**Exposure to a competitive social environment activates
an epigenetic mechanism that limits pheomelanin
synthesis in zebra finches**

Running title: Epigenetic mechanism limits pheomelanin synthesis

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Abstract

Competitive environments promote high testosterone levels, oxidative stress and, consequently, impair cellular homeostasis. The regulation of genes involved in the synthesis of the pigment pheomelanin in melanocytes seems to help to maintain homeostasis against environmental oxidative stress. Here, we experimentally increased social interactions in some zebra finch *Taeniopygia guttata* males by keeping them in groups of six birds during feather growth, while others were kept alone, to test if melanocytes show epigenetic lability under a competitive social environment. As these changes may depend on the oxidative status, we administrated buthionine sulfoximine (BSO) to decrease the antioxidant capacity of some birds. The competitive environment downregulated a gene involved in pheomelanin synthesis (*Slc7a11*) by changing the level of DNA methylation in feather melanocytes. In other genes involved in pheomelanin synthesis (*Slc45a2*, *MC1R* and *AGRP*), DNA methylation was also affected, but no changes in expression were detected. The exposure to the competitive environment did not affect systemic oxidative stress and damage, indicating that a protective epigenetic mechanism that changes the expression of *Slc7a11* may have been activated. However, no changes on the pigmentation phenotype of birds were found, likely due to the short duration or low intensity of the competitive environment. BSO treatment did not affect the epigenetic mechanism, suggesting that the antioxidant capacity of birds was high enough to deal with the competitive environment. An epigenetic mechanism limiting pheomelanin synthesis gets therefore activated under exposure to a competitive environment in male zebra finches, which may help avoiding damage caused by competitive interactions.

Keywords: pheomelanin synthesis, oxidative stress, gene expression, competitive social environment, epigenetic mechanism

Introduction

The social environment is an important factor that may induce a physiological stress response (Creel, Dantzer, Goymann, & Rubenstein, 2013). To maintain homeostasis under exposure to stressors caused by social interactions, organisms mount a diversity of physiological and/or behavioral responses (Tamashiro, Nguyen, & Saka, 2005). Agonistic social encounters, in particular, are a source of physiological stress (Masis-Calvo et al., 2018). In this regard, it has been shown that agonistic interactions affect DNA methylation patterns (i.e., Turecki & Meaney, 2016; Cardoso-Júnior, Eyer, Dainat, Hartfelder & Dietemann, 2018). Epigenetic changes lead phenotypic plasticity to adjust physiology response to novel conditions (Duncan, Gluckman & Dearden, 2014). Social competition may thus induce phenotypic plasticity (Hofmann, Benson, & Fernald, 1999) through epigenetic mechanisms (Champagne, 2010).

One of the main physiological mechanisms that mediate the adaptive responses of animals to environmental information is the hormonal regulation (Adkins-Regan, 2005). This regulation may lead testosterone levels to increase in response to social interactions (Wingfield, 1985), particularly those derived from a high density of competing males (Lacava, Brasileiro, Maia, Oliveira & Macedo, 2011). Maintaining high testosterone levels increases the chance to succeed in those interactions, but may also represent a physiological cost in terms of oxidative stress, i.e. the imbalance between the production of reactive oxygen

and nitrogen species (ROS and RNS) and the availability of antioxidant compounds that combat them (Alonso-Alvarez, Bertrand, Faivre, Chastel & Sorci, 2007). This cost arises because testosterone impairs the activity of antioxidant compounds that protect cells against oxidative damage (Chainy, Samantaray, & Samanta, 1997). Thus, agonistic interactions can increase testosterone levels, produce oxidative stress and, consequently, impair cellular homeostasis.

Testosterone may also affect the synthesis of melanins (Slominski, Tobin, Shibahara & Wortsman, 2004), responsible for black (eumelanin) and orange-reddish (pheomelanin) coloration (Ozeki, Ito, Wakamatsu & Ishiguro, 1997). Pheomelanin synthesis implies the consumption of cysteine, a semi-essential amino acid that is a constituent of glutathione (GSH), the main cellular antioxidant (Galván, Ghanem & Møller, 2012). This makes that, under high environmental stress conditions, intense pheomelanin synthesis favors that organisms suffer chronic stress (Napolitano, Panzella, Monfrecola & d'Ischia, 2014). However, cysteine can be toxic if in excess, as its oxidation promotes oxidative stress (Munday, 1989). Thus, under low environmental stress conditions or high availability of dietary cysteine, pheomelanin synthesis may be an adaptive mechanism that helps to maintain cysteine homeostasis (Galván et al., 2012). Indeed, it has recently been demonstrated that excess cysteine activates an epigenetic mechanism that favors pheomelanin synthesis and may protect melanocytes, the cells where melanin synthesis takes place, from oxidative stress (Rodríguez-Martínez, Márquez, Inácio & Galván, 2019).

