

Adaptive downregulation of pheomelanin-related *Slc7a11* gene expression by environmentally induced oxidative stress

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Abstract

Pheomelanin is a sulphur-containing yellow-to-reddish pigment whose synthesis consumes the main intracellular antioxidant (glutathione; GSH) and its precursor cysteine. Cysteine used for pheomelanogenesis cannot be used for antioxidant protection. We tested whether the expression of *Slc7a11*, the gene regulating the transport of cysteine to melanocytes for pheomelanogenesis, is environmentally influenced when cysteine/GSH are most required for antioxidant protection. We found that zebra finches *Taeniopygia guttata* developing pheomelanin-pigmented feathers during a 12-day exposure to the pro-oxidant diquat dibromide downregulated the expression of *Slc7a11* in feather melanocytes, but not the expression of other genes that affect pheomelanogenesis by mechanisms different from cysteine transport such as *MC1R* and *Slc45a2*. Accordingly, diquat-treated birds did not suffer increased oxidative stress. This indicates that some animals have evolved an adaptive epigenetic lability that avoids damage derived from pheomelanogenesis. This mechanism should be explored in human *Slc7a11* to help combat some cancer types related to cysteine consumption.

Keywords: environmental epigenetics, oxidative stress, pheomelanin, pigmentation, *Slc7a11*

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Introduction

Melanins are the most widespread biological pigments, accounting for a significant part of variation in the appearance of organisms (Solano 2014). These molecules mainly originate from the oxidation and polymerization of the amino acid tyrosine. In animals, this occurs within specialized organelles in melanocytes termed melanosomes, which are transferred to surrounding epidermal keratinocytes and hence provide colour to skin

and associated structures such as scales, feathers and hairs (d'Ischia *et al.* 2015). Two subgroups of melanins are differentiated, both deriving from the common precursor dopaquinone that results from tyrosine oxidation. When the sulphhydryl compounds cysteine or glutathione (GSH) are absent or below certain levels in melanocytes, dopaquinone gives rise to indole units that are polymerized to form black, brown and grey pigments termed eumelanins. However, when sulphhydryls are above a threshold level, they are incorporated to the process forming yellow-to-reddish sulphur-containing heterocycles termed pheomelanins (Ito & Wakamatsu 2008).

Pheomelanin synthesis therefore requires a constant supply of cysteine. Cysteine is one of the three amino acid constituents of GSH, which is considered the most important intracellular antioxidant (Wu *et al.* 2004). As GSH also functions as a source of cysteine,

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pheomelanogenesis represents a consumption of an important antioxidant, making it unavailable for antioxidant protection (Pavel *et al.* 2011). Furthermore, once formed, pheomelanin produces oxidative stress independently of exposure to ultraviolet (UV) radiation, promoting oxygen-dependent depletion of GSH (Panzella *et al.* 2014). These processes may actually be responsible for an increased risk of diseases such as melanoma independent of UV radiation (Galván *et al.* 2012; Mitra *et al.* 2012; Morgan *et al.* 2013). This association between pheomelanin-based pigmentation and diseases has probably contributed to the fact that the genetic control of pheomelanogenesis is currently relatively well known (Hearing 2011). This in turn allows investigating whether the expression of genes involved in regulating cysteine availability in melanocytes for pheomelanogenesis is open to environmental influences. Such lability may allow organisms to modulate pheomelanin synthesis according to prevailing environmental conditions and to avoid GSH depletion when its antioxidant activity is highly required for vital processes. This possibility has never been explored.

Slc7a11 is the gene responsible for the transport of cysteine to melanocytes to produce pheomelanin by encoding the plasma membrane cystine/glutamate exchanger xCT (Chintala *et al.* 2005). As cysteine used for pheomelanogenesis is no longer available for antioxidant protection, the level of expression of this gene may have physiological consequences. These consequences should depend on the prevailing conditions of environmental (i.e. exogenous) oxidative stress, which determine the needs for antioxidant protection (Galván & Alonso-Alvarez 2009). Under relatively high levels of exogenous oxidative stress, there would be a greater need for cysteine and GSH, so that the production of pheomelanin would constitute a physiological cost (Galván *et al.* 2014a). With such conditions, two consequences may arise: (i) if *Slc7a11* is not permeable to environmental factors, a high expression of this gene should lead to increased cellular oxidative stress, and (ii) alternatively, if *Slc7a11* expression is sensitive to environmental factors through epigenetic responses, a downregulation of this gene should be observed with no influence on oxidative stress.

