Differential influence of *Slc7a11* expression and body condition on pheomelanin-based pigmentation in two Eurasian nuthatch *Sitta europaea* populations with different predation risk

Ismael Galván^{1*} and Juan José Sanz²

¹Department of Evolutionary Ecology, Doñana Biological Station, CSIC, Sevilla, Spain. ORCID: 0000-0002-6523-8592.

²Department of Evolutionary Ecology, National Museum of Natural Sciences, CSIC, Madrid, Spain. ORCID: 0000-0003-2576-4050.

*Correspondence (galvan@ebd.csic.es).

Declarations

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Permits

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27 Abstract

28 The expression of the gene *Slc7a11* promotes the antioxidant capacity of cells by providing
29 them with cysteine that can be used for the synthesis of glutathione (GSH), the most important
30 intracellular antioxidant. In melanocytes, intracellular cysteine can also enter melanosomes and
31 get incorporated in the pigment pheomelanin synthesis pathway, thus decreasing cysteine
32 availability for GSH synthesis and potentially creating chronic oxidative stress. We thus
33 hypothesized that a mechanism limiting the use of intramelanocytic cysteine for pheomelanin
34 synthesis in environmental conditions generating oxidative stress may be physiologically
35 advantageous and favored by natural selection. Here we searched for evidence of such a
36 mechanism by comparing the influence of melanocytic *Slc7a11* expression on pheomelanin-
37 based pigmentation in developing Eurasian nuthatch *Sitta europaea* nestlings from two
38 populations differing in predation risk, a natural source of oxidative stress. Pheomelanin
39 synthesis and pigmentation tended to increase with *Slc7a11* expression in the low-risk
40 population as expected from the activity of this gene, but decreased with *Slc7a11* expression in
41 the high-risk population. The same was not observed in the expression of five other genes
42 influencing pheomelanin synthesis without affecting cysteine availability in melanocytes. The
43 influence of body condition on the intensity of pheomelanin-based pigmentation also differed
44 between populations, being positive in the low-risk population and negative in the high-risk
45 population. The resulting pigmentation of birds was more intense in the high-risk population.
46 These findings suggest that birds perceiving high predation risk may limit the use of cysteine for
47 pheomelanin synthesis, which becomes independent of *Slc7a11* expression. Some birds may
48 have thus evolved the ability to adjust their pigmentation phenotype to environmental stress.

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50 **Keywords:** Eurasian nuthatch, gene expression, pheomelanin, pigmentation, predation risk.

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54 Introduction

55 In recent years, evidence for a key role of the gene *Slc7a11* (solute carrier family 7 member 11)
56 in antioxidant capacity and health has increased. This gene encodes the cystine/glutamate
57 antiporter xCT (system x_c⁻) and is thus responsible for providing cells with cysteine (Chintala et
58 al. 2005). As cysteine is one of the three constituent amino acids of glutathione (GSH), the main
59 intracellular antioxidant (Wu et al. 2004), *Slc7a11* expression increases the availability of
60 cysteine for GSH synthesis and the antioxidant capacity of cells. Accordingly, a high expression
61 of *Slc7a11* in tumorous cells of many cancer types hampers their apoptosis (Liu et al. 2011,
62 Drayton et al. 2014, Robert et al. 2015), while *Slc7a11* inhibition induces ferroptosis upon
63 oxidative stress (Jiang et al. 2015).