The response of cells to environmental changes often includes the modulation of gene expression (López-Maury, Marguerat & Bähler, 2008). In this

regard, Galván (2018) has recently demonstrated that an environmental source of stress (predation risk) in a bird leads to the downregulation of genes that favor pheomelanin synthesis, which avoids oxidative stress. The regulation of pheomelanin synthesis therefore represents a mechanism against environmental oxidative stress. It remains unknown, however, whether environmental factors other than predation risk can induce similar effects in pheomelanin synthesis and pigmentation, and which underlying mechanisms allow cells and organisms to adjust gene expression according to those environmental factors. The underlying mechanisms may be represented by changes in DNA methylation (Champagne, 2010; Weaver et al., 2004; Zhang & Meaney, 2010), as oxidative stress modulates the activity of enzymes responsible for DNA demethylation (Niu, DesMarais, Tong, Yao & Costa, 2015). As changes in DNA methylation can be influenced by the social context and hormone levels (Weaver et al., 2004; Dhiman, Attwood, Campbell & Smiraglia, 2015), a similar adaptive response to that observed in response to predation risk (Galván, 2018) could be expected from competitive environments that promote high testosterone levels and increase the susceptibility to oxidative stress.

Here we explore this possibility by experimentally inducing a competitive social environment in male zebra finches *Taeniopygia guttata*. We investigated if the expression of genes involved in pheomelanin synthesis changes in response to environmental stress mediated by competitive interactions and if these are associated to changes in DNA methylation. As these changes may depend on the intrinsic oxidative status of birds, those with lowest antioxidant capacity being in higher need of limiting pheomelanin synthesis in response to stress (Galván, 2018), we simultaneously administrated buthionine sulfoximine (BSO) to deplete

GSH levels in some birds. We also investigated the potential consequences of exposure to a competitive social environment on systemic oxidative stress and damage and on the body condition of birds. We expect that oxidative stress and damage are higher in birds kept in groups than in solitary birds. A potential epigenetic mechanism mediated by DNA methylation changes is therefore expected in birds that are both kept in groups and treated with BSO.

Material and Methods

Experimental design

The study was carried out at Estación Biológica de Doñana (Seville, Spain) with 78 captive adult male zebra finches, a bird that displays patches of pheomelanin-based pigmentation in flank feathers (McGraw & Wakamatsu, 2004). All birds were marked with a numbered metal ring and housed in individual cages (60 x 40 x 25 cm) during ten days to achieve acclimation to the new conditions. Temperature (average: 24 ± 0.5 °C) and light-dark daily cycle (13L: 11D) were controlled. After the acclimation period, flank feathers were plucked to stimulate the growth of new ones and to measure pheomelanin-based color intensity, following previous studies (Galván, Wakamatsu, Camarero, Mateo & Alonso-Alvarez, 2015; Galván, Inácio, Romero-Haro & Alonso-Alvarez, 2017a). The birds were weighted with a digital balance, and their tarsus length measured with a digital calliper as an index of body size. Blood samples were collected to quantify systemic oxidative stress (reduced-to-oxidized glutathione ratio, GSH:GSSG), centrifuged, and plasma and cell fractions immediately stored at -80 °C. Values obtained at this stage are referred to as 'initial values'.

After initial measurements, the birds were randomly assigned to one of four different treatments: group vs. solitary and BSO vs. control. To manipulate the competitive social environment, 30 individuals were kept alone in individual cages, whereas other 48 birds were kept in groups of six individuals. To manipulate oxidative capacity, half of the birds received BSO at a dose of 1.11 g/l in the drinking water (Romero-Haro & Alonso-Alvarez, 2015), while the other half received only water and thus served as controls.

After 15 days with treatments, the growing flank feathers were collected to measure gene expression in melanocytes and pheomelanin-based color intensity (Galván et al., 2017a; Rodríguez-Martínez et al., 2019). We also quantified DNA methylation on these samples. The birds were weighted, and blood samples were collected to quantify systemic oxidative stress and oxidative damage (malondialdehyde, MDA). Values obtained at this stage are referred to as 'final values'.

