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THE RUSTY PLUMAGE COLORATION OF JUVENILE GYRFALCONS IS PRODUCED BY PHEOMELANIN AND ITS EXPRESSION IS AFFECTED BY AN INTRACELLULAR ANTIOXIDANT

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ABSTRACT.—Juveniles of many diurnal raptors exhibit a characteristic rusty plumage coloration whose biochemical basis has never been determined. Using the Gyrfalcon (*Falco rusticolus*) as a model species, we analyzed feathers by Raman spectroscopy and showed that the rusty color is due to the presence of the pigment pheomelanin, which was also observed in the feathers of a juvenile Peregrine Falcon (*Falco peregrinus*). We experimentally modified the expression of the rusty plumage coloration by treating four developing Gyrfalcons with buthionine sulfoximine (BSO), a specific and nontoxic inhibitor of glutathione (GSH) synthesis. Because cysteine, one of the three constitutive amino acids of GSH, is required for pheomelanin synthesis and GSH is the most important intracellular antioxidant, these findings indicate that the expression of rusty plumage coloration can be affected by environmental oxidative stress. Our results suggest that the rusty plumage coloration of at least some diurnal raptors is pheomelanin-based, and the dependence on GSH levels opens the possibility that the evolution of this trait in some species and the age-related variation in its expression across species may be explained by interspecific and intraspecific variation in exposure to environmental factors that generate oxidative stress and by age-related variations in endogenous levels of oxidative stress.

KEY WORDS: *Gyrfalcon*; Falco rusticolus; *age-related variation*; *oxidative stress*; *pheomelanin*; *plumage coloration*.

LA COLORACIÓN FERRUGINOSA DEL PLUMAJE JUVENIL DE *FALCO RUSTICOLUS* ES PRODUCIDA POR FEOMELANINA Y SU EXPRESIÓN ES AFECTADA POR UN ANTIOXIDANTE INTRACELULAR

RESUMEN.—Los juveniles de numerosas especies de rapaces exhiben una coloración ferruginosa típica del plumaje, cuya base bioquímica nunca ha sido determinada. Usando a *Falco rusticolus* como una especie modelo, analizamos sus plumas mediante espectroscopia Raman y demostramos que el color ferruginoso se debe a la presencia del pigmento feomelanina, el cual también fue observado en las plumas de un individuo juvenil de *Falco peregrinus*. Modificamos experimentalmente la expresión de la coloración ferruginosa del plumaje tratando a cuatro individuos juveniles de *F. rusticolus* con butionina sulfoximina (BS), un inhibidor específico y no tóxico de la síntesis del glutatión (GSH). Debido a que se requiere cisteína (uno de los tres aminoácidos constituyentes del GSH) para la síntesis de feomelanina y el GSH es el antioxidante intracelular más importante, estos hallazgos indican que la expresión de la coloración ferruginosa del plumaje puede ser afectada por el estrés oxidativo ambiental. Nuestros resultados sugieren que la coloración ferruginosa del plumaje de al menos algunas especies de rapaces diurnas se debe a la feomelanina. Por otro lado, la dependencia en los niveles de GSH abre la posibilidad de que la evolución de este rasgo y la variación de su expresión relacionada con la edad en diferentes especies puede explicarse por la variación inter e intra-específica en la exposición a factores ambientales que generan un estrés oxidativo y por variaciones en los niveles endógenos de estrés oxidativo relacionados con la edad.
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The plumage patterns exhibited by diurnal raptors (Order Falconiformes) is among the most diverse in all groups of birds (Ferguson-Lees and Christie 2001). However, the pigments that color their plumage seem to be mostly limited to the large chemical class of melanins, with porphyrins (another class of pigments) so far described only in Black-winged Kites (*Elanus caeruleus*; Negro et al. 2009). A common characteristic of the juvenal plumage of many raptors is the presence of chesnut, pinkish, or rusty coloration that is lost after subsequent molts, as exemplified by most species of the genus *Falco* (Ferguson-Lees and Christie 2001). This kind of coloration is typically conferred by pheomelanin, one of the two main chemical forms of melanin (Galván et al. 2012a), although confirming analyses of feathers of juvenile birds has not been reported in the published literature.

