An integrative approach to the evolution of melanin-based traits in birds

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An integrative approach to the evolution of melanin-based traits in birds

Memoria presentada por la Licenciada en Ciencias Biológicas María Sol Rodríguez Martínez para optar al título de Doctora por la Universidad de Sevilla

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Fdo. María Sol Rodríguez Martínez

El Dr. Ismael Galván Macías, Científico Titular en el Departamento de Ecología Evolutiva del Museo Nacional de Ciencias Naturales (MNCN-CSIC) como director de la tesis; y el Dr. Javier Balbontin Arenas, Profesor Titular del Departamento de Zoología en la Universidad de Sevilla, como tutor

CERTIFICAN

Que María Sol Rodríguez Martínez, Licenciada en Ciencias Biológicas, ha realizado bajo su dirección la presente Memoria de Tesis Doctoral titulada "An integrative approach to the evolution of melanin-based traits in birds", y que a su juicio reúne los méritos suficientes para optar al grado de Doctora por la Universidad de Sevilla ante el Tribunal que se designe a tal efecto.

Y para que a tal manera conste, firman el presente documento en Sevilla, a 26 de octubre de 2021.

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A mi familia,

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ABSTRACT

Organisms can respond to changes in environmental conditions by modulating gene expression through the action of epigenetic modifications to maintain cellular homeostasis. This adaptive response can lead to phenotypic plasticity and thus avoid the oxidative stress that environmental changes may induce. In that sense, the evolution of phenotypic traits may be mediated by environmental oxidative stress. Pigmentation phenotypes, in particular, are regulated by genes whose expression may be modulated by environmental conditions, helping organisms to deal with stressful environments. Therefore, understanding environmental influences on gene expression that may be adaptive is key to obtain a comprehensive view of pigmentation phenotype evolution.

Melanin is the most common pigment in animals. The synthesis of melanin is a physiological process intimately related to oxidative stress. Particularly, the synthesis of the yellow-reddish form of the melanin is dependent on the concentration of the main antioxidant in the cell, glutathione. Therefore, the consumption of a valuable antioxidant makes pheomelanin synthesis a constraining process when antioxidants are required for other vital processes. However, in the absence of environmental oxidative stress, pheomelanin synthesis may contribute to cysteine homeostasis, which is toxic if in excess. In that sense, it seems that pheomelanin synthesis is a physiological mechanism intimately related to oxidative stress that may help organisms to adapt to stressful environments. If the genes that regulate pheomelanin synthesis are susceptible to epigenetic modifications, their expression, and that of the associated pigmentation phenotype, may be modified according to environmental conditions and thus drive adaptive responses. Here I used a multidisciplinary and integrative approach to get a better understanding of the evolution of melanin-based traits. In particular, the aim of this thesis is to determine if the synthesis of melanin is susceptible to epigenetic modifications that provide birds phenotypic plasticity to

adjust pigmentation phenotypes to the prevailing environmental conditions (**Section 1**) and thus obtain a comprehensive view of plumage diversity evolution (**Section 2**).

To determine if birds adjust the pigmentation phenotype to the prevailing environmental conditions, experiments in captivity and natural populations under varying environmental conditions were conducted (**Section 1**). Environmental conditions that induce oxidative stress modulated the expression of genes involved in pheomelanin synthesis (**Chapter III**) through the action of epigenetic modifications (**Chapters I** and **II**), and this avoided the expected oxidative damage. Phenotypes resulting from a genetic predisposition to produce large amounts of pheomelanin creates weak physiological conditions but also more lability in pigment production, making pheomelanin-based coloration phenotypically plastic (**Chapter IV**). Thus, it seems that although pheomelanin synthesis induces chronic oxidative damage in birds with plumage profusely covered by this pigment, its plastic regulation under stress can even decrease damage levels. Dealing with stressful environments can then have consequences for both physiology and the pigmentation phenotype. To avoid changes in the pigmentation phenotype, however, the expression of the main gene involved in pheomelanin synthesis can be reversed after stress disappearance (**Chapter V**). This makes the regulation of pheomelanin synthesis an adaptive strategy to avoid the evolutionary consequences of external phenotypic changes, as mating preferences can be based on the intensity of pheomelanin-based plumage coloration (**Chapter VI**). Therefore, pheomelanin synthesis seems to represent an adaptive physiological mechanism that allows animals (birds) to deal with environmental stress and maintain cellular homeostasis, which may or may not result in a modification of the pigmentation phenotype.

A comprehensive view of the evolution of melanin-based plumage was obtained by developing phylogenetic analyses (**Section 2**). At an interspecific level, a negative association between color heterogeneity

and the level of expression of different melanin forms seems to provide a physiological solution to obtain diverse plumage coloration (**Chapter VII**). This is because producing several forms of melanin, which is required to generate several colors, is more physiologically constraining than producing a single or few melanin forms. However, differences in plumage coloration do not only occur between species of birds, but also through different life stages within the same species. In particular, during juvenile stages, organisms suffer less relative physiological stress than adults and are more prone to experience systemic excess cysteine, as they may have lower requirements for antioxidant protection. Thus, juvenile pheomelanin-based plumage coloration has evolved more frequently in birds with strictly carnivorous diets, which may make them more susceptible to experience excess cysteine, likely as a detoxifying strategy (**Chapter VIII**). Pheomelanin pigmentation is not only favored under certain physiological conditions, but also under exposure to certain environmental factors. The exposure to high levels of elemental sulfur in the soil favors the production of pheomelanin in the plumage of different species of birds, likely by an influence of increased intracellular thiols during plumage development, at least in areas with high volcanic activity such as Iceland (**Chapter IX**). Together, these three final chapters provide novel views of melanin-based pigmentation in a phylogenetic context, thus helping to understand how bird phenotypic diversity has evolved.

In sum, this thesis provides an integrative approach to the evolution of melanin-based traits. In particular, pheomelanin synthesis seems to have evolved as a physiological mechanism that provides phenotypic plasticity to birds and allows them to adjust the pigmentation phenotype to environmental conditions, which can be mediated by epigenetic modifications. This thesis thus contributes to a better understanding of bird phenotypic diversity by adopting a multidisciplinary approach that integrates phylogenetic analyses and experimental procedures conducted in natural and experimental populations.

RESUMEN

Los organismos pueden responder a cambios ambientales modulando la expresión de algunos genes mediante la acción de modificaciones epigenéticas para mantener así la homeostasis celular. Esta respuesta adaptativa puede alterar la morfología, fisiología y/o comportamiento (plasticidad fenotípica) y en consecuencia evitar el estrés oxidativo que los cambios en el ambiente pueden desencadenar. Es por ello que se ha propuesto que la evolución de los rasgos fenotípicos podría estar regulada por el estrés oxidativo ambiental. Los fenotipos de pigmentación, en particular, están regulados por genes cuya expresión puede estar modulada por las condiciones ambientales, lo que ayuda a los organismos a lidiar con ambientes estresantes. Por lo tanto, comprender las influencias ambientes en la expresión génica que pueden ser adaptativas es clave para obtener una visión integral de la evolución del fenotipo de pigmentación.

La melanina es el pigmento más común presente en los animales. Su síntesis representa un proceso fisiológico íntimamente relacionado con el estrés oxidativo. En particular, la síntesis de la forma rojiza de la melanina está íntimamente relacionada con la concentración plasmática del principal antioxidante celular, el glutatión. Por lo tanto, el consumo de un antioxidante valioso durante la síntesis de la feomelanina convierte a este proceso fisiológico en un limitante cuando los antioxidantes circundantes son requeridos para otros procesos vitales. Sin embargo, en ausencia de estrés oxidativo ambiental, la síntesis de feomelanina podría contribuir a mantener la homeostasis de cisteína, la cual es tóxica si está en exceso. En este sentido, la síntesis de feomelanina parece ser un mecanismo fisiológico íntimamente relacionado con el estrés oxidativo que puede estar implicado en la adaptación a ambientes estresantes. Si los genes que regulan la síntesis de feomelanina son susceptibles a modificaciones epigenéticas, su expresión y el fenotipo de pigmentación asociado podrían ser modificados de acuerdo con las condiciones

ambientales y así desencadenar respuestas adaptativas. Es por ello que utilicé un enfoque integrativo y multidisciplinario para obtener una visión global de la evolución de los rasgos melánicos. En particular, el objetivo de esta tesis es determinar si la síntesis de melanina es susceptible a modificaciones epigenéticas que proporcionen a las aves plasticidad fenotípica para ajustar el fenotipo de pigmentación a las condiciones ambientales predominantes (**Sección 1**) y así obtener una visión integral de la evolución de la diversidad del plumaje (**Sección 2**).

Para determinar si las aves ajustan la coloración del plumaje de acuerdo con las condiciones ambientales se realizaron procedimientos experimentales tanto en cautividad como en poblaciones naturales bajo diferentes condiciones ambientales (**Sección 1**). En condiciones ambientales que promueven el estrés oxidativo, la expresión de los genes implicados en la síntesis de feomelanina fue modulada (**Capítulo III**) mediante modificaciones epigenéticas (**Capítulos I** y **II**), lo cual evita el daño oxidativo esperado. Los fenotipos que resultan de una predisposición genética a producir grandes cantidades de feomelanina crean condiciones fisiológicas débiles, pero también más labilidad en la producción de diferentes pigmentos, lo que hace que la coloración a base de feomelanina sea fenotípicamente plástica (**Capítulo IV**). Por lo tanto, a pesar de que la síntesis de feomelanina induce daño oxidativo crónico en las aves con un plumaje profusamente cubierto por este pigmento, su regulación bajo condiciones que inducen estrés puede incluso disminuir los niveles de daño. Enfrentarse a entornos estresantes puede tener consecuencias tanto en la fisiología como en el fenotipo de pigmentación. Para evitar cambios en el fenotipo de pigmentación, sin embargo, la expresión del gen principal involucrado en la síntesis de feomelanina puede revertirse después de la desaparición del estrés (**Capítulo V**).Esto hace que la regulación de la síntesis de feomelanina sea una estrategia adaptativa para evitar las consecuencias evolutivas que conllevarían los cambios en el fenotipo externo, ya que las preferencias de apareamiento en algunas especies de aves parecen basarse en la intensidad de la

coloración del plumaje a base de feomelanina (**Capítulo VI**). Por lo tanto, la síntesis de feomelanina representaría un mecanismo fisiológico adaptativo que le permite a los animales (aves) lidiar con el estrés ambiental y mantener la homeostasis celular, lo cual puede resultar o no en una modificación del fenotipo de pigmentación.

En la **Sección 2**, se obtuvo una visión completa de la evolución del plumaje a base de melanina mediante el desarrollo de análisis filogenéticos. A nivel interespecífico, una asociación negativa entre la heterogeneidad del color y el nivel de expresión de las diferentes formas de melanina parece proporcionar una solución fisiológica para obtener diversa coloración del plumaje (**Capítulo VII**). Esto se debe a que producir varias formas de la melanina, lo cual es necesario para sintetizar varios colores, es más limitante que producir una sola o pocas formas de melanina para pigmentar el plumaje. Sin embargo, las diferencias en la coloración del plumaje no solo ocurren entre especies, sino que también suelen darse a lo largo de diferentes estadios en la vida de una misma especie. En particular, durante la etapa juvenil los organismos son menos propensos a sufrir estrés fisiológico que los adultos y por tanto más vulnerables a sufrir un exceso de cisteína a nivel sistémico ya que tienen menos requisitos de protección antioxidante. Por lo tanto, la coloración del plumaje juvenil a base de feomelanina ha evolucionado más frecuentemente en aves con dietas estrictamente carnívoras, lo que las hace más susceptibles a experimentar un exceso de cisteína, probablemente como una estrategia desintoxicante (**Capítulo VIII**). La pigmentación feomelánica no solo se ve favorecida frente a ciertas condiciones fisiológicas, sino también ante la exposición de ciertos factores ambientales. La exposición a altos niveles de azufre en el suelo favorece la producción de feomelanina en el plumaje de diferentes especies de aves, probablemente por una influencia de los altos niveles intracelulares de compuestos tiol durante el desarrollo del plumaje, al menos en áreas con una alta actividad volcánica como Islandia (**Capítulo IX**). Estos tres capítulos aportan una visión novedosa sobre la

pigmentación a base de melanina en un contexto filogenético, lo que ayuda a comprender cómo ha evolucionado la diversidad fenotípica de las aves.

En resumen, esta tesis proporciona una visión integral de la evolución de los rasgos melánicos. En particular, la síntesis de feomelanina parece haber evolucionado como un mecanismo fisiológico que brinda plasticidad fenotípica a las aves y les permite ajustar su fenotipo de pigmentación a las condiciones ambientales, lo cual puede estar modulado por modificaciones epigenéticas. Por lo tanto, esta tesis contribuye a una mejor compresión de la diversidad fenotípica en aves mediante un enfoque multidisciplinario que integra tanto análisis filogenéticos como procedimientos experimentales realizados en poblaciones experimentales y naturales.

GENERAL INTRODUCTION

Organisms have the ability to respond to changes and adapt to new conditions. To restore cellular homeostasis, the morphology, physiology, and/or behavior can be regulated (Wingfield, 2003) in accordance with the physical (abiotic) and biological (biotic, social, and/or internal) environmental conditions (Wingfield, 2013). In particular, modulation of gene expression plays a central role in cellular adaptation (López-Maury et al., 2008) to changing environments (Schulte, 2001) by leading to changes in the phenotype (Gabriel, 1999). However, this mechanism must be plastic over time to trigger a reversible response during short stressful periods (Gabriel, 2005). The adaptive response to changes in the environment is accepted as phenotypic plasticity, meaning the ability of a genotype to express different phenotypes (behavioral, physiological, or other environmentally sensitive traits) under varied environmental conditions (Pigliucci, 2005). As phenotypic plasticity has been recognized as a key strategy that enables organisms to respond adaptively to changing environments (Bradshaw, 1965), understanding the genetic mechanisms that regulate phenotype is essential to obtain a comprehensive view of phenotypic traits evolution.

Epigenetic changes are an important mechanism by which the genome responds to novel conditions (Duncan et al., 2014). The epigenetic response is mediated by changes in gene expression that do not affect the DNA sequence (Jaenisch & Bird, 2003). These epigenetic modifications are based on molecular processes that include methylation of DNA and RNA nucleobases, acetylation or methylation of histone proteins, and regulatory processes mediated by small RNA molecules (Bossdorf et al., 2008). In DNA, the best-known epigenetic modification is the methylation of cytosine to form 5-methylcytosine (m^5C), which leads to transcriptional silencing (Schübeler, 2015). Epigenetic changes may thus regulate gene expression in cells by gene silencing. In this regard, it is possible that epigenetic variation only viewed in a particular region of

DNA sequence does not reflect an effect on gene expression (Duncan et al., 2014). Therefore, it is not enough to find changes only in the methylation patterns, but it is also necessary to detect changes in gene expression and their impact on the phenotype to understand if the epigenetic variation has a functional effect. Indeed, it is well known that epigenetic variation defines phenotypic variation (Massicotte et al., 2011; Lea et al., 2016) providing the basis for an adaptive plastic response (Bossdorf et al., 2008). In that sense, the epigenetic modifications that induce genetic changes over time may be relevant to the evolution of phenotypic traits (Bateson & Gluckman, 2012). However, a reversible genetic response allows organisms to deal with transient environmental conditions without consequences in the phenotype (Gabriel, 2005). Thus, epigenetic plasticity seems to be a powerful mechanism of adaptation by leading to reversible changes in gene expression (Schuebel et al., 2016) that can rapidly return to the basal state after stress disappearance (Sørensen et al., 2005; Yale, 2001). Nevertheless, much work is still required to understand the epigenetic mechanisms that allow organisms to adapt to changing conditions (Hawkins & Storey, 2020).

Adaptive responses to environmental changes are induced by oxidative stress (Crawford & Davies, 1994), i.e. the imbalance between reactive oxygen species (ROS) and the availability of antioxidants to combat them (Finkel & Holbrook, 2000). Prolonged oxidative stress exposure causes damage to proteins, lipids, and DNA, leading to cellular damage, tissue injury, and inflammation (Halliwell & Gutteridge, 1999). Oxidative damage can occur both by endogenous processes related to the normal cellular metabolic activity and by environmental stimuli that generate high levels of ROS that perturb redox balance (Finkel & Holbrook, 2000). Besides the deleterious effects of oxidative stress on the normal cellular state, it has been demonstrated that a certain amount of oxidative stress can improve cellular function (Pandey & Rizvi, 2011). In particular, low concentrations of ROS enhance endogenous antioxidant response, while a massive level of ROS inhibits enzyme activity and causes

cellular apoptosis (Radak et al., 2005). Some environmental factors are known to produce oxidative stress (Schröder et al., 2005). The cellular response to stressful environments is mediated by diverse regulatory mechanisms (Kültz, 2005) that allow organisms to rapidly adapt to changes in stress levels (Pickering et al., 2013). In response to environmental oxidative stress, cells can restore homeostasis and avoid oxidative damage (Dalton et al., 1999) by modulating the expression of several genes (Causton et al., 2001). Therefore, survival is dependent on the ability of the cell to adapt to or deal with stressful conditions, and to repair cellular damage by regulating gene expression.

Oxidative stress has been proposed as a key mediator of the evolution of phenotypic traits (Metcalfe & Alonso-Alvarez, 2010). Particularly, oxidative stress experienced early in the development of birds affects the phenotype in adulthood (Romero-Haro & Alonso-Alvarez, 2015). In fact, exposure to a stressful environment during a significant period in the ontogeny of a bird induces changes in the expression of genes that regulate the pigmentation phenotype at later stages of life (Galván, 2018). Animal coloration seems to be physiologically regulated under environmental conditions that promote stress (Fitze et al., 2009; Loiseau et al., 2008), thus it can be considered a phenotypic trait with strong implications for adaptation (Manceau et al., 2011). Indeed, the evolution and diversity of animal coloration can result from the balance between sexual selection and natural selection (Dunn et al., 2015) within the constraints of a given environment (Endler, 1992). In particular, sexually selected traits are phenotypically plastic indicators of the physical condition or quality of an individual (Price, 2006). Recent findings in a bird species reveal that the color intensity of nestlings reflects body condition during development, suggesting that plumage coloration in adulthood may be a consequence of sexual selection operating in juveniles (Galván, 2017). Therefore, understanding mating preferences may help to explain how phenotypes evolve (Jennions & Petrie 1997). In that sense, an individual could obtain adaptive benefits

from their choices based on the pigmentation phenotype, which seems to reflect the physical condition.

Melanin is the most common pigment in animals. The synthesis of melanin occurs in specialized cells called melanocytes (Lin & Fisher, 2007) localized in the epidermis and some internal structures of virtually all organisms. Melanin synthesis is a physiological process intimately related to oxidative stress (Galván & Alonso-Álvarez, 2008) that may constraint the adaptation (and hence, the evolution) of organisms. The diversity of colors that melanin confers to feather results from the sulphurated form termed pheomelanin (yellowish to chestnut coloration) and the nonsulphurated form termed eumelanin (black, brown, and grey coloration; Galván & Wakamatsu, 2016). Particularly, the synthesis of pheomelanin occurs when the concentration of the amino acid cysteine in melanosomes is above a certain threshold and thus kinetic conditions favor the incorporation of the sulfhydryl group of cysteine to the reaction, which results in the formation of sulfur-containing heterocycles (García-Borrón & Olivares Sánchez, 2011). The yellow-reddish pigments are then transferred to surrounding keratinocytes, thus conferring pigmentation to the skin and associated structures such as hair, feathers, and scales (Lin & Fisher, 2007). Therefore, the cysteine used for pheomelanin synthesis cannot be incorporated back into cysteine metabolism nor be used to synthesize the main intracellular antioxidant glutathione (GSH; Wu et al., 2004). The consumption of a valuable antioxidant makes pheomelanin synthesis a constraining process when antioxidants are required for other vital processes (Napolitano et al., 2014). This might explain the increased risk of melanoma observed in humans and mice expressing phenotypes that result from high pheomelanin production (Mitra et al., 2012), or the diminished antioxidant capacity in wild birds exposed to ionizing radiation that pigment their feathers with large amounts of pheomelanin (Galván et al., 2014). However, in the absence of environmental factors that induce oxidative stress and make cysteine limiting, pheomelanin synthesis may represent an adaptive mechanism to maintain

homeostasis (Galván et al., 2012), avoiding excess cysteine, which promotes oxidative stress (Munday, 1989). This is because feathers are inert structures in which the incorporated cysteine during pheomelanin synthesis cannot exert any physiological effect. Therefore, there is a potential physiological trade-off between the use of cysteine for pheomelanin synthesis and its use for GSH synthesis, and the outcome of this trade-off can be determined by environmental conditions that induce oxidative stress. In sum, in the absence of environmental oxidative stress, pheomelanin synthesis may contribute to cysteine homeostasis. However, under environmental conditions that generate oxidative stress and make cysteine limiting during pheomelanin synthesis, available cysteine may contribute to antioxidant protection.