64 However, when *Slc7a11* is expressed in melanocytes, the specialized cells that
65 synthesize melanin pigments, the supplied cysteine is not only used for GSH synthesis, but also
66 to synthesize pheomelanin as the sulfhydryl group of cysteine is incorporated into the structure
67 of the melanin that is formed (García Borrón and Olivares-Sánchez 2011). If cysteine enters the
68 pheomelanin synthesis pathway instead of the GSH synthesis pathway in melanocytes, the
69 reduced GSH production can lead to chronic oxidative stress in the organism (Napolitano et al.
70 2014). The use of cysteine for melanin synthesis can also affect the pigmentation of organisms,
71 as pheomelanin produces orange and light yellow colours when deposited in integumentary
72 structures such as feathers and hairs (Galván and Wakamatsu 2016). The regulation of
73 *Slc7a11*-dependent, pheomelanin-based pigmentation may therefore affect the capacity of
74 organisms to cope with environmental oxidative stress. Indeed, some but not all bird species
75 have evolved environmental plasticity in *Slc7a11* that allows downregulating its expression in
76 melanocytes and avoiding cellular oxidative damage under exposure to oxidative stress (Galván
77 et al. 2017a,b). This is because the limitation of pheomelanin production in melanocytes may
78 save cysteine for GSH synthesis and antioxidant capacity in other cells. Indeed, a high
79 expression of *Slc7a11* in normal epidermal melanocytes increases melanoma risk in humans
80 (Galván et al. 2019). In addition to the potentially adaptive strategy of *Slc7a11* lability, it may

81 also be possible that organisms cope with environmental oxidative stress by modifying the
82 amount of cysteine that is used for pheomelanin synthesis and for GSH synthesis in
83 melanocytes. This possibility has never been explored.

84 Here we test if the influence of *Slc7a11* expression on pheomelanin-based pigmentation
85 phenotype differs between populations with different predation risk. Predation risk is a natural
86 environmental source of physiological stress (Newman et al. 2013, Kondoh et al. 2016) that
87 ultimately produces oxidative stress and damage to cells (Janssens and Stoks 2014). The
88 expected trend if most cysteine in melanocytes was used for pheomelanin synthesis is that
89 pheomelanin-based color intensity increases with *Slc7a11* expression (Galván et al. 2017b).
90 Any deviation from that influence of *Slc7a11* expression may be explained by a possible
91 physiological trade of cysteine for pheomelanin and GSH synthesis, which we hypothesize to
92 differ between populations differing in predation risk. In particular, we predict that, if a
93 physiologically advantageous lability has evolved, the expected influence of *Slc7a11* on
94 pigmentation should not be patent in the population with high predation risk. No mutations in
95 *Slc7a11* coding sequence are known to affect pigmentation (Ito and Wakamatsu 2011), thus it is
96 more relevant to study the expression of *Slc7a11* than its structure.

97 We tested our hypothesis in the Eurasian nuthatch *Sitta europaea*, a ca. 20 g hole-
98 nesting songbird that pigments their flank covert feathers with pheomelanin to create dark
99 orange coloration. This plumage patch that is already present in the plumage of nestlings (Fig.
L00 1). In at least one of our study populations, the intensity of pheomelanin-based color in nuthatch
L01 nestlings is negatively related to their body condition (Galván 2017). Therefore, possible
L02 variations in the influence of *Slc7a11* expression on pheomelanin-based pigmentation may also
L03 affect the condition-dependence of this trait, thus we also investigated this possible effect. In the
L04 same population, it has also recently been found that an experimental increase of perceived
L05 predation risk induces a downregulation of *Slc7a11* expression and changes the *Slc7a11*-
L06 mediated consumption of GSH in feather melanocytes of developing Eurasian nuthatches

(Galván 2018). Eurasian nuthatch nestlings perceive predation risk by hearing the alarm calls emitted by parents when they detect a potential predator (Galván 2018).

We also measured the expression of other genes that affect pheomelanin synthesis in melanocytes by mechanisms different from cysteine transport and thus not influence the availability of cysteine/GSH in melanocytes. These genes, which therefore served as control genes, were: *CTNS*, *MC1R*, *ASIP*, *AGRP* and *Slc45a2*. The gene *CTNS* codes for a cystine/H⁺ symporter that exports cystine (the oxidized dimer of cysteine) out of melanosomes (the specialized organelles where melanogenesis takes place in melanocytes; Chiaverini et al. 2012), thus potentially affecting the amount of cysteine/GSH available for pheomelanin synthesis. The melanocortin 1 receptor (*MC1R*) codes for the melanocortin 1 receptor in the membrane of melanocytes, to which melanocortins bind to influence pheomelanogenesis by changing intracellular cyclic adenosine monophosphate (cAMP) concentration. The agouti-signaling (*ASIP*) and the agouti-related protein (*AGRP*) genes code for the antagonists of *MC1R* in birds (Nadeau et al. 2008). The function of *Slc45a2* is not fully clear, but it most likely regulates the processing and trafficking of tyrosinase (a key enzyme in the melanogenesis pathway) by acting as a sucrose/H⁺ symporter (Xu et al. 2013).

