

Eumelanin- and pheomelanin-based colour advertise resistance to oxidative stress in opposite ways

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Abstract

The control mechanisms and information content of melanin-based colourations are still debated among evolutionary biologists. Recent hypotheses contend that molecules involved in melanogenesis alter other physiological processes, thereby generating covariation between melanin-based colouration and other phenotypic attributes. Interestingly, several molecules such as agouti and glutathione that trigger the production of reddish-brown pheomelanin have an inhibitory effect on the production of black/grey eumelanin, whereas other hormones, such as melanocortins, have the opposite effect. We therefore propose the hypothesis that phenotypic traits positively correlated with the degree of eumelanin-based colouration may be negatively correlated with the degree of pheomelanin-based colouration, or vice versa. Given the role played by the melanocortin system and glutathione on melanogenesis and resistance to oxidative stress, we examined the prediction that resistance to oxidative stress is positively correlated with the degree of black colouration but negatively with the degree of reddish colouration. Using the barn owl (*Tyto alba*) as a model organism, we swapped eggs between randomly chosen nests to allocate genotypes randomly among environments and then we measured resistance to oxidative stress using the KRL assay in nestlings raised by foster parents. As predicted, the degree of black and reddish pigmentations was positively and negatively correlated, respectively, with resistance to oxidative stress. Our results reveal that eumelanin- and pheomelanin-based colourations can be redundant signals of resistance to oxidative stress.

Introduction

Melanins are the most common pigments in animal integuments, and they serve as the background body colour in many taxa. Apart from their role in predator avoidance (e.g. camouflage) and physiological processes such as thermoregulation, melanic traits are also displayed during social interactions in several species, suggesting that they act as social or sexual signals of quality. Following Zahavi's handicap principle (1975), evolutionary biologists have often assumed that if melanic traits honestly reveal quality, their expression

must be costly and expressed in proportion to the phenotypic condition of the bearer. In line with this latter assumption, the expression of melanic traits can be influenced by ectoparasitism (e.g. Fitze & Richner, 2002), food supply (Fargallo *et al.*, 2007) and various sources of stress (Roulin *et al.*, 2008a). Therefore, positive correlations between the degree of melanin-based colouration and individual quality observed in wild populations may result from an evolutionary process consistent with the handicap principle. Interestingly, however, several studies have shown high heritability (e.g. Norris, 1993; review in Roulin, 2004a) and weak condition-dependent expression of melanic traits in various organisms (e.g. Roulin & Dijkstra, 2003; Bize *et al.*, 2006). These results allow for the possibility that covariations between the degree of melanism and aspects of individual quality result from pleiotropy.

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Associations between brown to black eumelanin pigmentation and physiology may arise from the pleiotropic effects of key regulators of melanogenesis (i.e. a few hormones affecting multiple traits). In vertebrates, the very well-conserved melanocortin system comprises the proopiomelanocortin gene (*POMC*) encoding the melanocortins (melanin-stimulating hormones α - and β -MSHs and the adrenocorticotrophin hormone ACTH) that bind to five melanocortin receptors (MC1-5Rs) and the agouti signalling protein (ASIP) that binds to a subset of the same receptors (see Fig. 3 in Ducrest *et al.*, 2008). The melanocortins and ASIP are among the prime regulators of the amount and type of melanin pigments synthesized (Slominski *et al.*, 2004). Binding of the melanocortins to MC1R triggers the synthesis of brown to black eumelanin, whereas the binding of ASIP to MC1R results in the production of yellow to reddish-brown pheomelanin (Prota, 1992; see Fig. 2 in Ducrest *et al.*, 2008). In addition to MC1R, melanocortins and ASIP bind to four other melanocortin receptors (MC2-5R), each involved in the regulation of several physiological processes including sexual behaviour, aggressiveness, exocrine gland activity, hypothalamic-pituitary-adrenal stress response, immune function, energy homeostasis and resistance to oxidative stress. The antagonist ASIP has the opposite pharmacological effect of the melanocortin agonists, and thus, we predict that eumelanin- and pheomelanin-based colouration is correlated in opposite ways with the latter phenotypic traits. Because glutathione, which is an intracellular oxidant, inhibits eumelanogenesis while triggering pheomelanogenesis (Galván & Alonso-Alvarez, 2008), we can propose a similar prediction for resistance to oxidative stress. Thus, whatever the exact underlying physiological mechanism, we predict eumelanin- and pheomelanin-based colouration to be inversely correlated with resistance to oxidative stress (e.g. the degree of eumelanin is positively correlated with resistance and pheomelanin is negatively correlated).