The identification of pheomelanin as the pigment responsible for the rusty color of the juvenal plumage of birds of prey is important for understanding why this coloration has evolved and why it is lost as birds age. Pheomelanin is produced when the amino acid cysteine is present at levels above certain thresholds in melanocytes, the cells where melanins are synthesized, as it is incorporated into the structure of the pigment (García-Borrón and Olivares Sánchez 2011). Cysteine is one of the three amino acid constituents of glutathione (GSH), the most important intracellular antioxidant, so the production of pheomelanin represents a consumption of an antioxidant resource, as cysteine can no longer be used for antioxidant purposes once it is incorporated into the structure of the pigment (Galván et al. 2012b). Thus, the expression of melanin-based plumage coloration is open to environmental influences mediated by factors generating oxidative stress (i.e., the imbalance between the production of reactive oxygen species [ROS] and the state of the antioxidant and repair machinery, with the balance tipped toward the former; Finkel and Holbrook 2000). Indeed, the pheomelanin content of feathers is associated with environmental conditions during development, even in species such as the Northern Goshawk (*Accipiter gentilis*), in which pheomelanin apparently does not contribute to plumage coloring, because the darker colors produced by eumelanin, the other form of melanin, dominate (Galván et al. 2010). Therefore, the synthesis of pheomelanin may represent a physiological cost or an adaptive benefit depending on the prevailing conditions of environmental oxidative stress to which birds are exposed (Galván et al. 2012b), which may constitute the basis of an evolutionary explanation for why the rusty plumage coloration is only exhibited by juvenile raptors.

Here we test if the rusty coloration exhibited by juvenile falcons is generated by the pigment pheomelanin, using the Gyrfalcon (*Falco rusticolus*) as a model species. The plumage color of the adult Gyrfalcon varies from white to gray to black along its natural distribution range and this range is explained by allelic variation at the melanocortin-1 receptor (MC1R) gene, indicating that plumage color variation in this species is due to differences in the levels and type of melanins in feathers (Johnson et al. 2012, Zhan et al. 2012). However, in addition to gray and black colors that form the plumage variability, white juvenile Gyrfalcons (i.e., birds that are predominantly white with some black plumage patches as adults) exhibit, as do the juveniles of most species of falcons, rusty coloration in all parts of the plumage, and some Gyrfalcons retain this color as adults, mainly in the feathers of head and mantle (Ford 1999). As mentioned above, the pigments of the plumage of diurnal raptors seem to be limited primarily to melanins, as to our knowledge no other pigments (e.g., carotenoids) have been described in the feathers of this taxa of birds, but a formal demonstration of the presence of pheomelanin can only be made by pigment analyses of feathers. Additionally, the possibility that the rusty coloration is generated by porphyrins should not be dismissed, as this pigment produces colors that are similar to those of pheomelanin (Toral et al. 2008, Negro et al. 2009). To increase the generality of our study, we also wished to analyze feathers of other falcon species that exhibit the characteristic rusty plumage coloration as juveniles (Ferguson-Lees and Christie 2001).

Because the synthesis of pheomelanin should depend on cysteine levels in cells, we also tested if the rusty plumage coloration of Gyrfalcons is affected by the levels of GSH, the main physiological reservoir of cysteine (Wu et al. 2004). With this aim we conducted an experiment with Gyrfalcon nestlings bred in captivity, artificially reducing GSH levels during the development of the first plumage. If the rusty plumage coloration is generated by pheomelanin and dependent upon the levels of cysteine, we hypothesized that the expression of the rusty coloration should be less intense in Gyrfalcon nestlings with reduced GSH levels compared to control nestlings in which GSH levels were not manipulated.

METHODS AND MATERIALS

Experimental Procedures. We conducted the experiment in May–June 2013 in a breeding center located in Camarma de Esteruelas (Spain), where white Gyrfalcons are bred in captivity for falconry. Adult birds are inseminated and the hatching of eggs occurs in incubators. We used four nestlings hatched on nearly the same date from eggs from the same breeding pair in the experiment. Two nestlings (Aviornis metal ring numbers 24p7723AE13 and 26p7723AE13, hereafter 24p and 26p, respectively) received the experimental treatment to reduce GSH levels, while two other nestlings (Aviornis metal ring numbers 17p7723AE13 and 20p7723AE13, hereafter 17p and 20p, respectively) served as controls.