Here, we experimentally test these predictions in the zebra finch *Taeniopygia guttata*, a bird that develops orange flank feathers pigmented by pheomelanin (McGraw & Wakamatsu 2004; Fig. 1). Flank feathers were plucked from adult male zebra finches kept in captivity to induce the growth of new feathers. During the development of new feathers, some birds were exposed during 12 days to an exogenous source of oxidative stress by providing diquat dibromide, a bipyridylum aquatic herbicide that produces intracellular superoxide anion (e.g. Sewalk *et al.* 2000; Zeman *et al.* 2005; Xu *et al.* 2007), in the

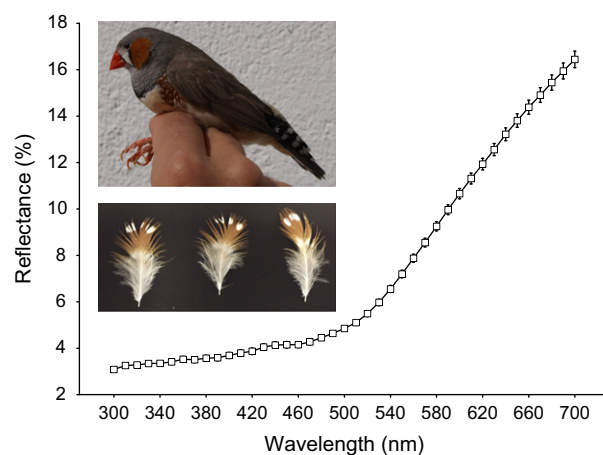


Fig. 1 Mean reflectance (\pm SE) of the pheomelanin-based feathers of zebra finches used in the study. Values correspond to the reflectance measurements taken on the fully grown feathers plucked before the beginning of the experimental treatment. Inserts are photographs of a male zebra finch showing fully grown flank feathers and a detail of three flank feathers. [Colour figure can be viewed at wileyonlinelibrary.com].

drinking water. Other birds not receiving diquat served as controls (see Materials and methods). We measured the expression of *Slc7a11* in melanocytes of the follicles of the developing feathers by quantitative real-time PCR, and the levels of cysteine and reduced (GSH) and oxidized (GSSG) glutathione in erythrocytes before and after the experimental treatment.

Our second alternative prediction considers that exogenous oxidative stress affects the mechanism that regulates cysteine availability during pheomelanogenesis. Thus, it should affect the expression of *Slc7a11*, but not the expression of genes that affect pheomelanogenesis through mechanisms not related to cysteine regulation. Therefore, we also measured the expression of the genes *MC1R* and *Slc45a2*. *MC1R* codes for the melanocortin 1 receptor in the membrane of melanocytes, to which melanocortins bind to influence pheomelanogenesis by changing intracellular cyclic adenosine monophosphate (cAMP) levels (Bowers *et al.* 1997). On the other hand, *Slc45a2* seems to affect pheomelanin synthesis by regulating the processing and trafficking of tyrosinase, a key enzyme in the melanogenesis pathway (Xu *et al.* 2013). Exogenous oxidative stress should thus affect the expression of *Slc7a11*, but not the expression of *MC1R* and *Slc45a2*.

Materials and methods

Experimental design

The study was carried out in August–September 2013 on 79 captive male adult zebra finches at the Dehesa

Galiana experimental facility (Diputación Provincial de Ciudad Real; Ciudad Real, Spain). The birds were the second generation of zebra finches raised in the experimental facility and were obtained from 38 different breeding couples. All birds were marked with a numbered metal ring. On August 8, the birds were randomly assigned to one of the two different treatments and transferred from individual cages to one of four different indoor aviaries ($4 \times 3 \times 3$ m), so that the body condition of birds (i.e. size-independent body mass) was equally distributed in each aviary. The light regime was 13 h:11 h (L:D), obtained with two conventional light bulbs and one UVA-UVB bulb in each aviary. Each aviary had two troughs where ad libitum food for tropical birds was provided. The birds were left 2 weeks in the aviaries with no experimental treatment to achieve acclimation to these conditions.

On 22 August, the birds were sampled for blood and their orange flank feathers were plucked ('initial values'). The same was done at the end of the experiment, 12 days later ('final values'). Blood samples were stored at 4 °C and centrifuged within 5 h after extraction. Plasma and cell fractions were immediately stored at -80 °C. Plucked feathers were immersed in RNAlater solution (Sigma-Aldrich, St. Louis, MO, USA) to stabilize and protect RNA, and stored at -20 °C.

The birds received diquat in drinking water or only water (controls). The commercial product (Reglone, Syngenta, Madrid, Spain) consisted of 20% w/v of diquat dibromide in water (consulted with the company). The treatment with diquat in water lasted 12 days, just before the new flank feathers were fully developed. Each aviary contained two 1-l water dispensers, which were replaced every 4 days to avoid the disintegration of the diquat molecule (i.e. following product properties; Syngenta). Therefore, each aviary contained a single drinking treatment (i.e. diquat plus water or only water), so that two aviaries contained diquat treatment and two aviaries were controls. Diquat and control aviaries alternated in space.