This study was approved by the Bioethics Subcommittee of Consejo Superior de Investigaciones Científicas (CSIC) (ref. 651/2018) and local authorities (Consejería de Agricultura, Pesca y Medio Ambiente, Junta de Andalucía; ref. 23/02/2018/016).

Measurement of gene expression in melanocytes

Melanocytes at the dermal papillae show intense melanogenesis activity during feather development (Lin et al., 2013). 15 melanin units from the bottommost portion of the feather follicles of each bird were used to obtain nucleic acid

164 samples. Total RNA was extracted using TRI Reagent (Ambion, Thermo Fisher
165 Scientific, Waltham, MA), and residual genomic DNA carry over was removed with
166 TURBO DNA-free kit (Ambion). Complementary DNA (cDNA) was prepared from
167 total RNA using RevertAid Reverse Transcriptase provided in the RevertAid First
168 Stand cDNA Synthesis kit (Thermo Scientific, Thermo Fisher Scientific). qPCR
169 was performed on cDNA of genes involved in cysteine transport through
170 melanocytes (*Slc7a11* and *Slc45a2*), a gene coding for the mediators of cysteine
171 metabolism (*CTNS*) and genes that regulate pheomelanin synthesis by changing
172 the intracellular concentration of cyclic adenosine monophosphate (cAMP)
173 (*MC1R*, *ASIP* and *AGRP*). *Slc7a11* (solute carrier family 7 member 11) encodes
174 the cysteine/glutamate antiporter xCT, a protein localized in the plasma
175 membrane (Conrad & Sato, 2012; He, Li, Zhou, Zhao & Li, 2012) that is
176 responsible of cysteine transport into melanocytes (Chintala et al., 2005). *Slc45a2*
177 (solute carrier family 45 member 2) was also quantified, as it has been suggested
178 to play a similar function in transporting cysteine to cells (Gunnarsson et al.,
179 2007). *CTNS* gene codes for a protein (cystinosis) responsible for the transport
180 of cysteine out of lysosomes (Town et al., 1998). Lastly, genes that regulate
181 intracellular concentration of cAMP and thus influence the activity of tyrosinase,
182 the key enzyme in the melanogenesis pathway, were quantified. These genes
183 code for the melanocortin 1 receptor in the membrane of melanocytes (*MC1R*;
184 Naysmith et al., 2004) and peptides that bind to it and act as their antagonists:
185 agouti-signaling (*ASIP*) and agouti-related (*AGRP*) proteins (Nadeau et al., 2008).
186 Also the gene *NFE2L2* was quantified to obtain a measure of intrinsic oxidative
187 capacity (see below).

qPCR reactions were performed using SYBR Green I Master in a LightCycler 480 System, employing denaturation at 95 °C, annealing at 55 °C and extension at 72 °C, with 50 amplification cycles. As the housekeeping glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is the most suitable endogenous reference gene (Silver, Best, Jiang & Thein, 2006) and the most common reference used in gene expression analyses in feathers (Nadeau et al., 2008; Walsh, Dale, McGraw, Pointer & Mundy, 2011), it was used for normalization. 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'-CACAGCAATGCCACGGTCCGG-3' and 5'-GTAGGTGGGCGAGTGCAGGT-3' for *MC1R*; 5'-AACGTGTCCAGAAGGAGCAA-3' and 5'-TGCAGGTTTTGAAGGTTGGC-3' for *ASIP*; and 5'-TGGGGTTTGAGCAGATGACC-3' and 5'-CCCTCAGCTTGCAGTTCTGT-3' for *AGRP*; and 5'-GGACCAGGTTGTCTCCTGTG-3' and 5'-TCCTTGGATGCCATGTGGAC-3' for *GAPDH*.

Gene expression was measured by means of cycle threshold (C_t) levels, with lower C_t values indicating higher gene expression and mRNA levels.

211 Normalization was made by subtracting C_t values for *GAPDH* from C_t values for
212 the target genes (ΔC_t).

214 **DNA methylation data**

215 DNA was extracted using a Quick-DNA Plus kit (Zymo Research, Irvine, CA) from
216 follicular melanin units. We quantified 5-methylcytosine (m^5C) at the target genes
217 by immunoprecipitation and real-time qPCR following a procedure previously
218 used with the same type of samples (Rodríguez-Martínez et al., 2019). The
219 proportion of DNA with m^5C was calculated by dividing C_t of the input DNA control
220 between C_t of the immunoprecipitated test sample (Rodríguez-Martínez et al.,
221 2019).