The modulation of gene expression serves as a regulatory mechanism that can maintain cellular homeostasis despite environmental conditions that induce oxidative stress (Causton et al., 2001), through the action of epigenetic modifications (Hala et al., 2014). In particular, the regulation of the genes involved in melanin production is dependent on cysteine availability and the need for antioxidant protection (Figure 1). In birds, it has been found lability in the expression of the main genes involved in pheomelanin synthesis under a stressful situation to avoid GSH consumption (Galván, 2018) and also under conditions generating excess cysteine (Galván et al., 2017). If the genes that regulate pheomelanin synthesis are susceptible to epigenetic modifications, organisms may modify the expression of genes involved in melanin synthesis and the associated pigmentation phenotype according to environmental conditions and thus adapt to them (Figure 2). Therefore, opposite effects on pigmentation are expected in response to factors that affect pheomelanin synthesis through opposing effects: increased environmental oxidative stress reduces pheomelanin synthesis and pigmentation, and increased dietary cysteine favor synthesis and pheomelanin-based pigmentation. In this regard, pheomelanin synthesis seems to represent an adaptive mechanism that allows birds to adjust

their pigmentation phenotype to the prevailing environmental conditions to avoid oxidative stress.

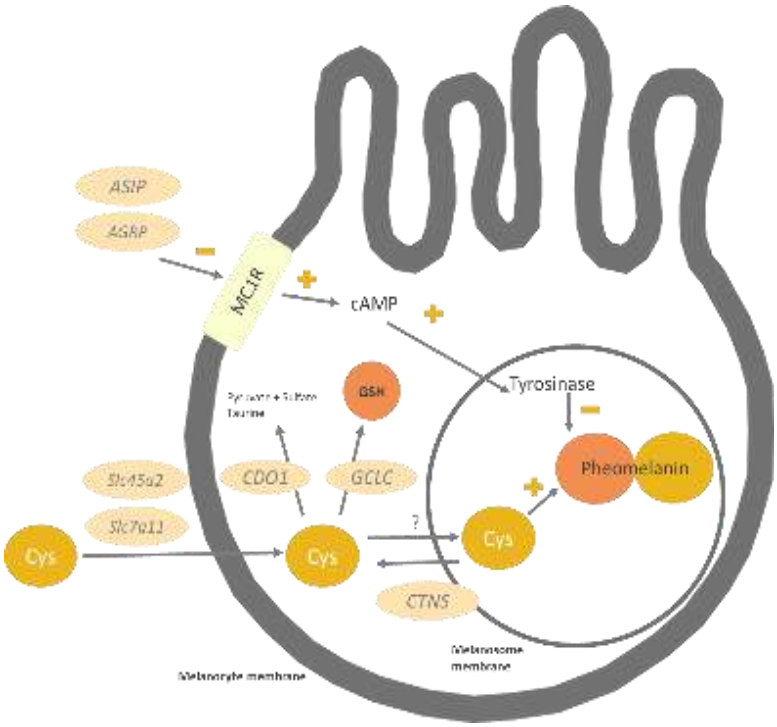


Figure 1. Scheme of the regulation of genes involved in pheomelanin synthesis in melanocytes. Gene names are shown in italics. The genes coding for the mediators of cysteine metabolism are cysteine dioxygenase type I (*CDO1*; McCann et al., 1994), the glutamatecysteine ligase catalytic subunit (*GCLC*; Meister, 1995) and cystinosin (*CTNS*; Town et al., 1998). *CDO1* and *GCLC* code, respectively, for the enzymes cysteine dioxygenase (CDO) and γ-glutamylcysteine synthetase (GCS), which compete for cysteine as a substrate (Stipanuk et al., 2002, 2009). The enzyme CDO catalyzes the addition of molecular oxygen to the sulfhydryl group of cysteine to form less toxic products such as sulfate and taurine (Stipanuk et al., 2006) while GCS catalyzes the binding of cysteine to glutamate in GSH synthesis (Stipanuk et al., 2002; Stipanuk, et al., 2009). *CTNS* codes for a protein that exports cystine out of lysosomes inhibiting pheomelanin synthesis in melanocytes (Chiaverini et al., 2012). Pheomelanin synthesis is also regulated by genes that change the intracellular concentration of cyclic adenosine monophosphate (cAMP) and thus influence the activity of tyrosinase, the key enzyme in the melanogenesis pathway. 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). The most important gene involved in pheomelanin synthesis is the gene encoding the cystine/glutamate antiporter xCT (solute carrier family 7 member 11, *Slc7a11*), a protein localized in the plasma membrane (Conrad & Sato, 2012; He, et al., 2012) that is responsible for providing cells with cysteine (Chintala et al., 2005). The gene *Slc45a2* (solute carrier family 45 member 2) has also been suggested to transport cysteine to cells (Gunnarsson et al., 2007).

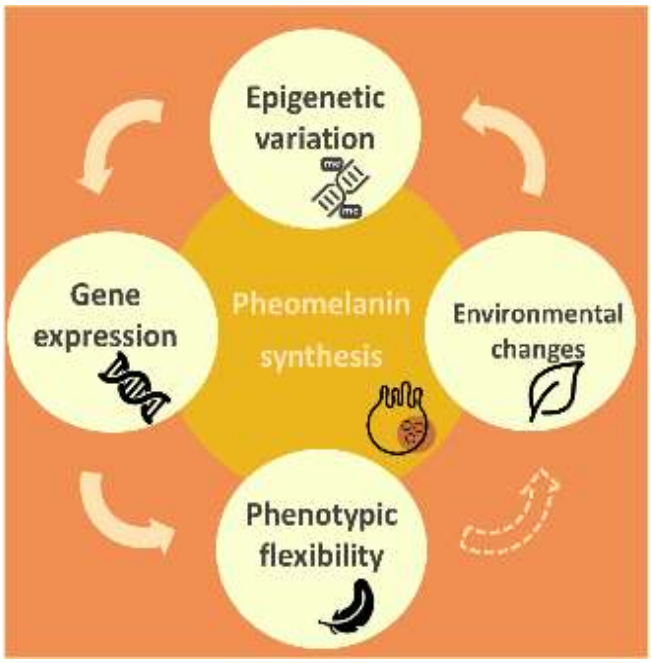


Figure 2. The interaction between pheomelanin synthesis, the environment, epigenetic regulation, and phenotype. Pheomelanin synthesis is a physiological mechanism regulated by the environment, gene expression and epigenetic variation. The synthesis of pheomelanin is susceptible to changes by environmental conditions. Environmental changes can lead to epigenetic modifications that alter the expression of genes involved in the synthesis of pheomelanin. The gene expression regulation does not only affect the pigmentation phenotype, but also affects the capacity to adapt to environmental changes. A flexible phenotype represents an adaptive strategy to deal with changes in environmental conditions.

Phenotypic traits are not fixed but are subject to genetic variation and evolutionary adaptation. In particular, the pigmentation phenotype represents an ideal trait for studying the genetic and physiological mechanisms that leads to phenotypic diversity and evolutionary change (Hubbard et al., 2010). Phenotypic differences among individuals are caused by a combination of differences in genotype, ontogeny, and environment (Stearns, 1989, 1992; van Noordwijk, 1989). Therefore, an adaptive phenotypic trait may be explained proximately by the nteraction of the genotype with environmental conditions during the expression of

the trait, as well as ultimately, by an interaction of the genotype with environmental conditions under which selection for the trait occurs (Nager & van Noordwijk, 1995). The difference between proximate and ultimate causes of phenotypic traits is central to evolutionary explanations (Mayr, 1963; Tinbergen, 1963). Proximate mechanisms refer to the physiological mechanisms that control the expression of a certain trait and its development (ontogeny), while ultimate mechanisms refer to evolutionary explanations, in particular the adaptive function and phylogenic history of a trait. Therefore, a major goal of Evolutionary Ecology is obtaining an integrative view of the mechanisms that modulate the evolution of traits, considering both proximate and ultimate causes. As pheomelanin synthesis seems to represent a physiological mechanism that allows organisms to adapt to environmental conditions by avoiding oxidative stress, understanding the epigenetic modifications that drive adaptation to the environment is key to obtain an integrative view of pheomelanin-based traits evolution.

OBJECTIVES

The overall aim of this thesis is to understand the epigenetic modifications that provide birds phenotypic plasticity to adjust the synthesis of melanin to the prevailing environmental conditions and obtain a comprehensive view of plumage diversity evolution. To fulfil this objective, this thesis explores at the intraspecific level the epigenetic and physiological responses induced by a range of environmental influences on the synthesis of pheomelanin and the effects on phenotype expression (**Section 1**). At the interspecific level, this thesis explores the phylogenetic context and the adaptive value of expressing pheomelanin-based pigmentation phenotypes (**Section 2**). Overall, understanding both the proximate mechanisms of pheomelanin synthesis and the ultimate causes of the expression of pheomelanin-based plumage phenotypes may lead to a global view of the evolution of melanin-based traits.

In **Section 1**, the epigenetic plasticity of genes involved in pheomelanin synthesis was explored by experimentally inducing environmental conditions that promote oxidative damage. As mentioned before, there is a potential physiological trade-off between the use of cysteine for pheomelanin synthesis and its use for antioxidant protection under environmental stress conditions. However, in the absence of environmental factors that induce oxidative stress, pheomelanin synthesis may represent a form of cysteine excretion. Therefore, it is essential to investigate if the genes involved in the synthesis of melanin are open to epigenetic influences (RNA and DNA methylation) that allow organisms to adapt to the prevailing environmental conditions, thus avoiding oxidative damage. **Chapter I** determines if melanocytes from pheomelanin pigmented feathers are epigenetically labile to respond to an increment in cysteine availability (objective 1). After evaluating if pheomelanin synthesis represents a mechanism that contributes to cysteine homeostasis, it is necessary to determine if gene regulation also occurs when available cysteine is required for antioxidant protection

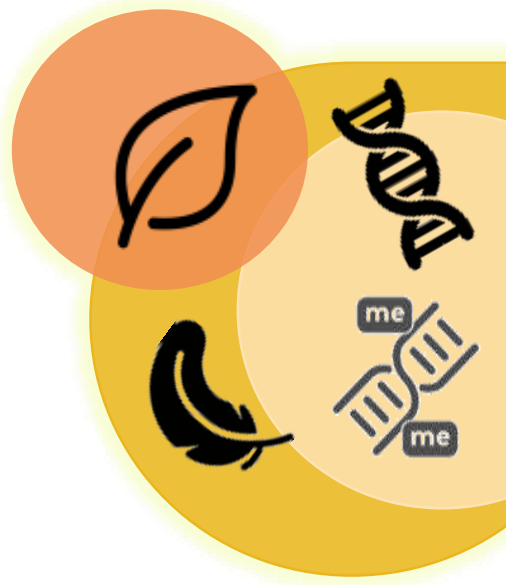
against environmental stress. Thus, different sources of oxidative stress were experimentally induced to examine if epigenetic modifications on genes involved in melanin synthesis occur and are equally efficient with any source and intensity of environmental oxidative stress. **Chapter II** explores the possibility that a competitive environment that increases the susceptibility to suffer oxidative stress modulates the expression of genes involved in pheomelanin synthesis to avoid the expected oxidative damage that social interactions may cause in male zebra finches *Taeniopygia guttata* (objective 2). In **Chapter III**, environmental oxidative stress was experimentally induced by the administration of the herbicide diquat dibromide to mimic the effects of any exogenous source of oxidative stress during the growth of pheomelanin-based feathers in male zebra finches (objective 3). To infer the direct physiological effects of pheomelanin synthesis, it is necessary to evaluate the implications of melanin production when antioxidant protection is required to combat environmental oxidative stress (objective 4). Thus, in **Chapter IV**, oxidative damage in DNA was quantified in birds with plumage profusely pigmented by pheomelanin during experimental induction of oxidative stress. Also, a detailed genotypic characterization among zebra finches with distinct color morphs was performed to directly infer oxidative damage implications of pheomelanin synthesis. Furthermore, to test if the synthesis of pheomelanin pigmentation can be considered an adaptive plastic response, **Chapter V** evaluates if the expression of the main gene involved in pheomelanin synthesis (*Slc7a11*) can be reversed after stress disappearance (objective 5). Finally, as the evolution of phenotypic traits can be driven by sexual selection, it is necessary to determine the variation in mating success in relation to pheomelanin-based plumage coloration (objective 6). Therefore, in **Chapter VI**, female preferences of Eurasian nuthatch *Sitta europaea* for the pheomelanin-based plumage coloration of males was explored as an indicator of their physical condition during plumage development. Overall, these six chapters aim to determine if pheomelanin synthesis actually represents an adaptive physiological mechanism that allows animals to adjust their

pigmentation phenotype in accordance with the prevailing environmental conditions, thus providing a better knowledge of the evolution of plumage coloration.

In **Section 2**, the interspecific differences in plumage coloration were investigated to get a better understand of the evolution of bird phenotypic diversity. The production of the different forms of melanin has different physiological limitations, thus it is necessary to investigate those differences in a phylogenetic context to understand how melanin-based plumage diversity may have evolved. The different chemical forms of melanin lead to different plumage colors and different amounts of those forms lead to different color intensities. Therefore, **Chapter VII** determines the association between color heterogeneity and the level of expression (i.e., intensity) of plumage coloration on birds (objective 7). After determining the physiological constraints of different melanin forms, it is necessary to evaluate if pheomelanin pigmentation is favored under certain physiological and environmental conditions. In **Chapter VIII**, the effect of the availability of dietary cysteine on the expression of pheomelanin-based pigmentation was investigated to test if the synthesis of pheomelanin may have evolved as a result of detoxifying strategy (objective 8). Pheomelanin synthesis may not only be influenced by dietary cysteine, but also by exposure to environmental sulfur. The sulfur content of soil may thus influence pheomelanin synthesis, as high contents may increase sulfur contents in organisms that interact with the soil. In that sense, pheomelanin synthesis may be favored under exposure to soils with high sulfur contents. Therefore, in **Chapter IX**, the possibility that the sulfur content of soil also exerts an influence on pheomelanin-based phenotypic diversity was investigated (objective 9). Together, these three final chapters provide a better understanding of the adaptive value of expressing melanin-based plumage coloration in accordance with the prevailing environmental conditions, thus helping to understand how bird phenotypic diversity has evolved.

SECTION 1

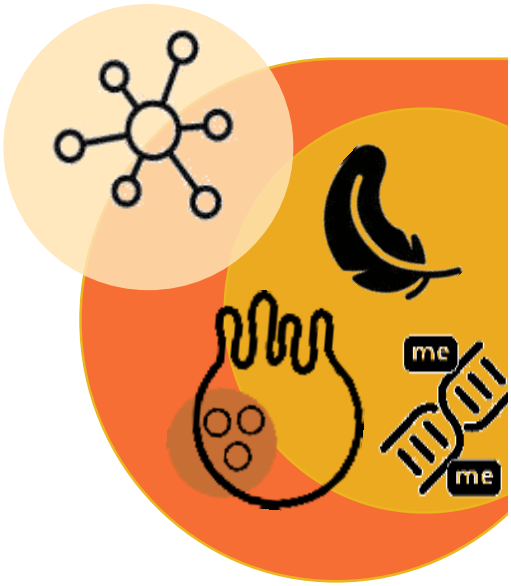
Epigenetic and physiological responses induced by environmental stress on the synthesis of pheomelanin



CHAPTER I

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

Sol Rodríguez-Martínez, Rafael Márquez, Ângela Inácio and Ismael Galván



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ABSTRACT

Cysteine plays essential biological roles, but excessive amounts produce cellular oxidative stress. Cysteine metabolism is mainly mediated by the enzymes cysteine dioxygenase and γ-glutamylcysteine synthetase, respectively coded by the genes *CDO1* and *GCLC*. Here we test a new hypothesis posing that the synthesis of the pigment pheomelanin also contributes to cysteine homeostasis in melanocytes, where cysteine can enter the pheomelanogenesis pathway. We conducted an experiment with the Eurasian nuthatch Sitta europaea, a bird producing large amounts of pheomelanin for feather pigmentation, to investigate if melanocytes show epigenetic lability under exposure to excess cysteine. We increased systemic cysteine levels in nuthatches by supplementing them with dietary cysteine during growth. In feather melanocytes this led to the downregulation of genes involved in intracellular cysteine metabolism (*GCLC*), cysteine transport to the cytosol from the extracellular medium (*Slc7a11*) and from melanosomes (*CTNS*), and regulation of tyrosinase activity (*MC1R* and *ASIP*). These changes were mediated by increases in DNA m^5C in all genes except *Slc7a11*, which experienced RNA m^6A depletion. Birds supplemented with cysteine synthesized more pheomelanin than controls, but did not suffer higher systemic oxidative stress. These results suggest that excess cysteine activates an epigenetic mechanism that favours pheomelanin synthesis and may protect against oxidative stress.

KEYWORDS

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

INTRODUCTION

Cysteine is a semi-essential amino acid that cells metabolize to produce glutathione (GSH), the major cellular antioxidant (Wu, Fang, Yang, Lupton, & Turner, 2004). Cysteine metabolism also leads to the production of another amino acid, cysteinesulfinate, which is further converted to either taurine or pyruvate and inorganic sulfur (Stipanuk, Londono, Lee, Hu, & Yu, 2002). These metabolites play a role in several essential cellular processes, ranging from energy supplementation to antioxidant protection (Bender & Martinou, 2016; Lambert, Kristensen, Holm, & Mortensen, 2015). However, excess cysteine can occur when cysteine availability is above the rate of cysteine metabolism, which favours the auto-oxidation to the disulfide (cystine), a redox cycling that generates reactive oxygen species (ROS) and thus produces oxidative stress (Munday, 1989). As a consequence, excess cysteine is responsible for several, often lethal oxidative stress-based cytotoxic effects (Dilger & Baker, 2008; Janaky, Varga, Hermann, Saransaari, & Oja, 2000).

The maintenance of cysteine homeostasis is mainly mediated by two enzymes that compete for cysteine as a substrate: cysteine dioxygenase (CDO), which catalyses the addition of molecular oxygen to the sulfhydryl group of cysteine to form cysteinesulfinate, and γ-glutamylcysteine synthetase (GCS), which catalyses the rate-limiting step in GSH synthesis involved in the binding of cysteine to glutamate (Stipanuk et al., 2002; Stipanuk, Ueki, Dominy, Simmons, & Hirschberger, 2009). CDO and GCS are therefore essential enzymes in the maintenance of cysteine homeostasis. In spite of this process, CDO and GCS activity does not seem sufficient to avoid excess cysteine. This is shown by the fact that a dysfunction in cystinosin, a cystine/H^+ symporter that exports cystine out of lysosomes, causes intralysosomal excess cysteine and the corresponding disease (cystinosis) despite apparent functionality of CDO and GCS (Chiaverini et al., 2012).

Cystinosin can thus be considered another essential component for the maintenance of cysteine homeostasis (Figure 1).