Diquat was provided at a dose of 0.125 mL/L. To calculate this dose, we took advantage of a pilot study previously performed in other granivorous passerine bird (i.e. house sparrow *Passer domesticus* adult males), which was performed to be applied in another study. In that pilot experiment, sparrows were kept in four cages (three birds per cage), each containing six water dispensers and four troughs with ad libitum food, during 24 days. In three of these cages, diquat was provided to drinking water at a dose of either 0.125, 0.25 or 0.50 mL/L, while birds in the fourth cage were provided with water only. The highest diquat dose (0.50 mL/L) was here chosen because it was the dose used in a previous experiment with red-legged

partridges (Galván & Alonso-Alvarez 2009). House sparrows receiving diquat at 0.25 mL/L were those showing a mean change in body condition (calculated as the residuals of body mass regressed against tarsus length) closer to that of control birds at the end of the pilot study (control: 1.68; 0.125 mL/L diquat: -1.90; 0.25 mL/L diquat: -0.67; birds receiving diquat at 0.50 mL/L died during the study). Mean total GSH levels in erythrocytes (measured as explained below) after the study, while being lower in birds receiving diquat at 0.25 mL/L (3.48 mmol/g) than in controls (3.89 mmol/g) as expected due to the pro-oxidant nature of diquat, were still higher than those of birds receiving diquat at 0.125 mL/L (2.81 mmol/g). Therefore, 0.25 mL/L was considered the optimal diquat dose for house sparrows. As the body mass of zebra finches is approximately half the body mass of house sparrows (30 g), we used a half-dose (0.125 mL/L) in zebra finches. No zebra finch died during the definitive experiment. These studies were performed under the legal permits provided by the Consejo Superior de Investigaciones Científicas (CSIC) Ethics Committee, Universidad de Castilla La Mancha (UCLM) Ethics and Animal Experimentation Committee (ref. 1201_08) and Consejería de Agricultura (Junta de Comunidades de Castilla La Mancha, Spain).

Measurement of gene expression in melanocytes

During feather growth, melanocytes at the dermal papillae experience intense melanogenesis, while they migrate towards the developing barbs (Jimbow & Takeuchi 1979). Therefore, we cut the bottommost portion of the feather follicles corresponding to the melanin unit, which can be identified by a dark colour contrasting with the remaining follicle. Similar to hair follicles (Mohanty *et al.* 2011), the follicular melanin units of feathers contain cell populations different from melanocytes such as keratinocytes, but they represent an important reservoir of melanocytes so that the genetic material obtained from these samples might always correspond to these cells to a large extent. As there is no melanogenesis in fully grown feathers, we could only measure gene expression in the final feather samples.

Twenty follicular melanin units were pooled per bird, and total RNA was extracted using TRI Reagent (Ambion, Thermo Fisher Scientific, Waltham, MA, USA). 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). Quantitative real-time PCR (qPCR) was performed for *Slc7a11*, *MC1R* and *Slc45a2* on

cDNA. Reactions were performed using SYBR Green I Master in a LightCycler 480 System (Roche, Basel, Switzerland). The housekeeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used for normalization. Cycle threshold (C_t) levels were used as a measure of gene expression. *Slc7a11*, *MC1R*, *Slc45a2* and *GAPDH* primers were designed based on *Taeniopygia guttata* refseq sequences (GenBank) using 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'-TCTCCTTCCTGGGGGTCATT-3' and 5'-TGATCAAGACGGTGCTGGAC-3' for *MC1R*; 5'-GAAATGCACGGTTGCGTCAC-3' and 5'-GGTACACAACCTGTCCCATGAA-3' for *Slc45a2*; and 5'-GGACCAGGTTGTCTCCTGTG-3' and 5'-TCCTTGATGCCATGTGGAC-3' for *GAPDH*.

For each gene and session, we made three negative controls: (i) no-template control during cDNA synthesis – no RNA, to discard extraneous nucleic acid contamination during cDNA synthesis, (ii) no reverse transcriptase control – absence of reverse transcriptase, to discard genomic DNA contamination in the RNA extracts, and (iii) no-template control during PCR amplification – no cDNA added for PCR to discard extraneous nucleic acid contamination during PCR amplification. Two replicates were obtained for each sample, and the mean value was used in the statistical analyses. The repeatability (Lessells & Boag 1987) of these estimates of gene expression was very high (*Slc7a11*: $r = 0.86$, $F_{66,69} = 13.63$, $P < 0.0001$; *MC1R*: $r = 0.95$, $F_{65,68} = 41.75$, $P < 0.0001$; *Slc45a2*: $r = 0.88$, $F_{63,72} = 15.57$, $P < 0.0001$; *GAPDH*: $r = 0.91$, $F_{66,71} = 21.26$, $P < 0.0001$).

The measurements of gene expression in feather follicles did not allow us to conduct additional analyses of cysteine, GSH and GSSG in feather follicles because of limitations in the number of samples. Therefore, the latter were only conducted in erythrocytes, which allowed us to consider more general systemic effects.