Although many animals vary in both black/grey eumelanin colouration and reddish-brown pheomelanin colouration and these traits are often used as signals (Jawor & Breitwisch, 2003), very few researchers have studied these two types of traits within individuals (e.g. Vergara & Fargallo, 2011). This is unfortunate because in many species, variation in colouration is related to the ratio of deposited eumelanin to pheomelanin pigments and varies from dark eumelanin to dark pheomelanin (Walker & Gunn, 2010). Even more interesting are those species for which the degree of darkness of one trait is primarily because of variation in the deposition of eumelanin, whereas the degree of darkness of another trait is primarily because of variation in the deposition of pheomelanin. In such species, it would be particularly interesting to investigate whether different eumelanin- and pheomelanin-based traits are associated with different phenotypic traits (multiple messages) or whether they are back-up signals of the same phenotypic attribute

(Candolin, 2003). Selection could promote the evolution of back-up signals in order to provide more accurate information about a specific aspect of individual condition. Alternatively, different traits could be selected to signal alternative attributes, which would allow females to select the best males with respect to a large number of phenotypic or genotypic qualities. Thus, based on the knowledge of the melanocortin system and the intracellular antioxidant glutathione, our aim here was to determine how resistance to oxidative stress covaries within individuals with both eumelanin- and pheomelanin-based colouration.

To this end, we considered the barn owl as a model organism. This bird is well suited for the purpose of the intended study because individuals from the same population vary from immaculate to heavily marked with eumelanin black spots of varying size and from white to reddish-brown, a pheomelanin-based trait (Roulin *et al.*, 2008a). The size of black spots is sexually antagonistically selected (Roulin *et al.*, 2010, 2011a) and a criterion in male mate choice (Roulin, 1999; Roulin *et al.*, 2010). To disentangle origin related (genetic and/or maternal effects) from environmental/parental influences on the covariation between plumage traits and resistance to oxidative stress, we considered nestling barn owls that were previously swapped between randomly chosen nests. Because the two melanin traits are highly heritable and their expression is weakly sensitive to environmental quality and body condition (Roulin *et al.*, 1998, 2010; Roulin & Dijkstra, 2003), any covariation between resistance to oxidative stress measured in the cross-fostered nestlings and plumage traits measured in the nestlings themselves or in their biological parents may be genetically determined.

Materials and methods

The barn owl is a medium-sized bird with adult females weighing between 264 and 515 g (mean \pm SE: 367 \pm 0.8 g) and males between 241 and 380 g (290 \pm 1.6 g). Small mammals form the bulk of the diet. Incubation starts as soon as the first egg is laid and additional eggs are added every 2–3 days generating a pronounced within-brood age hierarchy. In 2006, the number of breeding pairs was the smallest in our Swiss study area for the last 20 years. Considering the 18 broods used in this study, clutches were laid between 23 April and 25 May (7 May \pm 2 days), included 4–9 eggs (6.1 \pm 0.3 eggs), and mean brood size at fledging was small with only 3.6 \pm 0.3 offspring per breeding pair. Nestlings leave their nest at *ca.* 55 days of age. Nestling sex was determined using molecular markers (Py *et al.*, 2006).

Assessment of plumage traits

A. Roulin measured eumelanin- and pheomelanin-based plumage traits in 66 nestlings and their parents on the

breast, belly, flanks and underside of the wings. Pheomelanin-based plumage colouration was compared with eight colour chips with 1 for dark reddish-brown to 8 for white, and the mean value of all body parts was used in the statistical analyses. The resulting plumage colour score is associated with the amount of pheomelanin pigments packed in feathers (Roulin *et al.*, 2008a). The negative correlation between reddish colouration and the pheomelanin/eumelanin ratio (Pearson's correlation: $r = -0.81$, $n = 10$, $P = 0.005$; same data as in Roulin *et al.*, 2008a) indicates that the production of a darker reddish plumage necessitates a disproportionately larger amount of pheomelanin than eumelanin. For this study, we multiplied the index of reddish colouration by -1 in order to have a scale from pale to dark reddish pigmentation (we did not use this methodology in previous studies). A 60×40 mm frame was placed on the same four body parts within which black eumelanin spots were counted and their diameter measured using a calliper to the nearest 0.1 mm. Number of spots and spot diameters found on the two flanks were averaged, as well as values found on the two wings. The mean of the four body parts was used in the statistical analyses. Methods of assessing plumage traits are reliable (Roulin, 1999, 2004b).