223 **Systemic oxidative stress, oxidative damage and body condition**

224 Oxidative stress was quantified in blood by measuring reduced (GSH) and
225 oxidized (GSSG) glutathione levels. As glutathione is constantly experiencing
226 redox process in cells (Lu, 2009), the ratio GSH:GSSG reflects the proportion of
227 glutathione that is oxidized which is an indicator of the level of oxidative stress. In
228 contrast, GSH indicates glutathione level available for antioxidant protection.
229 Total glutathione level in erythrocytes was determined by following the methods
230 described by Tietze (1969) and Griffith (1980) with some modifications (see e.g.,
231 Galván & Alonso-Alvarez, 2009 for details of this technique applied to bird blood
232 samples). GSSG levels were determined from an aliquot (200 μ l) of the
233 supernatant obtained for the assessment of total glutathione adjusted to a pH of

7.5 by adding 6 N NaOH. Subsequently, 2-vinylpyridine (4 μ l) was added to the aliquot and vigorously shaken at ambient temperature in darkness to promote glutathione derivatization. Finally, the mixture was centrifuged (3,500 g for 10 min) and the change in absorbance of the supernatant was assessed at 405 nm using COBAS Integra 400 plus analyzer (Roche). The ratio GSH:GSSG was used as an index of systemic oxidative stress, with higher ratios indicating lower oxidative stress levels. The gene *NFE2L2* encodes for the transcription factor NRF2, a regulator of the cellular antioxidant response (Huppke et al., 2017). Thus, the effect of competitive social environment on the GSH:GSSG ratio was analyzed controlling for the intrinsic antioxidant capacity of birds by including ΔC_t for *NFE2L2* as a covariate in the linear mixed-effects models (see statistical analysis section).

Oxidative damage at a systemic level was measured by quantifying MDA in final plasma samples. MDA is a product of the peroxidation of cellular lipids induced by ROS (e.g., Weismann et al., 2011). MDA levels in plasma (picomoles of MDA per μ l of plasma) were quantified by measuring the absorbance at 532 nm of the product generated in the reaction of MDA with thiobarbituric acid, using the lipid peroxidation assay kit of Sigma Aldrich (St. Louis, MO, USA).

We also investigated potential effects on the physical condition of birds. This was made by running linear mixed-effects models (see Statistical analyses section below) with body mass as a response variable and tarsus length as a covariate.

Plumage color expression and pheomelanin content in feathers

The relative amount of pheomelanin content in flank feathers was quantified by measuring color intensity by UV-Vis reflectance spectrophotometry (Galván & Rodríguez-Martínez, 2018) using an Ocean Optics Jaz spectrophotometer (range 220-1000 nm) with ultraviolet (deuterium) and visible (tungsten-halogen) lamps and a bifurcated 400 micrometer fiber optic probe. The fiber optic probe both provide illumination and obtained light reflected from the sample, with a reading area of ca. 1 mm². 10 to 15 flank feathers were mounted on a light absorbing foil sheet (Metal Velvet coating, Edmund Optics, Barrington, NJ) to avoid any background reflectance. Measurements were taken at a 90° angle to the samples. All measurements were relative to a diffuse reflectance standards tablet (WS-1, Ocean Optics, Dunedin, FL), and reference measurements were frequently made. An average spectrum of five readings of different points of the feathers was obtained for each bird, removing the probe between each measurement. Reflectance curves were determined by calculating the median of the percent reflectance in 10 nm intervals. Spectral data was summarized as the summed percent reflectance values across the 300-700 nm range. As an alternative measure of pheomelanin-based color expression, the slope of percent reflectance regressed against wavelength was also calculated. In both summed reflectance and reflectance slope, lower values denoted darker colors and higher color intensity (Galván & Rodríguez-Martínez, 2018).

The flank feathers collected at the beginning of the experiment (i.e., initial values) were analyzed by micro-Raman spectroscopy to calculate their relative content of melanin. Both pheomelanin and the non-sulphurated form of melanin (eumelanin) exhibit distinctive Raman signal that can be used to non-invasively identify and quantify them (Galván, Jorge, Ito, Tabuchi, Solano & Wakamatsu,

2013; Galván & Jorge, 2015). 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 a NIR excitation laser of 780 nm were used. Laser power was set at 5-7 mW, integration time at 1 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. A total of five flank feathers were analyzed per bird, and five Raman spectra were obtained from each feather. An average Raman spectrum was calculated with the five spectra obtained from each bird.