Materials and methods

Study populations and field methods

We studied two wild populations of Eurasian nuthatches breeding in nest boxes. The populations are 250 km apart in southern and central Spain (Fig. 1). In the study, we included information collected in the period 2015-2017 in the southern population, and information collected in 2017 in the northern population.

The southern population is located in an extensive agro-ecosystem (Iberian dehesa) mainly composed of scattered holm oaks *Quercus ilex* and cork oaks *Quercus suber* at 450 m above sea-level in Sierra Norte de Sevilla Natural Park (37°47' N, 06°04' W), which belongs to Sierra Morena mountain range. Since 2015, 246 wooden nest boxes were mounted on tree

trunks at breast height without any particular protection from predators. Predation risk in this population is very high, as shown by the fact that 32 % of nuthatch pairs ($n = 68$) that initiated clutches in 2015-2017 suffered breeding failure due to predation by mammals and snakes (identified on the basis of predation signs in nests) on eggs or nestlings. Therefore, predation risk perceived by birds in this population might be high, thus we consider this population a high-risk population.

The northern population is located at 800 m above sea-level in Quintos de Mora (Toledo province; 39°24' N, 04°04' W), a government-owned game reserve with a similar ecosystem to the previous population. This location belongs to Montes de Toledo mountain range, which is the next main mountain range to Sierra Morena from the South, and habitat characteristics in both populations are similar. 200 wooden nest boxes distributed in two plots (Barranco de Gil García and Arroyo de Valdeyernos) were monitored during the spring of 2017. These plots were located on a riverbank dominated by Pyrenean oak *Quercus pyrenaica* and, to a lesser extent, zeen oaks *Quercus fagynae*. Since 2006, nest boxes were mounted on tree trunks at heights of 3-4 m and protected from predators (woodpeckers and mustelids) with wire mesh (Mainwaring and Hartley 2008) and a PVC elbow fixed to the hole entrance. Predation risk in this population is very low as compared with the southern population, as shown by the fact that no predation event was observed among the nuthatch pairs that initiated clutches in 2017 ($n = 17$). As predation risk perceived by birds in this population might be relatively low (as compared to the other population), we consider this population a low-risk population.

In both populations, frequent checks of nestboxes provided data on dates of clutch initiation and clutch size for all breeding pairs. At the age of 17 days, developing nestlings were weighed with a portable electronic balance to the nearest 0.1 g and their tarsus length measured to the nearest 0.01 mm with a digital calliper as a measure of body size. We plucked 15-20 pheomelanin-pigmented, orange flank feathers of nestlings to measure the intensity of pheomelanin-based pigmentation and pheomelanin content and to quantify gene expression in extracted melanocytes. The sex of nestlings was determined by a visual evaluation of the color

intensity of these feathers, as male nestlings display a more intense orange coloration than females, which is validated by an analysis of the CHD gene using the primer pair CHD1F/CHD1R (5'-TATCGTCAGTTTCCTTTTCAGGT-3' and 5'-CCTTTTATTGATCCATCAAGCCT-3') (Galván, 2017). Plucked feathers were immersed in RNAlater solution (Ambion, Thermo Fisher Scientific, Waltham, MA, USA) and stored at -80 °C until the analyses. The study comprised a total of 54 Eurasian nuthatch nestlings from 18 different nests in the high-risk population (25 males and 29 females) and 100 nestlings from 17 different nests (40 males, 37 females and 23 birds in which sex could not be determined) in the low-risk population.

We calculated the body condition of nuthatch nestlings as the residuals of body mass regressed against tarsus length, a measure that is often a good predictor of body fat content in birds (Labocha and Hayes 2012). Previous studies on juvenile European nuthatches showed that a similar index of residual body mass (mass divided by tarsus length) was higher in resident birds than in birds that did not secure a territory, the former also achieving higher local survival during some periods of the year than the latter (Matthysen 1989). This indicates that residual mass is a body condition index that is biologically relevant for Eurasian nuthatches, as it reflects survival prospects.

