model for eumelanic score: $F_{1,50} = 0.25$, $P = 0.618$). Thus, the GLMs to detect confounding variables made up models for pheomelanic color score and eumelanic score (response variables) that included body mass, diet PC1 and PC2, and EV1–EV6 as covariates and another model for annual survival rate (response variable) that included diet PC1 and PC2 and EV1–EV6 as covariates (table 1). The model for pheomelanic color score resulted in significant effects of body mass ($b = 2.55$, $F_{1,48} = 7.42$, $P = 0.009$) and diet PC2 ($b = 1.48$, $F_{1,48} = 5.41$, $P = 0.024$) and a nonsignificant effect of diet PC1 ($b = 0.21$, $F_{1,48} = 0.13$, $P = 0.718$). The model for eumelanic color score resulted in significant effects of diet PC1 ($b = -1.25$, $F_{1,48} = 5.73$, $P = 0.021$) and marginally nonsignificant effects of diet PC2 ($b = -1.12$, $F_{1,48} = 3.78$, $P = 0.058$) and body mass ($b = -1.55$, $F_{1,48} = 3.37$, $P = 0.072$). Diet variables had no significant effects in the model for annual survival rate (PC1: $b = 0.00$, $F_{1,49} = 0.00$, $P = 0.940$; PC2: $b = -0.02$, $F_{1,49} = 0.47$, $P = 0.498$). Therefore, diet does not represent a confounding variable because it is not related to survival rate, although it is related to both pheo- and eumelanic color, and only the effect of body mass was controlled for in subsequent analyses. (In models with body mass as response variable and color scores and EV1–EV6 as covariates, body mass was not related to either pheomelanic [$b = 0.28$, $F_{1,50} = 2.88$, $P = 0.096$] or eumelanic [$b = 0.11$, $F_{1,50} = 0.27$, $P = 0.603$] scores, thus avoiding multicollinearity effects in the analyses.) It is thus not likely that the analyses are affected by interspecific differences in levels of excess protein, because these should be related to differences in diet.

Table 1: Results of three generalized linear models (GLMs) with survival rate, pheomelanic color score, or eumelanic color score for 58 species of birds as response variables and body mass and diet as explanatory variables

Explanatory variable	Survival rate	Pheomelanic color score	Eumelanic color score
Body mass	...	Yes	Yes
Diet (PC1 or PC2)	No	Yes	Yes

Note. The model for survival rate did not include body mass because it is already known that these variables are tightly associated (Blueweiss et al. 1978). The GLMs also included six phylogenetic eigenvectors (EV1–EV6) to control for phylogenetic effects. These GLMs were made to determine whether body mass and diet represented confounding variables in models with survival rate as response variable and melanic color scores as explanatory variables. We considered that confounding variables were so if they were related to both response (i.e., survival rate) and explanatory (i.e., melanic color score) variables. Because diet was related to melanic color but not to survival rate, while body mass was related to both variables, only body mass was included as a covariate in the final models testing for an association between survival rate and melanic color scores. Yes = significant ($P < 0.05$) or marginally significant ($0.05 < P < 0.1$) effect; no = nonsignificant ($P > 0.1$) effect.

Results

Intraspecific Analyses

The model for barn swallow survival was statistically significant ($\chi^2_4 = 10.86$, $P = 0.028$) and explained 15% of deviance for this variable. Survival was significantly negatively related to the brightness of the melanic throat ($b = -4.03$, $\chi^2_1 = 10.50$, $P = 0.001$), indicating that survivors had higher pheomelanin concentration in feathers than nonsurvivors (fig. 1*B*). The other terms in the model did not reach significance (uric acid levels: $b = 1.03$, $\chi^2_1 = 0.46$, $P = 0.499$; age: $b = 0.28$, $\chi^2_1 = 0.86$, $P = 0.353$; sex: $b = 0.33$, $\chi^2_1 = 0.27$, $P = 0.606$).

