Changes in melanocyte RNA and DNA methylation favor pheomelanin synthesis and may avoid systemic oxidative stress after dietary cysteine supplementation in birds

Running title: Epigenetics of pheomelanin-based pigmentation

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Abstract

Cysteine plays essential biological roles, but excessive amounts produce cellular oxidative stress. Cysteine metabolism is mainly mediated by the enzymes cysteine dioxygenase and γ -glutamylcysteine synthetase, respectively coded by the genes *CDO1* and *GCLC*. Here we test a new hypothesis posing that the synthesis of the pigment pheomelanin also contributes to cysteine homeostasis in melanocytes, where cysteine can enter the pheomelanogenesis pathway. We conducted a experiment in the Eurasian nuthatch *Sitta europaea*, a bird producing large amounts of pheomelanin for feather pigmentation, to investigate if melanocytes show epigenetic lability under exposure to excess cysteine. We increased systemic cysteine levels in nuthatches by supplementing them with dietary cysteine during growth. This caused in feather melanocytes the downregulation of genes involved in intracellular cysteine metabolism (*GCLC*), cysteine transport to the cytosol from the extracellular medium (*Slc7a11*) and from melanosomes (*CTNS*), and regulation of tyrosinase activity (*MC1R* and *ASIP*). These changes were mediated by increases in DNA m⁵C in all genes excepting *Slc7a11*, which experienced RNA m⁶A depletion. Birds supplemented with cysteine synthesized more pheomelanin than controls, but did not suffer higher systemic oxidative stress. These results suggest that excess cysteine activates an epigenetic mechanism that favors pheomelanin synthesis and may protect from oxidative stress.

KEYWORDS

cysteine homeostasis, epigenetic mechanisms, gene expression, melanocytes, methylation, pheomelanin-based pigmentation

1. INTRODUCTION

Cysteine is a semi-essential amino acid that cells metabolize to produce glutathione (GSH), the major cellular antioxidant (1). Cysteine metabolism also leads to the production of another amino acid, cysteinesulfinate, which is further converted to either taurine or pyruvate and inorganic sulfur (2). These metabolites play a role in several essential cellular processes, ranging from energy supplementation to antioxidant protection (3,4). However, excess cysteine can occur when cysteine availability is above the rate of cysteine metabolism, which favors the autooxidation to the disulfide (cystine), a redox cycling that generates reactive oxygen species (ROS) and thus produces oxidative stress (5). As a consequence, excess cysteine is responsible for several, often lethal oxidative stress-based cytotoxic effects (6,7).

The maintenance of cysteine homeostasis is mainly mediated by two enzymes that compete for cysteine as a substrate: cysteine dioxygenase (CDO), which catalyzes the addition of molecular oxygen to the sulfhydryl group of cysteine to form cysteinesulfinate, and γ -glutamylcysteine synthetase (GCS), which catalyzes the rate-limiting step in GSH synthesis consisting in the binding of cysteine to glutamate (2,8). CDO and GCS are therefore essential enzymes in the maintenance of cysteine homeostasis. In spite of this process, CDO and GCS activity does not seem sufficient to avoid the occurrence of excess cysteine. This is shown by the fact that a dysfunction in cystinosin, a cystine/H⁺ symporter that exports cystine out of lysosomes, causes intralysosomal excess cysteine and corresponding disease (cystinosis) despite apparent functionality of CDO and GCS (9). Cystinosin can thus be considered another essential component for the maintenance of cysteine homeostasis (Figure 1).

71 In addition to CDO, GCS and cystinosis, another mechanism of cysteine
72 homeostasis specific to melanocytes has recently been proposed. Melanocytes are
73 cells that contain lysosome-like organelles, termed melanosomes, where the
74 synthesis of melanin pigments takes place (10). Melanin synthesis consists in the
75 oxidation of the amino acid tyrosine and the polymerization of the resulting indole
76 compounds. If intramelanosomal cysteine concentration is above a certain threshold,
77 kinetic conditions favor the incorporation of the sulfhydryl group of cysteine to the
78 reaction, which results in the formation of sulfur-containing heterocycles, reddish or
79 yellowish pigments that are termed pheomelanins (11). Pheomelanin is then
80 transferred to surrounding keratinocytes, thus conferring pigmentation to the skin and
81 associated structures such as hair, feathers and scales (10). Therefore, cysteine
82 used in pheomelanin synthesis cannot be incorporated back into cysteine
83 metabolism, which means that the production of large amounts of pheomelanin in
84 melanocytes can lead to chronic systemic oxidative stress if cysteine is limiting
85 because sufficient GSH cannot be produced (12,13). This could actually explain the
86 increased risk of melanoma observed in humans and mice expressing phenotypes
87 that result from a high pheomelanin production (14,15), or the diminished antioxidant
88 capacity observed in wild birds exposed to ionizing radiation that pigment their
89 feathers with large amounts of pheomelanin (16). However, under the absence of
90 environmental factors that induce oxidative stress and make cysteine limiting,
91 pheomelanin synthesis may represent a form of cysteine excretion, thus helping to
92 avoid excess cysteine. Accordingly, pheomelanin synthesis has been proposed as a
93 mechanism contributing to cysteine homeostasis (17). In sum, there is a potential
94 physiological trade-off between the use of cysteine for pheomelanin synthesis and its

use for GSH synthesis, and the outcome of this trade-off can be determined by environmental oxidative stress.

