**A source of exogenous oxidative stress improves
oxidative status and favors pheomelanin synthesis in
zebra finches**

Running title: Environmental stress enhance oxidative status

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Keywords: birds, environmental stress, epigenetic changes, gene expression,
melanocytes, plumage coloration

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Abstract

Some organisms can modulate gene expression to trigger physiological responses that help adapt to environmental stress. The synthesis of the pigment pheomelanin in melanocytes seems to be one of these responses, as it may contribute to cellular homeostasis. We experimentally induced environmental oxidative stress in male zebra finches *Taeniopygia guttata* by the administration of the herbicide diquat dibromide during feather growth to test if the expression of genes involved in pheomelanin synthesis shows epigenetic lability. As pheomelanin synthesis implies decreasing the availability of the main cellular antioxidant (glutathione), it is expected to cause oxidative stress unless a protective mechanism limits pheomelanin synthesis and thus favors the antioxidant capacity. However, diquat exposure did not only improve the antioxidant capacity of birds, but also upregulated the expression of a gene (*AGRP*) that promotes pheomelanin synthesis in feather melanocytes, leading to the development of darker plumage coloration. No changes in the expression of other genes involved in pheomelanin synthesis (*Slc7a11*, *Slc45a2*, *MC1R*, *ASIP* and *CTNS*) were detected. DNA methylation levels only changed in *MC1R*, suggesting that epigenetic modifications other than changes in methylation may

regulate *AGRP* expression lability. Our results suggest that exogenous oxidative stress induced a hormetic response that enhanced their oxidative status and, consequently, promoted pheomelanin-based pigmentation, supporting the idea that birds adjust pheomelanin synthesis to their oxidative stress conditions.

Keywords: environmental stress, epigenetic changes, gene expression, melanocytes, plumage coloration

Introduction

Some environmental factors are well known sources of oxidative stress (Schröder and Krutmann 2005), i.e. the imbalance resulting from the production of reactive oxygen species (ROS) over the ability of cells to trigger an effective antioxidant response to combat them (Finkel, 2000). To restore homeostasis after the exposure to stressful environmental conditions, different cellular responses can activate diverse physiological mechanisms (Kültz, 2005). As environments are highly dynamic, organisms have to rapidly adapt their physiological responses to temporary changes in stress levels (Pickering et al., 2013). In fact, phenotypic plasticity is a key strategy that allows organisms to adapt to environmental stress conditions by altering physiological or behavioral functioning (Jones and Relyea, 2015). Adaptation to new environmental conditions can be achieved, for example, by changes in DNA methylation, which may in turn modulate gene expression (López-Maury et al., 2008). Thus,

environmental stressors may trigger phenotypic plasticity by inducing adaptive responses through epigenetic mechanisms.

The expression of pheomelanin-based plumage coloration has been proposed as a physiological mechanism that helps maintaining cellular homeostasis and avoiding oxidative stress (Galván et al., 2012). Pheomelanin is a pigment responsible for orange-reddish coloration (Ozeki et al., 1997), and is synthesized by the incorporation of the semi-essential amino acid cysteine, a constituent of the main cellular antioxidant (glutathione, GSH; Galván et al., 2012), to the melanogenesis pathway that takes place in melanocytes. Cysteine used during pheomelanin synthesis is no longer available for an antioxidant response, thus its synthesis under stressful conditions may lead to chronic oxidative stress (Napolitano et al., 2014). However, cysteine in excess can be toxic, as its oxidation to the disulfide cystine promotes oxidative stress (Munday, 1989). Therefore, pheomelanin synthesis may be an adaptive mechanism to maintain cysteine homeostasis under low environmental stress conditions (Galván et al., 2012). Indeed, it has recently been demonstrated that excess cysteine activates an epigenetic mechanism that favors pheomelanin synthesis and consequently avoids oxidative damage in birds (Rodríguez-Martínez et al., 2019). Furthermore, competitive social interactions also activate an epigenetic mechanism mediated by DNA methylation, which leads to a change in the expression of a gene involved in pheomelanin synthesis and to the avoidance of oxidative stress (Rodríguez-Martínez and Galván, 2019). However, the possibility that exposure to environmental oxidative stress also promotes an epigenetic mechanism that regulates pheomelanin synthesis by changes in gene expression to avoid cellular damage remains unexplored.