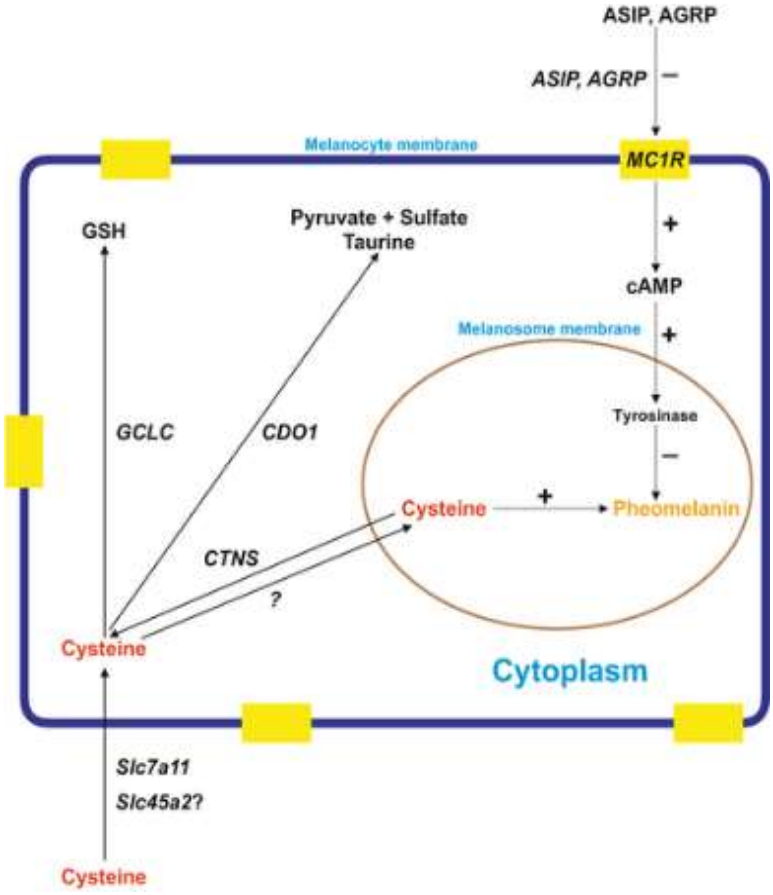

Figure 1. Scheme of cysteine metabolism in melanocytes. Gene names are shown in italics. Solid arrows represent transport or conversion. Dashed arrows represent influences of the indicated sign. Cysteine is transported from the extracellular medium by the cystine/glutamate antiporter xCT, which is encoded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

In addition to CDO, GCS and cystinosin, another mechanism of cysteine homeostasis specific to melanocytes has recently been proposed. Melanocytes are cells that contain lysosome-like organelles, termed melanosomes, where the synthesis of melanin pigments takes place (Lin & Fisher, 2007). Melanin synthesis involves oxidation of the amino acid tyrosine and polymerization of the resulting indole compounds. If intramelanosomal cysteine concentration is above a certain threshold, kinetic conditions favour incorporation of the sulfhydryl group of cysteine to the reaction, which results in the formation of sulfur-containing heterocycles, reddish or yellowish pigments that are termed pheomelanins (García-Borrón & Olivares Sánchez, 2011). Pheomelanin is then transferred to surrounding keratinocytes, thus confering pigmentation to the skin and associated structures such as hair, feathers and scales (Lin & Fisher, 2007). Therefore, cysteine used in pheomelanin synthesis cannot be incorporated back into cysteine metabolism, which means that the production of large amounts of pheomelanin in melanocytes can lead to chronic systemic oxidative stress if cysteine is limiting because sufficient GSH cannot be produced (Napolitano, Panzella, Monfrecola, & d'Ischia, 2014; Panzella et al., 2014). This might explain the increased risk of melanoma observed in humans and mice expressing phenotypes that result from high pheomelanin production (Mitra et al., 2012; Wang et al., 2016), or the diminished antioxidant capacity observed in wild birds exposed to ionizing radiation that pigment their feathers with large amounts of pheomelanin (Galván, Bonisoli-Alquati et al., 2014). However, in the absence of environmental factors that induce oxidative stress and make cysteine limiting, pheomelanin synthesis may represent a form of cysteine excretion, thus helping to avoid excess cysteine. Accordingly, pheomelanin synthesis has been proposed as a mechanism contributing to cysteine homeostasis (Galván, Ghanem, & Møller, 2012). In sum, there is a potential physiological trade-off between the use of cysteine for pheomelanin synthesis and its use for

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

The functionality of pheomelanin-producing melanocytes in the context of excess cysteine avoidance remains unexplored. If such a function is physiologically advantageous, we hypothesize that melanocytes would favour pheomelanin synthesis under an increase in cysteine availability. Here we investigate this possibility by experimentally increasing the dietary uptake of cysteine to developing Eurasian nuthatches *Sitta europaea*, a passerine bird that deposits large amounts of pheomelanin in flank feathers (Galván, 2017). Specifically, we tested if melanocytes from growing pheomelanin-pigmented feathers show epigenetic lability and respond to the increase in cysteine availability by favouring pheomelanin synthesis.

To test our hypothesis, we quantified the expression of genes coding for the mediators of cysteine metabolism (CDO, GCS and cystinosin), which are, respectively, cysteine dioxygenase type I (*CDO1*; McCann, Akbari, Williams, & Ramsden, 1994), glutamate-cysteine ligase catalytic subunit (*GCLC*; Meister, 1995) and (CTNS (Town et al., 1998). Additionally, we quantified the expression of the gene encoding the cystine/glutamate antiporter xCT (solute carrier family 7 member 11, *Slc7a11*), a protein localized in the plasma membrane (Conrad & Sato, 2012; He, Li, Zhou, Zhao, & Li, 2012) that is thus responsible for providing cells with cysteine (Chintala et al., 2005). We also quantified expression of the gene *Slc45a2* (solute carrier family 45 member 2), for which a similar function in transporting cysteine to cells has been suggested (Gunnarsson et al., 2007). Last, we quantified expression of the main genes that regulate pheomelanin synthesis by changing the intracellular concentration of cyclic adenosine monophosphate (cAMP), thereby influencing the intramelanosomal activity of tyrosinase, the key enzyme in the melanogenesis pathway. These are the genes coding for the melanocortin 1 receptor in the membrane of melanocytes (*MC1R*; Naysmith et al., 2004) and peptides that bind to it and act as their

antagonists: agouti-signalling (*ASIP*) and agouti-related (*AGRP*) proteins (Nadeau et al., 2008).

The genes described above and their influence on cysteine metabolism and pheomelanin synthesis are summarized in Figure 1. We investigated if the expression of these genes is sensitive to increased cysteine availability in a manner that favours pheomelanin synthesis in melanocytes. To date, the genes that regulate intramelanocytic cysteine transport to melanosomes (Figure 1) are unknown (García-Borrón et al., 2011), but investigation of the genes considered here should reflect a potential favouring of the genetic pathway to synthesis of pheomelanin in response to an increase in cysteine uptake. Any potential increase in pheomelanin synthesis by feather melanocytes should result in an increase in plumage colour intensity, which reflects the amount of pheomelanin deposited in feathers in our model species (Galván & Rodríguez-Martínez, 2018). We also investigated if these potential effects on gene expression are mediated by changes in RNA and DNA methylation. Recent developments in analytical methods have unveiled a key role of internal modifications in mRNA mediated by N^6-methyladenosine (m^6A) in the regulation of gene expression in eukaryotes (Dominissini et al., 2012; Fu, Dominissini, Rechavi, & He, 2014). In DNA, the best known epigenetic modification is that mediated by 5-methylcytosine (m^5C), which leads to transcriptional silencing (Schübeler, 2015). We therefore quantified m^6A in mRNA and m^5C in DNA at the target genes using antibody-mediated capture methods to investigate potential differential roles of these epigenetic markers in possible changes in gene expression after experimental cysteine supplementation. Last, we investigated the potential consequence of cysteine supplementation on cellular oxidative stress at a systemic level and on the physical condition of animals.

METHODS

Experimental design

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

Seventeen nuthatch nestlings from eight nests were used in the study (Supporting information Figure S1). The number of nestlings in nests ranged from one to five, the mean being 2.1. All nestlings in each nest were used. On day 6 after hatching, the nestlings were banded with numbered metal rings for identification and weighed to the nearest 0.1 g with a portable digital balance. On days 6, 7 and 8 after hatching, L-cysteine (Sigma-Aldrich) was orally administered to some nestlings at a dose of 0.1 g/L in a total volume of 100 µl of water using a syringe, while other nestlings that served as controls only received 100 µl of water. After 2 days without any treatment, the same administration of L-cysteine or water was repeated on days 11, 12 and 13 after hatching. A single dose was administered per nestling and day. The nestlings were assigned to these treatments (cysteine or control) following the order of body weights but changing the start of the sequence of treatments in each nest so that all positions in the sequence of weights received the same number of the different treatments. Eleven birds were supplemented with cysteine and six birds were controls.

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

Molecular sex determination

To determine the sex of nestling nuthatches, we extracted DNA from the blood with the ISOLATE II Genomic DNA kit (Bioline) and used real-time quantitative PCR (qPCR) combined with melting curve analysis (Chang et al., 2008). Reactions were performed with SYBR Green I Master in a LightCycler 480 System (Roche) with the primer pair CHD1F/CHD1R (5′-TATCGTCAGTTTCCTTTTCAGGT-3′ and 5′-CCTTTTATTGATCCATCAAGCCT-3′; Lee et al., 2010). The melting curve analyses differentiated males and females through a peak of melting temperature at 81°C in males and a peak at 78°C in females.

Cysteine levels in erythrocytes by gas chromatography

To investigate the effect of experimental treatment on systemic cysteine levels, we measured the levels of cysteine in erythrocytes following the method developed by Švagera, Hanzlíková, Šimek, and Hušek (2012) for plasma. To induce cell lysis and thus facilitate the extraction of intracellular cysteine, erythrocytes were first diluted to 1:10 with a carbonate-buffered saline (5 mM Na_2CO_3 in saline). Then, 10 µl of internal standard (4-chloro-DL-phenylalanine, PCP; Sigma-Aldrich) and 10 µl of reducing agent (dithiothreitol, DTT; Sigma-Aldrich) were

added to 40 µl of supernatant of the homogenate of cell pellet and buffer. Samples were then deproteinized by adding 40 µl of 0.6 M trichloroacetic acid (TCA). After centrifugation, the supernatant was aspirated and transferred into glass culture tubes (J. Jimeno). The supernatant was derivatized with an organic phase consisting of a mixture of isooctane, butyl acetate and ethyl chloroformate in a 10:6:1 volume ratio. Then, 130 µl of this reactive organic phase was added to the supernatant in the glass tubes after adding 40 µl of a mixture of pyridine and ethanol in a 1:3 volume ratio. After 10 min of incubation, the organic phase was aspirated and transferred to vials for chromatography.

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

Isolation of feather melanocytes

Flank feathers were cut at the rachis and the plumulaceous part was stored in the dark until analysis of pigmentation. We extracted melanocytes from the melanin unit of feathers, which corresponds to the bottommost portion of the feather follicles. Like hair follicles (Mohanty, Kumar, Dhawan, Sreenivas, & Gupta, 2011), the melanin unit of feathers represents an important reservoir of melanocytes. Melanocytes at the dermal papillae show intense melanogenesis during feather development (Lin et al., 2013), meaning that the melanin unit

of feathers represents the main source of integumentary melanins in birds. Fifteen follicular melanin units were pooled per bird. Nucleic acids obtained from these samples therefore correspond to melanocytes to a large extent.

Extraction of RNA and DNA from feather melanocytes

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

mRNA expression

After extracting total RNA, residual genomic DNA carry over was removed using the TURBO DNA-free kit (Ambion). Complementary DNA (cDNA) was prepared from total RNA using RevertAid Reverse Transcriptase provided in the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Thermo Fisher Scientific). qPCR was performed on cDNA for the target genes: *Slc7a11*, *Slc45a2*, *GCLC*, *CDO1*, *CTNS*, *MC1R*, *ASIP* and *AGRP*. Additionally, we quantified expression of the gene *NFE2L2* to obtain a measure of intrinsic antioxidant capacity (see below). Reactions were performed using SYBR Green I Master in a LightCycler 480 System. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used for normalization, as this is the most suitable endogenous reference gene (Silver, Best, Jiang, & Thein, 2006) and most commonly used in the analysis of gene expression in bird feathers (Nadeau et al., 2008; Walsh, Dale, McGraw, Pointer, & Mundy, 2012). Gene primers were designed based on refseq sequences (GenBank) using the Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

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

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

To quantify m^{6}A in RNA from feather melanocytes, we followed the immunoprecipitation method developed by Dominissini, Moshitch-Moshkovitz, Salmon-Divon, Amariglio, and Rechavi (2013), with some modifications. After DNAse digestion (see above), 15 µl from each total RNA sample was separated and stored at −80°C until later use as an input RNA control of the immunoprecipitation procedure. The remaining RNA was split in two 1.7-ml tubes. One of these tubes was subject to the complete immunoprecipitation procedure, while the other tube was used as a no-antibody control.

The samples were heat-denatured (65°C, 10 min) and immediately placed on ice. Then, 200 U of RNasin Plus RNase Inhibitor (Promega Corporation), 2 mM ribonucleoside vanadyl complexes (RVC; Sigma-Aldrich), 2 mg of m^{6}A-antibody (Synaptic Systems) and the remaining volume up to 1 ml of IP buffer (10 mM Tris-HCl, 150 mM NaCl and 0.1% [v/v] Igepal CA-630 [Sigma-Aldrich]) were added to each sample. In the no-antibody control tube, the volume of antibody was replaced by the same volume of water. The mixtures were incubated on a rotating platform at 4°C for 2 hr. The 200 µl of beads with immobilized recombinant Protein A (IPA-300; Repligen) was blocked with a 0.5 mg/ml bovine serum albumin solution (Sigma-Aldrich) in immunoprecipitation buffer (IP buffer) supplemented with RNAsin (5%, v/v) and RVC (5% v/v) for 2 hr on a rotating wheel. After two washes

with IP buffer, the previously incubated samples were added and keep on a rotating wheel at 4°C for 2 hr. Then, the supernatant was removed, followed by four washing steps with IP buffer. After the final wash, 100 µl of elution buffer (10 mM Tris-HCl, 150 mM NaCl, 0.1% [v/v] Igepal CA-630, RNAsin [5% v/v], RVC [5% v/v] and 6.7 mM m^6A 5'-monophosphate sodium salt [Sigma-Aldrich]) was added to the sedimented beads. The mixture was incubated at 4°C for 1 hr with continuous shaking. The eluted RNA was recovered by precipitation, converted to cDNA and analysed by real-time qPCR as described in the previous section.

The proportion of RNA with m^6A at the target genes was calculated by dividing C_t of the input RNA control by C_t of the immunoprecipitated test sample. As this is a proportion, no additional controls are necessary, although we included the ratio (C_t control/C_t test sample) for the gene *GAPDH* as a covariate in the linear mixed-effects models used for analysing the data (see Statistical analyses) to account for a possible covariation with *GAPDH* RNA methylation. However, the results were not affected by the inclusion or exclusion of the *GAPDH* ratio in the analyses (see Results), confirming that it was not necessary to use houskeeping genes as controls in the analysis of the methylated fraction of genes.

Quantification of m^5C in DNA by immunoprecipitation and real-time qPCR

2 µg of genomic DNA diluted in 130 µl of water was fragmented in a Covaris E220 Focused-ultrasonicator specifying a fragment size range of 300–1,000 bp. Sonicated DNA was split into three tubes (test and controls—input DNA and no-antibody). The samples (test and no-antibody control) were heat-denatured (95°C, 10 min) and immediately placed on ice for 5 min. Then, 1 µg of m^5C monoclonal antibody 33D3 (Diagenode) was added to each sample. The corresponding volume of water was added to the no-antibody control. IP buffer (10 mM $NaPO_4$,

pH 7.0; 140 mM NaCl and 0.05% Triton X-100) was then added to each sample to a final volume of 500 µl. The mixture was incubated on a rotating platform at 4°C for 2 hr. Pre-blocked Protein A/G beads (Diagenode) were then added and incubated on a rotating wheel at 4°C for 2 hr. The supernatant was removed, followed by four washing steps with IP buffer. After the final wash, the beads were resuspended in 400 µl of digestion buffer (10 mM Tris, pH 8.0; 100 mM EDTA [ethylenediaminetetraacetic acid], 0.5% SDS [sodium dodecyl sulfate] and 50 mM NaCl) and 100 µg of proteinase K was added. The digestion mixture was incubated overnight at 50°C. DNA was purified with a DNA Clean & Concentrator utility (Zymo Research) and analysed by real-time qPCR. Gene primers were designed based on refseq sequences (GenBank) using the Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The proportion of DNA with m^5C at the target genes was calculated by dividing C_t of the input DNA control by C_t of the immunoprecipitated test sample.

Systemic oxidative stress and body condition

To obtain a measure of oxidative stress at a general, systemic level, reduced (GSH) and oxidized (GSSG) glutathione were quantified in the blood of nestling nuthatches. Total glutathione levels in erythrocytes were determined by following the method described by Tietze (1969) and Griffith (1980) with some modifications (see, e.g., Galván & Alonso-Alvarez, 2009 for details of this technique applied to bird blood samples). To determine GSSG levels, an aliquot (200 µl) of the supernatant obtained for the assessment of total glutathione was adjusted to a pH of 7.5 by adding 6 M NaOH. Afterward, 4 µl of 2-vinylpyridine was added to the aliquot, and the mixture was vigorously shaken at ambient temperature in the dark to promote the chemical masking of GSH. The mixture was then centrifuged (3,500 *g* for 15 min), and the change in absorbance of the supernatant was assessed at 405

nm using a COBAS Integra 400 plus analyser (Roche). The GSH:GSSG ratio was used as an index of systemic oxidative stress. Oxidative stress increases as the GSH:GSSG ratio decreases. The gene *NFE2L2* encodes the transcription factor NRF2, the master regulator of the cellular antioxidant response (Huppke et al., 2017). Thus, the effect of the experimental treatment on the GSH:GSSG ratio was investigated controlling for the intrinsic antioxidant capacity of birds, determined by including ΔC_t for *NFE2L2* as a covariate in the linear mixed-effects models (see Statistical analyses).

On the other hand, we investigated the effect of the experimental treatment on the physical, body condition of nuthatch nestlings, a predictor of survival prospects in the species (Matthysen, 1989). For this, we used body mass corrected by (i.e., independent of) body size, as this measure is a good indicator of subcutaneous fat content in nuthatches and other birds (Galván, 2017). This was analysed by linear mixed-effects models with body mass as a response variable and tarsus length as a covariate (see Statistical analyses).

Pheomelanin synthesis and pigmentation

To determine the relative amount of pheomelanin produced and deposited in flank feathers by nuthatch nestlings, we quantified the intensity of the colour of feathers by UV–visual reflectance spectrophotometry (Galván & Rodríguez-Martínez, 2018). We used an Ocean Optics Jaz spectrophotometer (range: 220–1,000 nm) with UV (deuterium) and visible (tungsten–halogen) lamps and a bifurcated 400 µm fibre optic probe (Ocean Optics). The fibre optic probe both provided illumination and received light reflected from the sample, with a reading area of ~1 mm^2. Fifteen flank feathers were mounted on a light-absorbing foil sheet (Metal Velvet coating, Edmund Optics) to avoid any background reflectance, such that they resembled the natural appearance of the plumage patch. Measurements were taken at a 90°

angle to the sample. All measurements were relative to a diffuse reflectance standard tablet (WS-1, Ocean Optics), and reference measurements were frequently made. An average spectrum of five readings on different points of the orange, pheomelanin-pigmented portion of feathers was obtained for each bird, removing the probe after each measurement. Reflectance curves were determined by calculating the median of percentage reflectance in 10-nm intervals.

Using Raman spectroscopy (Galván et al., 2013), we have previously shown that, in Eurasian nuthatch flank feathers, the slope of lines fitted to reflectance spectra (i.e., the slope of percentage reflectance regressed against wavelength) across the range 300–700 nm is a good predictor of the pheomelanin content, with lower slopes denoting higher colour intensity and higher pheomelanin contents (Galván & Rodríguez-Martínez, 2018). We therefore summarized reflectance spectral data as a measure of reflectance slope. Although feathers usually contain eumelanin, the dark nonsulphurated melanin form, no detectable amounts of eumelanin have been found in the flank feathers of nuthatches (Galván & Rodríguez-Martínez, 2018). This means that eumelanin is absent or present at very low, undetectable levels, so variation in the colour expression of flank feathers mainly reflects variation in their relative pheomelanin content.

Statistical analyses

We used linear mixed-effects models (LMMs) fit with restricted maximum likelihood (REML) estimation, including experimental treatment (cysteine vs. control) as a fixed factor and nest identity as a random factor to account for the common origin of nuthatch nestlings belonging to the same nests. In the analysis of pheomelanin content of feathers (reflectance slope), sex was included as an additional fixed factor in the models because male Eurasian nuthatches exhibit darker feathers than females (Galván, 2017). In the analysis of systemic

oxidative stress (GSH:GSSG ratio), ΔCt for NFE2L2 was included as an additional covariate in the models to account for the intrinsic antioxidant capacity of birds. Table 1 summarizes the predictor effects that were considered in the model of each response variable. All variables were log10-transformed to fulfil the normality assumption of parametric tests.