Pheomelanin shows a distinctive Raman spectrum comprising three diagnostic bands located at 500, 1500 and 2000 cm^{-1} , and eumelanin shows different spectra with other diagnostic bands (Galván et al., 2013; Galván & Jorge 2015; Wang et al., 2016; Polidori, Jorge & Ornos, 2017). In the flank feathers of male zebra finches, we only detected Raman signal from pheomelanin together with bands that can be assigned to keratin (Hsu, Moore & Krimm, 1976). The three diagnostic bands of pheomelanin and four diagnostic bands of keratin were thus used to fit Voigt deconvolution functions to the Raman curves, from which spectral parameters were obtained.

The Raman parameter that better predicts pheomelanin concentration in feathers is the intensity (maximum value at the vertical axis) of the pheomelanin band at 2000 cm^{-1} (Galván et al., 2013). This band is located within the so-called Raman “silent region” of biomolecules (i.e. 1800- 2700 cm^{-1} ; Wang et al., 2016).

The intensity of this band increases with increasing the concentration of pheomelanin in feathers (Galván et al., 2013).

We regressed reflectance slope and summed reflectance against the intensity of the 2000 cm^{-1} Raman band to determine which color trait is better related to the pheomelanin content of feathers. Reflectance slope was significantly correlated with the intensity of the 2000 cm^{-1} Raman band ($r = -0.31$, $n = 67$, $P = 0.009$), while summed reflectance was not ($r = -0.16$; $n = 67$; $P = 0.20$). We thus used reflectance slope as an index of pheomelanin-based color expression, with lower slopes indicating higher pheomelanin contents and more intense color values.

Statistical analyses

Linear mixed-effects models (LMM) fit with restricted maximum likelihood (REML) estimation were performed using the R package *lme4* (Bates, Mächler, Bolker & Walker, 2015). P-values were calculated through the analysis of deviance of the models on the basis of Wald χ^2 tests using the package *car* (Fox et al., 2011). In these models, exposure to a competitive social environment (group vs. solitary) and antioxidant capacity (BSO vs. control) were included fixed factors, while cage identity was a random factor to account for the common conditions of birds in the same cages.

To investigate the effect of competitive social environment and antioxidant capacity on the change in the expression of genes involved in pheomelanin synthesis, we conducted LMMs as described above with final gene expression

level (ΔC_t) as response variable and initial gene expression level as a covariate. The interaction between exposure to competitive environment and antioxidant capacity was only significant in the model for *ASIP* (see Results), thus this interaction was removed from the other models. Similar models were conducted to investigate effects on final DNA methylation values.

LMMs conducted to investigate effects on the change in oxidative stress and on final oxidative damage levels included initial GSH:GSSG ratio and final MDA values as response variables, respectively. To account for the intrinsic antioxidant capacity of birds, *NFE2L2* ΔC_t values were included as an additional covariate in these models.

Similar LMMs were conducted to investigate effects on the change in body condition and plumage color intensity. These models included initial values of body mass and reflectance slope, respectively, as covariates.

All variables were $\log_{10}$ -transformed prior to analyses to normalize their distributions. Inspections of residuals confirmed that the normality assumption was fulfilled in all models.

Results

Systemic oxidative stress, oxidative damage and body condition

The GSH:GSSG ratio was not affected by the competitive social environment ($\chi^2_1 = 0.277$, $P = 0.598$), nor by the manipulation of the antioxidant capacity with BSO ($\chi^2_1 = 0.083$, $P = 0.773$). A similar absence of effects was found in MDA levels (social environment: $\chi^2_1 = 0.972$, $P = 0.324$; antioxidant capacity: $\chi^2_1 = 0.082$, $P =$

0.773) and in the body condition of birds (social environment: $\chi^2_1 = 0.046$, $P = 0.828$; antioxidant capacity: $\chi^2_1 = 0.423$, $P = 0.515$). This indicates that the competitive social environment did not induce systemic oxidative stress or damage, nor had a negative effect on the body condition of birds (Figure 1).