Analysis of pheomelanin-based pigmentation

To analyze the color expression of feathers, we used an Ocean Optics Jaz spectrophotometer (range 220-1000 nm) with ultraviolet (deuterium) and visible (tungsten-halogen) lamps and a bifurcated 400 µm fiber optic probe. The fiber optic probe both provided illumination and obtained light reflected from the sample, with a reading area of ca. 1 mm². Feathers were mounted on a light absorbing foil sheet (Metal Velvet coating, Edmund Optics, Barrington, NJ, USA) to avoid any background reflectance, such that they resembled the natural appearance of the feather patch. Measurements were taken at a 90° angle to the sample. All measurements were relative to a diffuse reflectance standard tablet (WS-1, Ocean Optics, Dunedin, FL, USA),

and reference measurements were frequently made. An average spectrum of five readings on different points of the feathers was obtained for each bird, removing the probe after each measurement. Reflectance curves were determined by calculating the median of the percent reflectance in 10 nm intervals.

Like any other biological tissue pigmented by melanins, the reflectance of pheomelanin-based orange feathers of nuthatches steadily increases from the UV spectral range to the beginning of the near-infrared (NIR), thus creating almost linear spectral curves with no defined peaks that mainly vary in their slope and in the total amount of reflectance (Galván 2017). We therefore summarized spectral data as a measure of the slope of reflectance regressed against wavelength across the 300-700 nm range and as a measure of summed reflectance in that range. Both measures are highly correlated and thus convey similar information (Galván 2017), but to determine which one better reflects the pheomelanin content of feathers, we regressed them against the concentration of pheomelanin in feathers in a subset of samples (see section below).

Analysis of feather pheomelanin content

The feathers of 16 nuthatch nestlings from the high-risk population sampled in 2017 were analyzed by micro-Raman spectroscopy to determine their relative content of pheomelanin, as this pigment exhibits a distinctive Raman signal that can be used to non-invasively identify and quantify them (Galván et al. 2013, Galván and Jorge 2015). Results show that only Raman signal from pheomelanin is detected in the nuthatch flank feathers, and that the slope of the regression of feather reflectance values against wavelength is a significant predictor of pheomelanin content because the slope decreases as the Raman signal from pheomelanin increases (Galván and Rodríguez-Martínez 2018). Therefore, the non-sulphurated form of melanin (i.e., eumelanin) was not present, or was present in undetectable amounts, in the flank feathers of nuthatches, meaning that pheomelanin was the main pigment responsible for their

color. We thus used reflectance slope as a measure of pheomelanin-based color expression in nuthatch flank feathers.

Gene expression in melanocytes

We extracted RNA from the melanin unit of the feather follicles, which is an important reservoir of melanocytes (Mohanty et al. 2011). Fifteen follicular melanin units were pooled per bird. Total RNA was extracted using TRI Reagent (Ambion) and quantified with a Qubit 4 Fluorometer (Invitrogen, Thermo Fisher Scientific). Residual genomic DNA carry over was removed using the TURBO DNA-free kit (Ambion). Complementary DNA (cDNA) was prepared from total RNA using RevertAid Reverse Transcriptase provided in the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Thermo Fisher Scientific). qPCR was performed on cDNA for the following genes: *Slc7a11*, *Slc45a2*, *CTNS*, *MC1R* and *ASIP*. Reactions were performed using SYBR Green I Master in a LightCycler 480 System (Roche, Basel, Switzerland). Cycle threshold (C_t) levels were used as a measure of gene expression, with lower C_t values indicating higher mRNA levels and higher gene expression levels. The housekeeping glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used for normalization by subtracting its expression level from the expression level of the other genes measured in the same sample (ΔC_t). Two C_t replicates were obtained for each sample, and the mean value was used in the statistical analyses. Normalized expression levels were unrelated to RNA concentration for most genes (*Slc7a11*: $r = 0.06$, $n = 107$, $p = 0.547$; *Slc45a2*: $r = 0.03$, $n = 107$, $p = 0.788$; *CTNS*: $r = 0.13$, $n = 107$, $p = 0.177$; *ASIP*: $r = 0.07$, $n = 107$, $p = 0.475$; *AGRP*: $r = 0.02$, $n = 107$, $p = 0.805$; *GAPDH*: $r = 0.11$, $n = 111$, $p = 0.252$; RNA concentration could not be quantified in all samples).