Environmental stressors can activate a beneficial response that allows organisms to adapt to the new environmental conditions (Mirbahai and Chipman, 2014). At high doses, stressful stimuli are deleterious, but low doses may induce adaptive responses that increases cell resistance (Calabrese and Baldwin, 2001) by epigenetic mechanisms (Scott et al., 2009). In particular, oxidative stress products (ROS) at low concentrations enhance endogenous antioxidant responses, while a massive level of ROS inhibits enzyme activity and causes cellular apoptosis (Radak et al., 2005). In response to oxidative stress induced by the environment, cells can maintain homeostasis (Samet and Wages, 2018) by modulating the expression of several genes (Causton et al., 2001). This response is regulated by epigenetic mechanisms (Hala et al., 2014), leading to changes in phenotypic traits (Metcalf and Alonso-Álvarez, 2010). In this regard, it has been shown that an environmental stressor (predation risk) alters plumage coloration in birds by changing the expression of genes involved in pheomelanin synthesis, which acts as a protective mechanism against oxidative stress (Galván, 2018). However, it remains unknown which mechanisms regulate the expression of genes involved in pheomelanin synthesis to maintain cellular homeostasis under stressful conditions.

Here, we induced environmental oxidative stress by the administration of a chemical stressor (diquat dibromide) in male zebra finches *Taeniopygia guttata*, a bird that displays patches of pheomelanin-based pigmentation in flank feathers (Mcgraw and Wakamatsu, 2004). Diquat is a bipyridilium aquatic herbicide commonly used in agriculture that has a negative impact on aquatic environments (Peterson et al., 1997). This pesticide produces intracellular superoxide anion and increases the production of ROS in organisms (Di Giulio

et al., 1989). Birds were treated with diquat to simulate environmental stress exposure during the growth of feathers, thereby testing oxidative stress effects on pheomelanin synthesis. We tested if the expression of genes involved in pheomelanin synthesis in feather melanocytes is affected by environmental oxidative stress and if this is activated by changes in DNA methylation level. We expected that, if such physiological response was activated by birds treated with diquat, cellular homeostasis would be maintained and this would in turn avoid oxidative stress.

Material and Methods

Experimental design

The study was carried out with 40 captive adult male zebra finches at Estación Biológica de Doñana (Seville, Spain). All birds were marked with a numbered metal ring and housed in individual cages (60 x 40 x 25 cm) at controlled temperature (average: 24 ± 0.5 °C) and light-dark daily cycle (13L: 11D). After 10 days of acclimation, the orange flank covert feathers were plucked to stimulate the growth of new ones and to measure pheomelanin-based color intensity. To calculate an index of body size, we weighted the birds with a digital balance, and measured their tarsus length with a digital calliper. We collected blood samples to quantify systemic oxidative stress (reduced-to-oxidized glutathione ratio, GSH:GSSG) and oxidative damage (malondialdehyde, MDA). The blood was centrifuged to separate plasma and cell fractions, which were stored at -80°C. These values are referred to as 'initial values'.

After initial measurements, to manipulate environmental oxidative stress, half of the birds received diquat at a dose of 0.125 ml/l in drinking water (Galván et al., 2017a), while the other half received water only and thus served as controls.

'Final values' were obtained after 15 days of treatment. We plucked the growing flank feathers to measure gene expression in follicular melanocytes and pheomelanin-based color intensity. We also quantified DNA methylation in melanocytes in these feathers, as well as systemic oxidative stress and oxidative damage in blood samples. The birds were also weighted.

This study was performed under the legal permits provided 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 were isolated from the dermal papillae where intense melanogenesis activity occurs during feather development (Lin et al., 2013). We used 15 melanin units from the bottommost portion of the feather follicles of each bird to obtain nucleic acid samples. Total RNA was extracted using TRI Reagent (Ambion, Thermo Fisher Scientific, Waltham, MA). Genomic DNA carry over was removed with TURBO DNA-free kit (Ambion). After extracting total RNA, complementary DNA (cDNA) was prepared using RevertAid Reverse Transcriptase provided in the RevertAid First Stand cDNA Synthesis kit (Thermo

Scientific, Thermo Fisher Scientific). qPCR was performed on cDNA of genes involved in pheomelanin synthesis, as explained below.