Gene expression and DNA methylation in melanocytes

The exposure to the competitive social environment induced a downregulation of *Slc7a11* expression in the melanocytes of growing feathers ($\chi^2_1 = 5.749$; $P = 0.016$) (Figure 2), but no significant changes were found on the expression of the other genes during the experiment (*Slc45a2*: $\chi^2_1 = 0.721$, $P = 0.396$; *CTNS*: $\chi^2_1 = 2.039$, $P = 0.15$; *MC1R*: $\chi^2_1 = 3.252$, $P = 0.071$; *ASIP*: $\chi^2_1 = 1.348$, $P = 0.245$; *AGRP*: $\chi^2_1 = 3.611$, $P = 0.060$). BSO treatment induced an upregulation of *AGRP* ($\chi^2_1 = 6.324$, $P = 0.01$) and *ASIP* ($\chi^2_1 = 14.365$, $P = 0.0001$), but did not lead to significant changes in the expression of the other genes (*Slc7a11*: $\chi^2_1 = 1.687$, $P = 0.193$; *Slc45a2*: $\chi^2_1 = 0.688$, $P = 0.406$; *CTNS*: $\chi^2_1 = 3.473$, $P = 0.062$; *MC1R*: $\chi^2_1 = 2.427$, $P = 0.119$). The interaction between exposure to competitive environment and antioxidant capacity was significant in the model for *ASIP* ($\chi^2_1 = 4.57$, $P = 0.032$).

The proportion of DNA with m^5C was higher in solitary birds than in birds kept in groups in *Slc7a11* ($\chi^2 = 5.86$, $P = 0.015$), *Slc45a2* ($\chi^2_1 = 21.28$, $P < 0.001$), *MC1R* ($\chi^2_1 = 15.07$, $P < 0.001$) and *AGRP* ($\chi^2_1 = 4.13$, $P = 0.042$), while no differences were found in *CTNS* ($\chi^2_1 = 2.13$, $P = 0.144$) nor *ASIP* ($\chi^2_1 = 0.91$, $P = 0.33$). BSO treatment had no effect on the proportion of DNA m^5C in any gene (*Slc7a11*: $\chi^2_1 = 0.20$, $P = 0.652$; *Slc45a2*: $\chi^2_1 = 2.81$, $P = 0.093$; *MC1R*: $\chi^2_1 = 0.26$, P

= 0.605; *AGRP*: $\chi^2_1 = 2.45$, $P = 0.117$; *CTNS*: $\chi^2_1 = 0.035$, $P = 0.851$; *ASIP*: $\chi^2_1 = 0.004$, $P = 0.947$) (Figure 3).

Pheomelanin-based pigmentation in feathers

The exposure to the competitive social environment had no significant effect on the intensity of pheomelanin-based plumage coloration ($\chi^2_1 = 0.02$, $P = 0.870$). However, the reflectance slope of the flank feathers of birds treated with BSO (mean $\pm$ SE: 0.023 ± 0.0011) was significantly higher ($\chi^2_1 = 5.01$, $P = 0.025$) than that of control birds (0.019 ± 0.0012), indicating a lower production of pheomelanin and a lighter pigmentation in birds treated with BSO (Figure 4).

Discussion

Organisms typically respond to stressors with physiological changes that reestablish homeostasis (Avitsur, Powell, Padgett & Sheridan, 2009). Particularly, environmental stressors can affect gene expression patterns by reversible epigenetic mechanisms that lead to phenotypic changes (Tammen, Friso & Choi, 2013). Our study indicates that the exposure to stress derived from a competitive social environment in male zebra finches leads to a change in the expression of a gene involved in pheomelanin synthesis (*Slc7a11*) in melanocytes of growing feathers through an epigenetic mechanism mediated by DNA methylation, which may avoid oxidative stress. Competitive social interactions, which were necessarily higher in male zebra finches kept in groups in relation to solitary males, increase testosterone levels (Eisenegger, Haushofer & Fehr, 2011), which

in turn causes oxidative stress (Alonso-Alvarez et al., 2007). We therefore expected to find higher oxidative stress and damage in male zebra finches kept in groups. However, we did not find differences in GSH:GSSG ratio nor MDA levels between males in groups and solitary birds. This suggests that birds kept in groups may have activated a physiological mechanism, related to a change in the expression of *Slc7a11*, that protects from oxidative stress and damage.

