Pheomelanin synthesis varies with protein food abundance in developing goshawks

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Abstract

The accumulation of the amino acid cysteine in lysosomes produces toxic substances, which are avoided by a gene (*CTNS*) coding for a transporter that pumps cystine out of lysosomes. Melanosomes are lysosome-related organelles that synthesize melanins, the most widespread pigments in animals. The synthesis of the orange melanin, termed pheomelanin, depends on cysteine levels because the sulfhydryl group is used to form the pigment. Pheomelanin synthesis may therefore be affected by cysteine homeostasis, although this has never been explored in a natural system. As diet is an important source of cysteine, here we indirectly tested for such an effect by searching for an association between food abundance and pheomelanin content of feathers in a wild population of Northern goshawk *Accipiter gentilis*. As predicted on the basis that *CTNS* expression may inhibit pheomelanin synthesis and increase with food abundance as previously found in other strictly carnivorous birds, we found that the feather pheomelanin content in nestling goshawks, but not in adults, decreased as the abundance of prey available to them increased. In contrast, variation in the feather content of the non-sulphurated melanin form (eumelanin) was only explained by sex in both nestlings and adults. We also found that the feather pheomelanin content of nestlings was negatively related to that of their mothers, suggesting a relevant environmental influence on pheomelanin synthesis. Overall, our findings suggest that variation in pheomelanin synthesis may be a side effect of the maintenance of cysteine homeostasis. This may help explaining variability in the expression of pigmented phenotypes.

Keywords: Animal pigmentation · Cysteine homeostasis · Melanogenesis · Phenotypic plasticity · Raptors

Introduction

The regulation of cysteine levels is essential for the physiological performance of organisms. This is due to the double-sided nature of this amino acid. On one hand, cysteine is a key component of protein structure (Giles et al. 2003), and one of the three constituent amino acids of glutathione (GSH), the most important intracellular antioxidant (Wu et al. 2004). On the other hand, when cysteine levels are higher than needed for protein and GSH synthesis, its autooxidation to cystine generates hydrogen peroxide, decreases GSH levels and causes oxidative DNA damage (Viña et al. 1983; Park and Imlay 2003). Thus, maintaining cysteine homeostasis is a vital process that is mainly mediated by the enzyme cysteine dioxygenase (CDO), which catalyzes the addition of molecular oxygen to the sulfhydryl group of cysteine to form less toxic products such as sulfate and taurine (Stipanuk et al. 2006, 2009). The capacity of CDO to maintain cysteine homeostasis is probably limited, however, as exemplified by the fact that a dysfunction in the cystine/H^+ symporter cystinosin (i.e., not in CDO) that exports cystine out of lysosomes causes the intralysosomal accumulation of cystine and a disease called cystinosis (Chiaverini et al. 2012).

Melanosomes are lysosome-related cellular organelles that synthesize melanins, the most abundant pigments in animals. Melanosomes are thus susceptible to accumulation of excess cysteine, with the possible physiological implications that this may have. Intramelanosomal cysteine levels affect the form of melanins that are synthesized, as when cysteine levels are above a certain threshold, the sulfhydryl group of the amino acid is incorporated into the resulting pigment, which is then termed pheomelanin (García-Borrón and Olivares Sánchez 2011). Therefore, any changes in intramelanosomal cysteine levels produced as a consequence of the cellular maintenance of cysteine homeostasis can affect the amounts of pheomelanin that is synthesized.

The cystine/H^+ symporter cystinosin is encoded by the gene *CTNS*, whose expression thus contributes to the avoidance of cysteine accumulation in lysosomes, including melanosomes (Chiaverini et al. 2012). Therefore, *CTNS* expression may also affect

pheomelanin synthesis and pigmentation, though it has been suggested that this effect may only occur under high systemic levels of cysteine in the organism (Chiaverini et al. 2012). However, the capacity of pheomelanin synthesis to reflect *CTNS* actitvity, and thus the performance of cysteine homeostasis, remains unexplored. As cysteine is a semi-essential amino acid, diet represents an important source of cysteine for animals (Klasing 1998; Stipanuk et al. 2006). In this regard, a recent study shows that *CTNS* expression in feather melanocytes increases with food abundance in an strict carnivorous bird (the gyrfalcon *Falco rusticolus*), hence having a high protein diet, suggesting an advantageous mechanism to avoid excess cysteine (Galván et al. 2017a). Nevertheless, possible consequences of this mechanism on pheomelanin synthesis have not been investigated. An association between *CTNS* activity and pheomelanin synthesis would however be highly relevant for the determination of the appearance of organisms, as pheomelanin confers orange and light brown colorations to the integumentary structures (skin, hairs and feathers), where it is deposited after its synthesis (Galván and Wakamatsu 2016).