Interspecific Analyses

The effect of the pheomelanic color score on the annual adult survival rate of species was significant and positive as predicted (fig. 2; $b = 0.02$, $F_{1,48} = 7.35$, $P = 0.009$) after controlling for uric acid levels ($b = -0.07$, $F_{1,48} = 2.40$, $P = 0.128$), body

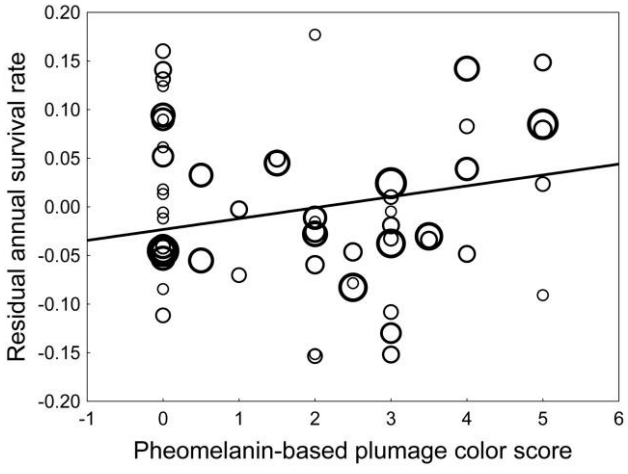

Figure 2. Residual annual survival rate (i.e., partial effects after applying the full model explained in "Material and Methods" without pheomelanin-based color score) plotted against pheomelanin-based plumage color score. The relationship between these two variables is significant (generalized linear model weighted by sample size: $b = 0.01$, $F_{1,56} = 4.92$, $P = 0.030$). The size of the data points increases logarithmically with sample size for illustrative purposes only. The line is the regression line.

mass ($b = 0.08$, $F_{1,48} = 3.31$, $P = 0.075$), and similarity in phenotype due to common phylogenetic descent (EV1–EV6 effects: $-0.01 < b < 0.01$, $0.11 < F_{1,48} < 16.38$, $0.001 < P < 0.737$). This model explained 59% of the variance in annual survival rate across species.

The same model, but including the eumelanic color score instead of pheomelanic color score, resulted in a significant and negative effect of the eumelanic score on annual adult survival rate ($b = -0.02$, $F_{1,48} = 8.69$, $P = 0.005$) as expected given the strong negative correlation between pheo- and eumelanic color scores (see "Material and Methods"). In this model, both uric acid level and body mass had significant effects (uric acid level: $b = -0.10$, $F_{1,48} = 5.04$, $P = 0.029$; body mass: $b = 0.09$, $F_{1,48} = 4.58$, $P = 0.037$; EV1–EV6 effects: $-0.01 < b < 0.01$, $0.18 < F_{1,48} < 19.77$, $0.001 < P < 0.672$). This model explained 60% of interspecific variance in annual survival rate.

Discussion

We found that the expression of pheomelanin-based plumage coloration is positively related to the survival prospects of adult birds after controlling for uric acid levels and the confounding effects of age, sex, body size, and phylogeny at both intra- and interspecific levels. In other words, when individuals or species with similar uric acid levels are compared, those having a higher intensity or greater proportion of plumage colored by pheomelanin survive better. This was the prediction made on the assumption that birds degrade excess dietary amino acids by channeling their amino group to the synthesis of uric acid (Klasing 1998), and pheomelanogenesis may represent a parallel excretory mechanism of excess amino acids for the particular case of cysteine, because thiol groups (either from free cysteine or from the cysteine-containing GSH; Potterf et al. 1999) are incorporated into the polymers of pheomelanin during the process (García-Borrón and Olivares Sánchez 2011; Galván et al. 2012*b*). By contrast, the effect of the eumelanin-based color score was negative in our comparative analysis because of the negative relationship present between the two color scores among species.

As a sulfur-containing amino acid, cysteine is susceptible to oxidation to the corresponding disulphide, which can generate oxidative stress because hydrogen peroxide is produced in the process (Viña et al. 1983; Munday 1989). In fact, the oxidation of excess dietary cysteine produces acidosis in birds and a diversity of associated problems such as poor bone mineralization, thinning of egg shells, and poor growth (Klasing 1998). Therefore, it is likely that adaptive mechanisms have evolved in vertebrates to avoid the toxicity of excess sulfur-containing amino acids. Indeed, disulphide reductases are enzymes that convert the oxidized form of cysteine back to its unmodified form (Berlett and Stadtman 1997), and the synthesis of uric acid represents an important excretory mechanism for excess dietary amino acids in the case of birds (Klasing 1998). However, the adaptive mechanisms to avoid the toxicity of excess cysteine may be more diverse than those so far mentioned. It has recently been proposed that pheomelanogenesis represents an alternative mechanism to remove excess cysteine, which may provide a biological explanation for the evolutionary origin and persistence of pheomelanin (Galván et al. 2012*b*). Although birds deposit pheomelanin in feathers mainly during a relatively short period of time (i.e., moult), the molecular basis of melanogenesis is genetically fixed so that the mechanism by which cysteine is incorporated into the pheomelanogenesis pathway is present all year round, as suggested by the fact that the development of pheomelanin-containing feathers can be induced at any time outside the molting period and pheomelanin not transferred to integumentary structures can be degraded through specific physiological mechanisms (Wakamatsu et al. 1991). Thus, any benefit of pheomelanin related to the removal of excess cysteine should be prevalent during the entire life cycle of birds.