Nestling resistance to oxidative stress

In 2006, we matched 18 nests in pairs with the criterion that eggs were laid on a similar date. As soon as the first egg had been laid, we visited nests at night every 3 days to mark each egg with a number corresponding to its laying order. Ten days later, we exchanged as many eggs as possible between pairs of nests without altering clutch size. For instance, when nests of the same pair contained four and six eggs, respectively, we exchanged the four eggs first laid from the nest with six eggs against the four eggs from the other nest. In total, we swapped 106 eggs. Around hatching, we visited broods every 3 days at night to assign each nestling to the egg it hatched from. We recognized nestlings individually by cutting off different combinations of claw tips before they were large enough to be ringed with a numbered aluminium band. Biological and foster parents did not resemble each other with respect to pheomelanin-based colouration (four Pearson's correlations, P -values > 0.54), number of spots (P -values > 0.09) and spot diameter (P -values > 0.56). Females displaying more black spots bred earlier in the season ($r = -0.50$, $n = 17$, $P = 0.038$) but because laying date was not associated with nestling resistance to oxidative stress (P -value > 0.44), this variable cannot confound any covariation between plumage traits and resistance to oxidative stress. A parallel study aimed at investigating the effect of the stress hormone corticosterone on innate and humoral components of immunity in barn owls used the same experimental broods as ours (Stier *et al.*, 2009). However, because these corticosterone and vaccination treatments were assigned randomly

with respect to plumage nestling traits (mixed model ANOVA with the three nestling plumage traits as dependent variable in three separate analyses and treatment as factor, P -values > 0.15), they cannot confound our interpretations.

We measured resistance to oxidative stress as the time needed to hemolyse 50% of the red blood cells ($T_{1/2}$ expressed in minutes) exposed to a controlled attack by reactive oxygen species (ROS) using the KRL[®] bioassay (Brevet Spiral V02023, Courernon, France). A quick lysis of red blood cells indicates low resistance of the red cell membrane to ROS attack. Resistance of the red blood cell membrane is shaped by membrane lipid composition and past exposure of unsaturated lipid bonds to oxidative stress (Brzezinska-Slebodzinska, 2001) as well as by levels of intra- and extracellular antioxidant defences (i.e. antioxidant compounds and enzymes) against ROS (Lesgards *et al.*, 2002). Thus, our measurement of resistance to oxidative stress is likely to be influenced by genetic factors (i.e. membrane composition and antioxidant enzymes) and environmental factors (i.e. dietary antioxidants and past exposure of the cell membrane to ROS). The high resistance of red blood cells to oxidative stress of some individuals compared with others can therefore reflect the aspects of individual quality. The KRL test provides an integrative measure of the balance between defence against oxidative stress and the amount of ROS. We can therefore consider this measure as a surrogate of resistance to ROS given that we assess the speed of red cell lysis. Although the KRL assay is carried out *in vitro*, it reflects the aspects of *in vivo* resistance to oxidative stress. Numerous studies have indeed shown that resistance to oxidative stress, as measured by the KRL assay, is genetically associated with senescence (Kim *et al.*, 2010) and many life-history traits as expected from theory (e.g. Bize *et al.*, 2008; Alonso-Alvarez *et al.*, 2006).

To measure red blood cell resistance to oxidative stress, 16 μL of blood was immediately diluted in 584 μL of KRL buffer adjusted to avian cell osmolarity. Samples were stored at 4 °C in the field before being analysed in the laboratory, which occurred on average 18.6 (SD = 12.0) hours later (range: 2–48 h). In previous studies on the same topics (e.g. Alonso-Alvarez *et al.*, 2007), samples are analysed within 24 h after collection, but in our case we could not analyse all samples within 24 h because the field station is 50–100 km away from the university and we were often working in the field from the morning to midnight. For this reason, we considered only the 167 of 331 samples analysed within 24 h; we thus had data on 66 cross-fostered nestlings from 18 origins and from the 76 initial nestlings from 20 origins. Note, however, that if we consider all blood samples and control statistically for the time span between blood collection and laboratory analysis (see also Bize *et al.*, 2008), we obtain qualitatively similar results. We incubated 90 μL of KRL-diluted blood at 40 °C and submitted it to a controlled ROS attack by adding a solution of 150 mmol of 2,

2'-azobis-(aminodinopropane) hydrochloride diluted in 153 μL of KRL buffer. The time required to lyse 50% of the red blood cells was assessed with a microplate reader device that follows the decrease in optical density at the wavelength of 540 nm; values were Box-cox-transformed to normalize the data set. Samples were run in duplicates or triplicates (repeatability computed on all 364 samples \pm SE = $97.9 \pm 0.3\%$; $F_{363,681} = 135.13$, $P < 0.0001$), and mean values were calculated for the analyses.