The functionality of pheomelanin-producing melanocytes in the context of excess cysteine avoidance remains unexplored. If such function is physiologically advantageous, we hypothesized that melanocytes would favor pheomelanin synthesis under an increase in cysteine availability. Here we investigate this possibility by experimentally increasing the dietary uptake of cysteine to developing Eurasian nuthatches *Sitta europaea*, a passerine bird that deposits large amounts of pheomelanin in flank feathers (18). Specifically, we tested if melanocytes from growing pheomelanin-pigmented feathers show epigenetic lability and respond to the increment in cysteine availability with a genetic favoring of pheomelanin synthesis.

To test our hypothesis, we quantified the expression of genes coding for the mediators of cysteine metabolism (CDO, GCS and cystinosin), which are, respectively, cysteine dioxygenase type I [*CDO1* (19)], glutamate-cysteine ligase catalytic subunit [*GCLC* (20)] and *CTNS* (21). Additionally, we quantified the expression of the gene encoding the cystine/glutamate antiporter xCT (solute carrier family 7 member 11, *Slc7a11*), a protein localized in the plasma membrane (22,23) that is thus responsible for providing cells with cysteine (24). We also quantified the expression of the gene *Slc45a2* (solute carrier family 45 member 2), for which a similar function in transporting cysteine to cells has been suggested (25). Lastly, we quantified the expression of the main genes that regulate pheomelanin synthesis by changing the intracellular concentration of cyclic adenosine monophosphate (cAMP) and thus influence the intramelanosomal activity of tyrosinase, the key enzyme in the melanogenesis pathway. These are the genes coding for the melanocortin 1 receptor

in the membrane of melanocytes [*MC1R* (26)] and peptides that bind to it and act as their antagonists: agouti-signalling (*ASIP*) and agouti-related (*AGRP*) proteins (27).

The genes described above and their influence on cysteine metabolism and pheomelanin synthesis are summarized in Figure 1. We investigated if the expression of these genes is sensitive to increase in cysteine availability in a manner that favors pheomelanin synthesis in melanocytes. To date, the genes that regulate intramelanocytic cysteine transport to melanosomes (Figure 1) are unknown (11), but the investigation of the genes considered here should reflect a potential favoring of the genetic pathway to synthesis of pheomelanin in response to an increase in cysteine uptake. Any potential increase in pheomelanin synthesis by feather melanocytes should result in an increase of plumage color intensity, which reflects the amount of pheomelanin deposited in feathers in our model species (28). We also investigated if these potential effects on gene expression are mediated by changes in RNA and DNA methylation. Recent developments in analytical methods have unveiled a key role of internal modifications in mRNA mediated by *N*⁶-methyladenosine (m⁶A) in the regulation of gene expression in eukaryotes (29,30). In DNA, the best known epigenetic modification is that mediated by 5-methylcytosine (m⁵C), which leads to transcriptional silencing (31). We therefore quantified m⁶A in mRNA and m⁵C in DNA at the target genes using antibody-mediated capture methods to investigate potential differential roles of these epigenetic marks in possible changes in gene expression after the experimental cysteine supplementation. Lastly, we investigated the potential consequence of cysteine supplementation on cellular oxidative stress at a systemic level and on the physical condition of animals.

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144 **2. METHODS**145 **2.1 Experimental design**

146 The experiment was conducted in a wild population of Eurasian nuthatch in Sierra
147 Norte de Sevilla Natural Park, southern Spain. Frequent checks of wood nest boxes
148 placed in the study area provided data on dates of clutch initiation, which allowed us
149 to follow the breeding activity of all nuthatch pairs. Nuthatch nestlings leave the nest
150 (i.e., the developmental period is complete) about 21 days after hatching. This
151 research was approved by the Bioethics Subcommittee of the Spanish National
152 Research Council (CSIC) and by local authorities (authorization #06-04-15-227 by
153 Consejería de Agricultura y Pesca y Desarrollo Rural, Junta de Andalucía).

154 17 nuthatch nestlings from eight nests were used in the study (Figure S1). The
155 number of nestlings in nests ranged from 1 to 5, the mean being 2.1. All nestlings in
156 each nest were used. At day 6 after hatching, the nestlings were banded with
157 numbered metal rings for identification and weighed to the nearest 0.1 g with a
158 portable digital balance. At days 6, 7 and 8 after hatching, L-cysteine (Sigma-Aldrich,
159 St. Louis, MO) was orally administered to some nestlings at a dose of 0.1 g/l in a total
160 volume of 100 µl of water using a syringe, while other nestlings that served as
161 controls only received 100 µl of water. After two days without any treatment, the
162 same administration of L-cysteine or water was repeated on days 11, 12 and 13 after
163 hatching. A single dose was administered per nestling and day. The nestlings were
164 assigned to these treatments (cysteine or control) following the order of body weights
165 but changing the start of the sequence of treatments in each nest so that all positions

in the sequence of weights received the same number of the different treatments. 11 birds were supplemented with cysteine and six birds were controls.

On day 17, the nestlings were weighed again and their tarsus length was measured to the nearest 0.01 mm with a digital calliper as an index of body size. 15-20 pheomelanin-pigmented, orange flank body feathers were plucked from each nestling, immersed in RNAlater solution (Ambion, Thermo Fisher Scientific, Waltham, MA) to stabilize and protect RNA, and stored at -80 °C. Blood samples were taken from the brachial vein and stored at -80 °C after separating cell and plasma fractions by centrifugation.