Gene primers were designed based on refseq sequences (GenBank) using the Primer-BLAST tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

The oligonucleotide primer sets used were as follows: 5'-GCTGGGGAATTGCTGCTTTC-3' and 5'-TGCAACGAAGAACATCCTGGA-3' for *Slc7a11*; 5'-GAAATGCACGGTTGCGTCAC-3' and 5'-GGTACACAACCTGTCCCATGAA-3' for *Slc45a2*; 5'-CCTACCCTCTCTGAGGGCTT-3'

and 5'-ACAGTTTGTGTTCCAGCCCA-3' for *CTNS*; 5'-TCTCCTTCCTGGGGGTCATT-3' and 5'-TGATCAAGACGGTGCTGGAC-3' for *MC1R*; 5'-AACGTGTCCAGAAGGAGCAA-3' and 5'-TGCAGGTTTTGAAGGTTGGC-3' for *ASIP*; 5'-TGGGGTTTGAGCAGATGACC-3' and 5'-CCCTCAGCTTGCAGTTCTGT-3' for *AGRP*; and 5'-GGACCAGGTTGTCTCCTGTG-3' and 5'-TCCTTGGATGCCATGTGGAC-3' for *GAPDH*.

Statistical analyses

We used linear mixed-effects models (LMM) fit with restricted maximum likelihood (REML) estimation to investigate how *Slc7a11* expression and body condition influenced pheomelanin-based color expression in nuthatch nestlings. The response variable was thus the slope of reflectance spectra, fixed terms were *Slc7a11* ΔC_t and body condition, and nest identity was added as a random term to account for the common origin of nuthatch nestlings belonging to the same nests. We tested for the possible effect of perceived predation risk on the influence of *Slc7a11* expression and body condition on pheomelanin-based pigmentation by including the interactions between population identity (high-risk vs. low-risk), which was a fixed factor, and *Slc7a11* expression and body condition. The sex of birds was an additional fixed factor to control for pheomelanin-based color variability between male and female nestlings (Galván 2017). Year was also included as a fixed factor to control for a possible effect of the increased variance caused by yearly variation in the high-risk population, but it was removed as the effect was not significant ($\chi^2_1 = 1.15$, $p = 0.284$). The same was done for the control genes (*CTNS*, *MC1R*, *ASIP*, *AGRP* and *Slc45a2*), which, due to a high level of intercorrelation in expression levels, were investigated in separate models. LMM analyses were made in R environment (R Core Team 2018) using the package *lme4* (Bates et al. 2015) to calculate fixed term estimates (b) and the package *car* (Fox and Weisberg 2011) to calculate p -values from the analyses of deviance of the models on the basis of Wald χ^2 tests. ΔC_t values were $\log_{10}$ -transformed prior to analyses to normalize their distributions.

Results

The model for *Slc7a11* resulted in a significant effect of this gene's expression on pheomelanin-based color intensity ($\chi^2_1 = 5.90$, $p = 0.015$), but this effect differed between populations, as shown by a nearly significant interaction between population identity and *Slc7a11* expression ($\chi^2_1 = 3.70$, $p = 0.054$). This interaction was due to an association between reflectance slope and *Slc7a11* ΔC_t being negative in the high-risk population ($b = -0.10$) and positive ($b = 0.03$) in the low-risk population (Fig. 2). Correlation tests of raw data for these variables showed that the association was significant in the high-risk population ($r = -0.34$, $p = 0.021$) and non-significant in the low-risk population ($r = 0.03$, $p = 0.128$). This suggests some tendency of pheomelanin-based color to increase with *Slc7a11* expression in the low-risk population, while a clear reversed pattern was observed in the high-risk population (Fig. 2).