Linear mixed models analyses were made in R environment (R Core Team, 2018) using the package *lme4* (Bates, Maechler, Bolker, & Walker, 2015). Probability values (*p*) were calculated through the analysis of deviance of LMMs on the basis of Wald χ^2 tests using the package *car* (Fox & Weisberg, 2011).

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

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

RESULTS

Effect on cysteine levels in erythrocytes

The concentration of cysteine in the erythrocytes of birds supplemented with dietary cysteine (mean ± *SE*: 442.62 ± 28.11 µmol/g) was significantly higher than that of control birds (373.15 ± 1.98 µmol/g; χ^2_1= 23.89, $p < 0.0001$; Figure 2). This indicates that the experimental cysteine supplementation during development increased cysteine levels in birds at a systemic level.

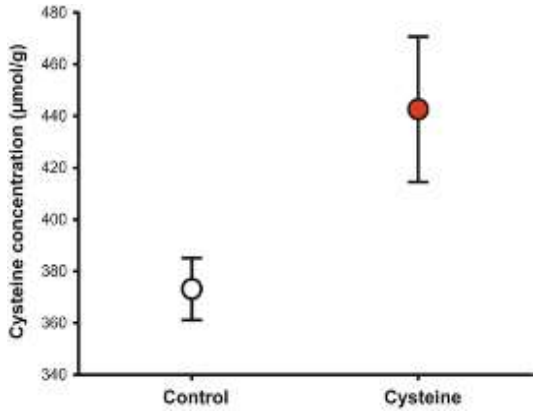

Figure 2. Mean ± *SE* systemic (erythrocyte) concentration of cysteine in developing Eurasian nuthatches experimentally supplemented with dietary cysteine (red circle) and in controls (open circle). Cysteine levels were higher in birds supplemented with cysteine than in controls (χ^2_1 = 23.89, $p < 0.0001$).

Effects on gene expression and RNA and DNA methylation in melanocytes

Cysteine supplementation induced a downregulation of four genes in feather melanocytes: *Slc7a11* (χ^2_1 = 9.92, $p = 0.002$), *CTNS* (χ^2_1 = 36.26, $p < 0.0001$), *MC1R* (χ^2_1 = 6.06, $p = 0.014$) and *ASIP* (χ^2_1 = 6.75, $p = 0.009$). *GCLC* was also downregulated, although the difference of mean

expression level with controls was marginally nonsignificant (χ^2_1= 3.78, p = 0.051; Figure 3). In contrast, there was no significant differences in gene expression level between cysteine-supplemented and control birds for *Slc45a2* (χ^2_1 = 1.73, p = 0.188), *CDO1* (χ^2_1 = 0.72, p = 0.394) and *AGRP* (χ^2_1 = 0.53, p = 0.464; Figure 3). According to the regulatory functions of the genes considered here (Figure 1), these results suggest a change in physiological conditions favouring pheomelanin synthesis.

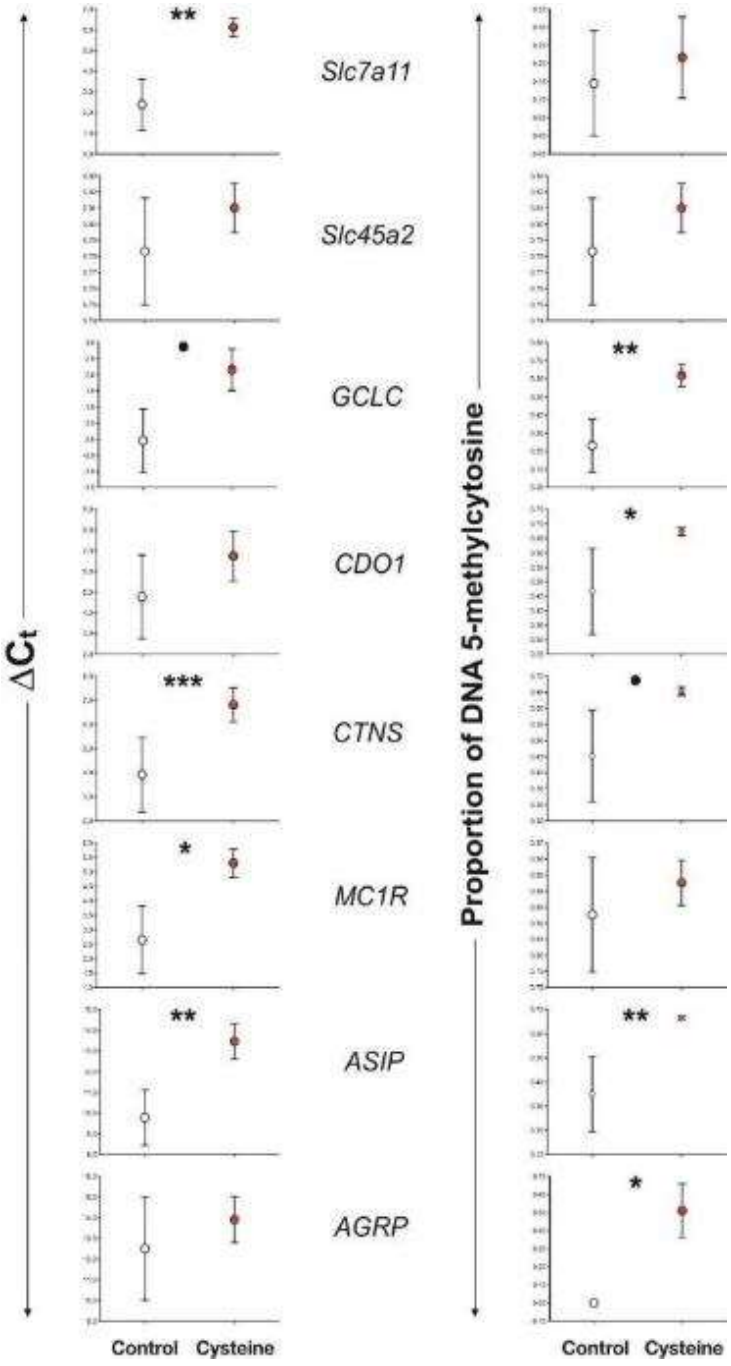

Figure 3. Normalized gene expression levels (ΔC_t, left column) and proportion of DNA with 5-methylcytosine bases (right column) in eight genes regulating cysteine transport and metabolism and pheomelanin synthesis in melanocytes. Values are mean ± *SE* obtained in melanocytes of growing flank feathers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

No difference in the proportion of RNA with m^6A nucleosides was found between cysteine-supplemented and control birds for any gene (*Slc45a2*: χ^2_1 = 0.05, *p* = 0.818, *GCLC*: χ^2_1 = 1.54, *p* = 0.215, *CTNS*: χ^2_1 = 0.04, *p* = 0.834, *MC1R*: χ^2_1 = 0.05, *p* = 0.825, *AGRP*: χ^2_1 = 0.15, *p* = 0.701), with the exception of *Slc7a11* and *CDO1*. In the case of *Slc7a11*, the proportion of RNA with m^6A was higher in controls (χ^2_1 = 6.84, *p* = 0.009) because no m^6A was detected in any cysteine-supplemented bird (Figure 3). In the case of *CDO1*, the proportion of RNA with m^6A was higher in cysteine-supplemented birds (χ^2_1 = 8.08, *p* = 0.004). No RNA m^6A was found in the gene *ASIP* among the samples. These results did not change when the proportion of m^6A in RNA for *GAPDH* was excluded from the analyses.

An increase in the proportion of DNA with m^5C bases was observed in the same genes that were downregulated after cysteine supplementation, with the exception of *Slc7a11* (χ^2_1 = 0.42, *p* = 0.518) and *MC1R* (χ^2_1 = 0.42, *p* = 0.514): *GCLC* (χ^2_1 = 8.28, *p* = 0.004), *CTNS* (χ^2_1 = 3.59, *p* = 0.058) and *ASIP* (χ^2_1 = 8.14, *p* = 0.004) (Figure 3). Additionally, an increase in the proportion of DNA with m^5C was detected in *CDO1* (χ^2_1 = 3.92, *p* = 0.048) and *AGRP* (χ^2_1 = 6.33, *p* = 0.012). No differences were found in *Slc45a2* (χ^2_1 = 0.76, *p* = 0.383) (Figure 3).

Effects on oxidative stress in erythrocytes and body condition

The GSH:GSSG ratio in erythrocytes did not differ between cysteine-supplemented (mean ± *SE*: *2*9.84 ± 9.08) and control birds (24.01 ± 3.03; $\chi^2_1 = 10^{-3}$, *p* = 0.975). No effect of experimental treatment was observed in the body condition of birds (χ^2_1 = 0.29, *p* = 0.587). This indicates that the dietary supplementation of cysteine did not induce systemic oxidative stress nor a negative effect on the physical condition of birds.

Effect on pheomelanin synthesis and pigmentation

The reflectance slope of the flank feathers of birds supplemented with cysteine (mean ± *SE*: *0*.017 ± 0.002) was significantly lower than that of controls (0.027 ± 0.005) (χ^2_1 = 4.29, p = 0.038; Figure 4a). As reflectance slope decreases as the concentration of pheomelanin in feathers increases, this indicates that cysteine-supplemented birds produced greater amounts of pheomelanin that was deposited in feathers. This effect was reflected in a perceptible difference in the colour of feathers, which was more intense in cysteine-supplemented birds than in controls (Figure 4b).

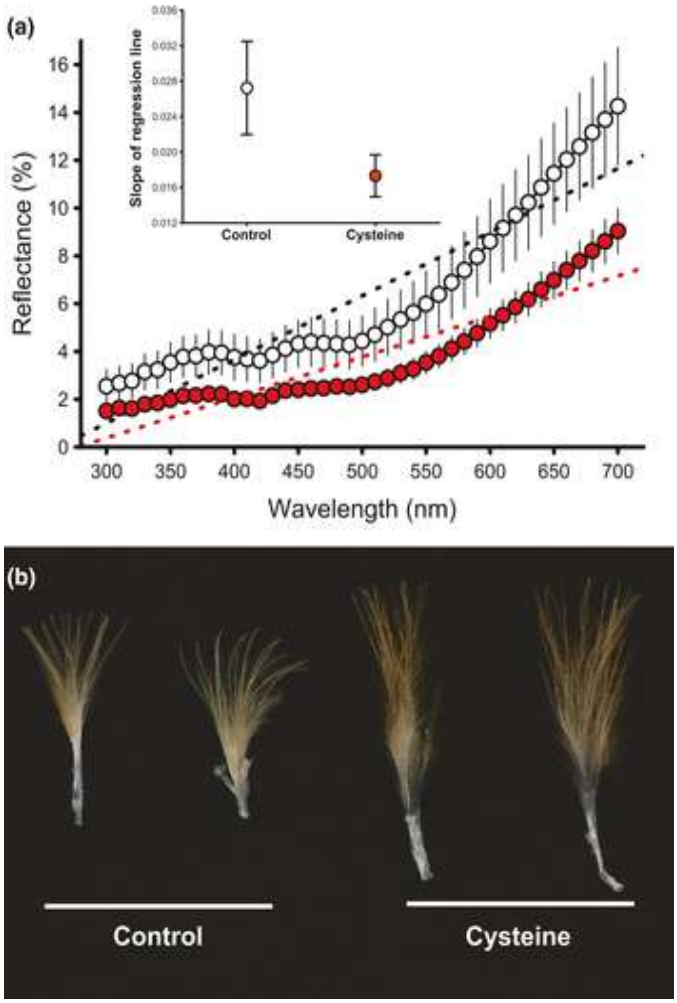

Figure 4. (a) Average UV-visible reflectance spectra of flank feathers from developing Eurasian nuthatches experimentally supplemented with dietary cysteine (red circles) and from controls (open circles). Values are median ± *SE* percentage reflectance in 10-nm intervals. Dashed lines are the result of fitting regression lines to the reflectance spectra. The slope of these lines decreases as the concentration of pheomelanin in the flank feathers increases. The mean ± *SE* reflectance slope values for cysteine-supplemented birds and controls are shown in the inset. Slope values in cysteine-supplemented birds are lower than in the controls (χ^2_1 = 4.29, p = 0.038). (b) Image of flank feathers from a female nuthatch supplemented with cysteine and from a female control nuthatch. Note the more intense orange colour in the feathers of the cysteine-supplemented bird as compared to the control, indicative of a lower reflectance slope and a higher pheomelanin content.

DISCUSSION

This study indicates that a dietary supplementation of cysteine leads to an increase in pheomelanin production that is mediated by methylation

changes in some genes involved in cysteine metabolism and pheomelanin synthesis in melanocytes. Our experiment produced a significant increase in systemic cysteine levels in developing birds despite the increment in pheomelanin production, suggesting that excess cysteine occurred at the organismal level. Excess cysteine causes cellular oxidative stress that leads to glutathione depletion (Viña et al., 1983). Consequently, excess cysteine is cytotoxic and neurotoxic and has been shown to exert detrimental effects in mammals and birds (Dilger & Baker, 2008; Janaky et al., 2000; Roman et al., 2013). In humans, oxidative stress mediated by elevated systemic cysteine levels has even been proposed as a causative factor of cancer (Lin et al., 2010). Our study shows, however, that nestling nuthatches with experimentally induced excess cysteine levels did not exhibit lower reduced-to-oxidized glutathione ratios (GSH:GSSG) nor poorer body condition than controls despite a downregulation of the gene that controls glutathione synthesis in melanocytes (*GCLC*), suggesting that the increase in pheomelanin production represents an advantageous epigenetic mechanism that protects against oxidative stress. Given the molecular similarity between the melanogenesis pathway in melanocytes of birds and mammals (d'Ischia et al., 2015), this epigenetic mechanism is also of relevance to humans. The oxidation of GSH to GSSG occurs immediately after the exposure to the source of oxidative stress (Sastre et al., 1992), and thus it is not likely that the lack of decrease in GSH:GSSG ratio found here was due to an early measurement of this parameter in birds.

The favouring of the genetic pathway to synthesis of pheomelanin induced by cysteine supplementation can be inferred from the regulatory roles of the genes considered here (Figure 1). *CDO1* and *GCLC* code, respectively, for the enzymes CDO and GCS, which compete for cysteine as a substrate (Stipanuk et al., 2002, 2009), and it should thus be expected that a downregulation of these genes favoured pheomelanin synthesis because greater amounts of cysteine would be available to be transported to melanosomes (Figure 1). In fact, we found

that *GCLC* was downregulated in feather melanocytes of cysteine-supplemented birds as compared to controls. According to the well-known repressing effect of DNA methylation on transcription (Schübeler, 2015), we also found that feather melanocytes of cysteine-supplemented birds increased the proportion of DNA m^5C in *GCLC*. An increase in DNA m^5C was also found in *CDO1*, but this was not reflected in a downregulation of the gene. Interestingly, *CDO1* also exhibited an increase in RNA m^6A in cysteine-supplemented birds. Recent studies have linked high DNA methylation levels in *CDO1* with several tumour types in humans (Deckers et al., 2015; Kojima et al., 2018), although also showing a decrease in gene expression (Meller et al., 2016). To our knowledge, this is the first time that an increase in RNA methylation has been observed in *CDO1* as a response to a physiologically damaging effect, and future studies should investigate if this is associated with the lack of downregulation of this gene despite increased DNA m^5C (see also below).

The gene *Slc7a11* codes for the cell membrane protein xCT, which transports cysteine (in the form of cystine) from the extracellular medium to the cytosol, its expression in melanocytes thus resulting in an increase in pheomelanin synthesis (Chintala et al., 2005). Transgenic sheep overexpressing xCT develop patches of hair pigmented by pheomelanin (He et al., 2012), and a tendency for pheomelanin-based colour intensity to increase with *Slc7a11* mRNA expression in feather melanocytes has also been shown in some birds (Galván, Moraleda, Otero, Álvarez, & Inácio, 2017). In the present study, however, the increase in pheomelanin synthesis in cysteine-supplemented birds was associated with a downregulation of *Slc7a11* in feather melanocytes. However, it must be borne in mind that cysteine in the cytosol of melanocytes can equally enter the cysteine metabolism pathway or the melanogenesis pathway in melanosomes (Figure 1), while the limiting source for pheomelanin synthesis is the concentration of cysteine inside melanosomes (García-Borrón et al., 2011). In this regard, *CTNS*, which codes for a protein that exports cystine out of lysosomes and expression

of which in melanocytes thus inhibits pheomelanin synthesis (Chiaverini et al., 2012), was also downregulated in feather melanocytes of cysteine-supplemented birds. In fact, other birds exposed to an environmental source of oxidative stress (diquat dibromide), which is expected to induce a reduction of pheomelanin synthesis that avoids a decrease of glutathione and antioxidant capacity, downregulated *Slc7a11* but not *CTNS* in feather melanocytes (Galván, Inácio, Romero-Haro, & Alonso-Alvarez, 2017). It therefore seems reasonable that a physiologically advantageous mechanism favouring pheomelanin synthesis under excessive cysteine levels in cells includes downregulation of *Slc7a11*, which limits uptake and further accumulation of cysteine in cells, and downregulation of *CTNS*, which maximizes the accumulation of the already high intramelanocytic levels of cysteine in melanosomes. In contrast, expression and methylation levels in *Slc45a2* were not affected by cysteine supplementation, arguing against a role of this gene in cysteine transport to melanocytes as findings in other birds suggest (Galván, Moraleda et al., 2017).

CTNS downregulation was associated with an increase in DNA m^5C, but the same was not found in *Slc7a11*. Instead, *Slc7a11* downregulation was accompanied by a depletion of RNA m^6A. It is accepted that DNA methylation generally leads to a decrease of gene expression (Schübeler, 2015), but the effect of RNA methylation on gene expression has only recently begun to be explored. Some authors have reported decreases of gene expression with increases in mRNA m^6A (Wang et al., 2014), but more recent studies show that increases in mRNA m^6A or m^5C lead to increases in the expression of several genes (Min et al., 2018; Wang, Tang, Wang, Wang, & Feng, 2017). This may therefore be in accordance with the depletion of RNA m^6A and expression downregulation of *Slc7a11* in feather melanocytes of cysteine-supplemented birds. This may also explain why *CDO1* was not downregulated despite increased DNA m^5C in cysteine-supplemented birds, as these animals also showed increased RNA m^6A in *CDO1*. Interestingly, these results may therefore suggest that the genes

regulating cysteine metabolism and transport are differentially affected by RNA and DNA methylation under exposure to excess cysteine.

Last, *MC1R*, *ASIP* and *AGRP* are the main genes affecting pheomelanin synthesis by regulating the activity of tyrosinase. *MC1R* was downregulated in feather melanocytes of cysteine-supplemented birds, thus favouring synthesis of pheomelanin because this gene codes for the expression of the melanocortin 1 receptor in the membrane of melanocytes, to which melanocortins bind and stimulate the synthesis of eumelanin as opposed to that of pheomelanin (Naysmith et al., 2004). *MC1R* downregulation was not accompanied, however, by a change in RNA or DNA methylation. *MC1R* methylation has never been reported to affect melanin synthesis, as polymorphic variation in this gene is considered the base of its influence on melanin-based pigmentation (Lin & Fisher, 2007). However, our results suggest that cysteine-induced *MC1R* downregulation may be the result of covariation with the expression of the receptor antagonist, as in other receptor–ligand systems (Wang, Barone, Aiyar, & Feuerstein, 1997). Indeed, *ASIP* was downregulated in cysteine-supplemented birds, which was associated with an increase in DNA m^5C, consistent with findings in mice (Dolinoy, Weidman, Waterland, & Jirtle, 2006). A low expression of *ASIP* inhibits pheomelanin synthesis and pigmentation (Nadeau et al., 2008), but in our study *ASIP* downregulation and methylation may be indirectly inducing *MC1R* downregulation and, thus, promoting pheomelanin synthesis.