2.2 Molecular sex determination

To determine the sex of nestling nuthatches, we extracted DNA from the blood with the ISOLATE II Genomic DNA kit (Bioline, London, UK) and used real-time quantitative PCR (qPCR) combined with melting curve analysis (32). Reactions were performed with SYBR Green I Master in a LightCycler 480 System (Roche, Basel, Switzerland) with the primer pair CHD1F/CHD1R (5'-TATCGTCAGTTTCCTTTTCAGGT-3' and 5'-CCTTTTATTGATCCATCAAGCCT-3') (33). The melting curve analyses differentiated males and females through a peak of melting temperature at 81 °C in males and a peak at 78 °C in females.

2.3 Cysteine levels in erythrocytes by GC

To investigate the effect of experimental treatment on systemic cysteine levels, we measured the levels of cysteine in erythrocytes following the method developed by Švagera et al. (34) for plasma. To induce cell lysis and thus facilitate the extraction of

intracellular cysteine, erythrocytes were first diluted to 1:10 with a carbonate-buffered saline (5 mM Na_2CO_3 in saline). 10 μl of internal standard (4-chloro-DL-phenylalanine, PCP; Sigma-Aldrich) and 10 μl of reducing agent (dithiothreitol, DTT; Sigma-Aldrich) were then added to 40 μl of supernatant of the homogenate of cell pellet and buffer. Samples were then deproteinized by adding 40 μl of 0.6 M trichloroacetic acid (TCA). After centrifugation, the supernatant was aspirated and transferred into glass culture tubes (J. Jimeno, Valladolid, Spain). The supernatant was derivatized with an organic phase consisting of a mixture of isooctane, butyl acetate and ethyl chloroformate in a 10:6:1 volume ratio. 130 μl of this reactive organic phase were added to the supernatant in the glass tubes after adding 40 μl of a mixture of pyridine and ethanol in a 1:3 volume ratio. After 10 min of incubation, the organic phase was aspirated and transferred to vials for chromatography.

Samples were analyzed in a GC-2010 gas chromatography (GC) system (Shimadzu, Kyoto, Japan) with a hydrogen flame ionization detector (FID). A capillary column Agilent HP-1 (15 m x 0.25 mm x 0.25 μm ; Agilent Technologies, Santa Clara, CA) was used. Retention times were 3.2 min and 3.5 min for cysteine and PCP, respectively. Chromatographic peaks were integrated with the software GCsolution (Shimadzu). A standard curve was prepared using L-cysteine dissolved in carbonate-buffered saline at concentrations of 50, 100, 200 and 400 μM and processed as described above for erythrocyte samples. Cysteine levels are expressed as μmol per gram of pellet.

2.4 Isolation of feather melanocytes

Flank feathers were cut at the rachis and the plumulaceous part was stored in the dark until the analyses of pigmentation. We extracted melanocytes from the melanin unit of feathers, which corresponds to the bottommost portion of the feather follicles. Like hair follicles (35), the melanin unit of feathers represents an important reservoir of melanocytes. Melanocytes at the dermal papillae show intense melanogenesis during feather development (36), meaning that the melanin unit of feathers represents the main source of integumentary melanins in birds. 15 follicular melanin units were pooled per bird. Nucleic acids obtained from these samples therefore correspond to melanocytes to a large extent.

2.5 Extraction of RNA and DNA from feather melanocytes

Total RNA was extracted from follicular melanin units using TRI Reagent (Ambion). DNA was extracted using a Quick-DNA Plus kit (Zymo Research, Irvine, CA). RNA and DNA were quantified with a Qubit 4 Fluorometer (Invitrogen, Thermo Fisher Scientific).

2.6 mRNA expression

After extracting total RNA, 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 target genes: *Slc7a11*, *Slc45a2*, *GCLC*, *CDO1*, *CTNS*, *MC1R*, *ASIP* and *AGRP*. Additionally, we quantified the expression of the gene *NFE2L2* to obtain a measure of intrinsic antioxidant capacity (see 'Systemic oxidative

stress and body condition' section below). Reactions were performed using SYBR Green I Master in a LightCycler 480 System. The housekeeping glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used for normalization, as this is the most suitable endogenous reference gene (37) and most commonly used in the analysis of gene expression in bird feathers (27,38). Gene primers were designed based on refseq sequences (GenBank) using the Primer-BLAST tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

Cycle threshold (C_t), defined as the number of cycles at which fluorescence signal changes to an exponential increase, was used as a measure of gene expression. C_t is inversely related to the amount of amplicon in the reaction, thus lower C_t values indicate higher mRNA and gene expression levels. Normalization was made by subtracting C_t values for *GAPDH* from C_t values for the target genes (ΔC_t).

2.7 Quantification of m^6A in RNA by immunoprecipitation and real-time qPCR

To quantify m^6A in RNA from feather melanocytes, we followed the immunoprecipitation method developed by Dominissini et al. (39), with some modifications. After DNase digestion (see above), 15 μ l from each total RNA sample were separated and stored at -80 °C until later use as an input RNA control of the immunoprecipitation procedure. The remaining RNA was split in two 1.7 ml tubes. One of these tubes was subject to the complete immunoprecipitation procedure, while the other tube was used as a no-antibody control.