In the model, the effect of body condition was not significant ($\chi^2_1 = 0.45$, $p = 0.503$), but its interaction with population identity was strongly significant ($\chi^2_1 = 8.87$, $p = 0.003$). This was due to an association between reflectance slope and body condition being positive in the high-risk population ($b = 0.05$) and negative ($b = -0.12$) in the low-risk population (Fig. 3). Both associations were significant (high-risk: $r = 0.28$, $p = 0.047$; low-risk: $r = -0.37$, $p < 0.001$). This suggests that pheomelanin-based color intensity increased with the body condition of nestlings in the low-risk population, while the reversed pattern was observed in the high-risk population (Fig. 3).

The effect of population alone was significant in the model ($\chi^2_1 = 4.37$, $p = 0.036$), as the intensity of pheomelanin-based color was higher in the high-risk population (least squares mean $\pm$ SE for reflectance slope: 0.021 ± 0.002) than in the low-risk population (0.029 ± 0.002) (Fig. 4a). The effect of sex was also significant ($\chi^2_1 = 46.40$, $p < 0.0001$), as the intensity of pheomelanin-based color was higher in males (0.021 ± 0.002) than in females (0.035 ± 0.002) (Fig. 4b).

The same model but including ΔC_t values for the control genes instead of *Slc7a11* ΔC_t values never resulted in a significant interaction between population identity and gene

expression (*CTNS*: $\chi^2_1 = 1.54$, $p = 0.214$; *MC1R*: $\chi^2_1 = 0.93$, $p = 0.335$; *ASIP*: $\chi^2_1 = 0.01$, $p = 0.902$; *AGRP*: $\chi^2_1 = 0.36$, $p = 0.549$). The interaction was marginally significant for *Slc45a2* ($\chi^2_1 = 4.08$, $p = 0.043$), but correlation tests were not significant when data from both populations were separately analyzed (high-risk: $r = -0.05$, $p = 0.647$; low-risk: $r = 0.15$, $p = 0.137$).

Discussion

Our results show that the influence of both *Slc7a11* expression and body condition on the intensity of pheomelanin-based plumage coloration, differed between Eurasian nuthatch populations with different regimes of predation risk. Predation events on eggs and nestlings are common in the nest boxes of our southern population but are virtually absent in our northern population, which justifies considering the former a high-risk population and the latter a low-risk population. Although the correlational nature of our study prevents us from determining the cause, there is a clear difference in predation frequency between our two populations that otherwise present similar habitat characteristics. This makes likely that predation risk explains the difference in the influence of *Slc7a11* expression and body condition on pheomelanin-based coloration between populations. In fact, a recent experiment conducted in the southern population provides strong evidence that perceived predation risk (through alarm calls emitted by parents) is responsible for differences in the physiological regulation exerted by *Slc7a11* expression (Galván 2018). It is possible that nestlings in this population also perceive a higher rate of alarm calls from adults than in the other, northern population. Probably because of this differential influence, the resulting pigmentation phenotype of nuthatches from the high-risk population consisted of a more intense orange plumage coloration than that of nuthatches from the low-risk population. Future experimental manipulations of predation risk will be necessary to firmly conclude that this causes differences in the influence of *Slc7a11* expression and body condition on pheomelanin-based coloration.

Perceived predation risk is already known to be a natural source of physiological stress in songbirds (Newman et al. 2013) and to produce oxidative stress and damage in other animals