Measurement of cysteine levels in erythrocytes

We measured cysteine levels following the method developed by Švagera *et al.* (2012) for plasma. To apply this method to the analysis of erythrocytes, we added a first step consisting of a dilution of erythrocytes to 1:10 with a carbonate-buffered saline (5 mM Na_2CO_3 in saline) to induce cell lysis and thus facilitate the extraction of intracellular cysteine. The samples were analysed by gas chromatography (GC) coupled with an electronic impact-mass spectrometry detector. The chromatographic system consisted of a 6890N Network GC System with a 5973 Network Mass Selective Detector (Agilent Technologies, Santa Clara, CA, USA).

Mass spectra of the derivatized cysteine and an internal standard (*p*-chlorophenylalanine, PCP) were obtained in a continuous scanning mode from 50 to 450 m/z , using for quantification m/z ions 220 and 210 for cysteine and PCP, respectively (retention time - cysteine: 2.70, PCP: 3.38). The ions used for identification were 220, 102, 74 and 204 for cysteine and 210, 102, 125 and 212 for PCP. Cysteine levels are expressed as micromoles per gram of pellet.

Measurement of GSH levels in erythrocytes

Total GSH (tGSH) levels in erythrocytes were determined by following the method described by Tietze (1969) and Griffith (1980) with some particular modifications. Details of the use of this technique with bird samples are published elsewhere (e.g. Galván & Alonso-Alvarez 2009). Concentration is presented as micromoles of GSH per gram of pellet.

To determine oxidized GSH (GSSG) levels, 8 μL of 2-vinylpyridine was added to an aliquot (400 μL) of the supernatant obtained for tGSH assessment to promote GSH derivatization (e.g. Romero-Haro & Alonso-Alvarez 2015). The mixture was then centrifuged (3500 g for 10 min), and the change in absorbance of the supernatant was assessed at 405 nm. Reduced GSH levels were calculated by subtracting GSSG levels from tGSH levels. The ratio GSH:GSSG was used as an index of oxidative stress in cells.

Measurement of pheomelanin-based pigmentation in feathers

The level of pigmentation of flank feathers was measured using an Ocean Optics Jaz spectrophotometer (range 220–1000 nm) with ultraviolet (deuterium) and visible (tungsten-halogen) lamps and a bifurcated 400-micrometre fibre-optic probe. The fibre-optic probe both provided illumination and obtained light reflected from the sample, and it had a reading area of c. 1 mm^2 . For each bird, 10–20 feathers were mounted on a light-absorbing foil sheet (Metal Velvet coating, Edmund Optics, Barrington, NJ, USA) to avoid any background reflectance, such that they resembled the natural appearance of the feather patch. The measurements were taken at a 90° angle to the sample. All measurements were relative to a diffuse reflectance standard tablet (WS-1; Ocean Optics, Dunedin, FL, USA), and reference measurements were frequently made. An average spectrum of five readings on different points of the distal part of feathers was obtained for each bird, removing the probe after each measurement. Reflectance curves were determined by calculating the median of the per cent reflectance in 10 nm intervals

beginning at 300 nm and ending at 700 nm to cover the full spectral range that can be detected by birds (Cuthill *et al.* 2000).

Spectral data were assumed to represent a measure of total brightness, as this is the best predictor of pheomelanin content in reddish-orange feathers, with lower values (i.e. darker colours) denoting higher pheomelanin content (McGraw *et al.* 2005). Brightness was defined as the summed reflectance across the entire spectral range (Galván & Møller 2013; Fig. 1). The repeatability of the five brightness measurements made per bird was high ($r = 0.86$, $F_{78,316} = 32.99$, $P < 0.0001$). The flank feathers of male zebra finches contain eumelanin in a very low proportion ($< 8\%$) in relation to pheomelanin (McGraw & Wakamatsu 2004), so that the contribution of eumelanin to the colour measurements taken on the feathers is negligible.

Statistical analyses

General linear models (GLMs) were used to analyse variation in gene expression (response variable) in relation to treatment (control vs. diquat), which was added to the models as a fixed factor. To ensure that these effects were not influenced by gains or losses in the body condition of birds during the experiment, the change in body condition (i.e. final body condition - initial body condition; calculating condition as the residuals of body mass regressed against tarsus length) was added as a covariate, but it was subsequently removed as it was not significant in any model (*Slc7a11*: $F_{1,63} = 0.98$, $P = 0.326$; *MC1R*: $F_{1,62} = 2.01$, $P = 0.161$; *Slc45a2*: $F_{1,63} = 0.43$, $P = 0.516$). Similarly, initial cysteine level was added as a covariate to the model for *Slc7a11* expression, using its interaction with treatment to investigate whether gene expression during the experiment depended on the initial availability of cysteine, separately for each treatment.