In conclusion, our results show that an experimental increase in cysteine uptake induces a downregulation of genes involved in cysteine metabolism and pheomelanin synthesis in feather melanocytes that results in the favouring of pheomelanin production and avoids the expected systemic oxidative stress caused by excess cysteine levels. The downregulation of gene expression is mediated by changes in methylation of RNA or DNA, which differentially controls the expression of distinct genes. This epigenetic mechanism therefore seems

physiologically advantageous. However, more precise description of this mechanism will require future experiments in which cysteine supplementation is provided in increasing doses, thus allowing exact determination of the conditions that activate the pheomelanogenesis pathway and its physiological limitations. In particular, although our experiment suggests an induction of excess cysteine at the systemic level, future studies should try to block the epigenetic mechanism observed here to produce cysteine-mediated toxicity and thus firmly demonstrate the potential adaptiveness of this mechanism.

However, the mechanism is not expected to be functional in all animals, as other species of birds such as the house sparrow *Passer domesticus* show a decrease in systemic antioxidant capacity despite an increase in pheomelanin production after an experimental induction of excess dietary cysteine (Galván & Alonso-Alvarez, 2017). This may be due to the fact that the average concentration of the benzothiazole moiety of pheomelanin in the studied trait in house sparrows (65.7 ng per mg feather; Galván, Wakamatsu, & Alonso-Alvarez, 2014) is more than 1,000 times lower than that of the benzothiazole moiety of pheomelanin in the flank feathers of Eurasian nuthatches studied here (104.1 µg per mg feather; Galván & Wakamatsu, 2016). Similarly, the expression of *CTNS* in feather melanocytes tends to increase instead of decrease with protein food abundance in a strict carnivorous bird with a limited production of pheomelanin such as the gyrfalcon *Falco rusticolus* (Galván, Inácio, & Nielsen, 2017). Thus, the epigenetic mechanism that protects from oxidative stress by favouring pheomelanin synthesis may be functional only in species that already have a genetic basis leading to the production of large amounts of pheomelanin, which is reflected in pigmentation phenotypes consisting of light brown or orange colours (Galván & Wakamatsu, 2016). In this regard, it will be interesting to investigate if this mechanism is present in humans with the red hair/fair skin phenotype associated with the production of large amounts of pheomelanin for hair pigmentation (Ito et al., 2011), which may actually explain the evolution of this human

phenotype despite the constraints imposed by pheomelanin synthesis (Mitra et al., 2012; Napolitano et al., 2014; Panzella et al., 2014; Wang et al., 2016). Last, it is interesting to note that Eurasian nuthatch males with more intense pheomelanin-based pigmented flank feathers mate later in the season than males producing lower amounts of pheomelanin (Galván & Rodríguez-Martínez, 2018), indicating that a negative consequence of the epigenetic mechanism may be a cost in terms of sexual selection. This is because nuthatches avoided oxidative stress by developing more intensely pigmented feathers (Figure 4). Given recent knowledge of the influence of sexual selection on gene expression and genome evolution (Harrison et al., 2015), future studies should investigate how the mechanism shown here affects the evolution of traits.

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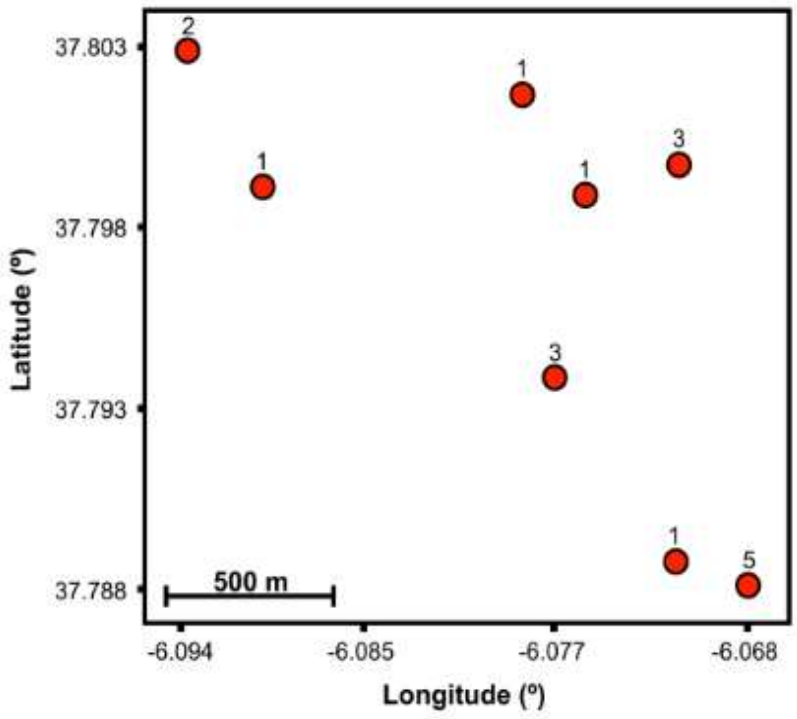
AUTHOR CONTRIBUTIONS

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

SUPPLEMENTARY MATERIAL

Figure S1. Spatial distribution of Eurasian nuthatch nests used in the study. Numbers above nests indicate the number of nestlings.



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CHAPTER II

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

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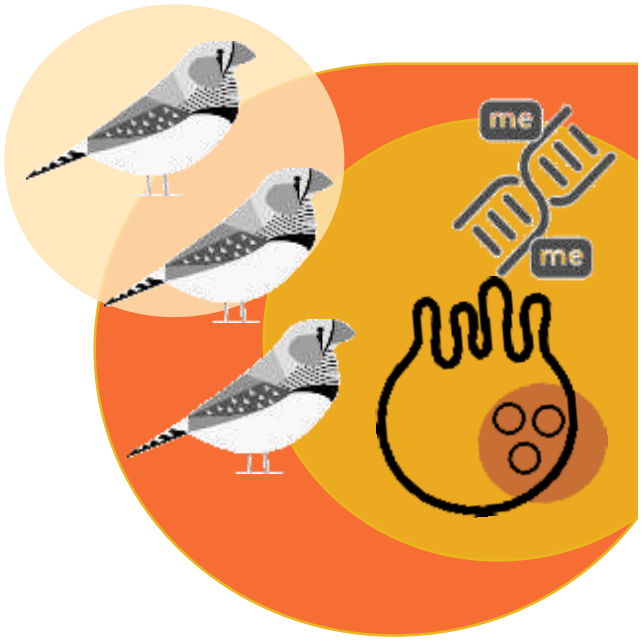

ABSTRACT

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 ºC. 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 Cientificas (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 samples. Total RNA was extracted using TRI Reagent (Ambion, Thermo Fisher Scientific, Waltham, MA), and residual genomic DNA carry over was removed with TURBO DNA-free kit (Ambion). Complementary DNA (cDNA) was prepared from total RNA 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 cysteine transport through melanocytes (*Slc7a11* and *Slc45a2*), a gene coding for the mediators of cysteine metabolism (*CTNS*) and genes that regulate pheomelanin synthesis by changing the intracellular concentration of cyclic adenosine monophosphate (cAMP) (*MC1R*, *ASIP* and *AGRP*). *Slc7a11* (solute carrier family 7 member 11) encodes the cysteine/glutamate antiporter xCT, a protein localized in the plasma membrane (Conrad & Sato, 2012; He, Li, Zhou, Zhao & Li, 2012) that is responsible of cysteine transport into melanocytes (Chintala et al., 2005). *Slc45a2* (solute carrier family 45 member 2) was also quantified,

as it has been suggested to play a similar function in transporting cysteine to cells (Gunnarsson et al., 2007). *CTNS* gene codes for a protein (cystinosin) responsible for the transport of cysteine out of lysosomes (Town et al., 1998). Lastly, genes that regulate intracellular concentration of cAMP and thus influence the activity of tyrosinase, the key enzyme in the melanogenesis pathway, were quantified. 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). Also the gene *NFE2L2* was 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, 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. Normalization was made by substracting C_t values for *GAPDH* from C_t values for the target genes (ΔC_t).

DNA methylation data

DNA was extracted using a Quick-DNA Plus kit (Zymo Research, Irvine, CA) from follicular melanin units. We quantified 5-methylcytosine (m^5C) at the target genes by immunoprecipitation and real-time qPCR following a procedure previously used with the same type of samples (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

Oxidative stress was quantified in blood by measuring reduced (GSH) and oxidized (GSSG) glutathione levels. As glutathione is constantly experiencing redox process in cells (Lu, 2009), the ratio GSH:GSSG reflects the proportion of glutathione that is oxidized which is an indicator of the level of oxidative stress. In contrast, GSH indicates glutathione level available for antioxidant protection. Total glutathione level in erythrocytes was determined by following the methods described by Tietze (1969) and Griffith (1980) with some modifications (see e.g., Galván & Alonso-Alvarez, 2009 for details of this technique applied to bird blood samples). GSSG levels were determined from an

aliquot (200 µl) of the supernatant obtained for the assessment of total glutathione adjusted to a pH of 7.5 by adding 6 N NaOH. Subsequently, 2-vinylpyridine (4 µl) 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 µl 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-Martinez, 2018) using an Ocean Optics Jaz spectrophometer (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 mm^2. 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-Martinez, 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 & Ornosa, 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 (χ_1^2 = 0.277, P = 0.598), nor by the manipulation of the antioxidant capacity with BSO (χ_1^2 = 0.083, P = 0.773). A similar absence of effects was found in MDA levels (social environment: χ_1^2 = 0.972, P = 0.324; antioxidant capacity: χ_1^2 = 0.082, P = 0.773) and in the body condition of birds (social environment: χ_1^2 = 0.046, P = 0.828; antioxidant capacity: χ_1^2 = 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).

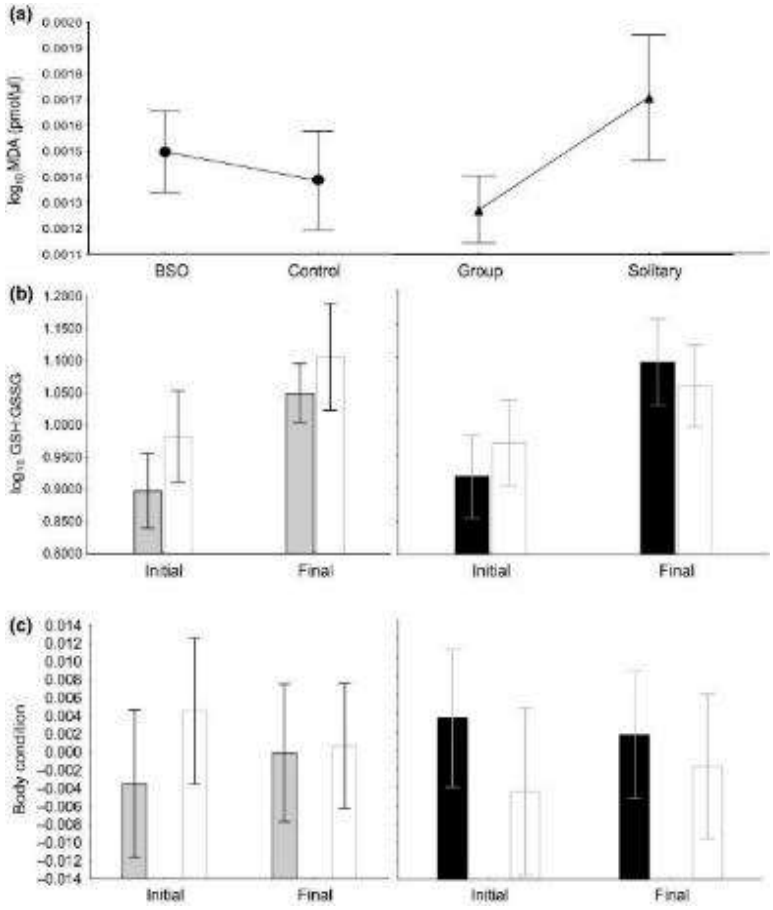

Figure 1. **(a)** Oxidative damage (malondialdeyde, MDA, levels from lipid peroxidation) of male zebra finches in relation to antioxidant capacity (BSO vs. controls; circles) and social environment (birds housed in groups vs. solitary birds; triangles) after experimental treatments. No differences were 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 ± SE.

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 (χ^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*: χ^2_1 = 0.721, P = 0.396; *CTNS*: χ^2_1 = 2.039, P = 0.15; *MC1R*: χ^2_1 = 3.252, P = 0.071; *ASIP*: χ^2_1 = 1.348, P = 0.245; *AGRP*: χ^2_1 = 3.611, P = 0.060). BSO treatment induced an upregulation of *AGRP* (χ^2_1 = 6.324, P = 0.01) and *ASIP* (χ^2_1 = 14.365, P = 0.0001), but did not lead to significant changes in the expression of the other genes (*Slc7a11*: χ^2_1 = 1.687, P = 0.193; *Slc45a2*: χ^2_1 = 0.688, P = 0.406; *CTNS*: χ^2_1 = 3.473, P = 0.062; *MC1R*: χ^2_1 = 2.427, P = 0.119). The interaction between exposure to competitive environment and antioxidant capacity was significant in the model for *ASIP* (χ^2_1 = 4.57, P = 0.032).

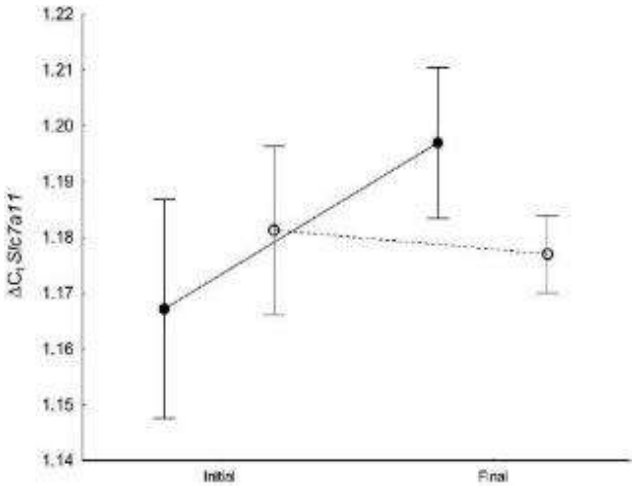

Figure 2. Normalized gene expression levels (ΔC_t) of *Slc7a11* (mean ± SE) in melanocytes of growing flank feathers of male zebra finches exposed to social stress (housed in groups; black circles) and solitary birds (open circles). Values before (initial) and after (final) experimental treatments are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

The proportion of DNA with m^5C was higher in solitary birds than in birds kept in groups in *Slc7a11* (χ^2 = 5.86, P = 0.015), *Slc45a2* (χ^2_1 =

21.28, P < 0.001), *MC1R* (χ_1^2 = 15.07, P < 0.001) and *AGRP* (χ_1^2 = 4.13, P = 0.042), while no differences were found in *CTNS* (χ_1^2 = 2.13, P = 0.144) nor *ASIP* (χ_1^2 = 0.91, P = 0.33). BSO treatment had no effect on the proportion of DNA m^5C in any gene (*Slc7a11*: χ_1^2 = 0.20, P = 0.652; *Slc45a2*: χ_1^2 = 2.81, P = 0.093; *MC1R* χ_1^2 = 0.26, P = 0.605; *AGRP*: χ_1^2 = 2.45, P = 0.117; *CTNS*: χ_1^2 = 0.035, P = 0.851; *ASIP*: χ_1^2 = 0.004, P = 0.947) (Figure 3).

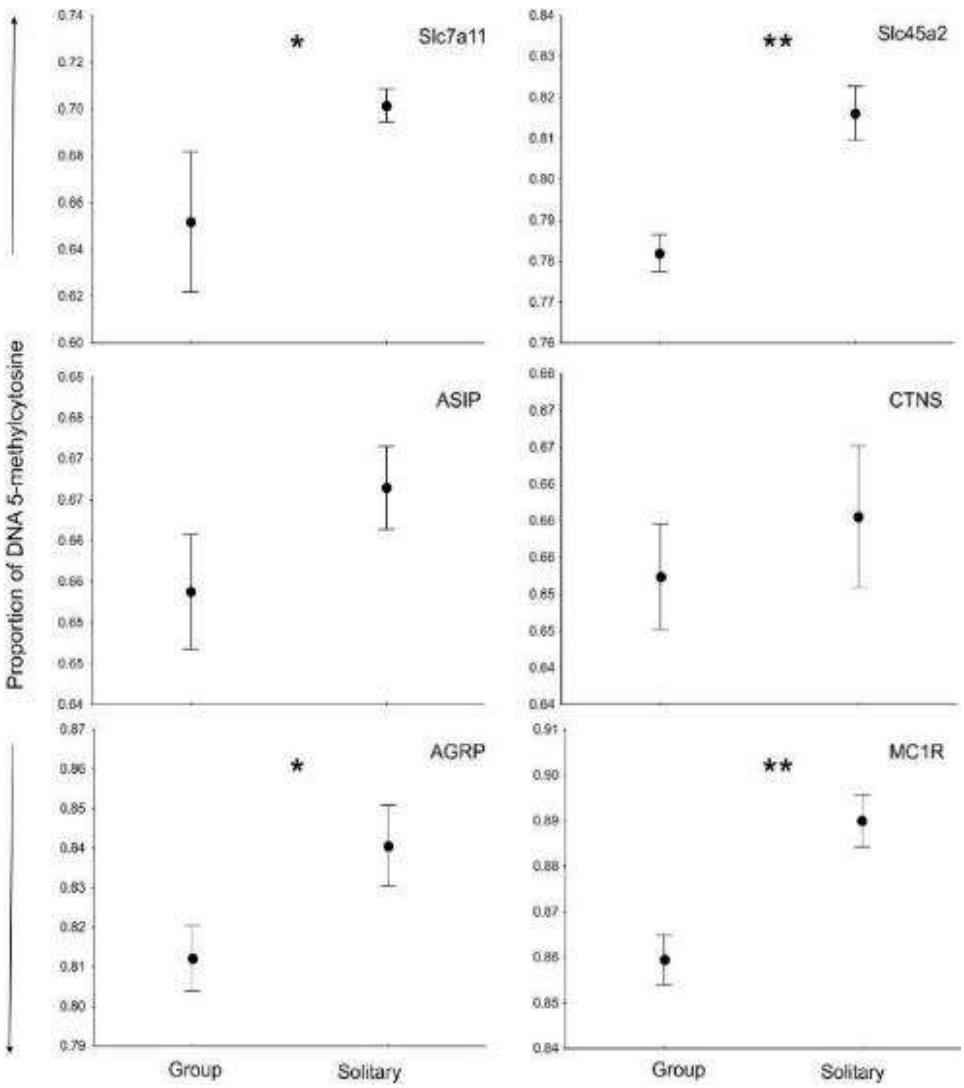

Figure 3. Proportion of DNA with 5-methylcytosine (mean ± SE) in six genes regulating pheomelanin synthesis in melanocytes of growing flank feathers of male zebra finches after exposure to social stress

conditions (housed in groups vs. solitary). Asterisks above graphs indicate statistically significant differences between grouped and solitary birds (*: $p < 0.001$, **: $p < 0.0001$).

Pheomelanin-based pigmentation in feathers

The exposure to the competitive social environment had no significant effect on the intensity of pheomelanin-based plumage coloration (χ^2_1 = 0.02, P = 0.870). However, the reflectance slope of the flank feathers of birds treated with BSO (mean ± SE: 0.023 ± 0.0011) was significantly higher (χ^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).

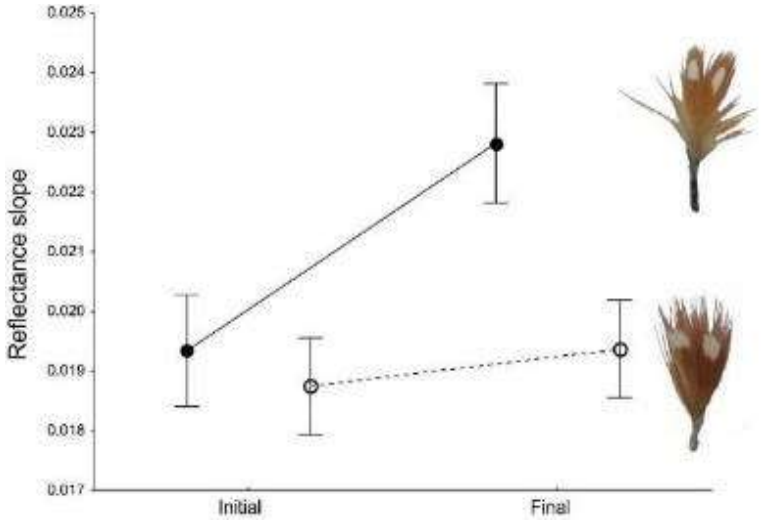

Figure 4. Reflectance slope (mean ± SE) of flank feathers of male zebra finches before (initial values) and after (final values) the experimental manipulation of their antioxidant capacity (BSO-treated: black circles, controls: open circles). Final slopes were higher in BSO-treated birds than in controls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

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 m^5C 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, m^5C 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.