Each individual was sampled between 1 and 5 times (2.5 ± 0.1) 1–35 days apart (mean \pm SE number of days between the first and last sample: 13.2 ± 1.4). The mean age at which siblings were sampled (31.3 ± 0.8 days; range: 14–61) was not significantly correlated with plumage traits measured in the nestlings themselves or in their biological parents (Pearson's correlations: all 9 P -values > 0.32) and not with mean resistance to oxidative stress ($r = 0.20$, $n = 66$, $P = 0.11$). Mean sampling date and brood size were also not associated with plumage traits (Pearson's correlations: all 18 P -values > 0.10) with the exception of nestlings being sampled earlier in the season when their biological mother displayed more black spots ($r = -0.57$, $n = 19$, $P = 0.017$).

Statistics

Any association between nestling plumage traits and resistance to oxidative stress could be caused by a within-nest effect (e.g. dark reddish-brown nestlings within a nest have lower resistance to oxidative stress than paler siblings) or a between-nest effect (e.g. families with dark reddish-brown nestlings on average have a lower resistance to oxidative stress than families with paler nestlings). To distinguish within- from between-nest effects, we used the technique of 'within-group centring' (van de Pol & Wright, 2009) and derived two new colour variables from each nestling melanin trait. A first variable that expresses only the within-nest variance component was computed by subtracting the mean value of a given melanin trait expressed by all related siblings raised by foster parents from each nestling value (e.g. nestling pheomelanin-based colouration minus the mean colouration of all siblings raised in the same foster nest, hereafter referred to as nestling colour deviation). A second variable that expresses only the between-nest variance component was given by the mean value calculated from all related siblings raised in a foster nest. All analyses were carried out with the software JMP (Sall & Lehman, 1996) and were two-tailed, and P -values smaller than 0.05 were considered significant. Means are quoted \pm SE.

Results

The time required to lyse 50% of the red blood cells (i.e. a proxy of resistance to oxidative stress) was on average

57 ± 0.4 min (range: 47.2 and 76.0 min). Resistance to oxidative stress measured on 2.5 ± 0.1 occasions within the same individuals was significantly repeatable ($r = 0.31 \pm 0.07$; $F_{65,101} = 3.28$, $P < 0.0001$).

Mean individual resistance to oxidative stress was associated with mean siblings' pheomelanin-based colouration (mixed model ANOVA with nest of origin as random variable: $F_{1,14.94} = 12.20$, $P = 0.0033$) and mean siblings' spot diameter (same model: $F_{1,14.47} = 13.36$, $P = 0.0025$) but not with the deviation of nestling plumage traits from the mean siblings' values (same model: pheomelanin: $F_{1,43.82} = 1.38$, $P = 0.24$; eumelanin: $F_{1,43.82} = 2.71$, $P = 0.11$; nestling sex was not significant and hence removed from the final analysis). Thus, variation in resistance to oxidative stress was related to between-nest variation in plumage traits but not to within-nest variation in plumage traits. For this reason, we calculated mean siblings' values after having removed variation in plumage traits explained by sex using one-way ANOVAs with each plumage trait as dependent variable in a separate model and sex as a factor. Using mean sibling values as the unit of statistical analysis, a stepwise multiple regression analysis showed that nestlings displaying a paler reddish plumage (first variable: $F_{1,15} = 10.42$, $P = 0.0056$; Fig. 1a) and larger black spots were more resistant to oxidative stress (same model, second variable: $F_{1,15} = 13.72$, $P = 0.0021$; Fig. 1b); date ($F_{1,11} = 0.08$, $P = 0.79$), mean siblings' number of spots ($F_{1,12} = 1.07$, $P = 0.32$), mean nestling mass ($F_{1,13} = 2.76$, $P = 0.12$) and mean nestling age ($F_{1,14} = 2.39$, $P = 0.14$) were not significant. Note that residual spot diameter was still significantly associated with resistance to oxidative stress if we do not include residual pheomelanin-based colouration in the model (Pearson's correlation: $r = 0.46$, $n = 18$ nests, $P = 0.05$); in contrast, pheomelanin-based colouration was no longer significant if not controlling for eumelanin-based colouration ($r = -0.40$, $n = 18$ nests, $P = 0.10$).