Here we indirectly test for an association between *CTNS* activity and pheomelanin synthesis in a wild population of Northern goshawk *Accipiter gentilis* with different food abundance regimes in breeding territories. The Northern goshawk is a raptorial, strict carnivorous bird (Fig. 1), thus variation in food abundance mainly reflects variation in protein availability in the diet (Klasing 1998). Protein intake, in turn, positively affects overall cysteine levels in the organism (Stipanuk et al. 2009). If raptorial birds are able to modulate *CTNS* expression according to food abundance (Galván et al., 2017a), and *CTNS* expression decreases pheomelanin synthesis as it pumps cystine out of melanosomes (Chiaverini et al. 2012), it is then expected that pheomelanin synthesis decreases with food abundance in raptors such as the Northern goshawk. We therefore tested this prediction by analyzing the pheomelanin content in feathers of both developing (nestling) and adult goshawks by searching for a relationship with prey abundance in the breeding territories. We made the same tests for the feather content of eumelanin, the non-sulphurated form of melanin whose synthesis does

not depend on cysteine (García-Borrón and Olivares Sánchez 2011) and, therefore, constitutes an internal control.

Materials and methods

Study model

The Northern goshawk is the most common large predator of birds in temperate and boreal forests in Europe and North America (Kenward 2006). This species is a resident relying almost exclusively on birds, in particular pigeons, grouse and pheasants as their main prey, although a large number of different species are consumed to some extent (Kenward 2006). There is a high degree of sexual size dimorphism, with females being around a third larger than males. Goshawks use traditional nest sites and nests that can reach more than 600 kg (Møller and Nielsen 2014). Males capture most prey during the breeding season and bring this for consumption to the female who may pass on the food to the offspring. Since males pluck all food items brought to the nest site, it is feasible to collect feathers from habitual prey plucking sites near the nest for later identification of prey items.

Goshawks molt during reproduction, allowing molted feathers to be collected in the immediate surroundings of the nest (Kenward 2006). Females start to capture prey when young emerge, and females likewise transport the prey to the neighborhood of the nest (Kenward 2006). Thus, feathers collected from the neighborhood of the nest provide a reliable sample of prey during the breeding season.

Goshawks have a specific plumage during their first two years of life, allowing breeders in their first year of reproduction to be aged reliably (Kenward 2006). Goshawks have individual specific patterns on the wing and tail feathers that remain stable throughout life (Opdam and Müskens 1976; Kühlapfel and Brune 1995; Nielsen and Drachmann 2003; Kenward 2006; Hoy et al. 2015). This claim is substantiated by capture of individual goshawks at their nests in subsequent years always resulting in a specific female of known identity being the captured adult at that specific nest.

Study sites, feather sampling and numbers of prey

We systematically visited more than 120 localities with nests of goshawks in northern Vendsyssel (57°10'-57°40'N, 9°50'-10°50'E), Denmark, during April-August 2012-2013, as part of a long-term population study since 1977. See Nielsen and Drachmann (2003) for a detailed description of the study areas. Each nest was visited 3-5 times during the breeding season. We recorded 72 occupied territories, but not all the sites produced nestlings because of predation, disturbance, and prosecution, hence accounting for the reduction in sample size to the final 48 nests with nestlings. Mean brood size was 2.9 nestlings (range: 1-6 nestlings).

All nest sites of goshawks were checked during March-August 2012-2013. Once having identified nest locations, all nests were visited when nestlings were 6-42 days old (mean ± SD: 22 ± 14 days). Nestlings were sexed and their wing length measured with a ruler to the nearest mm. A total of 3-4 covert feathers were plucked from the back of each nestling and stored in the dark until analyses. Only nestlings with almost fully developed dorsal plumage (i.e., feathers > 3/4 full length) were sampled for feathers. In total, we sampled for feathers 72 nestlings from 25 nests.

Prey remains of goshawks were systematically collected near the nests during the visits. All nest sites were visited three to five times during the breeding season, and sampling effort can therefore be considered to remain similar across territories. In the analyses, we considered the total number of prey collected in each goshawk territory (mean: 20.8 prey; range: 1-58 prey), as well as only the number of wood pigeons *Columba palumbus*, which is the preferred species prey for the goshawk in our study site (Møller and Nielsen 2007). This is because wood pigeons may exert a stronger influence on pigmentation than occasional prey, as the preferred prey is habitually consumed by goshawks. These analyses were made separately by excluding data on prey not represented by wood pigeons from the linear mixed-effect models (see Statistical analyses below).

Capture of adult goshawks

JTN captured adult goshawks with traps near nest sites during June-July 2012, in total 14 individuals of which nine were females and five males, while only two adult goshawks were captured during June-July 2013 with a similar capture effort and a similar timing of capture attempts relative to the age of nestlings. Adult goshawks were captured in large traps with clap nets placed near the nest site of the goshawks, and to attract the goshawks, live domestic pigeons were placed in a specially protected container with ad libitum food and water. We compared patterns in feathers at nests in subsequent years. If a new pattern emerged, this bird had the pattern of a young bird either 2 or 3 years, and the pattern in such a bird was maintained the rest of the years when this bird was present. These goshawks were sexed and sampled for back covert feathers. In total, we sampled for feathers 15 adults from 13 nests.