GLMs were also used to analyse the relationship between initial feather brightness in control birds (response variable) and gene expression (covariate). Three different GLMs were performed for each gene of interest, as the level of correlation between the expression of the three genes (*Slc7a11*-*Slc45a2*: $r = 0.41$, $N = 67$, $P < 0.001$; *MC1R*-*Slc45a2*: $r = 0.26$, $N = 66$, $P = 0.036$; *Slc7a11*-*MC1R*: $r = -0.05$, $N = 67$, $P = 0.683$) did not allow including them as predictor variables in the same model. These models included tarsus length (as a proxy for body size) as a covariate, because it showed a clear tendency to covary with feather brightness ($b = 722.61$, $F_{1,37} = 2.71$, $P = 0.108$).

To analyse the effect of treatment on the change in body condition, in the levels of cysteine, GSH and GSSG and in the GSH:GSSG ratio during the course of

the experiment, we used GLMs including the time at which measurements were taken (initial or final) as a repeated-measures (i.e. within-subjects) effect. Diquat-treated and control birds did not differ in the initial levels of neither cysteine ($F_{1,71} = 2.49$, $P = 0.119$), GSH ($F_{1,71} = 0.37$, $P = 0.542$), GSSG ($F_{1,71} = 0.06$, $P = 0.810$) nor GSH:GSSG ratio ($F_{1,70} = 0.05$, $P = 0.827$). Treatment was added as a fixed factor. In the sake of simplicity, we only show the results of the effect of the interaction between the within-subjects factor and treatment (i.e. all effects in these models refer to the interaction between the within-subjects factor and treatment). We also explored the possibility that the body condition of birds affected the predictive capacity of treatment in these models, so the change in body condition was also added as a covariate but subsequently removed as it was not significant in any model (interaction between the within-subjects factor and change in body condition: GSH: $F_{1,66} = 1.01$, $P = 0.318$; GSSG: $F_{1,67} = 2.34$, $P = 0.131$; GSH:GSSG ratio: $F_{1,67} = 2.99 \times 10^{-4}$, $P = 0.986$).

Similar repeated-measures GLMs were used to analyse the effect of gene expression on oxidative stress, with initial and final levels of GSSG and GSH:GSSG ratio as response variables. Gene expression and treatment were added to the models as covariate and fixed factor, respectively, also testing the interaction of these variables with the time at which measurements were taken (i.e. within-subjects factor). This allowed exploring the possibility that the effect of gene expression differed between diquat-treated and control birds.

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. All statistical analyses were made with STATISTICA 12 (StatSoft, Tulsa, OK, USA).

Results and discussion

We first tested whether the expression of *Slc7a11*, but not the expression of the other genes, was affected by the exposure to diquat. As postulated, the experimental treatment explained a significant proportion of variance in *Slc7a11* expression ($F_{1,66} = 4.99$, $P = 0.029$), but not in the expression of *MC1R* ($F_{1,65} = 0.01$, $P = 0.921$) nor *Slc45a2* ($F_{1,65} = 0.25$, $P = 0.617$). *Slc7a11* was downregulated in zebra finches exposed to diquat during the development of pheomelanin-based feathers (Fig. 2). These results were not affected when the body condition of birds was included in the analyses (see Materials and methods), but the effect of treatment on *Slc7a11* expression was more marked ($F_{1,58} = 7.46$, $P = 0.008$) when initial cysteine levels (i.e. those before the beginning of the experiment) were included in the model to

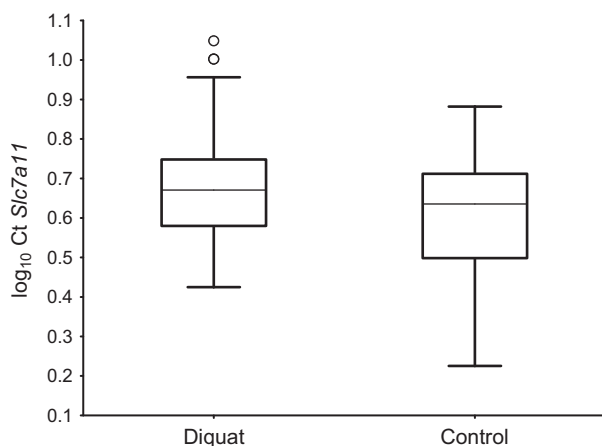


Fig. 2 Relative expression (C_t values) of *Slc7a11* when compared with GAPDH in follicular feather melanocytes. The graph indicates lower expression in diquat-treated than in control male zebra finches. Median, interquartile range and nonoutlier range are shown.

account for the amount of cysteine available for the development of new pheomelanin-based feathers. Accordingly, the interaction between initial cysteine levels in erythrocytes and treatment was significant in the same model ($F_{1,58} = 6.16$, $P = 0.016$), as the correlation between *Slc7a11* C_t values and initial cysteine levels was positive and significant for control birds ($b = 0.39$, $t = 2.35$, $P = 0.022$) but nonsignificant for diquat-treated birds ($b = -0.08$, $t = -0.89$, $P = 0.378$). This suggests that higher systemic cysteine availability before the experiment was indicative of lower levels of cysteine being allocated to pheomelanin production because of lower activation of the gene that regulates the transport of cysteine to melanocytes. By contrast, the exposure to diquat led to a downregulation of this gene, which may prevent making a greater allocation of cysteine to the process.