The evolution of pheomelanin has remained a mystery. This pigment is phototoxic (de Leeuw et al. 2001; Takeuchi et al. 2004; Samokhvalov et al. 2005), and its production consumes GSH, a key intracellular antioxidant (Meyskens et al. 1999; Pavel et al. 2011). Indeed, studies on birds have shown that the expression of pheomelanin-based plumage limits the capacity to cope with physiological processes or environmental conditions that generate high levels of oxidative stress (Keyser and Siefferman 2005; Almasi et al. 2008; Galván and Møller 2011; Galván et al. 2011, 2012*a*; Karell et al. 2011). Indeed, some authors have considered pheomelanin an "accident of nature" (Hill and Hill 2000). The results of our study suggest, from both intra- and interspecific perspectives, that under equality of performance of the excretory mechanism represented by uric acid, the production of pheomelanin for feathers may improve the survival prospects of birds. The amount of

pheomelanin produced in different species would thus depend at least partly on the efficiency of the other mechanisms to degrade excess cysteine (i.e., levels of disulphide reductases and uric acid). This may explain the persistence of pheomelanin in higher vertebrates. However, it must be considered that our analyses represent only indirect tests of Galván et al.'s (2012*b*) hypothesis, and future studies providing direct measurements of excess cysteine and pheomelanin content should be performed to corroborate the predictions made by the hypothesis. These studies should test the adaptive value of pheomelanin by exploring the relationship between viability and pheomelanin production along environmental gradients of oxidative stress.

We predicted that the removal of cysteine represented by pheomelanogenesis should be adaptive under conditions of relatively low environmental oxidative stress because cysteine needs for antioxidant protection would be lower and excess levels more likely to occur (Galván and Alonso-Alvarez 2009; Galván et al. 2012*b*). As we had no evidence of particularly high levels of thermal stress, parasite prevalence, food scarcity, or any other sources of physiological stress in the individuals or species included in our data set, we assume that our information is related to low environmental oxidative stress levels and thus that the results support our hypothesis. Under the opposite conditions (i.e., relatively high environmental oxidative stress), the needs of cysteine/GSH for antioxidant protection would be greater, and under such conditions the removal of this antioxidant source would represent a physiological cost. The recent study by Karell et al. (2011) would support the latter mechanism because they have shown that tawny owls of the pheomelanic morph have lower survival prospects than those of the eumelanic morph, but only during severe, cold winters, that is, under highly stressful conditions. It is interesting to note that the physiological cost that may represent the production of pheomelanin under conditions of high environmental oxidative stress should in turn increase the reliability of pheomelanic traits as signals of quality (i.e., condition-dependent traits), because only individuals of high genotypic quality would be able to afford the costs of producing large pheomelanic traits under such conditions (Galván and Alonso-Alvarez 2009; Galván and Solano 2009). Indeed, the capacity to face such physiological costs under high levels of environmental oxidative stress may explain why the intensity of pheomelanin-based coloration acts as an honest signal of quality in some of the species considered here, including the barn swallow (Ninni 2003; Safran and McGraw 2004; Safran et al. 2005). This capacity of pheomelanin-based traits to act as honest signals agrees with the definition of condition-dependent traits as those whose expression is related to the performance of vital physiological processes such as antioxidant mechanisms (Hill 2011).

We are aware, however, that our analyses represent only an indirect test of the hypothesis formulated by Galván et al. (2012*b*) because we did not have an estimate of the magnitude of excess dietary cysteine in different individual barn swallows or species or of levels of disulphide reductases that also help avoid oxidation of cysteine residuals of proteins. Our aim is to present this novel hypothesis to scientists who may collect more specific measurements related to cysteine intake and degradation in future studies. However, the fact that our prediction was fulfilled by both the intraspecific analysis of barn swallows and the comparative analysis of American birds is compelling and opens new perspectives for the study of melanin evolution.

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