The samples were heat-denatured (65°C, 10 min) and immediately placed on ice. 200 U of RNasin Plus RNase Inhibitor (Promega Corporation, Madison, WI),

2mM ribonucleoside vanadyl complexes (RVC; Sigma-Aldrich), 2 mg of m⁶A-antibody (Synaptic Systems, Goettingen, Germany) and the remaining volume up to 1ml of IP Buffer [10 mM Tris-HCl, 150 mM NaCl and 0.1% (vol/vol) Igepal CA-630 (Sigma-Aldrich)] were added to each sample. In the no-antibody control tube, the volume of antibody was replaced by the same volume of water. The mixtures were incubated on a rotating platform at 4 °C for 2 h. 200 µl of beads with immobilized recombinant Protein A (IPA-300; Repligen, Waltham, MA) were blocked with a 0.5 mg/ml bovine serum albumin solution (Sigma-Aldrich) in immunoprecipitation buffer (IP buffer) supplemented with RNAsin (5% vol/vol) and RVC (5% vol/vol) for 2 h on a rotating wheel. After two washes with IP buffer, the previously incubated samples were added and keep on a rotating wheel at 4 °C for 2 h. Then, the supernatant was removed, followed by four washing steps with IP buffer. After the final wash, 100 µl of elution buffer [10 mM Tris-HCl, 150 mM NaCl, 0.1% (vol/vol) Igepal CA-630, RNAsin (5% vol/vol), RVC (5% vol/vol) and 6.7 mM m⁶A 5'-monophosphate sodium salt (Sigma-Aldrich)] were added to the sedimented beads. The mixture was incubated at 4 °C for 1h with continuous shaking. The eluted RNA was recovered by precipitation, converted to cDNA and analyzed by real-time qPCR as described in the previous section.

The proportion of RNA with m⁶A at the target genes was calculated by dividing C_t of the input RNA control by C_t of the immunoprecipitated test sample. As this is a proportion, no additional controls are necessary, although we included the ratio (C_t control / C_t test sample) for the gene *GAPDH* as a covariate in the linear mixed-effects models used for analyzing the data (see Statistical Analyses) to account for a possible covariation with *GAPDH* RNA methylation. However, results were not

affected by the inclusion or exclusion of the *GAPDH* ratio in the analyses (see Results), confirming that it was not necessary to use housekeeping genes as controls in the analysis of the methylated fraction of genes.

2.8 Quantification of m⁵C in DNA by immunoprecipitation and real-time qPCR

2 µg of genomic DNA diluted in 130 µl of water were fragmented in a Covaris E220 Focused-ultrasonicator (Covaris, Woburn, MA) specifying a fragment size range of 300-1000 bp. Sonicated DNA was split in three tubes (test and controls – input DNA and no-antibody). The samples (test and no-antibody control) were heat-denatured (95°C, 10 min) and immediately placed on ice for 5 min. 1 µg of m⁵C monoclonal antibody 33D3 (Diagenode, Liege, Belgium) were added to each sample. The corresponding volume of water was added to the no-antibody control. IP buffer (10 mM NaPO₄, pH 7.0; 140 mM NaCl and 0.05% Triton X-100) was then added to each sample to a final volume of 500 µl. The mixture was incubated on a rotating platform at 4 °C for 2 h. Pre-blocked Protein A/G beads (Diagenode) were then added and incubated on a rotating wheel at 4 °C for 2 h. The supernatant was removed, followed by four washing steps with IP buffer. After the final wash, the beads were resuspended in 400 µl of digestion buffer (10 mM Tris, pH 8.0; 100 mM EDTA, 0.5% SDS and 50 mM NaCl) and 100 µg of proteinase K was added. The digestion mixture was incubated overnight at 50°C. DNA was purified with a DNA Clean & Concentrator utility (Zymo Research) and analyzed by real-time qPCR. Gene primers were designed based on refseq sequences (GenBank) using the Primer-BLAST tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The proportion of DNA with m⁵C at

the target genes was calculated by dividing C_t of the input DNA control by C_t of the immunoprecipitated test sample.

2.9 Systemic oxidative stress and body condition

To obtain a measure of oxidative stress at a general, systemic level, reduced (GSH) and oxidized (GSSG) glutathione were quantified in the blood of nestling nuthatches. Total glutathione levels in erythrocytes were determined by following the method described by Tietze (40) and Griffith (41) with some modifications (see, e.g., ref. 42 for details of this technique applied to bird blood samples). To determine GSSG levels, an aliquot (200 μ l) of the supernatant obtained for the assessment of total glutathione was adjusted to a pH of 7.5 by adding 6 N NaOH. Afterward, 4 μ l of 2-vinylpyridine were added to the aliquot, and the mixture was vigorously shaken at ambient temperature in the dark to promote the chemical masking of GSH. The mixture was then centrifuged (3500 g for 15 minutes), and the change in absorbance of the supernatant was assessed at 405 nm using a COBAS Integra 400 plus analyzer (Roche). The GSH:GSSG ratio was used as an index of systemic oxidative stress. Oxidative stress increases as the GSH:GSSG ratio decreases. The gene *NFE2L2* encodes the transcription factor NRF2, the master regulator of the cellular antioxidant response (43). Thus, the effect of the experimental treatment on the GSH:GSSG ratio was investigated controlling for the intrinsic antioxidant capacity of birds, which was made including ΔC_t for *NFE2L2* as a covariate in the linear mixed-effects models (see Statistical Analyses).

On the other hand, we investigated the effect of the experimental treatment on the physical, body condition of nuthatch nestlings, a predictor of survival prospects in

the species (44). For this, we used body mass corrected by (i.e., independent of) body size, as this measure is a good indicator of subcutaneous fat content in nuthatches and other birds (18). This was analyzed by linear mixed-effects models with body mass as a response variable and tarsus length as a covariate (see Statistical Analyses).