Slc7a11 is located on the surface of cell membranes and regulates the transport of cysteine into the cytosol, thus playing an important role in the synthesis of pheomelanin in melanocytes (Chintala et al., 2005). Consequently, *Slc7a11* expression also affects cellular oxidative stress, as cysteine consumed during pheomelanin synthesis is no longer available for antioxidant protection as a constituent of GSH (Pavel, Smit & Pizinger, 2011). *Slc7a11* downregulation in melanocytes of growing feathers has previously been observed in male zebra finches exposed to an exogenous pro-oxidant substance (Galván, Moraleda, Otero, Álvarez & Inácio, 2017b) and in other bird species (the Eurasian nuthatch *Sitta europaea*) exposed to a natural source of environmental stress (predation risk; Galván, 2018). This indeed resulted in a decrease in the intensity of pheomelanin-based plumage coloration (Galván, 2018). The present study confirms those results and shows that *Slc7a11* downregulation also occurs as a response to another natural source of stress: a competitive social environment. In our study, *Slc7a11* downregulation in birds kept in groups was associated to lower levels of DNA m⁵C than in control birds. DNA methylation generally leads to a decrease of gene expression (Schübeler, 2015), but some recent studies reveal that lower methylation levels may also be associated to lower gene expression (Wierczeiko et al., 2018). While the reasons why DNA methylation

may enhance instead of suppress gene expression must still be elucidated, our results may be related to these recent findings. In any case, our results suggest that *Slc7a11* epigenetic lability may have evolved in some birds as a mechanism sensitive to different sources of environmental stress that adjusts pheomelanin synthesis to stress conditions and thus protects from oxidative stress and damage. Despite *Slc7a11* downregulation, we did not find a decrease in the intensity of pheomelanin-based pigmentation in feathers, which may be due to the short duration of the experimental treatment (15 days).

Although *CTNS* also exerts an important role in pheomelanin synthesis, its expression did not differ between male zebra finches in groups and solitary males. *CTNS* encodes cystinosin, a protein that pumps cysteine out of melanosomes (the organelles where melanogenesis takes place inside melanocytes), thus its expression inhibits pheomelanin synthesis (Chiaverini et al., 2012). An absence of changes in *CTNS* expression in feather melanocytes has also been reported in another bird species (the lesser kestrel *Falco naumanni*) under exposure to a different natural source of environmental stress such as breeding colony size (Galván et al., 2017b). However, when dietary cysteine intake was experimentally increased in developing Eurasian nuthatches, a downregulation of *CTNS* that favors pheomelanin synthesis was detected (Rodríguez-Martínez et al., 2019). Indeed, *CTNS* expression has been suggested to influence pheomelanin synthesis only when systemic cysteine levels are above a certain threshold (Chiaverini et al., 2012). This may explain why we found a downregulation in *Slc7a11* but not in *CTNS* in male zebra finches kept in groups, as systemic cysteine levels were not manipulated.

On the other hand, *Slc45a2* has also been proposed to participate in the transport of cysteine in bird melanocytes (Galván, Inácio & Nielsen, 2017c), but a previous experimental increment of systemic cysteine did not affect its expression in nuthatches (Rodríguez-Martínez et al., 2019). Here, m⁵C DNA proportion in *Slc45a2* decreased in male zebra finches kept in groups as compared to solitary males, but no changes in its expression were found. It may be suggested that our experimental treatment activated the epigenetic mechanism (i.e., changes in DNA methylation) leading to changing the expression of *Slc7a11* and *Slc45a2*, but the short duration of the treatment only resulted in an effect in the expression of the former but not of the latter. This, in turn, may suggest that *Slc7a11* has a more significant role in protecting from environmental stress than *Slc45a2*. The same may be applied to the change in DNA methylation and lack of change in expression in other two genes that affects pheomelanin synthesis by regulating the activity of tyrosinase: *MC1R* and *AGRP* (García-Borrón, Sánchez-Laorden & Jiménez-Cervantes, 2005; Nadeau et al., 2008).

In addition to manipulating the competitive social environment of male zebra finches, we decreased the antioxidant capacity of some of them by administrating BSO, an inhibitor of γ -glutamylcysteine synthetase that consequently decreases GSH levels (Drew & Miners, 1984). We thus expected to find higher oxidative stress and damage, or a more intense activation of the epigenetic protective mechanism, in birds treated with BSO as derived from a higher need of antioxidant protection. However, the interaction between competitive social environment and antioxidant capacity was only significant for *ASIP* but not for the other genes. It may be speculated that the antioxidant

capacity of male zebra finches was high enough to deal with the stress of the competitive social environment induced here, even with BSO treatment, which prevented us from detecting particular limitations in birds both kept in groups and treated with BSO. The molecular basis of this antioxidant capacity may be related to relatively high levels of the antioxidant compounds that regulate the antioxidant machinery of vertebrates, particularly glutathione peroxidase, an enzymatic antioxidant that seems to have an important role in avoiding oxidative damage from normal metabolic activity in birds (Montgomery, Buttemer & Hulbert, 2012). Male zebra finches treated with BSO, however, developed less intense pheomelanin-based plumage coloration. This can be explained by the capacity of BSO to react with intermediate oxidation products of the melanogenesis pathway and consequently lead to the production of more soluble pigments that may be less stable when deposited in feathers (Galván, Wakamatsu, Alonso-Alvarez & Solano, 2014).