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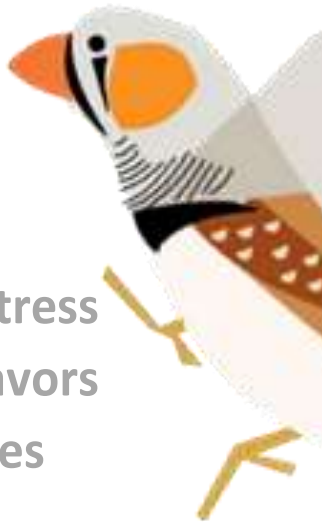
CHAPTER III



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

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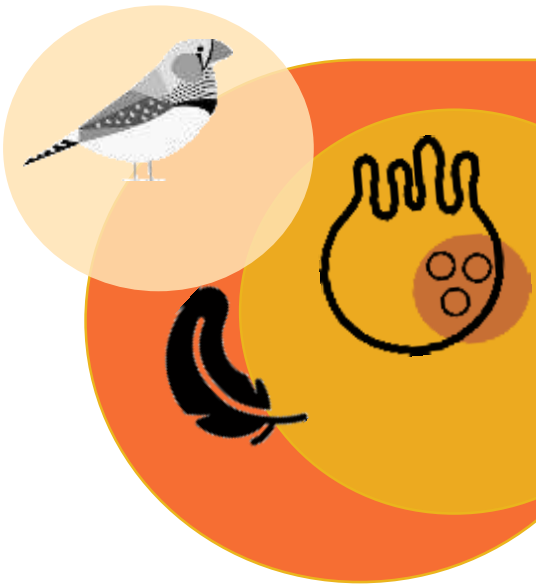

ABSTRACT

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 & 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 & 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 & 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 & Chipman, 2014). At high doses, stressful stimuli are deleterious, but low doses may induce adaptive responses that increases cell resistance (Calabrese & 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 & 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 (Metcalfe & 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 & 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 Cientificas (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 & 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 (*Ct*) levels were used to measure gene expression. Lower *Ct* values indicate higher mRNA levels and gene expression. Normalization was made by substracting *Ct* values for GAPDH from *Ct* values for the target genes (*ΔCt*).

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 m5C was calculated by dividing *Ct* of the input DNA control between *Ct* 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 & Alonso-Alvarez, 2009) for details of this technique applied to bird blood samples). To determine GSSG levels, an aliquot (200 µl) 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 µl) 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 ΔCt 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 µl 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 & Rodríguez-Martínez 2018), using an Ocean Optics Jaz spectrophometer (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 mm^2. 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 & Rodríguez-Martinez, 2018; Rodríguez-Martínez & 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 $\chi 2$ 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 (*ΔCt*) 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*: F1,33 = 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 ΔCt* 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 ± 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 ± SE = 0.040 ± 0.003; controls: 0.047 ± 0.002) nor body condition ($F_{1,35}$ = 0.406; P = 0.528; diquat-treated: 0.006 ± 0.013; controls: -0.005 ± 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; Figure 1), with higher ratios in birds treated with diquat (mean ± SE: 0.942 ± 0.084) than in control birds (mean ± SE: 0.626 ± 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 (Figure 2), but improved their systemic oxidative status.

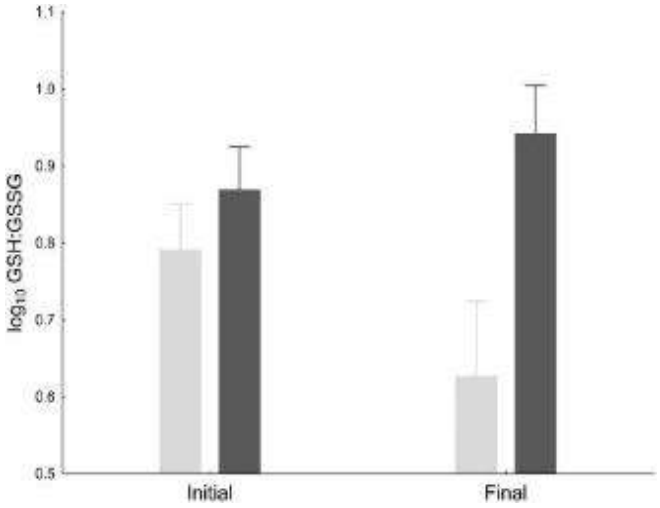

Figure 1: Initial and final oxidative status (GSH:GSSG ratio) of diquat-treated male zebra finches (dark grey) and control birds (grey) (n = 37). Values are means ± SE.

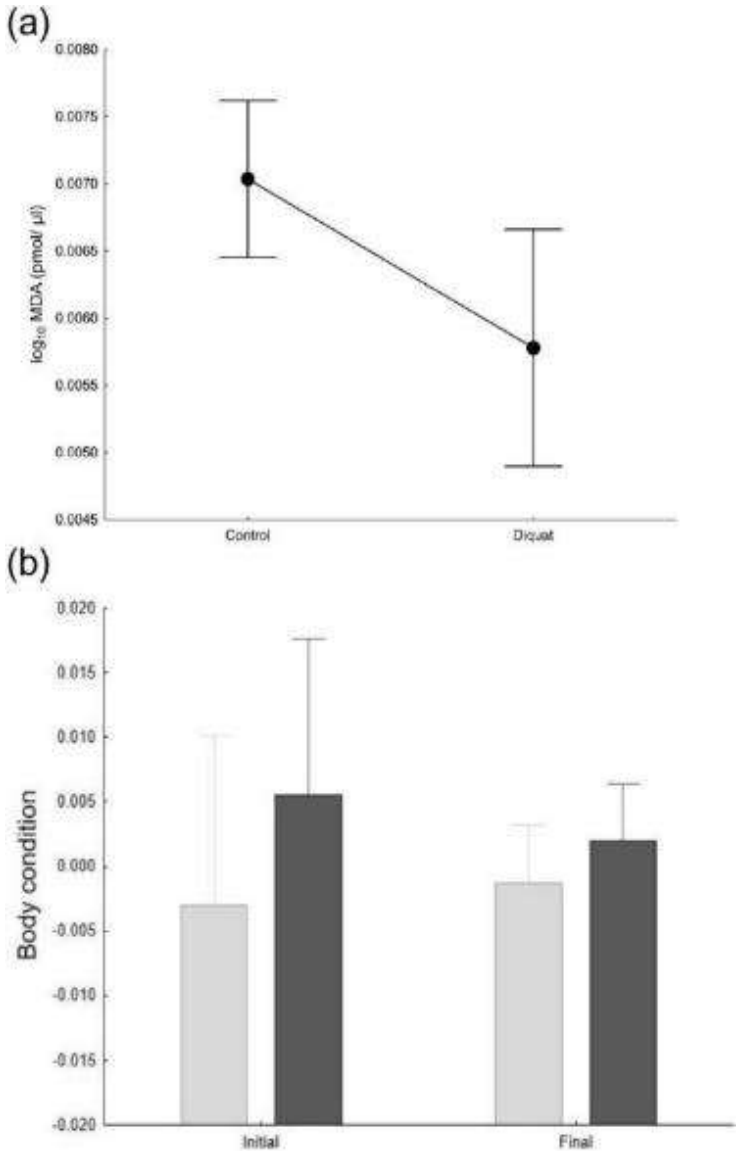

Figure 2: **(a)** Oxidative damage (malondialdeyde, MDA) in male zebra finches after treatment in diquat-treated birds and controls (n = 37). Values are means ± SE **(b)** Body condition before and after treatment of diquat-treated (dark grey) and control birds (grey). Body condition is expressed here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

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) (Figure 3), 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*: χ^2 = 1.519, P = 0.217; *CTNS*: χ^2 = 1.269, P = 0.259; *MC1R*: χ^2 = 3.626, P = 0.056; *ASIP*: χ^2 = 2.208, P = 0.137).

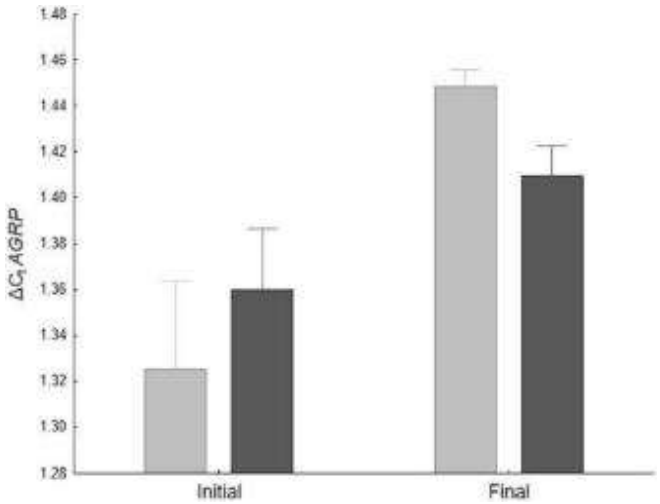

Figure 3. Initial and final values of normalized expression levels (ΔC_t) of *AGRP* in male zebra finches (n = 35) exposed to diquat (dark grey) and controls (grey). Lower ΔC_t indicates higher gene expression. Values are means ± SE.

The proportion of DNA with m^5C was significantly higher in controls than in diquat-treated birds in *MC1R* only (χ^2 = 4.554, P = 0.032), while no differences were found in the other genes (*Slc7a11*: χ^2 = 0.399, P = 0.527; *Slc45a2*: χ^2 = 0.039, P = 0.842; *CTNS*: χ^2 = 0.021, P = 0.884; *ASIP*: χ^2 = 2.481, P = 0.115; AGRP: χ^2 = 0.105, P = 0.745) (Figure 4).

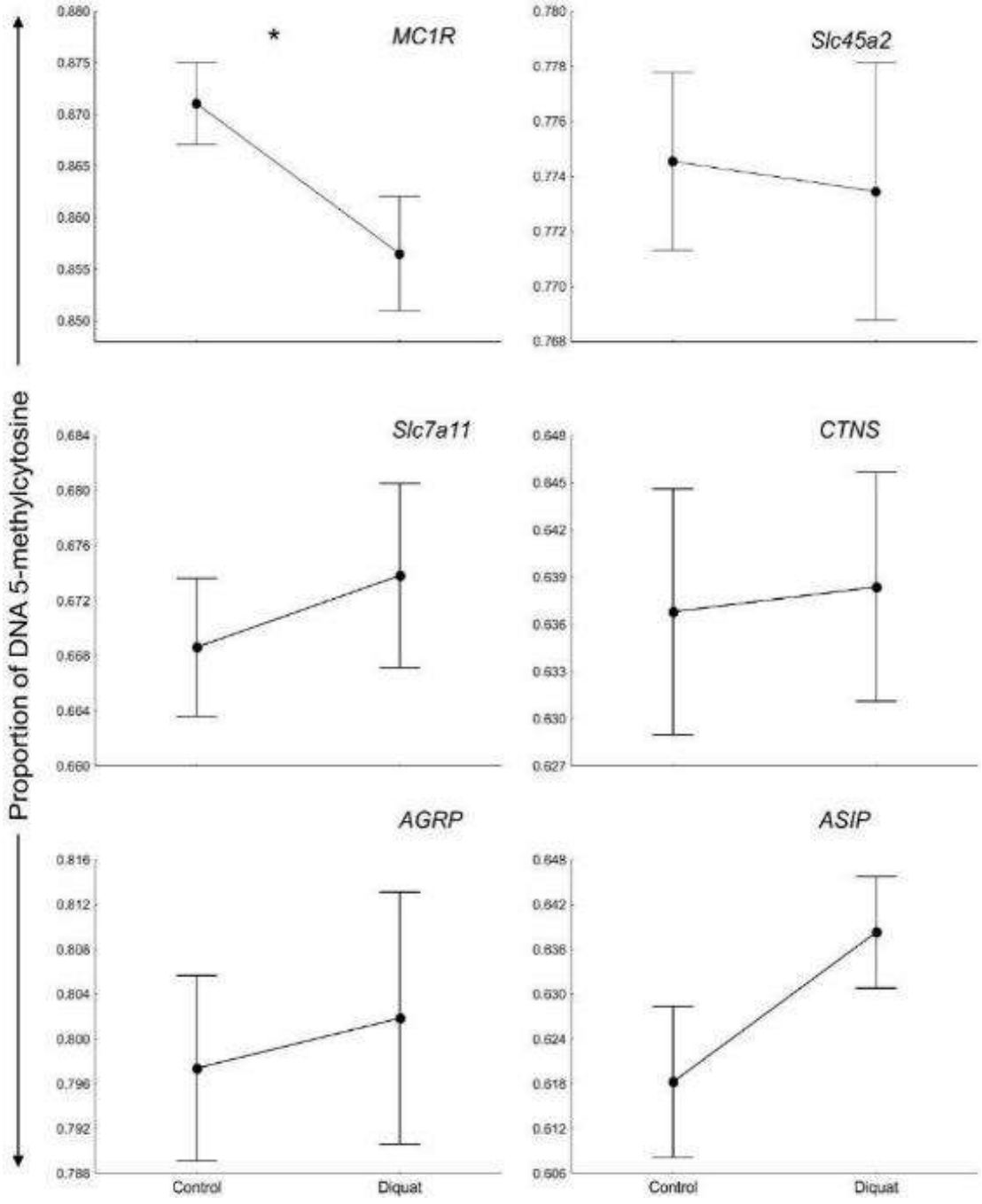

Figure 4. Proportion of DNA with 5-methylcytosine in six genes regulating pheomelanin synthesis (n = 30). DNA was obtained from melanocytes of growing feathers of male zebra finches exposed to diquat and control birds. Asterisk above graph indicates statistically significant differences between diquat-treated birds and controls. Values are means ± SE.

Pheomelanin-based pigmentation in feathers

Diquat exposure had a significant effect on the intensity of pheomelanin-based plumage coloration (χ^2 = 3.872, P = 0.049). The reflectance slope of the flank feathers of birds treated with diquat (mean ± SE: 0.039 ± 0.002) was lower than that of control birds (mean ± SE: 0.046 ± 0.002), indicating a higher production of pheomelanin and a darker pigmentation in birds treated with diquat (Figure 5).

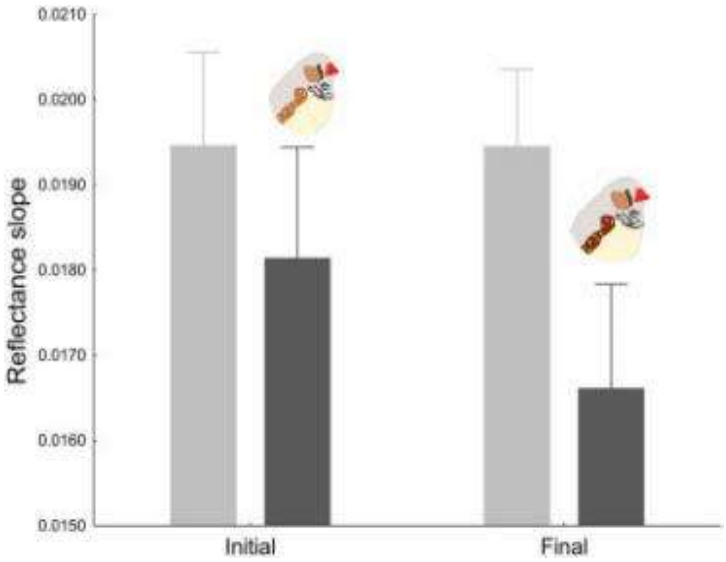

Figure 5. Initial and final mean ± SE reflectance slope (n = 32) of diquat-treated birds (dark grey) and controls (grey). Lower reflectance slopes indicate higher pheomelanin content and more intense color.

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 & 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 m^5C 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 & Galván, 2019). Thus, it is possible that a short exposure to a 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 & Bird 2003). Here, we did not found changes in the m^5C 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 m^5C DNA modifications but by changes in mRNA m^6 (Rodríguez-Martínez et al., 2019). This may suggest that epigenetic modifications other than DNA methylation (i.e., chromatin histone modifications or RNA methylation; Goldberg & 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 & 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 found 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 & 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.

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CHAPTER IV

Higher DNA damage but physiological plasticity in polymorphic zebra finches genetically selected to produce pheomelanin

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Introduction

Constituting a universal response of organisms against solar UV radiation, melanins are the most common pigments in animals and thus responsible for a high proportion of diversity in their color phenotypes and appearance (d'Ischia et al., 2015, Galván et al., 2017a). Melanins are synthesized in animal cells, hence their production unavoidably has physiological consequences, as any other metabolic process. In vertebrates, where melanins are synthesized in specialized cells termed melanocytes, one of these physiological consequences is the incorporation of sulfhydryl groups to the melanogenesis pathway when intramelanocytic levels of thiols are above certain threshold, resulting in the formation of a reddish pigment called pheomelanin (García-Borrón & Olivares Sánchez, 2011). As cysteine is the main thiol providing sulfhydryls to the process and glutathione (GSH), the main intracellular antioxidant, is the main physiological reservoir of cysteine (García-Borrón & Olivares Sánchez, 2011), a constant synthesis of pheomelanin may lead to cysteine/GSH consumption and chronic oxidative stress in humans (Napolitano et al., 2014). In mice, genotypes associated to abundant pheomelanin production increase melanoma risk independently of UV exposure (Mitra et al., 2012), probably due to oxidative stress induced by pheomelanin synthesis (Morgan et al., 2013). In birds, the same cause is hypothesized to limit physiological adaptation to environmental conditions that generate oxidative stress when pigmentation patterns are characterized by high pheomelanin levels (Galván et al., 2014, 2017b). Organisms pigmented by pheomelanin are therefore physiologically constrained, which raises the question of which responses allow to deal with such constraints and favor the evolution of these phenotypes.

An intrinsic difficulty to identify the consequences of pheomelanin production is the lack of conspecific animals that differ in pigmentation but not in the performance of other, independent

physiological processes (i.e., pleiotropic effects), and that therefore allow to make appropriate comparisons. Birds constitute the animal Class with most over-representation of genetic polymorphisms (i.e., the expression of single genes depending upon the alleles present) leading to distinct color phenotypes within the same interbreeding populations (White & Kemp, 2016), meaning that many species occur in morphs that mainly differ in pigmentation patterns. Birds thus offer unique opportunities to investigate the physiological implications of pheomelanin production.

In the Eurasian nuthatch *Sitta europaea*, a passerine bird with flank feathers pigmented by pheomelanin, an environmental factor that produces physiological and oxidative stress (perceived predation risk) induces changes in the expression of some genes involved in cysteine metabolism such as *Slc7a11* that result in a decrease in the production of pheomelanin and the expression of plumage color during development (Galván, 2018). Such phenotypic plastic response seems to be adaptive, as it avoids the cellular damage that is expected from oxidative stress (Galván, 2018). In wildtype zebra finches *Taeniopygia guttata*, another passerine bird with flank feathers pigmented by pheomelanin, a possible hormetic response to exposure to a pro-oxidant substance (diquat dibromide) improves the systemic oxidative status and, likely as a consequence, upregulates a gene (*AGRP*) that promotes pheomelanin synthesis and increases plumage color expression (Rodríguez-Martínez & Galván, 2020). In at least some species of birds, pheomelanin synthesis therefore appears to be limited under oxidative stress while the opposite occurs under favorable physiological conditions, affecting the pigmentation phenotype accordingly. Neither Eurasian nuthatches nor wildtype zebra finches, however, present birds that do not produce any pheomelanin, which limits the capacity to infer direct physiological effects of pheomelanin synthesis.