Diquat generates superoxide anion and as a consequence produces oxidative stress by depleting antioxidants and, ultimately, oxidative damage to cells. This is what has been found in the blood and other tissues of birds exposed to diquat (Sewalk *et al.* 2000; Zeman *et al.* 2005), and also in mammals (Xu *et al.* 2007; Fussell *et al.* 2011; Yin *et al.* 2015). This is actually the expectation if the expression of genes regulating cysteine consumption for pheomelanin production was not inhibited by exogenous oxidative stress. Such a lack of inhibition would make that producing large amounts of pheomelanin directly leads to a decreased antioxidant capacity under stressful environmental conditions or during the development of costly physiological processes. Interspecific comparisons of birds offer evidence of this physiological cost (Galván *et al.* 2014a). In the present

study, however, zebra finches downregulated the expression of *Slc7a11*, and accordingly, diquat-treated and control birds did not differ in the change of levels of neither cysteine ($F_{1,69} = 0.03$, $P = 0.856$), GSH ($F_{1,69} = 2.11 \times 10^{-3}$, $P = 0.963$), GSSG ($F_{1,69} = 0.43$, $P = 0.515$) nor in the change in GSH:GSSG ratio ($F_{1,68} = 0.23$, $P = 0.634$). The body condition of diquat-treated birds, however, decreased while that of controls increased (Fig. 3). This change in body condition does not affect the models cited above (Materials and methods). Overall, the results suggest that, by downregulating *Slc7a11*, the birds exposed to diquat experienced a decrease in body condition but avoided oxidative stress. This epigenetic change may therefore be advantageous, and future studies should investigate which species in addition to the zebra finch (a possibility may be that this is only observed in species producing large amounts of pheomelanin like the zebra finch; McGraw & Wakamatsu 2004) have evolved such epigenetic lability in *Slc7a11*. Alternatively, the effect of diquat may have not been strong enough to affect GSH and GSSG levels, although we believe that this possibility is unlikely because a clear decrease in the general condition of birds was observed. In any case, it must be noted that the benefits of the downregulation of *Slc7a11* might depend on the intensity of oxidative stress, and the limits of this epigenetic response should be explored.

If a high expression of *Slc7a11* actually represents a physiological cost, it should also be expected that

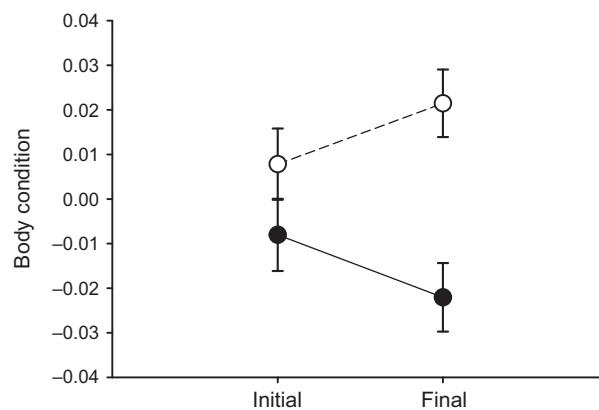


Fig. 3 Body condition of zebra finches before the beginning and at the end of the experiment. Values for diquat-treated (black symbols and solid line) and control birds (white symbols and dashed line) are shown. Body condition was calculated as the residuals of body mass regressed against tarsus length. Values are least squares means (\pm SE) obtained from a GLM with initial and final body condition as response variables, treatment (diquat vs. control) as a fixed factor and the moment at which measurements were taken (initial vs. final) as a within-subjects factor, testing the interaction between treatment and the within-subjects factor.