In conclusion, our study unveils an epigenetic mechanism that gets activated under exposure to a competitive social environment and creates physiological conditions that limits pheomelanin synthesis in male zebra finches, which may help avoiding oxidative stress caused by social interactions. Particularly, the mechanism seems to be mediated by changes in DNA methylation of some genes involved in pheomelanin synthesis, although only one of them (*Slc7a11*) was downregulated and thus blocked cysteine uptake by melanocytes, and no effects on the pigmentation phenotype of birds are observed. This may be explained by the short duration of the exposure to the competitive social environment and the low stress that this may have caused to birds, which may be sufficient to activate the protective epigenetic mechanism

(DNA methylation changes) but not to end up with changes in gene expression and pigmentation. Environmental factors are known sources of oxidative stress (Schröder & Krutmann, 2004), and can induce epigenetic changes that eventually result in adaptive responses (Jablonka & Lamb, 1989). Physiological responses mediate the adaptation to environmental changes, and our results suggest that the epigenetic regulation of pheomelanin synthesis constitutes one of such responses to stress caused by a competitive social environment.

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Author contributions

S.R-M. and I.G conceived the study, conducted the experiment and statistical analysis, and wrote the manuscript. S.R-M. performed laboratory analysis.

Data accessibility

All data obtained and used during this study are available on the Dryad Digital Repository (<https://doi.org/10.5061/dryad.5rj88j5>).

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Legends to figures:

(A) Oxidative damage (malondialdehyde, MDA, levels from lipid peroxidation) of male zebra finches in relation to antioxidant capacity (BSO vs. controls; circles) and social environment (birds housed in groups vs. solitary birds; triangles) after experimental treatments. No differences were found in any treatment (antioxidant capacity: $\chi^2_1 = 0.082$; $P = 0.773$; social environment: $\chi^2_1 = 0.972$; $P = 0.324$). **(B)** Oxidative status (GSH:GSSG ratio) of male zebra finches in relation to antioxidant capacity (left panel; BSO-treated: grey bars, controls: white bars) and social environment (right panel; groups: black bars, solitary: white bars) before (initial values) and after (final values) experimental treatments. No differences were found in any treatment (antioxidant capacity: $\chi^2_1 = 0.083$, $P = 0.773$; social environment: $\chi^2_1 = 0.277$; $P = 0.598$). **(C)** Body condition of male zebra finches in relation to antioxidant capacity (left panel; BSO-treated: grey bars, controls: white bars) and social environment (right panel; groups: black bars, solitary: white bars) before and after experimental treatments. Body condition was calculated as the residuals of body mass regressed against tarsus length. No changes were found in any treatment (social environment: $\chi^2_1 = 0.046$, $P = 0.828$; BSO: $\chi^2_1 = 0.423$, $P = 0.515$). In all graphs, values correspond to means $\pm$ SE.

Figure 2. Normalized gene expression levels (ΔC_t) of *Slc7a11* (mean $\pm$ SE) in melanocytes of growing flank feathers of male zebra finches exposed to social stress (housed in groups; black circles) and solitary birds (open circles). Values before (initial) and after (final) experimental treatments are shown. Higher ΔC_t final values for males housed in groups indicate lower gene expression ($\chi^2_1 = 5.749$; $P = 0.016$)

Figure 3. Proportion of DNA with 5-methylcytosine (mean $\pm$ SE) in six genes regulating pheomelanin synthesis in melanocytes of growing flank feathers of male zebra finches after exposure to social stress conditions (housed in groups vs. solitary). Asterisks above graphs indicate statistically significant differences between grouped and solitary birds (*: $p < 0.001$, **: $p < 0.0001$).

Figure 4. Reflectance slope (mean $\pm$ SE) of flank feathers of male zebra finches before (initial values) and after (final values) the experimental manipulation of their antioxidant capacity (BSO-treated: black circles, controls: open circles). Final slopes were higher in BSO-treated birds than in controls ($\chi^2_1 = 3.99$; $P = 0.045$). Higher reflectance slopes indicate lower pheomelanin content and lighter color in feathers.

Proportion of DNA 5-methylcytosine