Slc7a11 (solute carrier family 7 member 11) encodes the cysteine/glutamate antiporter xCT, a protein localized in the plasma membrane (Conrad and Sato, 2012; He et al., 2012) that is responsible of cysteine transport into melanocytes (Chintala et al., 2005). *Slc45a2* (solute carrier family 45 members 2) has been suggested to play a similar function in transporting cysteine to cells (Gunnarsson et al., 2007). We also quantified genes that regulate pheomelanin synthesis by changing the intracellular concentration of cyclic adenosine monophosphate (cAMP) and thus influence the activity of tyrosinase, the key enzyme in the melanogenesis pathway (*MC1R*, *ASIP* and *AGRP*). These genes code for the melanocortin 1 receptor in the membrane of melanocytes (*MC1R*; Naysmith et al., 2004) and peptides that bind to it and act as their antagonists: agouti-signaling (*ASIP*) and agouti-related (*AGRP*) proteins (Nadeau et al., 2008). Lastly, we quantified a gene coding for a protein (cystinosin) responsible for the transport of cysteine out of lysosomes (*CTNS*; Town et al., 1998). The expression of the gene *NFE2L2* was also quantified to obtain a measure of intrinsic oxidative capacity (see below).

qPCR reactions were performed using SYBR Green I Master in a LightCycler 480 System. To normalize gene expression, we used the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), as this is the most suitable endogenous reference gene (Silver et al., 2006) and the most common reference used in gene expression analyses in feathers (Nadeau et al., 2008; Walsh et al., 2011). 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) levels were used to measure gene expression. Lower C_t values indicate higher mRNA levels and gene expression. Normalization was made by subtracting C_t values for *GAPDH* from C_t values for the target genes (ΔC_t).

DNA methylation

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

Systemic oxidative stress, oxidative damage and body condition

To quantify oxidative stress, we measured reduced (GSH) and oxidized (GSSG) glutathione levels. Glutathione constantly experiences a redox process in cells (Lu, 2009). Therefore, the ratio GSH:GSSG reflects the proportion of glutathione that is oxidized by ROS and thus indicates the level of oxidative stress, with lower ratios indicating higher stress. To determine total glutathione in erythrocytes, we followed the method described by Tietze (1969) and Griffith

(1980), with some modifications (see e.g., Galván and Alonso-Alvarez, 2009) 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. Subsequently, 2-vinylpyridine (4 μ l) was added to the aliquot and vigorously shaken at ambient temperature in darkness to promote glutathione derivatization. Lastly, the mixture was centrifuged (3,500 g for 10 min) and the change in absorbance of the supernatant was assessed at 405 nm using a COBAS Integra 400 Plus analyzer (Roche). The gene *NFE2L2* encodes for the transcription factor NRF2, a regulator of the cellular antioxidant response (Huppke et al., 2017). Thus, the effect of environmental oxidative stress on the GSH:GSSG ratio was analyzed controlling for the intrinsic antioxidant capacity of birds by including initial ΔC_t for *NFE2L2* as a covariate in the general linear models (see Statistical analyses below).

To quantify oxidative damage at a systemic level, we measured final MDA levels in plasma. MDA is a product of the peroxidation of cellular lipids induced by ROS (e.g., Weismann et al., 2011). MDA levels (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).

The potential effects of treatment on the physical condition of birds was calculated by running linear mixed-effects models with body mass as a response variable and tarsus length as a covariate (see Statistical analyses below).

Plumage color expression and pheomelanin content in feathers

To quantify the relative amount of pheomelanin content in flank feathers, the color intensity was measured by UV-Vis reflectance spectrophotometry (Galván and 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. To avoid any background reflectance, measurements were made on 10-15 flank feathers mounted on a light absorbing foil sheet (Metal Velvet coating, Edmund Optics, Barrington, NJ) 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. For each bird, was obtained an average spectrum of five readings of different points of the feathers, removing the probe between each measurement. Reflectance curves were determined by calculating the median of percent reflectance in 10 nm intervals. The slope of percent reflectance regressed against wavelength across the 300-700 nm range was used as a measure of pheomelanin-based color expression. Reflectance slope is the best color indicator of the pheomelanin content of feathers in different passerine birds, including the zebra finch, with lower values denoting darker colors (i.e., higher color intensity) and higher pheomelanin contents (Galván and Rodríguez-Martínez, 2018; Rodríguez-Martínez and Galván, 2019).