Domesticated zebra finches, on the contrary, have given rise to multiple pigmentation patterns that appear as color morphs characterized by the combination of different body parts colored by pheomelanin (orange plumage patches), eumelanin (black patches) or with an absence of pigments (white patches) (Landry, 1997), thus constituting excellent models to identify melanin production implications. Here we conducted an experiment using male zebra finches of three distinct color morphs: a morph characterized by plumage profusely pigmented by pheomelanin (orange morph), a morph characterized by plumage profusely pigmented by eumelanin (black morph), and a morph with an absence of plumage pigmentation (white morph). By using cutting-edge liquid chromatography associated to quadrupole time-of-flight mass spectrometry (LC/MS Q-TOF), we quantified oxidative damage in DNA during an experimental induction of oxidative stress by exposing the birds to a pro-oxidative substance (diquat dibromide) when feathers were being developed and melanins produced (Figure 1). A detailed genotype characterization of the three color morphs allowed us to directly infer oxidative damage implications of pheomelanin synthesis.

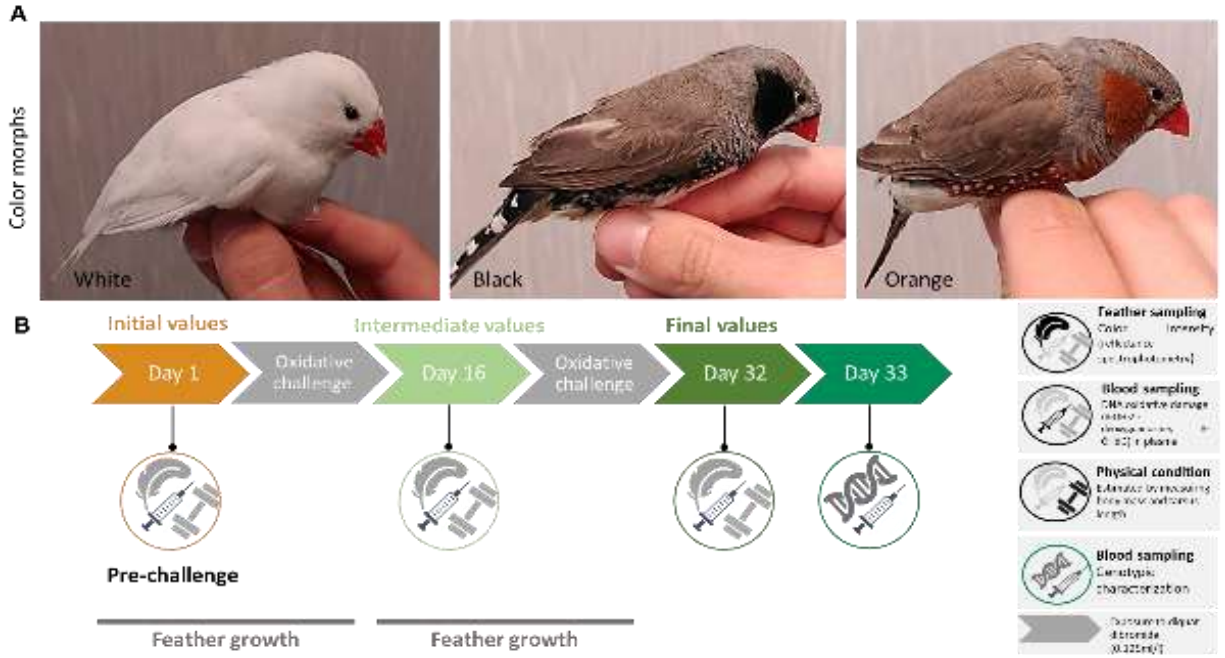

Figure 1. Experimental procedures. (A) Images of male zebra finches belonging to the three color morphs used in the study. (B) Schematic timeline for the experimental oxidative challenge and sampling of birds.

METHODS

Experimental design

The study was carried out at Doñana Biological Station (Seville, Spain) with 19 domesticated male zebra finches that were obtained from an authorized breeder. The birds belonged to three distinct color morphs with plumage pigmentation patterns than differ from that of wildtype birds: 6 birds belonged to an 'orange' morph (chest feathers are orange unlike the black breast of wildtype birds), 10 birds belonged to a 'black' morph (cheek and flank feathers are black unlike wildtype birds) and 3 birds belonged to a 'white' morph (all feathers are unpigmented). Given that the orange plumage of zebra finches is caused by the deposition of pheomelanin in feathers while the black plumage is caused by the deposition of eumelanin (Galván et al., 2013), 'orange' morph birds have been artificially selected to produce distinctively greater amounts of pheomelanin than 'black' morph birds, which have instead been selected to produce greater amounts of eumelanin. 'White' morph birds do not produce any pigment to color their plumage.

All birds were marked with a numbered metal ring and housed in individual cages (60 × 40 × 25 cm) at controlled temperature (average: 24 ± 0.5 °C) and light-dark daily cycle (13 L: 11D). After 10 days of acclimation, the pigmented flank, breast and cheek covert feathers were plucked to stimulate the growth of new ones and thus induce the production of melanins. The reflectance spectra of flank feathers were obtained to measure the expression of the pigmentation phenotype. Body mass and tarsus length were also measured to have an estimation of physical condition. Lastly, blood from the brachial veins of birds was collected to quantify systemic levels of DNA oxidative damage (8-OH-2'-deoxyguanosine, 8-OHdG) in plasma. Blood samples were stored at -80 ºC after centrifugation. Values from all these measurements are referred to as 'initial values' (i.e., pre-oxidative challenge) (Figure 1).

After initial measurements, the birds were exposed to an oxidative damage by treating them with diquat dibromide, a chemical stressor that produces intracellular superoxide anion and increases the production of reactive oxygen substances (ROS) in organisms (Di Giulio et al., 1989). All birds received diquat at a dose of 0.125 ml/l in drinking water (Rodríguez-Martínez & Galván, 2020). After 15 days of treatment, most of the pigmented part of feathers was developed. The feathers were thus plucked and the morphological and blood samples collected again. Values from these measurements are referred to as 'intermediate values'.

The oxidative challenge was prolonged during 15 days more to avoid possible hormetic effects (Rodríguez-Martínez & Galván, 2020). 'Final values' were thus obtained after 30 days of treatment, when the feathers were plucked and the morphological and blood samples collected again. Additional blood samples were collected one day after for the genotypic characterization of morphs (Figure 1).

Systemic DNA oxidative damage

8-OH-2'-deoxyguanosine (8-OHdG) was identified and quantified in plasma samples by using high-performance liquid chromatography (HPLC) coupled to electrospray ionization-quadrupole-time of flight mass spectrometry (AJS ESI-QTOF-MS) instruments. A Chromatography System Agilent series 1200 (Waldbronn, Germany) and a Luna 5u PFP(2) 100A (150 X 4.6 mm) column from Phenomenex® (Torrance, CA, USA) were used for the chromatographic separation of the analytes. This system consists of a degasser, a liquid chromatographic pump, an autosampler and a column compartment (Agilent series 1200). Detection was carried out with an Agilent 6546 LC/QTOF detector (Agilent Technologies, Santa Clara, USA.) which operated with a dual Agilent Jet Stream electrospray ionization (AJS ESI). The analysis of mass spectra was made with Agilent MassHunter Data Acquisition TOF/Q-

TOF 10.0 and Qualitative Analysis 10.0 softwares (see Supplementary Material for additional analytical and chromatographic methods and Table S1).

Expression of pigmentation phenotype and pheomelanin content of feathers

To quantify the relative amount of pheomelanin content of flank feathers, the color intensity was measured by UV-Vis reflectance spectrophotometry (Rodríguez-Martínez & Galván, 2019), using an Ocean Optics Jaz spectrophometer (range 220-1000nm) with ultraviolet (deuterium) and visible (tungsten-halogen) lamps and a bifurcated 400 µm fiber optic probe. The fiber optic probe both provide illumination and obtained light reflected from the sample, with a reading area of ca. 1 mm^2. 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 standard tablet (WS-1, Ocean Optics, Dunedin, FL), and reference measurements were frequently made. An average spectrum of three 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 percent reflectance in 10 nm intervals. Most of the pigmented part of flank feathers was not developed at the intermediate sampling point in one bird and at the final sampling point in five birds, was prevented to obtain the reflectance spectra of these flank feathers accurately.

The slope of percent reflectance regressed against wavelength across the 300-700 nm range was used as a measure of pheomelanin-based color expression in birds of the orange morph. Reflectance slope is the best color indicator of the pheomelanin content of feathers in the flank feathers of zebra finches, with lower values denoting darker colors

(i.e., higher color intensity) and higher pheomelanin contents (Rodríguez-Martínez and Galván 2019). As the reflectance spectra of pheomelanin and eumelanin have the same shape (a progressive increase in reflectance from 300 to 700 nm, with no defined peaks), and the slope of eumelanin reflectance spectrum predicts feather eumelanin content in several species of birds (Galván & Wakamatsu, 2016), reflectance slope was also used as a measure of expression of pigmentation phenotype and eumelanin content of feathers in zebra finches of the black morph.

Genetic mapping of zebra finches' color morphs

Inheritance of zebra finch color mutations

Zebra finches have been extensively bred by aviculturists for decades, which has given rise to a myriad of color mutations that mostly alter the type or deposition of melanins in specific body patches. We selected individuals from three well-established genetic color mutants – Black cheek, orange breasted and white zebra finches (Landry, 1997) to be contrasted with the wildtype form. In these domestication mutants, melanin biosynthesis/deposition is altered, causing either an overproduction of eumelanin (black cheek zebra finch), a complete switch from eumelanin to pheomelanin pigmentation (orange breasted zebra finch), or a total absence of this pigment from the plumage (white zebra finch). All three color morphs are known autosomal recessive mutations, following a simple Mendelian pattern of inheritance (Landry, 1997).

Whole-genome sequencing and read mapping

To study the genetic basis of zebra finches' melanin-based mutations, we generated whole-genome sequencing data. A total of 41 birds were sampled from black cheek (N=10), orange breasted (N=11), white (N=9) and wildtype (N=11) color morphs. Whole blood was drawn by brachial

venipuncture with a sterile needle and stored in 96% ethanol at -20 ºC until DNA extraction. To prevent ethanol contamination, blood was washed in PBS solution overnight prior to DNA extraction. Genomic DNA was then isolated from these samples using a modified salt-based protocol (Enbody et al., 2021) with an additional RNAse-A digestion step. Following extraction, DNA quality and integrity was assessed through spectrophotometry (Nanodrop), fluorometric quantitation (Qubit™ dsDNA BR Assay Kit, Thermo Fisher Scientific) and agarose gel electrophoresis visualization. High quality DNA was subsequently used to prepare individual whole-genome sequencing libraries using the TruSeq DNA PCR-free Library Preparation Kit (Illumina), following the manufacturer protocol. Libraries were sequenced using 150 bp reads in an Illumina instrument at Novogene UK to an average coverage of 3.3X. Whole-genome sequencing data are available in the Sequence Read Archive SRA, (www.ncbi.nlm.nih.gov/sra) under BioProject PRJNA773662.

After sequencing, read quality was checked with FastQC v0.11.8 and reads were mapped to the Zebra finch reference genome (Ensembl version taeGut3.2.4, Aug. 2008; Warren et al., 2010) using bwa-mem v0.7.17-r1188 (Li & Durbin, 2009) with default parameters. Duplicate reads were marked for removal using Picard MarkDuplicates (http://broadinstitute.github.io/picard). Summary mapping statistics per individual were computed with SAMtools v1.11 (Li et al., 2009) and are detailed in Table S2.

Genome-wide selective sweep mapping

To identify the regions of the genome underlying the different color morphs, we scanned the genome for regions of increased genetic differentiation and reduced genetic diversity. To do so, we estimated population branch statistics (PBS; Yi et al., 2010) and nucleotide diversity (π; Korneliussen et al., 2013) across the genome, using a sliding window approach (100kb windows, iterated in 20kb steps) in ANGSD v0.929-13-gb5c4df3 (Korneliussen, Albrechtsen & Nielsen, 2014). PBS

compares genetic differentiation between two populations and a target population allowing us to infer lineage-specific differentiation levels across the genome. All the mutations that explain our color morphs are recessive; therefore, genetic diversity should be strongly reduced around the causative locus when compared to wild-type birds.

For each morph, we calculated site allele frequencies followed by 2D site frequency spectra for all possible pairwise-comparisons, using realSFS. PBS and nucleotide diversity were then estimated for each of the populations (black, orange, white and wildtype) based on these priors. To account for natural variation in genetic diversity across the genome, we summarized nucleotide diversity as the ratio between wildtype and each of the remaining color morphs (πwild-type/ πmorph). It is expected that regions underlying the different color morphs should have high values of this ratio. Calculations for both statistics were based on genotype likelihoods to account for uncertainty in low-coverage data, as implemented in ANGSD v0.929-13-gb5c4df3 (Korneliussen, Albrechtsen & Nielsen, 2014). Reads with mapping quality <30, individual base quality <20, and multiple best hits were excluded from the analysis. When computing genotype likelihoods, we also excluded reads with mates not mapping correctly and tri-allelic positions. Only windows with at least 50% of the positions covered were more than 20% of the positions passing filters were retained.

Variant calling and functional impact

Having identified genomic regions of simultaneously heightened differentiation and low nucleotide diversity, we investigated its gene content for obvious candidate genes implicated in plumage color variation. We performed variant calling within candidate genomic regions, by means of the Bayesian haplotype-based method implemented in Freebayes v1.3.4-linux-static-AMD64 (https://github.com/ekg/freebayes). We excluded reads with mapping

qualities lower than 30 and read bases with quality lower than 20. We required at least 4 observations supporting the alternative allele and disabled left alignment of indels. Additionally, we used the "genotype-qualities" option to obtain a quality score associated with each individual genotype. All retrieved variants were functionally annotated using the genetic variant annotation and effect prediction toolbox SnpEff v4.3t (Cingolani et al., 2014) and posteriorly filtered for functional impact categories, such as nonsynonymous, frameshift and STOP mutations. We further complemented this analysis with visual inspection of candidate mutations on IGV v2.8.9 (Robinson et al., 2011). IGV was further used to identify potential structural rearrangements around the candidate loci.

Statistical analyses

Repeated-measures general linear models (GLM) were used to investigate differences between morphs in variation across sampling points in physical condition and DNA oxidative damage levels (response variables). The sampling point ('initial', 'intermediate' and 'final') was thus the within-subjects term in the models, while morph was a fixed term (factor). In the model for physical condition, tarsus length was an additional fixed term (covariate) to control for body size. Differences between groups were explored using Fisher LSD post hoc tests. As the reflectance spectra of some birds could not be obtained at intermediate and final sampling points, Fisher LSD tests were also used to explore differences in reflectance slopes between sampling points to avoid sampling size reduction in repeated-measures GLMs. Analyses were made with STATISTICA 13 (StatSoft, Tulsa, OK, USA).

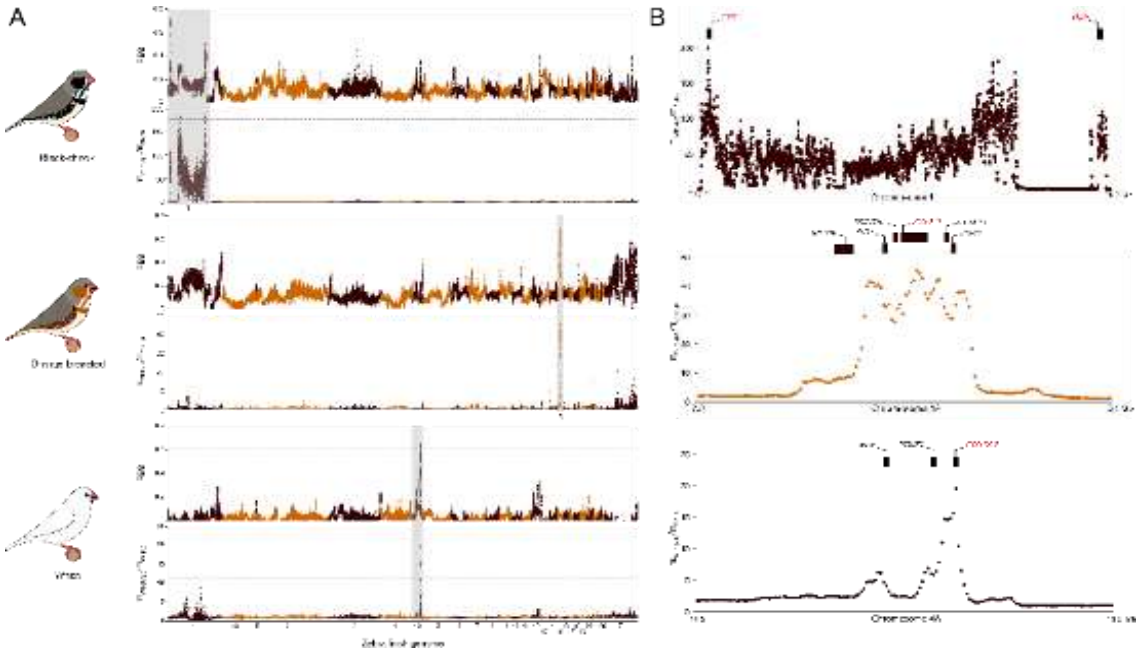

Figure 4. Genetic mapping of three melanin-based mutations in domestic zebra finches. (A) Genome-wide patterns of lineage-specific differentiation statistics (PBS) and ratios of nucleotide diversity (π) for black cheek (top), orange breasted (middle) and white (bottom) zebra finches. Each dot represents the averaged statistics across 100kb windows iterated over 20kb steps. The horizontal dashed lines indicate the 99.99th percentile threshold. Shaded areas highlight genomic regions associated with black cheek (top), orange breasted (middle) and white (bottom) phenotypes. The different chromosomes are shown in the x-axis following the same order as in the Zebra finch reference genome. Smaller and unplaced chromosomal regions are not displayed for visualization purposes. (B) Nucleotide diversity ratios within candidate regions identified for each zebra finch breed. Each dot represents the ratio averaged across the genome in 100kb windows, iterated in 20kb steps. Protein coding genes within each region are shown at the top of the graphs; Candidate genes for explaining coloration in each breed are highlighted in red.

RESULTS

Characterization of zebra finch melanin-based domestic mutations

Discrete variation in melanin pigmentation is typical of Zebra finches, with the wildtype combining black, grey, brown, and white plumage patches. We focused this study in three well-established breeds known to display melanin-based variation in restricted plumage patches (black-cheek, orange breasted and white zebra finches). The Black-cheek is a recent mutation (1970's) characterized by an overexpression of eumelanin in the cheeks and flanks, converting the originally brown cheeks seen in the wildtype, into black cheeks and darkening of the flanks. Orange breasted zebra finches differ from the black-cheek by a pigmentary shift from eumelanin to pheomelanin production in the chest and tail coverts, while maintaining the typical brown cheeks and flanks (Landry, 1997). While these two mutants vary in melanin type and concentration, the white zebra finch mutant is completely devoid of melanin in its plumage, maintaining dark eyes and carotenoid pigmentation in bare parts. These zebra finch color traits are inherited in an autosomal recessive fashion and are described as following a simple Mendelian segregation (Landry, 1997).

Genetic mapping of genomic regions implicated in altered melanin pigmentation

To study the genetic basis of the three color morphs used in our experiments, we performed whole-genome Illumina sequencing at low coverage for a total of 41 unrelated individuals (Table S1). We produced a total of ~1,13 billion Illumina reads that were mapped against the zebra finch reference genome, resulting in an average coverage of 2.4X per individual (range 1.0 – 5.0X; Table S1).

All three zebra finch breeds used in this study are described as following a Mendelian autosomal recessive segregation based on crosses (Landry, 1997). Therefore, increased genetic differentiation and low genetic diversity is expected around one causative locus. Using a sliding-window approach, we searched for regions of elevated differentiation and low genetic diversity across the genomes of wildtype and the three breeds by applying the population branch statistic (PBS; Figure 4A) and the ratio of nucleotide diversity between wildtype and each of the breeds (πwild-type/πbreed; Figure 4A).