Melanin content of plumage

Feathers were analyzed by micro-Raman spectroscopy to determine their relative content of pheomelanin and eumelanin, as these melanin forms exhibit a distinctive Raman signal that can be used to non-invasively identify and quantify them (Galván et al. 2013; Galván and Jorge 2015). We used a Thermo Fisher DXR confocal dispersive Raman microscope (Thermo Fisher Scientific, Madison, WI, USA) with a point-and-shoot Raman capability of 1 μm spatial resolution and using a near-infrared excitation laser of 780 nm. Laser power was set at 2 mW, integration time at 3 s, and number of accumulations at 8. The spectra were obtained using a 50x confocal objective and a slit aperture of 50 μm. The system was operated with Thermo Fisher OMNIC 8.1 software. Calibration and alignment of the spectrograph were checked using pure polystyrene. The feathers were exposed to photobleaching during 0.1 min to remove dirt before the analyses.

A total of 1-2 dorsal feathers were analyzed per bird. In each feather, the laser beam was focused at three barbs and three barbules. Up to two different pigmented areas (dark brown and light brown-orange) can be perceived in the dorsal feathers of goshawks (Fig. 2), and thus the

six Raman spectra were obtained from each of these areas. The two melanin forms show distinctive Raman spectra comprising three diagnostic bands each, located at 500, 1500 and 2000 cm^{-1} in the case of pheomelanin and at 500, 1380 and 1580 cm^{-1} in the case of eumelanin. These usually appear as two clearly distinguishable spectral shapes, and thus a single Raman spectrum can usually be assigned to either pheomelanin or eumelanin (Galván et al. 2013; Galván and Jorge 2015; Wang et al. 2016; Polidori et al. 2017). In the case of goshawk feathers, however, we found that Raman signals from pheomelanin and eumelanin appear mixed in the single spectra obtained from the dark brown areas of feathers, which show all six diagnostic bands of both pheomelanin and eumelanin with some displacement of the 500 cm^{-1} band for each form (Fig. 2). These spectra also show bands that can be assigned to keratin (Hsu et al. 1976). Therefore, we used the six diagnostic bands of pheomelanin and eumelanin and two diagnostic bands of keratin (Fig. 2) to fit Voigt deconvolution functions to the Raman curves to obtain spectral parameters derived from each spectrum. An average Raman spectrum was calculated for the six spectra obtained from each distinctive pigmentation areas of feathers.

In the analyses, we used the intensity (maximum value at the vertical axis) of the three diagnostic bands to estimate the content of eumelanin and pheomelanin (Fig. 2), as the intensity of these bands are the best predictors of both pheomelanin and eumelanin concentration in feathers (Galván et al. 2013). The intensity of these bands incrases with the concentration of pheomelanin and eumelanin in feathers (Galván et al. 2013). The average intensity was calculated for each of the six diagnostic Raman bands from the spectra obtained from different distinctive pigmentation areas of each feather. We used R environment (R core Team 2018) to conduct a Principal Components Analysis (PCA) that summarizes the information in the variability in the intensity of the three Raman bands across birds, separately for pheomelanin and eumelanin bands, and for nestlings and adults. In the analyses, we used the first extracted component (PC1) as an estimator of pheomelanin or eumelanin content in feathers. In nestlings, PC1 explained 62.5 and 67.1 % of variance in the intensity of

pheomelanin and eumelanin Raman bands, respectively, and it was negatively related to the intensity of Raman bands (factor loadings: pheomelanin: 500 cm^{-1} band: -0.60, 1500 cm^{-1} band: -0.53, 2000 cm^{-1} band: -0.60; eumelanin: 500 cm^{-1} band: -0.24, 1380 cm^{-1} band: -0.68, 1580 cm^{-1} band: -0.68). In adults, PC1 explained 61.2 and 80.4 % of variance in the intensity of pheomelanin and eumelanin Raman bands, respectively, and it was negatively related to the intensity of pheomelanin Raman bands (factor loadings: 500 cm^{-1} band: -0.53, 1500 cm^{-1} band: -0.49, 2000 cm^{-1} band: -0.69) and positively related to the intensity of eumelanin Raman bands (500 cm^{-1} band: 0.50, 1380 cm^{-1} band: 0.61, 1580 cm^{-1} band: 0.61). Thus, PC1 scores increase with decreasing pheomelanin and eumelanin content in the feathers of goshawk nestlings. In the feathers of adult goshawks, PC1 scores increase with decreasing pheomelanin content and with increasing eumelanin content. For each bird, we calculated the mean intensity of the Raman bands in the spectra obtained from the two feathers.