cellular oxidative stress increases with the expression of the gene under high environmental oxidative stress. Under low environmental oxidative stress, by contrast, less cysteine is used for antioxidant protection, being more likely to suffer from excess cysteine, which is toxic (Galván *et al.* 2012). An adaptive scenario would thus predict that cellular oxidative stress decreases with *Slc7a11* expression under relative low environmental stress. We evaluated this possibility by testing the effect of the interaction between treatment and *Slc7a11* expression in explaining the variance in the change in GSSG values and in GSH:GSSG ratio. We must note that these variables were measured in blood (erythrocytes), which should have a stronger association with overall systemic health than measures taken in the feather follicle. The effect was marginally nonsignificant in the model for GSSG ($F_{1,56} = 3.62$, $P = 0.062$) and significant in the model for GSH:GSSG ratio ($F_{1,55} = 7.62$, $P = 0.008$). The latter arose because the correlation between the change in GSH:GSSG ratio and C_t values for *Slc7a11* was negative and significant in control birds but nonsignificant in diquat-treated birds (Fig. 4). This means that, among control birds, the higher the expression of *Slc7a11*, the lower the oxidative stress. The downregulation of *Slc7a11* by oxidative stress is probably the reason why the correlation between oxidative stress and *Slc7a11* expression was not observed in diquat-treated birds, as it is likely that downregulation imposes an upper limit to *Slc7a11* expression and consequently a limit to covary with oxidative stress. The same effect explaining variance in the change in the GSH:GSSG ratio, by contrast, was not found considering the expression of *MC1R* ($F_{1,54} = 0.12$, $P = 0.735$) or *Slc45a2* ($F_{1,55} = 0.28$, $P = 0.599$). Thus, the results support the idea that cysteine consumption for pheomelanogenesis can be beneficial as it avoids the toxicity of excess cysteine under low environmental oxidative stress, providing some evidence that this benefit leads to the evolution of pheomelanin (Galván *et al.* 2012). High environmental stress conditions, by contrast, elicit an epigenetic change that avoids that this benefit becomes a physiological cost.

Fully developed flank feathers after the end of the experiment could not be assessed. Nonetheless, in control birds, the sign of the relationship between the brightness of the initial feathers and C_t values differs between *Slc7a11* (negative: $b = -65.96$, $F_{1,31} = 1.30$, $P = 0.263$) and *MC1R* expression (positive: $b = 65.00$, $F_{1,31} = 1.12$, $P = 0.297$), the effect being less clear (but also nonsignificant) for *Slc45a2* ($b = -52.75$, $F_{1,31} = 0.44$, $P = 0.511$). As the brightness of orange feathers is negatively related to their pheomelanin content (McGraw *et al.* 2005), this suggests that, unexpectedly (Chintala *et al.* 2005; Tian *et al.* 2015), *Slc7a11* expression may not be favouring the development of pheomelanin-rich

pigmentation patterns. It must be noted that original (initial) feathers were probably developed weeks or months before the experiment, so these associations were probably not clearer because feather colour was analysed before the experiment and gene expression was measured after the experiment.

To our knowledge, this represents the first direct evidence of an environmental epigenetic change in genes (which may be mediated by certain transcription factors; see below) that control pigmentation in animals, although Waterland & Jirtle (2003) already showed that another pigmentation-related gene (*Agouti*) is epigenetically labile in mice. *Slc7a11* expression was downregulated under exposure to diquat, and this prevented birds from suffering oxidative stress as usually observed with this substance. This means that at least some animals have evolved a epigenetic mechanism to inhibit cysteine consumption during pheomelanogenesis, which may be favoured by natural selection because of its adaptiveness. Interestingly, the epigenetic response can be produced very rapidly, as we detected the change in zebra finches after a period of only 12 days of exposure to diquat. Future studies should investigate the basis of this mechanism, although we may speculate that it is mediated by transcription factors such as Nrf2, which is implied in other epigenetic changes that avoid diquat-induced oxidative stress (Osburn *et al.* 2006).

Integumentary structures coloured by melanins frequently form the basis of honest signals used in animal communication (McGraw 2008; Guindre-Parker & Love 2014). In particular, there are examples of pheomelanin-based colour traits acting as honest signals in both mammals (Clough *et al.* 2009) and birds (Safran & McGraw 2004; Galván *et al.* 2014b). Following the handicap principle, signal honesty is based on costs of signal production or maintenance that are only affordable by signallers of high genotypic quality, and as a consequence, pheomelanin-based signals could evolve as honest signals under high levels of environmental oxidative stress, when allocating cysteine to pheomelanin synthesis instead of antioxidant protection is particularly costly (Galván & Alonso-Alvarez 2009). Our findings in the present study show that *Slc7a11* is environmentally labile in zebra finches, and then, pheomelanin-based coloration can change with environmental oxidative stress. This may be advantageous, but is incompatible with costly signalling from the perspective of the handicap principle, for which a fixed gene and signal expression level is expected if it is associated with the individual capacity of birds to display intense pheomelanin-based coloration under high environmental oxidative stress. Thus, the colour of flank feathers of male zebra finches is not likely to act as an honest