If we replaced the plumage traits of nestlings with those of their biological parents, the relationships between resistance to oxidative stress, reddish colouration and spot diameter disappeared (multiple regression analysis: P -values > 0.12). This also occurred if we used the plumage traits of foster parents (another multiple regression analysis: P -values > 0.18).

Discussion

Adaptive function of the covariation between melanin colouration and resistance to oxidative stress

Most of the studies on the adaptive function of melanin-based colour traits have concerned species with inter-individual variation in the degree of eumelanin colour but more rarely of pheomelanin colour. In this context, the barn owl is particularly interesting because plumage varies with respect to both pigment types. Previous

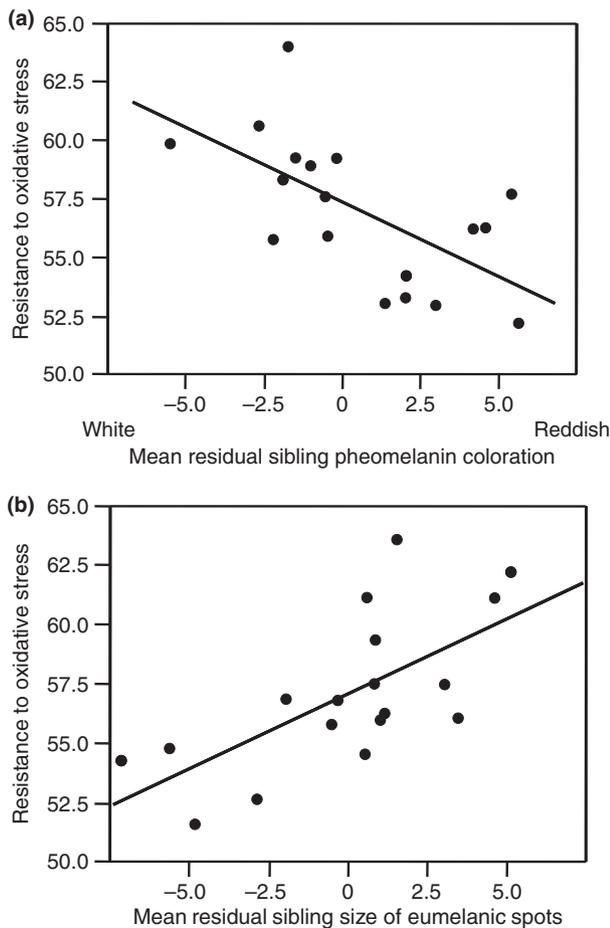


Fig. 1 Resistance to oxidative stress in relation to pheomelanin-based reddish colouration (a) and eumelanin-based black colouration (b). Data points are least squares extracted from a multiple regression analysis with mean siblings' resistance to oxidative stress as dependent variable and the two mean siblings' plumage traits as independent variables.

studies have shown that the degree of pheomelanin reddish colour is associated with variation in nestling body mass (Roulin *et al.*, 2008b), whereas eumelanin colour is correlated with immunocompetence (Roulin *et al.*, 2000, 2001) and the ability to cope with stressful factors that induce a glucocorticoid response (Schwabl, 1995; Kittilsen *et al.*, 2009; Almasi *et al.*, 2010). The present study shows that, at least with respect to resistance to oxidative stress, eumelanin- and pheomelanin-based colour traits can be redundant signals of resistance to oxidative stress. This finding has several implications on the coevolution of these two colour traits, as darker reddish owls commonly display larger black spots than paler reddish individuals (Roulin, 2004b). Depending on whether they are selected to signal the same or different qualities, the sign and

magnitude of the genetic correlation between the two traits could thus evolve. For instance, in populations where the two plumage traits signal similar qualities, their correlation may be stronger than in populations where the two traits are selected to signal different phenotypic attributes. This issue will be tackled using museum skin specimens collected worldwide.