Statistical analyses

We tested if the pheomelanin and eumelanin content of feathers (estimated by PC1 scores for pheomelanin and eumelanin, respectively) changed with the abundance of prey (log_{10}-transformed) using linear mixed-effect models (LMM) fit with restricted maximum likelihood (REML) estimation in R environment (R Core Team 2018). We included wing length as a fixed continuous term in the models to control for differences in the age of nestlings, as wing length increases linearly with age across the age classes of nestlings that we have investigated here (Kenward 2006). Year and sex were added as fixed factors, and territory identity was added as a random factor to account for the common origin of birds belonging to the same nests. In addition to considering total prey abundance in the models, we also tested the same models considering prey abundance divided by brood size (log_{10}-transformed) to account for possible unequal distributions of food between nestlings belonging to the same nests. Effect sizes (i.e., regression coefficients) for the fixed terms (*b*) were calculated with the R package *lme4* (Bates *et al*., 2015). *P*-values were calculated from the analysis of deviance of the models on the basis

of Wald χ^2 tests using the R package *car* (Fox and Weisberg 2011). Full models were compared with the models without non-significant predictors using the corrected Akaike information criterion (AICc), considering that models with ΔAICc ≤ 2 were equally probable (Burnham and Anderson 2002). In some nestlings for which we could obtain the information, we also investigated the association between their pheomelanin and eumelanin content in feathers and the pheomelanin and eumelanin content of their fathers (16 nestlings) and their mothers (10 nestlings), using similar models to those described above that included the sex of nestling as a fixed factor and its interaction with the pheomelanin or eumelanin content of their father or mother.

Results

Feather melanin content and prey abundance in nestlings

The full model for pheomelanin PC1 scores of nestling feathers resulted in a significant effect of total prey abundance (χ^2_1 = 4.39, *P* = 0.036; Fig. 3a) and non-significant effects of wing length (χ^2_1 = 0.02, *P* = 0.898), year (χ^2_1 = 0.21, *P* = 0.648) and sex (χ^2_1 = 0.12, *P* = 0.725; Fig. 4a). The reduced model after removing these non-significant terms was preferred (ΔAICc = 5.25) and again resulted in a significant effect of prey abundance (χ^2_1 = 5.06, *P* = 0.024). The estimate of the effect (*b* = 1.23) indicated a positive association between pheomelanin PC1 scores and prey abundance (Fig. 3a), suggesting that the pheomelanin content of nestlings' feathers decreased with the abundance of food available to them. The same was obtained when prey abundance divided by brood size was considered in the model instead of total prey abundance (*b* = 1.68, χ^2_1 = 9.58, *P* = 0.002). This significant effect, however, disappeared when data from three nestlings belonging to two different nests with very low prey abundance (Fig. 3a) were removed from the analyses (χ^2_1 = 1.15, *P* = 0.283). When only the number of pigeons, the preferred prey of goshawks, was considered, it did not significantly affect pheomelanin PC1 scores in neither the full model (χ^2_1 = 0.72, *P* = 0.395) nor the reduced model excluding wing length, year and sex (χ^2_1 = 1.03, *P* = 0.309; ΔAICc = -0.05).

In contrast, PC1 eumelanin scores of nestling feathers were not related to total prey abundance (*b* = 0.25, χ_1^2 = 0.22, *P* = 0.636). Considering prey abundance divided by brood size instead of total prey abundance, the effect remained non-significant (*b* = 0.88, χ_1^2 = 2.70, *P* = 0.100). The effects of wing length (χ_1^2 = 0.64, *P* = 0.423) and year (χ_1^2 = 3.39, *P* = 0.065) were neither significant, but the effect of sex was highly significant (χ_1^2 = 8.91, *P* = 0.003). The model excluding prey abundance and wing length was not better (ΔAICc = 0.88). Thus, the eumelanin content of nestlings' feathers did not covary with food abundance (Fig. 3b), but females deposited greater amounts of eumelanin in feathers than males (Fig. 4b). The same was found when only the number of pigeons was considered (number of pigeons: χ_1^2 = 0.03, *P* = 0.863, wing length: χ_1^2 = 0.79, *P* = 0.375, year: χ_1^2 = 3.76, *P* = 0.052, sex: χ_1^2 = 8.52, *P* = 0.003).

Feather melanin content and prey abundance in adults

PC1 pheomelanin scores of adult feathers were not related to neither total prey abundance (χ_1^2 = 1.17, *P* = 0.279) nor to sex (χ_1^2 = 0.74, *P* = 0.389; Fig. 4c). A better model excluding sex (ΔAICc = 2.74) also resulted in a non-significant effect of prey abundance (χ_1^2 = 2.15, *P* = 0.142). The same was found when only the number of pigeons was considered, as its effect was not significant (χ_1^2 = 3.51, *P* = 0.061).

PC1 eumelanin scores were not related to total prey abundance (χ_1^2 = 0.10, *P* = 0.752), but the effect of sex was highly significant (χ_1^2 = 13.80, *P* < 0.001). The model excluding prey abundance was not better (ΔAICc = 1.38). Thus, adult female goshawks deposited greater amounts of eumelanin in feathers than males (Fig. 4d). The same was found when only the number of pigeons was considered, as its effect was not significant (χ_1^2 = 0.61, *P* = 0.435).

Relationship between feather melanin content in nestlings and their parents

The model testing for an association between PC1 pheomelanin scores in the feathers of nestlings and those of their fathers did not result in a significant effect of the latter (χ_1^2 = 0.61, *P* = 0.435), nor its interaction with nestling sex (χ_1^2 = 3.75, *P* = 0.053). The model excluding

nestling sex and its interaction with PC1 pheomelanin scores of the father was better (ΔAICc = 3.94) but again resulted in a non-significant effect of PC1 pheomelanin scores of the father (χ_1^2 = 0.76, *P* = 0.382). However, the preferred model when PC1 pheomelanin scores of the mother were considered instead (ΔAICc = 19.58) only included this effect, which was significant (χ_1^2 = 5.09, *P* = 0.024). The estimate of the effect (*b* = -1.40) indicates that PC1 pheomelanin scores of nestlings and their mothers negatively covaried, suggesting that the higher the pheomelanin content of adult female goshawk feathers, the lower the pheomelanin content of their offspring's feathers (Fig. 5a).