(Janssens and Stoks 2014). If cysteine in melanocytes is used to synthesize pheomelanin pigment instead of GSH utilized for antioxidant activity, this can generate chronic oxidative stress (Napolitano et al. 2014, Panzella et al. 2014). This possibility is more likely if environmental conditions, such as elevated predation risk, in turn favor physiological and oxidative stress. This allows to hypothesize the evolution of mechanisms that are physiologically advantageous by limiting pheomelanin synthesis under such conditions. One of such mechanisms seems to be environmental lability in *Slc7a11*, which allows its downregulation in melanocytes under exposure to pro-oxidant substances. In some species of birds, this in turn allows avoiding oxidative damage by saving cysteine that can be used for GSH synthesis in other cells (Galván et al. 2017a). Our study suggests that an additional mechanism providing environmental lability to melanocytes may have evolved in birds. Instead of, or in addition to inducing the downregulation of *Slc7a11* under environmental oxidative stress, melanocytes may make a differential utilization of cysteine when exposed to stress to favor GSH instead of pheomelanin synthesis. This has actually been experimentally proved in our southern population, where an experimental exposure to predation risk downregulated *Slc7a11* expression in the feather melanocytes of those nuthatch nestlings with lowest GSH levels (i.e., in higher need of antioxidant capacity), thus avoiding oxidative damage to cells (Galván 2018).

We propose that our findings support the existence of this potential mechanism because of the following reasons. 1) The influence on pheomelanin-based feather color intensity of genes known to affect pheomelanin synthesis by mechanisms different from modifications of cysteine availability did not differ between populations. 2) In the low-risk population, pheomelanin-based feather color intensity tended to increase with *Slc7a11* expression in feather melanocytes, as expected from the fact that *Slc7a11* expression increases intramelanocytic cysteine levels (Chintala et al. 2005), but clearly decreased in the high-risk population. Thus, under high predation risk, pheomelanin production in nuthatch nestlings did not increase, and even decreased, with *Slc7a11* expression. This is most likely explained by an effect occurring after *Slc7a11* is expressed, i.e. after cystine is transported to melanocytes. It is

possible that greater amounts of cystine enter the GSH synthesis pathway instead of the pheomelanin synthesis pathway in melanosomes. 3) In the low-risk population, pheomelanin-based feather color intensity increased with the body condition of nestlings, as expected if only birds in good physical condition were able to use cysteine resources for pheomelanin production, but decreased with body condition in the high-risk population. This suggests that the sensitivity of pheomelanin synthesis to variations in body condition may be lost under a high risk of predation. It has previously been proposed that the negative association between pheomelanin-based feather color intensity and body condition in nuthatch nestlings that is found in our high-risk population may respond to a detoxifying benefit of pheomelanin synthesis, as this would help avoiding excess cysteine (i.e., levels higher than needed for GSH and protein synthesis; Galván 2017). This may be physiological advantageous, as excess cysteine is toxic and decreases GSH levels (Viña et al. 1983, Munday 1989). Nuthatch nestlings in poor condition may thus be suffering the toxicity of excess cysteine, hence their greater pheomelanin production (Galván 2017). In fact, a recent experiment in the high-risk population indicates that feather melanocytes of nuthatch nestlings receiving dietary cysteine show epigenetic modifications related to RNA and DNA methylation in some of the genes investigated in the present study, which promotes pheomelanin synthesis and pigmentation (Rodríguez-Martínez et al. 2019). It may be speculated that, if nestlings exposed to high predation risk limit the use of intramelanocytic cysteine for pheomelanin synthesis, they may avoid oxidative stress but in turn favor excess cysteine. This is likely, as cysteine is not incorporated into the GSH synthesis pathway immediately after entering cells (Lu 1999). This may then favor pheomelanin synthesis in a body condition-dependent manner and independently of *Slc7a11* expression. These considerations are summarized in Fig. 5.

We cannot envisage the mechanism that makes melanocytes sensitive to predation-induced stress, but our findings suggest that this type of stress affects the condition-dependence of pheomelanin-based color traits. This may be relevant to understand the evolution of honest communication, as the expression of large visual signals is often restricted