2.10 Pheomelanin synthesis and pigmentation

To determine the relative amount of pheomelanin produced and deposited in flank feathers by nuthatch nestlings, we quantified the intensity of the color of feathers by UV-Vis reflectance spectrophotometry (28). We used an Ocean Optics Jaz spectrophotometer (range 220-1000 nm) with UV (deuterium) and visible (tungsten-halogen) lamps and a bifurcated 400 μm fiber optic probe (Ocean Optics, Dunedin, FL). The fiber optic probe both provided illumination and obtained light reflected from the sample, with a reading area of ca. 1 mm^2 . 15 flank feathers were mounted on a light absorbing foil sheet (Metal Velvet coating, Edmund Optics, Barrington, NJ) to avoid any background reflectance, such that they resembled the natural appearance of the plumage 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), and reference measurements were frequently made. An average spectrum of five readings on different points of the orange, pheomelanin-pigmented portion of 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.

We have previously proved using Raman spectroscopy (45) that, in Eurasian nuthatch flank feathers, the slope of lines fitted to reflectance spectra (i.e., the slope of percent reflectance regressed against wavelength) across the 300-700 nm range is a good predictor of the pheomelanin content, with lower slopes denoting higher color intensity and higher pheomelanin contents (28). We therefore summarized reflectance spectral data as a measure of reflectance slope. Although feathers usually contain eumelanin, the dark non-sulphurated melanin form, no detectable amounts of eumelanin have been found in the flank feathers of nuthatches (28). This means that eumelanin is absent or in very low, undetectable levels, thus variation in the color expression of flank feathers mainly reflects variation in their relative pheomelanin content.

2.11 Statistical analyses

We used linear mixed-effects models (LMM) fit with restricted maximum likelihood (REML) estimation, including experimental treatment (cysteine vs. control) as a fixed factor and nest identity as a random factor to account for the common origin of nuthatch nestlings belonging to the same nests. In the analysis of pheomelanin content of feathers (reflectance slope), sex was included as an additional fixed factor in the models because male Eurasian nuthatches exhibit darker feathers than females (18). In the analysis of systemic oxidative stress (GSH:GSSG ratio), ΔC_t for *NFE2L2* was included as an additional covariate in the models to account for the intrinsic antioxidant capacity of birds. Table 1 summarizes the predictor effects that were considered in the model of each response variable. All variables were $\log_{10}$ -transformed to fulfill the normality assumption of parametric tests.

LMM analyses were made in R environment (46) using the package *lme4* (47). P-values were calculated through the analysis of deviance of LMMs on the basis of Wald χ^2 tests using the package *car* (48).

3. RESULTS

3.1 Effect on cysteine levels in erythrocytes

The concentration of cysteine in the erythrocytes of birds supplemented with dietary cysteine (mean $\pm$ SE: 442.62 $\pm$ 28.11 $\mu\text{mol/g}$) was significantly higher than that of control birds (373.15 $\pm$ 11.98 $\mu\text{mol/g}$; $\chi_1^2 = 23.89$, $P < 0.0001$) (Figure 2). This indicates that the experimental cysteine supplementation during development increased cysteine levels in birds at a systemic level.

3.2 Effects on gene expression and RNA and DNA methylation in melanocytes

The cysteine supplementation induced a downregulation of four genes in feather melanocytes: *Slc7a11* ($\chi_1^2 = 9.92$, $P = 0.002$), *CTNS* ($\chi_1^2 = 36.26$, $P < 0.0001$), *MC1R* ($\chi_1^2 = 6.06$, $P = 0.014$) and *ASIP* ($\chi_1^2 = 6.75$, $P = 0.009$). *GCLC* was also downregulated, although the difference of mean expression level with controls was marginally non-significant ($\chi_1^2 = 3.78$, $P = 0.051$) (Figure 3). In contrast, there was no significant differences in gene expression level between cysteine-supplemented and control birds for *Slc45a2* ($\chi_1^2 = 1.73$, $P = 0.188$), *CDO1* ($\chi_1^2 = 0.72$, $P = 0.394$) and *AGRP* ($\chi_1^2 = 0.53$, $P = 0.464$) (Figure 3). According to the regulatory functions of the genes considered here (Figure 1), these results suggest a change in physiological conditions favoring pheomelanin synthesis.

No difference in the proportion of RNA with m⁶A nucleosides was found between cysteine-supplemented and control birds for any gene (*Slc45a2*: $\chi^2_1 = 0.05$, $P = 0.818$, *GCLC*: $\chi^2_1 = 1.54$, $P = 0.215$, *CTNS*: $\chi^2_1 = 0.04$, $P = 0.834$, *MC1R*: $\chi^2_1 = 0.05$, $P = 0.825$, *AGRP*: $\chi^2_1 = 0.15$, $P = 0.701$), with the exception of *Slc7a11* and *CDO1*. In the case of *Slc7a11*, the proportion of RNA with m⁶A was higher in controls ($\chi^2_1 = 6.84$, $P = 0.009$) because no m⁶A was detected in any cysteine-supplemented bird (Figure 3). In the case of *CDO1*, the proportion of RNA with m⁶A was higher in cysteine-supplemented birds ($\chi^2_1 = 8.08$, $P = 0.004$). No RNA m⁶A was found in the gene *ASIP* among the samples. These results did not change when the proportion of m⁶A in RNA for GAPDH was excluded from the analyses.

An increase in the proportion of DNA with m⁵C bases was observed in the same genes that were downregulated after the cysteine supplementation with the exception of *Slc7a11* ($\chi^2_1 = 0.42$, $P = 0.518$) and *MC1R* ($\chi^2_1 = 0.42$, $P = 0.514$): *GCLC* ($\chi^2_1 = 8.28$, $P = 0.004$), *CTNS* ($\chi^2_1 = 3.59$, $P = 0.058$) and *ASIP* ($\chi^2_1 = 8.14$, $P = 0.004$) (Figure 3). Additionally, an increase in the proportion of DNA with m⁵C was detected in *CDO1* ($\chi^2_1 = 3.92$, $P = 0.048$) and *AGRP* ($\chi^2_1 = 6.33$, $P = 0.012$). No differences were found in *Slc45a2* ($\chi^2_1 = 0.76$, $P = 0.383$) (Figure 3).