We reduced GSH levels with DL-buthionine-(*S,R*)-sulfoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthetase, the enzyme that catalyzes the rate-limiting step in GSH synthesis, in which two of its three constitutive amino acids (glutamate and cysteine) are bonded, thus decreasing GSH levels with no side effects (Griffith 1982, Dizdar et al. 1997, Galván and Alonso-Alvarez 2008). BSO also increases cysteine catabolism, hence decreasing its levels (Griffith 1982, Dizdar et al. 1997), and reacts with intermediates of the melanogenesis pathway, generating more soluble and degradable pigments, thus impairing the synthesis of both eumelanin and pheomelanin (Galván et al. 2014). We administered BSO to experimental young orally in doses of 0.072 g/nestling/day dissolved in 1.5 ml of water, while we only administered 1.5 ml of water to control nestlings. We started the experiment when the young were 9 d old (19 May 2013), and administered the treatment (i.e., BSO or water only) to them daily with the help of a syringe until they were 30 d old. During this period the nestlings were housed in a plastic brooder and fed with quail, chicken, and pigeon. Then we introduced the four nestlings into the breeding chamber, where they were reared by their biological parents. During this period, the nestlings did not get any treatment. When the birds were 52 d old and they had completely developed plumage (1 July), we photographed the back and breast of birds and plucked two feathers from each part for analyses of pigments. Additionally, we analyzed one back feather with rusty coloration of one untreated juvenile Peregrine Falcon from the same breeding center.

Analysis of Melanins. The feathers were analyzed by Raman spectroscopy to identify and quantify melanins following Galván et al. (2013). This technique consists of detecting Raman signal from melanins by directly analyzing the pigmented tissues, so it does not require destruction of samples nor chemical decomposition of the melanins. The two main forms of melanin (i.e., pheomelanin and eumelanin) have distinctive Raman bands, so the identification of melanins can be done by determining the shape of the Raman spectra: pheomelanin has a large band about 1490 cm^{-1} and two smaller bands about 500 and 2000 cm^{-1} and eumelanin has two close bands about 1380 and 1580 cm^{-1} (similar to D and G bands, respectively, characteristic of disordered graphite) and a smaller band about 500 cm^{-1} (Galván et al. 2013). We used a Thermo Fisher DXR confocal dispersive Raman microscope (Thermo Fisher Scientific, Madison, Wisconsin, U.S.A.) operating in the Museo Nacional de Ciencias Naturales (MNCN-CSIC, Madrid, Spain) with a point-and-shoot Raman capability of 1-μm spatial resolution and using an excitation laser source at 780 nm of 1 mW power. The single spectra were obtained using a 100× confocal objective, a slit aperture of 50 μm and a grating of 400 lines/mm. These conditions produced an average spectral resolution of 2.2–4.4 cm^{-1} in the wavenumber range of 100–2500 cm^{-1}. An integration time of 2 s × 16 accumulations allowed getting an acceptable SNR (2.13). The system was operated with Thermo Fisher OMNIC 8.1 software. Calibration and aligning of the spectrograph were checked using pure polystyrene.

The Raman beam was focused at four barbs and four barbules of each feather, chosen at random. The back feathers of juvenile falcons exhibit a pattern of dark and light patches, with the rusty coloration present in the latter. Thus, we restricted the analyses of back feathers to the light patches where the rusty coloration appears. Two light patches were analyzed in each back feather (i.e., four barbs and four barbules of each light patch). The breast feathers of juvenile falcons exhibit a single dark drop-like patch surrounded by light color. We restricted the analyses of breast feathers to the dark patch. A single spectrum was obtained from each of these structures, which was either pheomelanin-based or eumelanin-based. A total of 192 Raman spectra were collected from the 16 Gyrfalcon feathers, and 8 Raman spectra from one Peregrine Falcon feather. From each spectrum we determined the intensity of the second Raman band (Y2), i.e., the band about 1490 cm^{-1} in pheomelanin spectra or about 1380 cm^{-1} in eumelanin spectra, as a measure of the concentration of melanins in feathers that is valid for comparative purposes. Y2 is the parameter that is most strongly