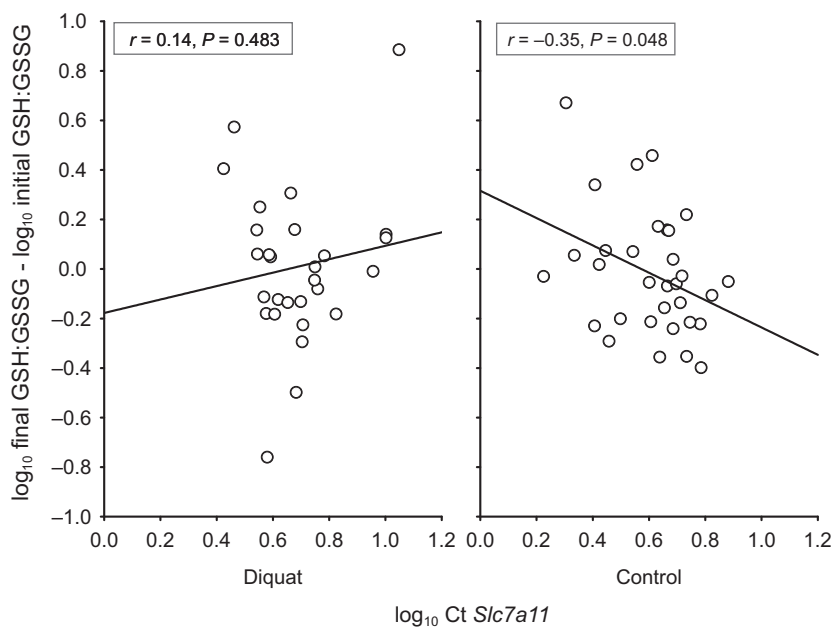


Fig. 4 Relationship between change in the oxidative status and relative *Slc7a11* expression. Expression measurements are C_t values for diquat-treated and control male zebra finches during the course of the experiment. Lower GSH:GSSG ratios indicate higher oxidative stress. Lower C_t values indicate higher gene expression. Inserts are results of correlation tests. Lines are regression lines.

signal of ability to cope with oxidative stress that evolves because of signal production costs, although this will have to be determined in future studies.

Alternatively, and although we detected a general downregulation of *Slc7a11* induced by diquat, this downregulation may be only produced in some birds. Our previous experimental investigation on the chest bib of male house sparrows, an honest signal produced by pheomelanin (Galván *et al.* 2014b), suggests that a physiological mechanism uncoupling cysteine and pheomelanin levels may have evolved in low-quality birds that prevents them from producing large dishonest signals even if cysteine levels change (Galván *et al.* 2015). Thus, pheomelanin synthesis (i.e. signal expression) directly depends on cysteine levels in high-quality birds only. With this mechanism, handicap costs would not be the basis of signal honesty, and the gene *Slc7a11* may determine the quality of birds (Galván *et al.* 2014b), so that gene expression may be fixed in low-quality birds but environmentally labile in high-quality birds. Therefore, the effect of diquat on *Slc7a11* expression detected in zebra finches may be the result of downregulation in high-quality birds only. These possibilities should now be investigated to understand the evolution of signal honesty in an scenario of lability in the genes that control signal expression.

Given the similarity of melanogenesis in birds and mammals (d'Ischia *et al.* 2015), these results have implications for understanding the evolution of human pigmentation. If *Slc7a11* was also epigenetically labile in humans, then phenotypes associated to large amounts of pheomelanin such as the red hair (Ito *et al.* 2011) may be the result of evolution favoured because of the

benefits of modulating gene expression in response to environmental stress. This may modify the current paradigm for the evolution of human pigmentation, which only considers a trade-off between protection against UV radiation and vitamin D₃ synthesis (Jablonski & Chaplin 2010) and is clearly insufficient (Galván *et al.* 2012). Thus, human pigmentation may vary in response to environmental stressors different from UV radiation, and it would be necessary to investigate the nature of such stressors. Furthermore, the physiological consequences of human pheomelanin-based pigmentation must be considered. Individuals with a genetic basis promoting the production of larger amounts of pheomelanin may experience chronic oxidative stress because of constant cysteine/GSH consumption (Napolitano *et al.* 2014). Once formed, pheomelanin also decreases GSH levels by inducing GSH autoxidation (Panzella *et al.* 2014). Induction of oxidative stress therefore continues after pheomelanin synthesis, probably favouring melanoma formation (Morgan *et al.* 2013; Napolitano *et al.* 2014). Cysteine transport regulated by *Slc7a11* also favours tumours unrelated to pigmentation, as shown by a negative association between *Slc7a11* expression and survival of patients with malignant glioma (Robert *et al.* 2015). If the beneficial downregulation of *Slc7a11* found in birds could be induced in humans, new strategies against melanoma and other cancer types may arise.

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Data accessibility

Data used in this study are available as Table S1 (Supporting information).

I.G. conceived of, designed and coordinated the study, conducted the experiment, carried out the laboratory work and data analyses, and wrote the manuscript. A.I. participated in the laboratory work and designed PCR primers. A.A.R.H. participated in the experiment. C.A.A. participated in the design of the study and in the experiment.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1. Dataset used in the study.