to individuals in good body condition that therefore denote high genetic quality (Hasson 1997). Indeed, the honesty of the bib size of male house sparrows *Passer domesticus*, a color plumage patch produced by pheomelanin (Galván et al. 2014), can be explained by a different sensitivity of high- and low-quality males to variations in cysteine availability (Galván and Alonso-Alvarez 2017). Some birds therefore seem to have evolved a mechanism that uncouples cysteine and pheomelanin synthesis when the latter is physiologically limiting, as it happens in low-quality birds (Galván and Alonso-Alvarez 2017) or in those perceiving high predation risk (this study). Within melanocytes, melanin synthesis exclusively occurs inside melanosomes, where it is considered that cysteine spontaneously enters the synthesis pathway that leads to pheomelanin formation when it is above a certain concentration (Land et al. 2003). A possible mechanism that allows derivating intramelanocytic cysteine to either GSH or pheomelanin synthesis pathways may therefore be related to the regulation of the transport of cysteine inside melanosomes. The gene *CTNS* regulates the ejection of cysteine from melanosomes (Chiaverini et al. 2012), but we did not find any difference in the influence of this gene's expression on pheomelanin-based pigmentation between populations. To date, the mechanisms that regulate intramelanocytic cysteine transport to melanosomes are virtually unknown (García Borrón and Olivares-Sánchez 2011), but our study suggests that these might be responsible for the potentially adaptive strategy that leads some birds to adjust their pigmentation phenotype to environmental stress. This, in turn, can make that pheomelanin-based coloration transfers different information depending on stress conditions experienced during development, which implies a differential potential for the action of sexual selection (Galván and Rodríguez-Martínez 2018).

Declarations

These are provided in the Title page.

Data availability

The datasets generated during and/or analyzed during the current study will be deposited in the Dryad Digital repository upon acceptance.

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Legends to figures:

Figure 1. Map showing the location of the two Eurasian nuthatch study populations. Insert photograph shows the flank plumage patch pigmented by pheomelanin of a nuthatch nestling.

Figure 2. Relationship between the slope of the reflectance spectra of Eurasian nuthatch feathers and *Slc7a11* expression (normalized C_t values: ΔC_t) in melanocytes from the same feathers in two populations differing in predation risk. Pheomelanin concentration in feathers increases as reflectance slope decreases. *Slc7a11* expression increases as C_t decreases. The lines are the best-fit lines.

Figure 3. Relationship between the slope of the reflectance spectra of feathers and the body condition of Eurasian nuthatch nestlings in two populations differing in predation risk. Pheomelanin concentration in feathers increases as reflectance slope decreases. Body condition is measured as the residuals of body mass regressed against tarsus length. The lines are the best-fit lines.

Figure 4. Least squares mean $\pm$ SE pheomelanin-based plumage color expression, measured by means of the slope of the reflectance spectra of feathers, in Eurasian nuthatch nestlings from two populations differing in predation risk (a) and in male and female nestlings (b).

Figure 5. Schematic representation of the hypothesis tested here. Nestling Eurasian nuthatches perceive predation risk through alarm calls emitted by their parents. Under relative low predation risk (upper figure), cysteine taken by the growing feather melanocytes of nestlings after expression of the gene *Slc7a11* is used for the synthesis of the main intracellular antioxidant (glutathione, GSH) and is also incorporated into the pheomelanin synthesis pathway in melanosomes. Accordingly, in the present study we found a clear tendency of *Slc7a11*

expression to positively affect the pheomelanin-based color intensity of feathers in the low-risk population. In contrast, nestlings under relative high predation risk (lower figure) are expected to require higher amounts of GSH for antioxidant protection, as perceived predation risk produces oxidative stress, and limiting the incorporation of intramelanocytic cysteine into the pheomelanin synthesis pathway may save cysteine for GSH synthesis. Accordingly, we found that *Slc7a11* expression was negatively related to pheomelanin-based feather color intensity in the high-risk population, suggesting an uncoupling (marked with a dashed arrow) between *Slc7a11* expression and pheomelanin synthesis under high levels of environmental oxidative stress. The fact that the pheomelanin-based feather color intensity of nestlings increased with their body condition in the low-risk population suggests that only nestlings in good physical condition were able to use cysteine resources for pheomelanin production. The fact that pheomelanin-based color intensity decreased with body condition in the high-risk population suggests that the relative limitation of pheomelanin production by nestlings under such conditions may avoid oxidative stress but create higher needs of excess cysteine removal, to which pheomelanin synthesis contributes.

Figure 1

Figure 2

Figure 3

Figure 4