Statistical analysis

We employed general linear models (GLM) using the R package *lme4* (Bates et al., 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., 2012). In these models, the effect of the exposure to environmental oxidative stress (diquat vs. control) was evaluated by including it as a fixed factor.

To investigate the effect of environmental oxidative stress on the change in the expression of genes involved in pheomelanin synthesis, final gene expression level (ΔC_t) was a response variable in the models, and initial gene expression level was included as a covariate. Similar models were conducted to investigate effects on final DNA methylation values. No differences were found between initial gene expression levels of diquat-treated birds and controls (*Slc7a11*: $F_{1,33} = 0.778$, $P = 0.081$; *Slc45a2*: $F_{1,33} = 1.697$, $P = 0.202$; *CTNS*: $F_{1,33} = 0.618$, $P = 0.438$; *MC1R*: $F_{1,33} = 0.265$, $P = 0.610$; *ASIP*: $F_{1,33} = 0.012$, $P = 0.912$; *AGRP*: $F_{1,33} = 0.543$, $P = 0.466$).

To investigate effects on the change in oxidative stress and damage induced, final GSH:GSSG ratio or MDA values were response variables, respectively. To account for the intrinsic antioxidant capacity of birds, these models included initial *NFE2L2* ΔC_t as an additional covariate. The initial GSH:GSSG ratio did not differ ($F_{1,35} = 0.908$; $P = 0.347$) between diquat-treated birds (mean $\pm$ SE: 0.869 ± 0.057) and controls (0.791 ± 0.058).

Similar GLMs were conducted to investigate effects on the change in body condition and plumage color intensity. In the model for body condition, tarsus length was an additional covariate. Initial values did not differ between treatments in neither color intensity ($F_{1,26} = 2.787$; $P = 0.107$; diquat-treated

mean $\pm$ SE = 0.040 $\pm$ 0.003; controls: 0.047 $\pm$ 0.002) nor body condition ($F_{1,35}$ = 0.406; P = 0.528; diquat-treated: 0.006 $\pm$ 0.013; controls: -0.005 $\pm$ 0.013).

All variables were log₁₀-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

After controlling for the initial values, the final GSH:GSSG ratio of birds was affected by diquat treatment (χ^2 = 6.785, P = 0.009; Fig1), with higher ratios in birds treated with diquat (mean $\pm$ SE: 0.942 $\pm$ 0.084) than in control birds (mean $\pm$ SE: 0.626 $\pm$ 0.082). However, no effect of diquat treatment was found on MDA levels (χ^2 = 1.382, P = 0.239) nor on body condition (χ^2 = 0.925, P = 0.336). This indicates that the exposure to diquat did not cause oxidative damage nor affected the body condition of birds (Fig2), but improved their systemic oxidative status.

Gene expression and DNA methylation in melanocytes

The exposure to diquat upregulated the expression of *AGRP* in melanocytes of growing feathers (χ^2 = 7.833, P = 0.005) (Fig3), but did not lead to significant changes in the expression of the other genes during the experiment (*Slc7a11*: χ^2

= 0.968, $P = 0.325$; *Slc45a2*: $\chi^2 = 1.519$, $P = 0.217$; *CTNS*: $\chi^2 = 1.269$, $P = 0.259$; *MC1R*: $\chi^2 = 3.626$, $P = 0.056$; *ASIP*: $\chi^2 = 2.208$, $P = 0.137$).

The proportion of DNA with m⁵C was significantly higher in controls than in diquat-treated birds in *MC1R* only ($\chi^2 = 4.554$, $P = 0.032$), while no differences were found in the other genes (*Slc7a11*: $\chi^2 = 0.399$, $P = 0.527$; *Slc45a2*: $\chi^2 = 0.039$, $P = 0.842$; *CTNS*: $\chi^2 = 0.021$, $P = 0.884$; *ASIP*: $\chi^2 = 2.481$, $P = 0.115$; *AGRP*: $\chi^2 = 0.105$, $P = 0.745$) (Fig4).

Pheomelanin-based pigmentation in feathers

Diquat exposure had a significant effect on the intensity of pheomelanin-based plumage coloration ($\chi^2 = 3.872$, $P = 0.049$). The reflectance slope of the flank feathers of birds treated with diquat (mean $\pm$ SE: 0.039 ± 0.002) was lower than that of control birds (mean $\pm$ SE: 0.046 ± 0.002), indicating a higher production of pheomelanin and a darker pigmentation in birds treated with diquat (Fig5).