The model testing for an association between PC1 eumelanin scores in the feathers of nestlings and those of their fathers resulted in a significant interaction between the latter and the sex of nestlings (χ_1^2 = 4.28, *P* = 0.038). This was due to a negative association in female nestlings (*r* = -0.86, *n* = 8, *P* = 0.005) and a lack of association in male nestlings (*r* = -0.52, *n* = 8, *P* = 0.187). Thus, the eumelanin content of feathers in male goshawk nestlings increased with that of their fathers (Fig. 5b). In contrast, PC1 eumelanin scores in the feathers of nestlings were not related to those of their mothers either alone (χ_1^2 = 0.02, *P* = 0.892) or in interaction with sex (χ_1^2 = 0.20, *P* = 0.651). The model excluding this interaction and sex was better (ΔAICc = 23.84), but again resulted in a non-significant effect of PC1 eumelanin scores of the mother (χ_1^2 = 0.02, *P* = 0.875).

Discussion

Our results show that the amount of pheomelanin in feathers of nestling goshawks decreases as the abundance of food available to them increases. As the expression of the gene *CTNS* increases with food abundance in nestlings of another raptor species (the gyrfalcon; Galván et al. 2017a), it is likely that the association found here is also due to *CTNS* activity. This is because *CTNS* expression favors the ejection of cysteine from melanosomes, the organelles where melanin synthesis takes place, thus decreasing intramelanosomal cysteine levels and potentially inhibiting pheomelanin synthesis (Chiaverini et al. 2012). The influence of *CTNS*

activity on pheomelanin synthesis, however, has remained unclear, as the only study so far reported that the influence is not always exerted (Chiaverini et al. 2012). Our findings thus suggest that pheomelanin synthesis might be affected by the maintenance of cysteine homeostasis, likely mediated by *CTNS* activity, in wild birds with a strict carnivorous diet and a consequent high need to avoid excess cysteine (Klasing 1998). This is the first time that pheomelanin synthesis is reported to covary with protein intake. However, Fargallo et al. (2007) showed that the proportion of male nestlings displaying the greyest plumage patches, which are produced by a low pheomelanin:eumelanin ratio in feathers, was higher in years of high prey abundance in another raptor species, the Eurasian kestrel *Falco tinnunculus*. This appears to be in agreement with our findings, suggesting that pheomelanin synthesis in strictly carnivorous birds may reflect their regulation of cysteine homeostasis. It must be considered, however, that the effect of prey abundance on the pheomelanin content of nestling feathers was not patent when data from three nestlings reared in territories with very low food abundance were excluded from the analyses. This may suggest that an effect of protein content on pheomelanin synthesis only emerge under strong food deficiency.

Although our study is correlational, two results support the hypothesis that pheomelanin synthesis is affected by the mechanism of cysteine homeostasis in goshawks. First, prey abundance was related to the feather content of pheomelanin but not to the content of eumelanin, which is the melanin form that is produced under low levels or absence of cysteine in melanosomes (García-Borrón and Olivares Sánchez 2011). Additionally, the association between feather pheomelanin content and number of prey was not apparent when only pigeons, the preferred prey of goshawks, was considered. This suggests that the association may be mediated by the total amount of protein (cysteine) in the diet, in agreement with the prediction that pheomelanin synthesis is only affected by *CTNS* expression under high systemic cysteine levels (Chiaverini et al. 2012).

In a previous study in a Dutch goshawk population, the eumelanin content of feathers was found to increase, while the pheomelanin content did not covary with food abundance in

coniferous deciduous mixed woodlands but not in other habitat types, where the eumelanin content was unrelated to food abundance (Galván et al. 2010). However, it must be considered that a spectrophotometric method unable to fully discriminate between pheomelanin and eumelanin was used in that previous study. Thus the results are not directly comparable. In any case, it suggests that the effect of habitat on the influence of food abundance of melanin synthesis deserves further investigation.