Several ecological factors and life-history traits are known to increase oxidative stress and its sensitivity. This includes reproductive and physical activities, immune challenges, age and UV light (Costantini, 2008). Considering these factors, it is important to examine whether oxidative stress could be a major factor promoting the evolution of melanin-based colouration as suggested for darker skin colouration in humans in regions where UV light is intense (Jablonski & Chaplin, 2010). Ample geographic variation in the degree of both eumelanin- and pheomelanin-based colouration has been reported in the barn owl (Roulin *et al.*, 2009; Roulin & Salamin, 2010), allowing us to discuss the potential importance of oxidative stress in the evolution of these colour traits. Because UV light and parasites are more prevalent in the tropics than in the temperate zones (Guernier *et al.*, 2004), we should expect plumage to be marked with larger black spots and to be lighter pheomelanin in barn owl species living in the tropics than in the temperate zones, if oxidative stress is the most important factor underlying the evolution of melanin-based colouration in this species. However, whereas barn owls indeed exhibit larger spots closer to the equator, at least in the northern hemisphere, variation in pheomelanin-based colouration was not associated with latitude (Roulin *et al.*, 2009). We thus suggest that oxidative stress may participate in the evolution of melanin-based colouration in the barn owl but it might not be the most important factor. Further studies are needed to evaluate this proposition.

Proximate mechanism underlying the covariation between melanin colouration and resistance to oxidative stress

As predicted by the model of Ducrest *et al.* (2008), we found that darker eumelanin nestlings (i.e. with larger black spots) had red blood cells more resistant to oxidative stress than their lighter counterparts. Assuming that variation in the degree of eumelanin pigmentation is in part attributed to variation in melanocortin levels, two nonmutually exclusive explanations may account for the beneficial effects of producing more melanocortins on resistance to oxidative stress. First, melanocortins may reduce the production of ROS within red blood cells following hydrochloride-induced oxidative stress. In line with this possibility, α -MSH reduces the generation of hydrogen peroxide (H_2O_2 ; a ROS) in UV-irradiated melanocytes through its binding to MC1R (Kadekaro *et al.*, 2005). Given that in vertebrates melanocortins

such as α -MSH are present in the blood stream (Catania & Lipton, 1993; and in the tawny owl *Strix aluco*, Roulin *et al.*, 2011b), our results suggest that nestlings with larger black spots have higher blood melanocortin levels that limit the production of ROS in cells submitted to an oxidative stress. Second, melanocortins may stimulate or enhance antioxidant defences within red blood cells following the oxidative attack. In apparent contrast with this hypothesis, α -MSH has been found to inhibit glutathione peroxidase in H₂O₂-stimulated keratinocyte and melanoma cell lines (Haycock *et al.*, 2000). Glutathione peroxidase is a family of antioxidant enzymes of primary importance for protecting cells and tissues against ROS (Nagata *et al.*, 2007). The data suggest that α -MSH reduces the amount of peroxide in the H₂O₂-stimulated cells by inducing a cellular mechanism that actively removes ROS. Hence, α -MSH probably inhibits glutathione peroxidase by reducing the availability of the enzyme's substrate (i.e. peroxide) and not by direct enzyme inhibition. Thus, assuming that melanocortins account for the relationship between melanin-based colouration and resistance to oxidative stress, this would be due to a reduced production (or improved intracellular removing) of ROS rather than to an increased resistance to ROS.

To our knowledge, no study in cellular biology has yet investigated the effect of ASIP (or a structurally related molecule such as agouti-related protein) on the regulation of cell redox state. This potential effect remains, however, to be established to determine whether the levels of melanocortin antagonists and inverse agonists can explain covariation between the degree of pheomelanin-based colouration and resistance to oxidative stress.

Two alternative hypotheses for the pleiotropic effects of melanocortins have recently been put forward to explain the occurrence of correlations between melanin-based colourations and resistance to oxidative stress. First, melanin pigments can act as antioxidants, and thus, individuals producing more pigments should be more resistant to oxidative stress (McGraw, 2005). Even though we agree that this hypothesis can apply in some organisms, it cannot explain our results found in the barn owl because both darker eumelanic and redder pheomelanic individuals should exhibit higher resistance to oxidative stress, a prediction not supported by our present results. Second, low levels of thiols (such as the antioxidant glutathione and cysteine) in melanocytes are required for eumelanogenesis (Ito, 2003), and it has been proposed that darker eumelanic individuals should display higher levels of circulating antioxidants in plasma to compensate for low intracellular levels of glutathione (Galván & Alonso-Alvarez, 2008). This hypothesis can explain why owlets with larger black spots had better blood resistance to oxidative stress. Additional empirical studies are therefore needed to evaluate the roles of this latter mechanism and the pleiotropic effects of melano-

cortins, respectively, on blood resistance to oxidative stress. Thus, it is particularly important to understand why resistance was related to plumage traits measured in the nestlings themselves and not in their parents.

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