3.3 Effects on oxidative stress in erythrocytes and body condition

The GSH:GSSG ratio in erythrocytes did not differ between cysteine-supplemented (mean $\pm$ SE: 29.84 ± 9.08) and control birds (24.01 ± 3.03 ; $\chi^2_1 = 10^{-3}$, $P = 0.975$). The effect of experimental treatment was neither observed in the body condition of birds ($\chi^2_1 = 0.29$, $P = 0.587$). This indicates that the dietary supplementation of cysteine did

not induce systemic oxidative stress nor a negative effect on the physical condition of birds.

3.4 Effect on pheomelanin synthesis and pigmentation

The reflectance slope of the flank feathers of birds supplemented with cysteine (mean $\pm$ SE: 0.017 ± 0.002) was significantly lower than that of controls (0.027 ± 0.005) ($\chi^2_1 = 4.29$, $P = 0.038$; Figure 4A). As reflectance slope decreases as the concentration of pheomelanin in feathers increases, this indicates that cysteine-supplemented birds produced greater amounts of pheomelanin that was deposited in feathers. This effect was reflected in a perceptible difference in the color of feathers, which were more intense in cysteine-supplemented birds than in controls (Figure 4B).

4. DISCUSSION

This study indicates that a dietary supplementation of cysteine leads to an increase in pheomelanin production that is mediated by methylation changes in some genes involved in cysteine metabolism and pheomelanin synthesis in melanocytes. Our experiment succeeded in producing a significant increase in systemic cysteine levels in developing birds despite the increment in pheomelanin production, suggesting that excess cysteine occurred at the organismal level. Excess cysteine causes cellular oxidative stress that leads to glutathione depletion (49). Consequently, excess cysteine is cytotoxic and neurotoxic and has been shown to exert detrimental effects in mammals and birds (6,7,50). In humans, oxidative stress mediated by elevated systemic cysteine levels has even been proposed as a causative factor of cancer (51). Our study shows, however, that nestling nuthatches with experimentally-

induced excess cysteine levels did not exhibit lower reduced-to-oxidized glutathione ratios (GSH:GSSG) nor poorer body condition than controls despite a downregulation of the gene that controls glutathione synthesis in melanocytes (*GCLC*), suggesting that the increase in pheomelanin production represents an advantageous epigenetic mechanism that protects from oxidative stress. Given the molecular similarity between the melanogenesis pathway in melanocytes of birds and mammals (52), this epigenetic mechanism is also of relevance to humans. The oxidation of GSH to GSSG occurs immediately after the exposure to the source of oxidative stress (53), thus it is not likely that the lack of decrease in GSH:GSSG ratio found here was due to an early measurement of this parameter in birds.

The favoring of the genetic pathway to synthesis of pheomelanin induced by cysteine supplementation can be inferred from the regulatory roles of the genes considered here (Figure 1). *CDO1* and *GCLC* code respectively for the enzymes CDO and GCS, which compete for cysteine as a substrate (2,8), and it should thus be expected that a downregulation of these genes favored pheomelanin synthesis because greater amounts of cysteine would be available to be transported to melanosomes (Figure 1). We actually found that *GCLC* was downregulated in feather melanocytes of cysteine-supplemented birds as compared to controls. According to the well known repressing effect of DNA methylation on transcription (31), we also found that feather melanocytes of cysteine-supplemented birds increased the proportion of DNA m⁵C in *GCLC*. An increase in DNA m⁵C was also found in *CDO1*, but this was not reflected in a downregulation of the gene. Interestingly, *CDO1* also exhibited an increase in RNA m⁶A in cysteine-supplemented birds. Recent studies associate high DNA methylation levels in *CDO1* with several tumor types in humans

(54,55), although also show a decrease in gene expression (56). To our knowledge, this is the first time that an increase in RNA methylation is observed in *CDO1* as a response to a physiologically damaging effect, and future studies should investigate if this is associated to the lack of downregulation of this gene despite increased DNA m⁵C (see also below).

The gene *Slc7a11* codes for the cell membrane protein xCT, which transports cysteine (in the form of cystine) from the extracellular medium to the cytosol, its expression in melanocytes thus resulting in an increase in pheomelanin synthesis (24). Transgenic sheep overexpressing xCT develop patches of hair pigmented by pheomelanin (23), and a tendency in pheomelanin-based color intensity to increase with *Slc7a11* mRNA expression in feather melanocytes has also been shown in some birds (57). In the present study, however, the increase in pheomelanin synthesis in cysteine-supplemented birds was associated with a downregulation of *Slc7a11* in feather melanocytes. It must be considered, however, that cysteine in the cytosol of melanocytes can equally enter the cysteine metabolism pathway or the melanogenesis pathway in melanosomes (Figure 1), while the limiting source for pheomelanin synthesis is the concentration of cysteine inside melanosomes (11). In this regard, *CTNS*, which codes for a protein that exports cystine out of lysosomes and its expression in melanocytes thus inhibits pheomelanin synthesis (9), was also downregulated in feather melanocytes of cysteine-supplemented birds. In fact, other birds exposed to an environmental source of oxidative stress (diquat dibromide), which is expected to induce a reduction of pheomelanin synthesis that avoids a decrease of glutathione and antioxidant capacity, downregulated *Slc7a11* but not *CTNS* in feather melanocytes (58). It seems reasonable, indeed, that a

physiologically advantageous mechanism favoring pheomelanin synthesis under excessive cysteine levels in cells includes downregulation of *Slc7a11*, which limits uptake and further accumulation of cysteine in cells, and downregulation of *CTNS*, which maximizes the accumulation of the already high intramelanocytic levels of cysteine in melanosomes. In contrast, expression and methylation levels in *Slc45a2* were not affected by cysteine supplementation, arguing against a role of this gene in cysteine transport to melanocytes as findings in other birds suggest (57).