When deposited in integumentary structures such as feathers, pheomelanin produces orange and light brown color hues (Galván and Wakamatsu 2016). In goshawks, this results in orange-yellowish patches in covert feathers of nestlings that are less expressed in adults (see Fig. 2). It then makes biological sense that the association between feather pheomelanin content and prey abundance was found in nestlings but not in adults. However, it has been suggested that the synthesis of pheomelanin may be adaptive under conditions of low oxidative stress. Under such conditions, excess cysteine is more likely to occur because GSH is less required for antioxidant protection, and the incorporation of the sulfhydryl group of cysteine into the structure of pheomelanin may help removing potentially toxic excess cysteine (Galván 2017). The nestling stage is a period of low physical activity in which developing birds are fed by parents, and as exercise can increase oxidative stress (Vaanholt et al. 2007, 2008), the low expenditure of energetic resources causes the nestling stage to be affected by relatively low stress as compared to post-fledging stages (Weimerskirch et al. 2003). As cysteine is a constituent amino acid of GSH, the most important intracellular antioxidant, it is likely that nestlings experience a relative low need of cysteine for GSH synthesis. This may explain why pheomelanin seems to be so abundant in juvenile birds, which often display distinctive plumage with colors that are characteristic of this pigment (Galván 2017). Unfortunately, we could not measure the physical condition of nestling goshawks to test the detoxifying function of pheomelanin, but this may well be the cause favoring the evolution of pheomelanin-based juvenile plumage patches in this species too. On the other hand, the present study shows that pheomelanin synthesis may be a side effect of cysteine homeostasis. Therefore, we suggest

376 that the presence of pheomelanin in juvenile animals is due to a potentially adaptive detoxifying
377 effect of this pigment, and its synthesis levels are then affected by variations in the maintenance
378 of cysteine homeostasis.

379 As mentioned above, the eumelanin content of feathers was not affected by food
380 abundance in neither nestlings nor adults. However, there was a clear difference in the
381 eumelanin content of male and female goshawks, the latter depositing higher amounts of
382 eumelanin in their dorsal feathers. Such a difference was not observed in pheomelanin content.
383 As no other pigments are present in goshawk feathers, differences in eumelanin synthesis
384 levels are thus responsible for the sexual dichromatism observed in adult goshawks (see Fig.
385 1). Interestingly, we found the same differences in the eumelanin content of feathers between
386 male and female nestling goshawks. Although goshawks can start breeding in their first year of
387 age (Nielsen and Drachmann 2003), the change to the distinctive adult plumage pattern that
388 occurs at the age of three (Kenward 2006) suggests that pigmentation of the first plumage
389 developed by goshawks does not play a role in sexual selection. As sexual dichromatism
390 generally indicates the existence of sexual selection (Heinsohn et al. 2005), it is likely that
391 plumage pigmentation is only involved in sexual selection in adult goshawks. Thus, the
392 eumelanin-mediated sexual dichromatism in nestling goshawks may be the result of genes
393 being expressed in all developmental stages and under sexual selection only in adults (Johnsen
394 et al. 2003).

395 Our comparisons of melanin contents of goshawk nestlings with those of their parents
396 also suggest differences between pheomelanin and eumelanin. Although our small sample size
397 emphasizes that these results must be taken with caution, we found evidence for a positive
398 correlation between eumelanin content of female nestlings with that of their fathers, and a
399 negative correlation between the eumelanin content of both male and female nestlings with that
400 of their mothers. To our knowledge, the heritability of melanin contents per se has never been
401 investigated. It is likely, however, that our correlative result for the eumelanin content agrees
402 with the relative high heritability, although also differing between sexes, reported for the

percentage of grey color in the rump plumage patch of Eurasian kestrels (Kim et al. 2013). This is because the percentage of grey color in this plumage patch increases with the eumelanin content of feathers (Fargallo et al. 2007). Other studies on birds also report high heritability values for plumage traits whose expression depends on feather pheomelanin contents (Saino et al. 2013), although these include some studies conducted with color polymorphic species (Roulin and Dijkstra 2003) that, by definition, have a fixed phenotypic variability (Wente and Phillips 2003). In fact, recent studies show that red human hair, which is mainly produced by pheomelanin (Ito et al. 2011), has a lower heritability than other hair types (Lin et al. 2015), and that the expression of a gene that controls the transport of cysteine to melanocytes (*Slc7a11*) changes with environmental oxidative stress (Galván et al. 2017b). Although we could not estimate the heritability of pheomelanin content in goshawks, the negative correlation that we found between the pheomelanin contents of nestlings and their mothers at least suggest that heritability might not be high, in agreement with earlier studies.

In conclusion, we show that, while eumelanin synthesis is independent of food abundance in both nestling and adult goshawks and may play a role in sexual selection in adults, pheomelanin synthesis depends on food abundance and is probably not involved in sexual selection in nestlings. This suggests that variability in pheomelanin synthesis levels are partly a side effect of the maintenance of cysteine homeostasis during development, may help explain variability in the expression of pigmentation phenotypes.

References

Bates D, Maechler M, Bolker B Walker S (2015) Fitting linear mixed-effects models using lme4. J Stat Softw 67:1-48.

Burnham KP, Anderson DR (2002) Model selection and multimodel inference. A practical information-theoric approach. Springer-Verlag, New York.

Chiaverini C, Sillard L, Flori E et al. (2012) Cystinosin is a melanosomal protein that regulates melanin synthesis. FASEB J 26:3779-3789.

Fargallo JA, Laaksonen T, Korpimäki E, Wakamatsu K (2007) A melanin-based trait reflects environmental growth conditions of nestling male Eurasian kestrels. Evol Ecol 21:157-171.