Discussion

Cells have the ability to respond and adapt to external sources of stress. High ROS levels alter cellular homeostasis and cause cellular damage (Cross et al., 1987), but low chronic doses may allow to activate a protective response (Dröge, 2002). Such response may not only avoid oxidative stress, but even improve the initial oxidative status if the response is stronger than the damaging effect. This hormetic response can be induced by low doses of toxic stressors (Calabrese, 2008), and may result adaptive, as it can improve the functionality

of cells (Vaiserman, 2011) and benefit fitness (Forbes, 2000). In some birds, environmental stress can inhibit pheomelanin synthesis, thereby avoiding a decrease in GSH availability and antioxidant capacity (Galván et al., 2017a). Therefore, we expected that male zebra finches treated with diquat would decrease the production of pheomelanin as a mechanism to avoid oxidative damage. In contrast, we found an improvement of the antioxidant status of diquat-treated birds, which accordingly increased their production of pheomelanin, resulting in a darker plumage color phenotype. This suggests that environmental oxidative stress may have activated a hormetic response that enhanced the oxidative status of birds sufficiently to even favor pheomelanin synthesis by upregulating the expression of *AGRP*.

Evidence of a similar hormetic effect on the oxidative status has previously been found in several species of birds experiencing chronic exposure to low-dose ionizing radiation, an environmental oxidative stress source, at Chernobyl, as the GSH:GSSG ratio increased with radiation levels (Galván et al., 2014). Interestingly, this improvement of the oxidative status was less marked in those species that produce greater amounts of pheomelanin to pigment their feathers (Galván et al., 2014). In fact, oxidative damage has been reported to decrease in developing red-legged partridges *Alectoris rufa* exposed to the same exogenous source of oxidative stress that has been used in the present study (diquat in drinking water; Galván and Alonso-Alvarez, 2009). At least some birds thus seem to be able to mount physiological responses that counteract the effects of environmental stress and even improve their initial status.

MC1R and *AGRP* are among the main genes responsible for pheomelanin synthesis in birds (Nadeau et al., 2008). Here, however, *MC1R* expression in feather melanocytes did not change after diquat exposure, but this gene showed an increase in DNA m⁵C levels. DNA methylation generally leads to a decrease of gene expression (Schübeler, 2015), but recent studies suggest that the expression of genes involved in pheomelanin synthesis are differentially affected by RNA and DNA methylation in feather melanocytes (Rodríguez-Martínez et al., 2019). Thus, an increase in DNA methylation levels induced by environmental oxidative stress (diquat exposure) may not alter the expression of *MC1R*. In fact, we have previously detected changes in DNA methylation without an effect on the expression of genes involved in pheomelanin synthesis in zebra finches exposed to a competitive social environment (Rodríguez-Martínez and Galván, 2019). Thus, it is possible that a short exposure to an stressful source may allow observing changes in DNA methylation in genes involved in pheomelanin synthesis, which may be indicative of the activation of a protective mechanism, but the low duration or intensity of the exposure may prevent that this process ends up with changes in gene expression.

AGRP products bind to the melanocortin 1 receptor (*MC1R*) in the membrane of melanocytes, decreasing the activity of tyrosinase and consequently promoting pheomelanin synthesis (Ozeki et al., 1997). Pheomelanin synthesis thus seems to be a physiological response that is sensitive to the oxidative status of birds, as *AGRP* was upregulated only when the antioxidant capacity of male zebra finches increased. Changes in gene expression allow organism to respond to the environment by epigenetic mechanisms (Jaenisch and Bird 2003). Here, we did not found changes in the

m⁵C DNA proportion for *AGRP*. Indeed, we have previously revealed changes in the expression of genes involved in pheomelanin synthesis in other birds (the Eurasian nuthatch *Sitta europaea*), but these changes were not explained by m⁵C DNA modifications but by changes in mRNA m⁶ (Rodríguez-Martínez et al., 2019). This may suggest that epigenetic modifications other than DNA methylation (i.e., chromatin histone modifications or RNA methylation; Goldberg and Bernstein, 2007) may regulate the sensitivity of *AGRP* and/or *MC1R* expression to environmental oxidative stress.