CTNS downregulation was associated with an increase in DNA m⁵C, but the same was not found in *Slc7a11*. Instead, *Slc7a11* downregulation was accompanied by a depletion of RNA m⁶A. It is accepted that DNA methylation generally leads to a decrease of gene expression (31), but the effect of RNA methylation on gene expression has only recently begun to be explored. Some authors have reported decreases of gene expression with increases in mRNA m⁶A (59), but more recent studies show that increases in mRNA m⁶A or m⁵C lead to increases in the expression of several genes (60,61). This may therefore be in accordance with the depletion of RNA m⁶A and expression downregulation of *Slc7a11* in feather melanocytes of cysteine-supplemented birds. This may also explain why *CDO1* was not downregulated despite increased DNA m⁵C in cysteine-supplemented birds, as these animals also showed increased RNA m⁶A in *CDO1*. Interestingly, then, these results may suggest that the genes regulating cysteine metabolism and transport are differentially affected by RNA and DNA methylation under exposure to excess cysteine.

Lastly, *MC1R*, *ASIP* and *AGRP* are the main genes affecting pheomelanin synthesis by regulating the activity of tyrosinase. *MC1R* was downregulated in

feather melanocytes of cysteine-supplemented birds, thus favoring pheomelanin synthesis because this gene codes for the expression of the melanocortin 1 receptor in the membrane of melanocytes, to which melanocortins bind and stimulate the synthesis of eumelanin as opposed to that of pheomelanin (26). *MC1R* downregulation was not accompanied, however, by a change in RNA or DNA methylation. *MC1R* methylation has never been reported to affect melanin synthesis, as polymorphic variation in this gene is considered the base of its influence on melanin-based pigmentation (10). Our results suggest, however, that cysteine-induced *MC1R* downregulation may be the result of a covariation with the expression of the receptor antagonist, like in other receptor-ligand systems (62). Indeed, *ASIP* was downregulated in cysteine-supplemented birds, which was associated with an increase in DNA m⁵C, consistent with findings in mice (63). A low expression of *ASIP* inhibits pheomelanin synthesis and pigmentation (27), but in our study *ASIP* downregulation and methylation may be indirectly inducing *MC1R* downregulation and, thus, promoting pheomelanin synthesis.

In conclusion, our results show that an experimental increase in cysteine uptake induces a downregulation of genes involved in cysteine metabolism and pheomelanin synthesis in feather melanocytes that results in the favoring of pheomelanin production and avoids the expected systemic oxidative stress caused by excess cysteine levels. The downregulation of gene expression is mediated by changes in methylation of RNA or DNA, that differentially controls the expression of distinct genes. This epigenetic mechanism therefore seems physiologically advantageous. A more precise description of this mechanism will require, however, future experiments in which cysteine supplementation is provided in increasing

doses, thus allowing to exactly determine the conditions that activate the pheomelanogenesis pathway and its physiological limitations. Particularly, although our experiment suggests an induction of excess cysteine at the systemic level, future studies should try to block the epigenetic mechanism observed here to produce cysteine-mediated toxicity and thus firmly demonstrate the potential adaptiveness of this mechanism.

However, the mechanism is not expected to be functional in all animals, as other species of birds like the house sparrow *Passer domesticus* show a decrease in systemic antioxidant capacity despite an increase in pheomelanin production after an experimental induction of excess dietary cysteine (64). This may be due to the fact that the average concentration of the benzothiazole moiety of pheomelanin in the studied trait in house sparrows [65.7 ng/mg feather (65)] is more than 1000 times lower than the concentration of the benzothiazole moiety of pheomelanin in the flank feathers of Eurasian nuthatches studied here [104.1 µg/mg feather (66)]. Similarly, the expression of *CTNS* in feather melanocytes tends to increase instead of decrease with protein food abundance in a strict carnivorous bird with a limited production of pheomelanin such the gyrfalcon *Falco rusticolus* (67). Thus, the epigenetic mechanism that protects from oxidative stress by favoring pheomelanin synthesis may be functional only in species that already have a genetic basis leading to the production of large amounts of pheomelanin, which is reflected in pigmentation phenotypes consisting of light brown or orange colorations (66). In this regard, it will be interesting to investigate if this mechanism is present in humans with the red hair/fair skin phenotype associated with the production of large amounts of pheomelanin for hair pigmentation (68), which may actually explain the evolution of

this human phenotype despite the constraints imposed by pheomelanin synthesis (12-15). Lastly, it is interesting to note that Eurasian nuthatch males with more intense pheomelanin-based pigmented flank feathers mate later in the season than males producing lower amounts of pheomelanin (28), indicating that a negative consequence of the epigenetic mechanism may be a cost in terms of sexual selection. This is because nuthatches avoided oxidative stress by developing more intensely pigmented feathers (Figure 4). Given the recent knowledge of the influence of sexual selection on gene expression and genome evolution (69), future studies should investigate how the mechanism shown here affects the evolution of traits.