Fox J, Weisberg S (2011) An {R} Companion to Applied Regression, Second Edition. Sage, Thousand Oaks. URL: http://socserv.socsci.mcmaster.ca/jfox/Books/Companion

Galván I (2017) Condition-dependence of pheomelanin-based coloration in nuthatches *Sitta europaea* suggests a detoxifying function: implications for the evolution of juvenile plumage patterns. Sci Rep 7:9138.

Galván I, Jorge A (2015) Dispersive Raman spectroscopy allows the identification and quantification of melanin types. Ecol Evol 5:1425-1431.

Galván I, Wakamatsu K (2016) Color measurement of the animal integument predicts the content of specific melanin forms. RSC Adv 6:79135-79142.

Galván I, Bijlsma RG, Negro JJ, Jarén M, Garrido-Fernández J (2010) Environmental constraints for plumage melanization in the northern goshawk *Accipiter gentilis*. J Avian Biol 41:523-531.

Galván I, Jorge A, Ito K, Tabuchi K, Solano F, Wakamatsu K (2013) Raman spectroscopy as a non-invasive technique for the quantification of melanins in feathers and hairs. Pigment Cell Melanoma Res 26:917-923.

Galván I, Inácio Â, Nielsen ÓK (2017a) Gyrfalcons *Falco rusticolus* adjust *CTNS* expression to food abundance: a possible contribution to cysteine homeostasis. Oecologia 184:779-785.

Galván I, Inácio Â, Romero-Haro AA, Alonso-Alvarez C (2017b) Adaptive downregulation of pheomelanin-related *Slc7a11* gene expression by environmentally induced oxidative stress. Mol Ecol 26:849-858.

García-Borrón JC, Olivares Sánchez MC (2011) Biosynthesis of melanins. In: Borovanský J, Riley PA (eds) Melanins and melanosomes: biosynthesis, biogenesis, physiological, and pathological functions. Weinheim, Wiley-Blackwell, pp 87-116.

157 Giles NM, Watts AB, Giles GI, Fry FH, Littlechild JA, Jacob C (2003) Metal and redox
158 modulation of cysteine protein function. Chem Biol 10:677-693.
159 Heinsohn R, Legge S, Endler JA (2005) Extreme reversed sexual dichromatism in a bird without
160 sex role reversal. Science 309:617-619.
161 Hoy SR, Ball RE, Lambin X, Whitfield DP, Marquiss M (2015) Genetic markers validate using
162 the natural phenotypic characteristics of shed feathers to identity individual northern
163 goshawk *Accipiter gentilis*. J Avian Biol 47:43-47.
164 Hsu SL, Moore WH, Krimm S (1976) Vibrational spectrum of the unordered polypeptide chain: a
165 Raman study of feather keratin. Biopolymers 15:1513-1528.
166 Ito S, Nakanishi Y, Valenzuela RK, Brilliant MH, Kolbe L, Wakamatsu K (2011) Usefulness of
167 alkaline hydrogen peroxide oxidation to analyze eumelanin and pheomelanin in various
168 tissue samples: application to chemical analysis of human hair melanins. Pigment Cell
169 Melanoma Res 24:605-613.
170 Johnsen A, Delhey K, Andersson S, Kempenaers B (2003) Plumage colour in nestling blue tits:
171 sexual dichromatism, condition dependence and genetic effects. Proc R Soc Lond B
172 270:1263-1270.
173 Kenward R (2006) The goshawk. London, Poyser.
174 Kim SY, Fargallo JA, Vergara P, Martínez-Padilla J (2013) Multivariate heredity of melanin-
175 based coloration, body mass and immunity. Heredity 111:139-146.
176 Klasing KC (1998) Comparative avian nutrition. Wallingford, CAB International.
177 Kühlapfel O, Brune J (1995) Die Mauserfeder als Hilfsmittel zur Altersbestimmung und
178 Individualerkennung von Habichten (*Accipiter gentilis*). Charadrius 31:120-125.
179 Lin BD, Mbarek H, Willemsen G, et al. (2015) Heritability and genome-wide association studies
180 for hair color in a Dutch twin family based sample. Genes 6:559-576.
181 Møller AP, Nielsen JT (2007) Malaria and risk of predation: a comparative study of
182 birds. Ecology 88:871-881.

Møller AP, Nielsen JT (2014) Large increase in nest size linked to climate change: An indicator of life history, senescence and condition. Oecologia 179:913-921.

Nielsen JT, Drachmann J (2003) Age-dependent reproductive performance in Northern Goshawks *Accipiter gentilis*. Ibis 145:1-8.

Opdam P, Müskens G (1976) Use of shed feathers in population studies of *Accipiter* hawks (Aves, Accipitriformes, Accipitridae). Beaufortia 24:55-62.

Park S, Imlay JA (2003) High levels of intracellular cysteine promote oxidative DNA damage by driving the Fenton reaction. J Bacteriol 185:1942-1950.

Polidori C, Jorge A, Ornosa C (2017) Eumelanin and pheomelanin are predominant pigments in bumblebee (Apidae: Bombus) pubescence. PeerJ 5:e3300.

R Core Team (2018) R: a language and environment for statistical computing. Vienna, R Foundation for Statistical Computing.