Although *Slc7a11* plays an important role in pheomelanin synthesis by transporting cysteine into melanocytes (Chintala et al., 2005), we did not found differences in its expression between diquat-treated birds and controls. *Slc7a11* expression is affected by oxidative stress, as cysteine consumed during pheomelanin synthesis is not available for antioxidant protection (Pavel et al., 2011). In fact, when exogenous oxidative stress was experimentally induced by diquat administration in a previous study on male zebra finches, a downregulation of *Slc7a11* in feather melanocytes occurred (Galván et al., 2017a). In this previous study, a hormetic response and the consequent promotion of pheomelanin synthesis was not observed in male zebra finches likely because, in contrast to the present study, the birds were kept in groups of 20 males (Galván et al., 2017a), which represents another source of stress mediated by social interactions (Rodríguez-Martínez and Galván 2019). Changes in *Slc7a11* expression have also been found in feather melanocytes of Eurasian nuthatches exposed to a natural source of environmental stress (predation risk; Galván, 2018). Overall, these results suggest that, at least in the zebra finch, different environmental stressful conditions affect pheomelanin

synthesis by regulating the expression of different genes, as the necessity of decreasing pheomelanin synthesis seems to lead to *Slc7a11* downregulation (Galván et al., 2017a), while the necessity of increasing pheomelanin synthesis seems to lead to *AGRP* upregulation (present study). Indeed, different sources of environmental stress are known to activate different adaptive responses that maintain cellular homeostasis by regulating the expression of different genes (De Nadal et al., 2011). This may thus explain why we did not find changes in the expression of the genes *Slc7a11*, *Slc45a2* and *CTNS* after treatment with diquat in male zebra finches.

It must be noted that, in other bird species (the lesser kestrel *Falco naumanni*), the expression of *Slc7a11* did not change in response to other source of environmental oxidative stress (colony size; Galván et al., 2017b). Thus, it seems that not all species are able to regulate the expression of genes involved in pheomelanin synthesis under environmental stress.

In conclusion, our study shows that environmental stress may enhance the oxidative status of male zebra finches and thus promote pheomelanin synthesis, leading to the development of a darker color phenotype. In particular, the mechanism seems to be mediated by changes in the expression of *AGRP*, which might be induced by epigenetic mechanisms other than changes in DNA methylation. Cells respond to different sources of stress by regulating the expression of specific sets of genes (Enjalbert et al., 2003). In particular, the expression of genes involved in pheomelanin synthesis seems to differentially respond to different conditions of environmental stress to maintain cellular homeostasis. Adjusting the level of pheomelanin production to stress conditions

by regulating the genes involved in pheomelanogenesis therefore seems to represent a mechanism that allow organisms to adapt to environmental changes, with consequences on their color phenotype, which may in turn affect behavioral processes in nature such as those involved in sexual selection (Galván and Rodríguez-Martínez, 2018).

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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 will be deposited on Dryad Digital Repository upon acceptance.

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Legends to figures:

Figure 1: Initial and final oxidative status (GSH:GSSG ratio) of diquat-treated male zebra finches (dark grey) and control birds (grey) (n = 37). Values are means $\pm$ SE.

Figure 2: **(a)** Oxidative damage (malondialdehyde, MDA) in male zebra finches after treatment in diquat-treated birds and controls (n = 37). Values are means $\pm$ SE **(b)** Body condition before and after treatment of diquat-treated (dark grey) and control birds (grey). Body condition is expressed here as the residuals of the regression of body mass against tarsus length. Values are means $\pm$ SE.

Figure 3. Initial and final values of normalized expression levels (ΔC_t) of *AGRP* in male zebra finches (n = 35) exposed to diquat (dark grey) and controls (grey). Lower ΔC_t indicates higher gene expression. Values are means $\pm$ SE.

Figure 4. Proportion of DNA with 5-methylcytosine in six genes regulating pheomelanin synthesis (n = 30). DNA was obtained from melanocytes of growing feathers of male zebra finches exposed to diquat and control birds.

Asterisk above graph indicates statistically significant differences between diquat-treated birds and controls. Values are means $\pm$ SE.

Figure 5. Initial and final mean $\pm$ SE reflectance slope (n = 32) of diquat-treated birds (dark grey) and controls (grey). Lower reflectance slopes indicate higher pheomelanin content and more intense color.

(a)

(b)