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AUTHOR CONTRIBUTION

S.R.M. and I.G. conceived the study. S.R.M., R.M. and I.G. conducted the experiment. S.R.M. and R.M. performed laboratory analyses. A.I. contributed to the development of methods for DNA and RNA methylation analyses. S.R.M. and I.G. conducted statistical analyses and wrote the manuscript.

DATA ACCESSIBILITY

All data obtained and used during this study is available on Dryad Digital Repository
(DOI: 10.5061/dryad.8j5j72t).

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Table 1. Summary of linear mixed-effects models (LMM) conducted to test for the effect of cysteine supplementation (Treat.) on the response variables considered in the study (upper row): systemic cysteine concentration, gene expression levels (ΔC_t) in melanocytes, proportion of RNA and DNA methylation (m^6A and m^5C) in melanocytes, systemic oxidative stress levels (GSH:GSSG ratio), body mass (condition) and feather pigmentation intensity (reflectance slope). Predictor variables (lower rows) other than treatment are included to control for potentially confounding effects on the responses: nest identity (random factor), sex (fixed factor), and C_t control: C_t test sample ratio for *GAPDH*, ΔC_t for *NFE2L2* and tarsus length (covariates).

Cysteine concentration	ΔC_t	m^6A RNA	m^5C DNA	GSH:GSSG	Body mass	Refl. slope
Treat.	Treat.	Treat.	Treat.	Treat.	Treat.	Treat.
Nest id.	Nest id.	Nest id.	Nest id.	Nest id.	Nest id.	Nest id.
		C_t control:		ΔC_t <i>NFE2L2</i>	Tarsus length	Sex
		C_t test sample				
		<i>GAPDH</i>				

Legends to figures:

Figure 1. Scheme of cysteine metabolism in melanocytes. Gene names are shown in italics. Solid arrows represent transport or conversion. Dashed arrows represent influences of the indicated sign. Cysteine is transported from the extracellular medium by the cystine/glutamate antiporter xCT, which is encoded by the gene *Slc7a11* (a similar role may be fulfilled by *Slc45a2*, but this is still not clear). In the cytosol of melanocytes, cysteine can be converted to either pyruvate + sulfate or taurine by the addition of molecular oxygen to its sulfhydryl group, which is catalyzed by the enzyme cysteine dioxygenase (CDO) whose synthesis is in turn coded by the gene *CDO1*. Cysteine can also enter the synthesis pathway of glutathione (GSH), the most important intracellular antioxidant. The rate-limiting step in GSH synthesis is the binding of cysteine to glutamate, which is catalyzed by the enzyme γ -glutamylcysteine synthetase (GCS) whose synthesis is in turn coded by the gene *GCLC*. Lastly, cysteine can also enter melanosomes, the organelles where pheomelanin synthesis takes place. The genetic regulation of cysteine transport to melanosomes is unknown, but the efflux of cysteine out of melanosomes is regulated by the gene *CTNS*. The synthesis of pheomelanin in melanosomes is favored by intramelanosomal cysteine levels. Additionally, pheomelanin synthesis is negatively affected by the activity of the enzyme tyrosinase, which is in turn positively affected by cyclic adenosine monophosphate (cAMP). cAMP levels are positively affected by the activation of the melanocortin 1 receptor (MC1R), coded by the *MC1R* gene, in the melanocyte membrane. The antagonists of MC1R are the agouti signaling protein (ASIP) and the agouti-related protein (AGRP), coded by the genes *ASIP* and *AGRP*, respectively.

Figure 2. Mean $\pm$ SE systemic (erythrocyte) concentration of cysteine in developing Eurasian nuthatches experimentally supplemented with dietary cysteine (red symbol) and in controls (hollow symbol). Cysteine levels were higher in birds supplemented with cysteine than in controls ($\chi^2_1 = 23.89$, $P < 0.0001$).

Figure 3. Normalized gene expression levels (ΔC_t , left column) and proportion of DNA with 5-methylcytosine bases (right column) in eight genes regulating cysteine transport and metabolism and pheomelanin synthesis in melanocytes. Values are mean $\pm$ SE obtained in melanocytes of growing flank feathers from developing Eurasian nuthatches experimentally supplemented with dietary cysteine (red symbols) and from controls (hollow symbols). Note that gene expression levels increase as ΔC_t decreases. Asterisks above graphs indicate statistically significant differences between cysteine-supplemented birds and controls (***: $P < 0.0001$, **: $P < 0.001$, *: $P < 0.05$). Black circles indicate marginally non-significant differences ($0.05 < P < 0.06$).

Figure 4. A: Average UV-Vis reflectance spectra of flank feathers from developing Eurasian nuthatches experimentally supplemented with dietary cysteine (red symbols) and from controls (hollow symbols). Values are median $\pm$ SE percent reflectance in 10 nm intervals. Dashed lines are the result of fitting regression lines to the reflectance spectra. The slope of these lines decreases as the concentration of pheomelanin in the flank feathers increases. The mean $\pm$ se reflectance slope values

912 for cysteine-supplemented birds and controls are shown in the insert graph. Slope
913 values in cysteine-supplemented birds are lower than in the controls ($\chi^2_1 = 4.29$, $P =$
914 0.038). B: Image of flank feathers from a female nuthatch supplemented with
915 cysteine and from a female control nuthatch. Note the more intense orange color in
916 the feathers of the cysteine-supplemented bird as compared to the control, indicative
917 of a lower reflectance slope and a higher pheomelanin content.