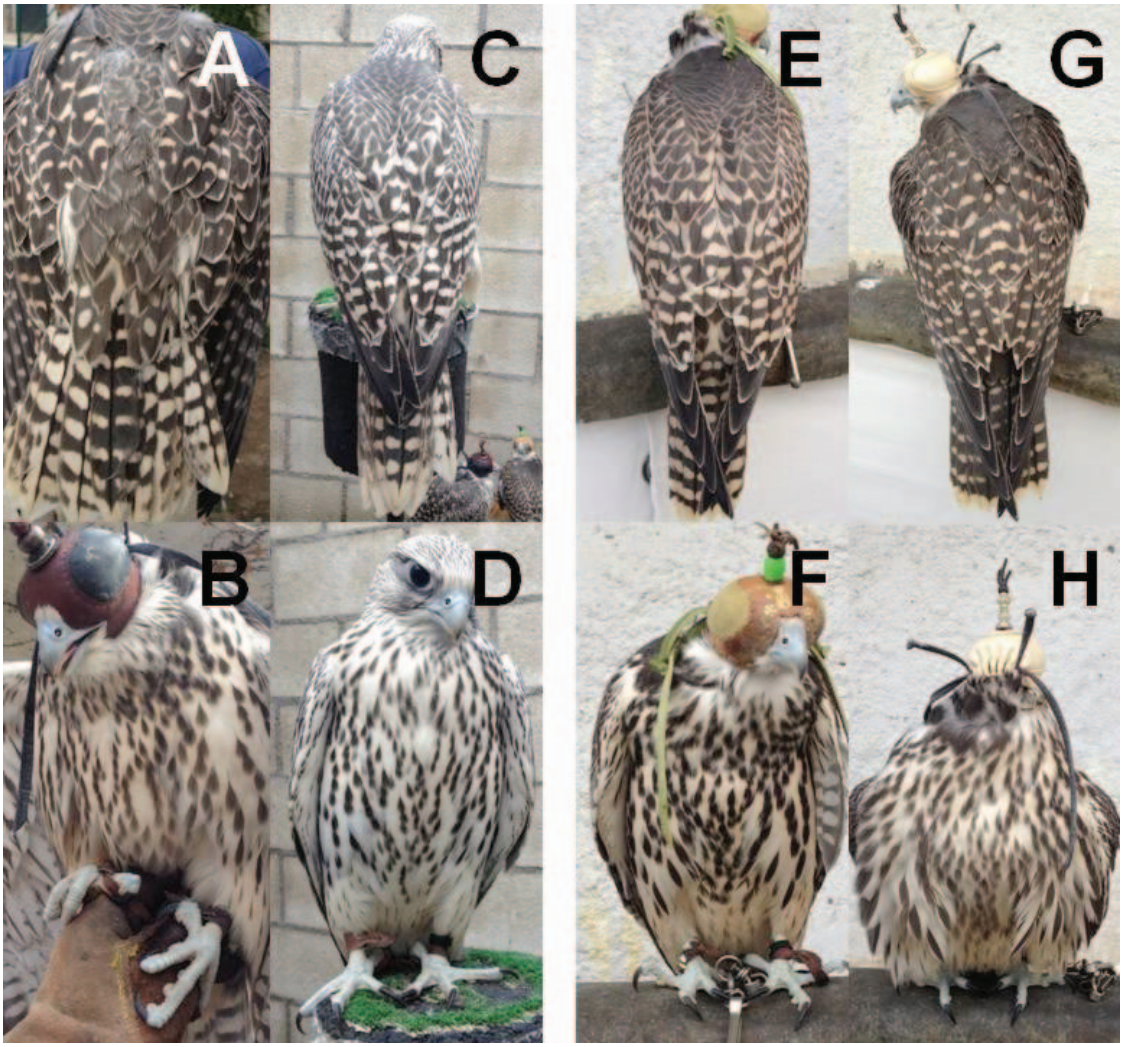

Figure 1. Photos of backs and breast of the four Gyrfalcons used in the experiment. Left panel: BSO-treated birds. Right panel: control birds. A-B: bird 24p, C-D: bird 26p, E-F: bird 17p, G-H: bird 20p.

associated with the melanin content measured by HPLC and the most repeatable parameter within feathers (Galván et al. 2013). For each bird we calculated the average Y2 for pheomelanin and eumelanin. We analyzed the Raman spectra with ORIGIN v.7 software (OriginLab Corporation, Northampton, Massachusetts, U.S.A.).