Roulin A, Dijkstra C (2003) Genetic and environmental components of variation in eumelanin and phaeomelanin sex-traits in the barn owl. Heredity 90:359-364.

Saino N, Romano M, Rubolini D, Teplitsky C, Ambrosini R, Caprioli M, Canova L, Wakamatsu K (2013) Sexual dimorphism in melanin pigmentation, feather coloration and its heritability in the barn swallow (*Hirundo rustica*). PLoS ONE 8:e58024.

Stipanuk MH, Dominy JE Jr, Lee JI, Coloso RM (2006) Mammalian cysteine metabolism: new insights into regulation of cysteine metabolism. J Nutr 136:1652S-1659S.

Stipanuk MH, Ueki I, Dominy JE Jr, Simmons CR, Hirschberger LL (2009) Cysteine dioxygenase: a robust system for regulation of cellular cysteine levels. Amino Acids 37:55-63.

Vaanholt LM, De Jong B, Garland T Jr, Daan S, Visser GH (2007) Behavioural and physiological responses to increased foraging effort in male mice. J Exp Biol 210:2013-2024.

508 Vaanholt LM, Speakman JR, Garland T Jr, Lobley GE, Visser GH (2008) Protein synthesis and
509 antioxidant capacity in aging mice: e ects of long-term voluntary exercise. Physiol
510 Biochem Zool 81:148-157.
511 Viña J, Saez GT, Wiggins D, Roberts AFC, Hems R, Krebs HA (1983) The effect of cysteine
512 oxidation on isolated hepatocytes. Biochem J 212:39-44.
513 Wang H, Osseiran S, Igras V, et al. (2016b) In vivo coherent Raman imaging of the
514 melanomagenesis-associated pigment pheomelanin. Sci Rep 6:37986.
515 Weimerskirch H, Ancel A, Caloin M, Zahariev A, Spagiari J, Kersten M, Chastel O (2003)
516 Foraging e ciency and adjustment of energy expenditure in a pelagic seabird provisioning
517 its chick. J Anim Ecol 72:500-508.
518 Wente WH, Phillips JB (2003) Fixed green and brown color morphs and a novel color-changing
519 morph of the Pacific tree frog *Hyla regilla*. Am Nat 162:461-473.
520 Wu G, Fang YZ, Yang S, Lupton JR, Turner ND (2004) Glutathione metabolism and its
521 implications for health. J Nutr 134:489-492.
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535 **Fig. 1.** Images of >3-year male (a) and 2-year female (b) Northern goshawks with their prey.
536 Note the different pigmentation of dorsal plumage in both sexes. c: Image of goshawk nestlings
537 in a nest.
538
539 **Fig. 2.** Photograph of a dorsal covert feather of a Northern goshawk nestling included in the
540 study, and Raman spectra obtained from its two differentially pigmented areas. Diagnostic
541 bands are marked with arrows. The spectrum on the left shows Raman signal from only
542 pheomelanin, thus including the three diagnostic bands of this pigment at 500, 1500 and 2000
543 cm^{-1}. Note the orange color in the feather that is only caused by the presence of pheomelanin.
544 The spectrum on the right shows mixed Raman signal from both melanin forms and includes the
545 three diagnostic bands of pheomelanin combined with the three diagnostic bands of eumelanin
546 at 500, 1380 and 1580 cm^{-1}, in addition to two bands produced by keratin at 1200 and 1310 cm^{-1}
547 . These spectra were deconvoluted using the marked bands to calculate the intensity of
548 pheomelanin and eumelanin bands. The curves shown here were smoothed by the adjacent
549 averaging technique using ORIGIN v.7 software (OriginLab Corporation, Northampton, MA,
550 USA).
551
552 **Fig. 3.** Relationship between prey abundance in the rearing territories and the relative content
553 of pheomelanin (a) and eumelanin (b) in dorsal covert feathers of Northern goshawk nestlings.
554 Melanin contents are measured by the scores of the first component (PC1) from a Principal
555 Components Analysis (PCA) made with the intensity of three diagnostic bands in the Raman
556 spectrum of each pigment. PC1 scores decrease as melanin content increases. Lines are best-
557 fit lines.
558
559 **Fig. 4.** Mean ± se melanin contents in feathers in male and female Northern goshawk nestlings
560 (a: pheomelanin, b: eumelanin) and adults (c: pheomelanin, d: eumelanin). Melanin contents
561 are measured by the scores of the first component (PC1) from a Principal Components Analysis

562 (PCA) made with the intensity of three diagnostic bands in the Raman spectrum of each
563 pigment. PC1 scores decrease as melanin content increases in a, b and c. In d, PC1 scores
564 increase with melanin contents.

565

566 **Fig. 5.** a: Relationship between the feather pheomelanin content of Northern goshawk nestlings
567 and that of their mothers. PC1 scores decrease as pheomelanin content increases. b:
568 Relationship between the feather eumelanin content of male and female Northern goahawk
569 nestlings and that of their fathers. In nestlings, PC1 scores decrease as eumelanin content
570 increases, while in their adult fathers PC1 scores increase with eumelanin content. Lines are
571 best-fit lines.