RESULTS

When the plumage of Gyrfalcons was completely developed, a difference in the color of back feathers was clearly visible (Fig. 1). Although the lighter areas of the back feathers of control birds were rusty, those of the birds that were treated with BSO were paler, with a more whitish color, particularly in the case of the bird 26p (Fig. 1). We detected the Raman signal only from pheomelanin in the back feathers, indicating that the rusty color of juvenile Gyrfalcons is produced by this pigment (Fig. 2A). The analyses of the concentration of pheomelanin in the back feathers confirmed the perceived differences in color, as the average values of Y2 were lower for BSO-treated birds (27.16 and 6.56 for birds 24p and 26p, respectively; mean = 18.86) than for control birds (20.80 and 31.34 for birds 17p and 20p, respectively; mean = 26.07); how-

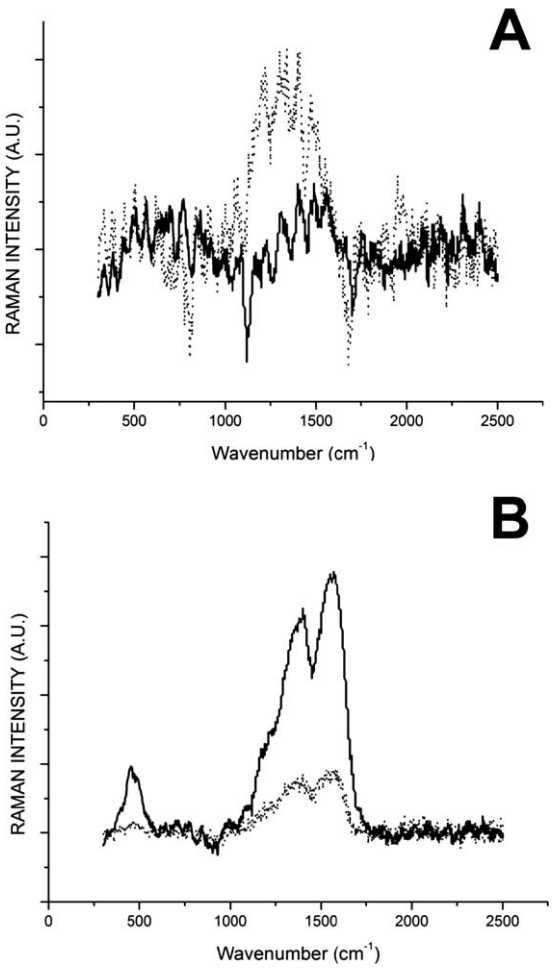

Figure 2. Examples of Raman spectra of (A) pheomelanin and (B) eumelanin from a juvenile Gyrfalcon (bird 17p, solid curves) and a juvenile Peregrine Falcon (dashed curves).

ever, variability within treatments was high and range of Y2 values overlapped between treatments.

The Raman signal from the dark drop-like patches of the breast feathers was attributed to eumelanin (Fig. 2B). We did not visually detect a difference in the color of these feathers between BSO-treated and control birds (Fig. 1), although the analyses of Y2 indicated that the concentration of eumelanin in these feathers was higher overall in BSO-treated birds (201.30 and 257.41 for birds 24p and 26p, respectively; mean = 229.36) than in control birds (183.82 and 215.34 for birds 17p and 20p, respectively; mean = 199.58); again, variability was high. We did not observe any anomalies in the body condition or health of the birds treated with BSO, neither during the treatment nor 6 mo after the experiment, suggesting that BSO did not cause any significant side effects on birds.

We detected the Raman signal only from pheomelanin in the light part of the dorsal feather of the Peregrine Falcon (Fig. 2A), which suggested that the rusty plumage coloration of juvenile birds is also generated by pheomelanin in this species. The dark patch of the same feather generated the Raman signal from eumelanin only (Fig. 2B).

DISCUSSION

Our study supported the assumption that the rusty plumage coloration exhibited by juvenile Gyrfalcons is generated by the pigment pheomelanin, while the dark patches of plumage are generated by eumelanin, although sample sizes were small and variability great. This was the expected finding because, to our knowledge, no pigments other than melanins have been described in the feathers of diurnal raptors, although this is the first time that a detailed chemical analysis to differentiate between the two main types of melanin (i.e., pheomelanin and eumelanin) has been made. Another class of pigments, porphyrins, has been identified in the feathers of juvenile Black-winged Kites, which exhibit a rusty coloration to which pheomelanin also contributes (Negro et al. 2009). Iron oxide from exogenous origin (acquired during mud-bathing) has also been identified in the breast feathers of adult Bearded Vultures (*Gypaetus barbatus*) exhibiting rusty coloration (Toral et al. 2008). Porphyrins and iron oxide are rare pigments, found thus far only in the species mentioned above (which interestingly have some common ecological attributes; Negro et al. 2009). The rusty coloration we investigated in juvenile Gyrfalcons is observed in most species of large typical falcons (genus *Falco*; Ferguson-Lees and Christie 2001); indeed, we also found that the rusty coloration of a juvenile Peregrine Falcon was generated by pheomelanin. It is therefore reasonable to speculate that the rusty coloration observed in the juvenile plumage of other falcon species is also due to the presence of pheomelanin.

As mentioned above, the rusty plumage coloration is present in many species of large falcons, but there is age-related variation in its expression across species. Although some species typically exhibit this coloration only before the first molt (e.g., the Prairie Falcon [*Falco mexicanus*] and the Gyrfalcon; Ferguson-Lees and Christie 2001), other species

exhibit this coloration during adulthood, when its expression is even more intense than in juveniles (e.g., the Eurasian Hobby [*F. subbuteo*]; Toral et al. 2008). This variation is also observed intraspecifically in the Peregrine Falcon, in which some subspecies (e.g., *F. p. peregrinator*) exhibit intense rusty coloration during adulthood whereas others exhibit this color only as juveniles (e.g., *F. p. peregrinus*; Ferguson-Lees and Christie 2001). Rusty plumage coloration is also exhibited by juvenile birds of several other groups of raptors such as Accipiters (e.g., the Northern Goshawk) and eagles (e.g., Spanish Imperial Eagle [*Aquila adalberti*]; Ferguson-Lees and Christie 2001). Interestingly, the age-related variation in the expression of rusty plumage coloration is also observed in these other groups of raptors. For example, in some eagle species, juveniles are rusty and become darker with age (e.g., the Spanish Imperial Eagle; Galván et al. 2012a) and in others, the opposite is observed (e.g., the Golden Eagle [*Aquila chrysaetos*]). Therefore, the presence of rusty plumage coloration and the age-related variation in its expression is not limited to falcons but may be common among diurnal raptors.

The results of our experiment with BSO in Gyrfalcons may constitute important baseline data for increasing our understanding of inter- and intraspecific variation in the expression of rusty plumage coloration in diurnal raptors. Although the questions of why rusty coloration has evolved in some species and why the age-related variation in its expression differs among and within species will have to be addressed in future studies, we have here confirmed that the rusty plumage coloration is generated by pheomelanin and its expression can be experimentally modified with a specific inhibitor of GSH synthesis. Thus, the expression of rusty plumage coloration may be affected by the levels of environmental oxidative stress. Indeed, pheomelanin may have evolved because its synthesis represents a consumption of cysteine, which may be adaptive when cysteine is in excess (probably under low levels of environmental oxidative stress) as very large amounts of this amino acid can be toxic (Galván et al. 2012b). By contrast, pheomelanin production may represent a physiological cost under high levels of environmental oxidative stress, as cysteine is required for antioxidant protection as part of GSH in such conditions (Galván et al. 2012b). Therefore, differential exposure to environmental factors that generate oxidative stress (e.g., extreme temperature or parasites) may explain the interspecific variation in the expression of rusty plumage coloration in diurnal raptors. Because senescence is a process in which endogenous oxidative stress increases with age, accordingly affecting the synthesis of pheomelanin (Galván and Møller 2009, Galván et al. 2012c), differences in environmental factors to which the birds are exposed during ontogeny may also help explain why the expression of rusty plumage coloration changes with age differentially across species and subspecies in diurnal raptors.

Lastly, the plumage color variation in adult Gyrfalcons, which is due to differences in the extent of black and gray colors, is explained by allelic variation at the MC1R gene (Johnson et al. 2012, Zhan et al. 2012). As black and gray colors are geneated by eumelanin (Galván et al. 2012a) and the expression of these colors also depends on GSH levels (Galván and Alonso-Alvarez 2008), our study opens the possibility that the plumage color of adult Gyrfalcons is affected by environmental oxidative stress, which may help to fix certain alleles under the same environmental conditions and thus explain why different plumage color types are geographically segregated (Potapov and Sale 2005) and this coincides with segregation of MC1R alleles (Johnson et al. 2012). Future studies should explore these possibilities.

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