EDNRB2 whole-gene deletion explains melanin loss in white zebra finches

Our sliding-window analysis of the white zebra finch mutation revealed a clear outlier genomic region characterized by increased genetic differentiation and low genetic diversity when compared to the rest of the genome. All windows above the 99.99th percentile of the empirical distribution map to this region. This region is ~80kb and is located on chromosome 4A (~15,270,500 - 15,350,500 bp). Next, we explored the gene content within the associated region and found a single gene – the endothelin receptor B subtype 2 (EDNRB2) encoding a 7-transmembrane G-protein coupled receptor paralog of the EDNRB gene (Lecoin et al.m 1998). EDNRB2 is expressed in neural-crest cells and is explicitly involved in melanoblast development and melanocyte differentiation (Lee et al., 2003; Pla et al., 2005; Harris et al., 2008; Krispin et al., 2010; Nitzan et al., 2013). It has been systematically implicated in abnormal melanin pigmentation in birds (Tsudzuki et al., 1993; Miwa et al., 2007; Kinoshita et al., 2014; Wu et al., 2017; Xi et al., 2020; Maclary et al., 2021; Xi et al., 2021). A careful analysis of this region revealed a large deletion (~7kb) encompassing the full EDNRB2 gene (~7kb). This full-gene deletion is a strong candidate to explain the white phenotype.

A splice-site mutation in ZNRF3 is associated with the orange breasted phenotype

We found a clear outlier ~180kb genomic region on chromosome 15 (~8,410,000 - 8,590,000bp) on both statistics. All top 0.1% windows were comprised within this interval. A closer inspection of the gene content within the interval identifies two consecutive genes - the kringle containing transmembrane protein 1 (*KREMEN1*) and zinc/ring finger protein 3 (*ZNRF3*) genes - as likely candidates to explain this phenotype.

Kremen proteins are high affinity Dikkopf receptors that functionally interact with DKK1 to inhibit Wnt/β-catenin signalling (Mao et al., 2002; Ellwanger et al., 2008; Zebisch et al., 2016), a key developmental pathway promoting neural crest cell differentiation into pigment cells (Dorsky et al., 1998; Dunn et al., 2000). ZNRF3 gene encodes a transmembrane E3 ubiquitin ligase that functions as a negative regulator of the same signaling pathway (MacDonald & He, 2012; Zebisch et al., 2015). In the presence of R-spondin, ZNRF3 is cleared from cell membranes and allows β-catenin to accumulate in cells, through enhancement of Wnt signaling (Hao et al., 2012). Increased transport of β-catenin into the nucleus will induce downstream target gene expression (e.g. melanogenesis genes) (Gajos-Michniewicz & Czyz, 2020). The role of these genes in regulating melanin production is largely unexplored and they have never been directly implicated in pigmentation phenotypes. Notwithstanding, their function in regulating Wnt signalling may be determinant in the cascade of events leading to melanocyte proliferation, differentiation, and survival through regulation of major melanin-related genes (Guo et al., 2012; Hwang et al., 2013; Lapedriza et al., 2014). In mammals, pigment-type switching from eumelanin to pheomelanin was linked to changes in β-catenin activity possibly through suppression of MITF, a major regulator of pigmentation (Enshell-Seijffers et al., 2010; Kaelin et al., 2021).

Another gene located within the outlier interval, solute carrier family 2 member 11b (*SLC2A11B*), is worthy of note, as it has recently been implicated in pigmentation differences in the iris of pigeons by three independent studies (Andrade et al., 2021; Maclary et al., 2021; Si et al., 2021). It has also been shown to be required for xanthophore differentiation in medaka fish (Kimura et al., 2014). This gene has been exclusively found to be involved in the pterin pigmentation pathway and xanthophore formation, functions seemingly unrelated to melanin production. Therefore, *SLC2A11B* is most likely not involved in the orange breasted zebra finch mutation.

We next screened the genomic interval containing *KREMEN1*, *SLC2A11B* and *ZNRF3* for possible causative mutations with potential impact on protein function. We found a splice-site mutation on *ZNRF3*, at the termini of intron 1 (15: 8,550,533bp), that likely affects mRNA processing. No mutations of significant impact and following inheritance expectations were found in surrounding coding regions. Splice-site disruptions usually reflect in the usage of distinct isoforms in affected tissues, but do not necessarily lead to protein loss-of-function. This supports the observation that in orange breasted zebra finches only restricted plumage patches show pigment-type switching. Individual genotypes of the candidate splice-site mutation in *ZNRF3* follow the expected inheritance pattern, being found in homozygosity exclusively in orange breasted individuals. This mutation is likely to be functionally relevant since it overlaps a position showing strong evolutionary conservation across the genomes of multiple bird species (Supplementary Figure S1).

NDP likely explains eumelanin overproduction in Black-cheek zebra finches

Our sliding window approach revealed a region of heightened differentiation (PBS) within chromosome 1 (~106,900,000-

107,200,000bp). Nucleotide diversity in this region was also among the lowest in the genome of the Black cheek mutant. Immediately upstream of the candidate genomic region of heightened differentiation, we identified the Norrie disease protein gene (NDP), which encodes a ligand molecule that triggers the Wnt signalling pathway by binding to FZD4/LRP5 receptors and has the potential to regulate the transcription of melanin-related genes. This gene has been associated to melanin-based pigmentation in crows (Poelstra et al., 2015) and rock pigeons (Vickrey et al., 2018). In an independent study, Sly (2019) suggests that the black cheek mutation in Zebra finches is explained by this same locus. We note, however, that the top outlier region using the ratio of nucleotide diversity contains a classic melanogenesis gene, Tyrosinase (TYR; 1: ~34,086,228-34,138,422bp). *Tyr* is the rate limiting enzyme that catalyzes the first fundamental step in melanin biosynthesis, the conversion of tyrosine into dopaquinone. Dopaquinone processing will generate pheomelanin until cysteine is depleted and eumelanin synthesis begins. In general, mutations in TYR have consistently resulted in albinism (Kulikova, 2021), however increased expression of TYR has been recently reported in melanic phenotypes (Xu et al., 2013).

Sly et al. (2019) also reported a downregulation of NDP in feather follicles of Black cheek zebra finches together with changes in the expression of other melanogenesis genes subsequently modulated by the Wnt/β-catenin signalling pathway. Consistent with these findings, we failed to detect any protein-coding changes in NDP that could explain the phenotype. However, we found a partial ~1.5kb intronic deletion within the first intron (1: ~107,361,432 - 107,362,950bp) that is a potential candidate variant to underlie the expression differences. Taken altogether, NDP is a strong candidate to explain eumelanin overproduction in the Black cheek mutant. Even so, we cannot discard with our current data that both NDP and TYR might be required for full expression of the Black cheek phenotype or if the individuals sampled were carriers for another melanin related mutation.

Physiological implications of pheomelanin synthesis

The physical condition of birds, measured as body mass corrected by size, was lower in the orange morph than in the black ($P = 0.025$) and white morphs ($P = 0.063$) before the beginning of the experimental treatment with diquat (Figure 2a). This significant lower physical condition of birds of the orange morph remained during the treatment ($0.003 < P < 0.045$; Figure 2a). Accordingly, the morph term was significant in the repeated-measures model ($P = 0.011$), while its interaction with the within-subjects (repeated measures) term was not ($F_{4,30} = 0.26$, $P = 0.899$).

The model for levels of deoxyguanosine in plasma resulted in a significant interaction of morph and within-subjects terms ($F_{4,32} = 3.66$, $P = 0.015$). This was due to higher levels in birds of the orange morph than in birds of the black morph ($P = 0.006$) and the white morph ($P = 0.066$) before the beginning of the diquat treatment (Figure 2b). During the experimental treatment, however, deoxyguanosine levels in birds of the orange morph suffered a progressive decrease, making that final levels were considerably lower ($P < 0.001$). In contrast, deoxyguanosine levels varied only slightly during diquat treatment in birds of the black morph, whose final levels did not differ from initial levels ($P = 0.293$), and the same was found in birds of the white morph ($P = 0.684$) (Figure 2b).

Changes in oxidative damage occurred together with changes in the pigmentation phenotype in birds of the orange morph. The slope of the reflectance spectrum of pheomelanin-pigmented flank feathers followed a tendency to decrease (i.e., pheomelanin concentration in feathers tended to increase) at the intermediate sampling point of the experiment ($t = 1.91$, $df = 10$, $P = 0.085$), but then it significantly increased (i.e., pheomelanin concentration decreased) at the end of the experiment ($t = 2.56$, $df = 9$, $P = 0.031$) (Figure 3). In contrast, the slope of the reflectance spectrum of eumelanin-pigmented flank feathers of

birds of the black morph did not change at the intermediate point of the experiment nor from this point to the end ($0.451 < P < 0.740$) (Figure 3).

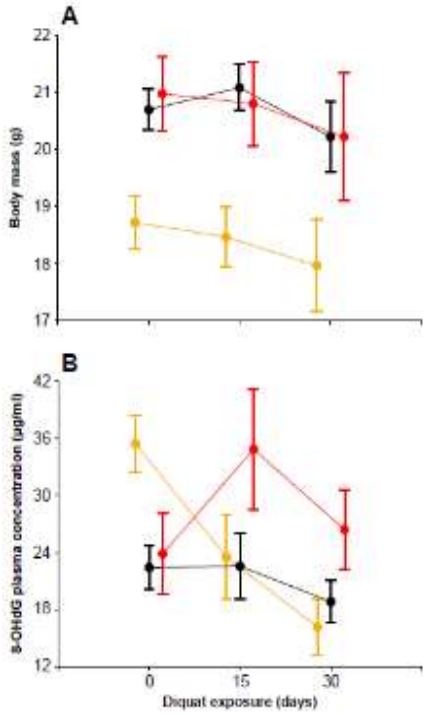

Figure 2. Physiological effects of oxidative challenge on male zebra finches of different color morphs. Physical condition (A) and DNA oxidative damage (B) measured while feathers were being developed and pigments produced are shown. Values (mean ± se) were obtained before an experimental exposure to diquat dibromide (0 days), at an intermediate point of the treatment (15 days) and at the end of the treatment (30 days). Physical condition is represented by body mass. DNA oxidative damage was measured as levels of 8-OH-2'-deoxyguanosine (8-OHdG) in plasma by LC/MS Q-TOF. Orange symbols: pheomelanin-based color (orange) morph. Black symbols: eumelanin-based color (black) morph. Red symbols: Unpigmented (white) morph.

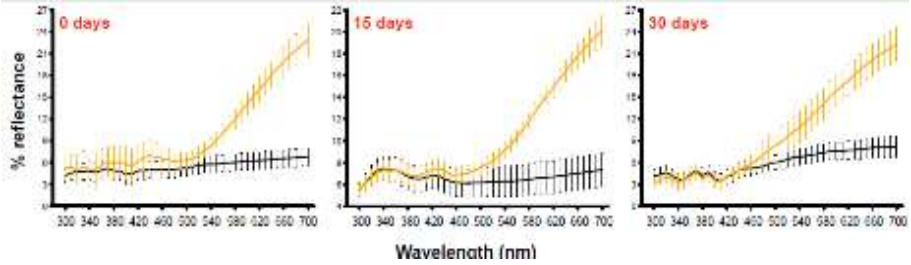

Figure 3. Phenotypic effects of oxidative challenge on orange and black male zebra finches. The reflectance spectra of flank feathers are shown in 10-nm intervals (mean ± se) for birds of the pheomelanin-based (orange) morph and the eumelanin-based (black) morph at different times of the experimental treatment with diquat dibromide. The slope of a linear fit for the % reflectance-wavelength regression across the 300-700 nm range provides a measure of the expression of the pigmentation phenotype, with lower levels indicating higher pheomelanin feather concentrations and darker (i.e., more intense) colors in male zebra finches (Rodríguez-Martínez & Galván, 2019). The same approach was used to quantify eumelanin feather concentration and color expression in birds of the black morph (Galván & Wakamatsu, 2016).

DISCUSSION

Our experiment reveals that domesticated male zebra finches that have artificially been selected to profusely pigment their plumage with pheomelanin show a lower physical condition and a higher oxidative damage in DNA than male zebra finches selected to pigment their plumage with eumelanin and males selected to develop unpigmented plumage. This agrees with physiological studies of birds and mammals, including humans, showing that a constant and abundant production of pheomelanin may lead to chronic oxidative stress and damage (Napolitano et al., 2014; Mitra et al., 2012; Galván et al., 2014, 2017b). This is probably caused by the decrease of antioxidant resources (cysteine/GSH) that pheomelanin synthesis implies (Morgan et al., 2013; Galván et al., 2014, 2017b). These results may constitute a physiological limitation for organisms expressing pheomelanin-based color phenotypes. However, our experiment also arised a surprising effect when birds were exposed to an oxidative challenge by treating them with diquat dibromide, as birds of the pheomelanin-based (orange) color morph steeply reduced their DNA damage levels during the treatment. Some increase in the degree of pheomelanin feather

pigmentation was also observed. In contrast, DNA damage levels did not change in birds of the eumelanin-based (black) color morph nor in unpigmented (white) birds. This unveils dual physiological properties of pheomelanin-pigmented animals: their oxidative status and condition seem to be weaker under physiological conditions, but they also show a phenotypic plasticity that allows them to adjust pheomelanin production that animals not producing pheomelanin do not possess. The genotypic characterization of the three zebra finch color morphs used in the experiment shows that these result from mutations in genes that mainly affect pigmentation and do not have known pleiotropic effects on the antioxidant metabolism, which allows us to be confident in assigning the morph-specific physiological effects to consequences of pheomelanin synthesis.

These findings unveil new consequences of color polymorphism, which to date have been limited to the enhancing role of evolutionary potential and speciation derived from reproductive isolation and geographic gradients of selection (Forsman et al., 2008; White & Kemp, 2016). Physiological consequences of color polymorphism, derived from pigment production, have been overlooked. Between-morph differences in systemic antioxidant status have previously been reported in other species of birds with melanin-based color polymorphisms, but considering morphs differing in the extension of body covered by a single pigment (eumelanin) instead of melanin forms, and without a genotypic characterization of morphs (Galván et al., 2010). Our genotypic characterization of zebra finch morphs shows clear physiological consequences for animals genetically selected to produce pheomelanin and for those selected to produce eumelanin or to block melanin synthesis, indicating a direct consequence of the pheomelanin biosynthesis pathway. While the chronic activation of this pathway implies a physiological constraint for birds as it leads to weak body condition and high DNA damage, it also seems to facilitate phenotypic plasticity. This plasticity may be considered as physiologically adaptive, as it leads to a decrease of oxidative damage

after a threshold of oxidative stress levels. In a previous experiment with wildtype male zebra finches exposed to diquat dibromide during half the duration of the present experiment, the antioxidant status of birds improved and pheomelanin production increased (Rodríguez-Martínez & Galván, 2020). In agreement, pheomelanin production in birds of the orange morph tended to increase at the intermediate point (15 days) of the oxidative challenge here. By doubling this challenging time, however, pheomelanin production decreased and the flank color expression changed accordingly. A likely explanation for this is that an oxidative stress threshold leading to DNA damage increase may have been exceeded during the 30 days of diquat exposure, which may have limited pheomelanin synthesis and, as a consequence, decreased DNA damage. This was observed only in birds of the pheomelanin-pigmented morph.

Ecological and evolutionary implications should derive from these results. Non-polymorphic (wildtype) zebra finches and other species of birds have previously been found to adjust pheomelanin production to oxidative stress caused by environmental conditions (Galván, 2018; Rodríguez-Martínez & Galván, 2020), probably promoted by methylation changes in genes of cysteine metabolism (Rodríguez-Martínez et al., 2019). These findings are here applied to melanin-based color polymorphisms, as domesticated zebra finches of the pheomelanin-pigmented morph, but not those of the eumelanin-pigmented or unpigmented morphs, show adaptive phenotypic plasticity against an exogenous source of oxidative stress. Given the abundance of color polymorphisms characterized by pheomelanin- vs. eumelanin-pigmented morphs in nature (Robbins et al., 1993; Baião et al., 2007; Pannkuk et al., 2010), this plasticity may provide pheomelanin-pigmented morphs a relative ecological advantage respecting other pigmentation phenotypes. The evolution of color polymorphism may be affected by this, as well as by potential interspecific differences in physiological plasticity (Galván et al., 2017b).

In conclusion, the regulatory activity of genotypes leading to the production of large amounts of pheomelanin creates weak physiological conditions but is more labile than other pigmentation genotypes, making pheomelanin-based color morphs more phenotypically plastic at least in the zebra finch. This gives pheomelanin-pigmented morphs a greater physiologically adaptive potential. Whether this also translates into a greater evolutionary potential should now be explored to elucidate the evolution of color polymorphism.

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SUPLEMENTARY MATERIAL

Analytical and chromatographic methods for DNA oxidative damage analyses

Analytical methods

8-OH-2'-deoxyguanosine (8-OHdG) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid (88%), sodium chloride and sodium carbonate were used to treat blood samples, and purchased from Sigma-Aldrich. LC-MS grade acetonitrile was used as mobile phase solvent and acetic acid as acidifier, and both were purchased from Fisher Scientific (Loughborough, Leics, UK). HPLC grade ethanol and hydrochlorid acid (37%) was purchased from Panreac (Spain, Barcelona). Ultrapure water was obtained from a Milli-Q water instrument from Millipore (Merck KGaA, Darmstadt, Germany).

Stock solutions were prepared at 1000 mg/L in ethanol and stored in absence of light at -20 ºC. Working standard solutions were daily prepared at 1 mg/L by appropriate dilution in the corresponding solvent and stored at -20 ºC as well.

Plasma samples were stored at -80 ºC before and after treatment, defrosted and treated to precipitate the proteins present according to Ludwig et al. (2015). Briefly, acetonitrile acidified with formic acid at -20 ° C was added to the samples in a proportion of 2.5:1 (solvent: sample) and mixed using a vortex mixer. Then the solution was centrifugated at 14500 rpm for 10 min. The supernatant was collected and evaporated, and the resulting pellet was resuspended with the mobile phase used in the high-performance liquid chromatograph-mass spectrometry (HPLC-MS) method.

Liquid chromatography-MS

Chromatographic analyses were carried out in isocratic mode using 0.02% acetic acid in water as mobile phase A and 0.02 % acetic acid in acetonitrile as mobile phase B. The percentages of the mobile phases were optimized to achieve the separation of the analytes from the elution peak, while reducing the separation time. Injection volume was 2 µL.

MS detection of analytes was carried out in positive ion mode under the following optimized conditions of the source: gas temperature of 305 ºC, drying gas flow of 12 L/min, nebulizer pressure of 55 psi, sheath gas temperature of 250 ºC, sheath gas flow of 8.5 L/min, capillary voltage of 4000 V, nozzle voltage of 250 V, fragmentor voltage of 115 V, skimmer voltage of 45 V and octupole voltage of 700 V. To correct the mass accuracy in continuous mode, two reference masses (m/z 121.050873 and 922.009798) were used. Before analyzing the samples, the standards were qualitatively determined to know their retention time and mass spectra. The m/z ion used for the identification of 8-OHdG was 284.0989.

The calibration data and validation parameters for this method are presented in Table S1. The calibration curve showed a linear range for 8-OHdG solutions from 5 to 600 mg/L and 615 to 1200 mg/L.

Table S1. Calibration data and validation parameters obtained for the determination of 8-OHdG in zebra finch plasma.

Linear range (mg/L)	$Y = (A \pm S_A^c) + (B \pm S_B^d)X$	R^2	$S_{x/y}^e$	LOD (mg/L)	LOQ (mg/L)
5-600	$Y = (216{,}657.9 \pm 738.1) + (196{,}314.3 \pm 75{,}940.4)X$	0.9992	100,447.5	1.5350	5.1167
615-1200	$Y = (11{,}916{,}184.5 \pm 26897.2) + (75{,}487.3 \pm 11{,}548.7)X$	0.9989	4,719,843.4	187.5750	625.2502

LOD: Limit of detection

LOQ: Limit of quantification

Table S2. Whole-genome sequencing and read mapping statistics.

Sample ID	Phenotype	No. reads	Mapped reads (%)	Sequencing depth	Final coverage
ZF_13	Black	22,757,862	96.16	2.77	1.72
ZF_14	Black	50,624,090	96.73	6.16	4.54
ZF_17	Black	14,491,464	96.35	1.76	1.32
ZF_18	Black	20,068,156	95.59	2.44	1.76
ZF_20	Black	15,439,034	95.70	1.88	1.38
ZF_21	Black	29,532,946	96.41	3.59	2.66
ZF_23	Black	11,551,688	95.16	1.41	1.02
ZF_25	Black	18,729,112	95.62	2.28	1.64
ZF_26	Black	27,534,182	95.64	3.35	2.41
ZF_30	Black	29,767,384	95.27	3.62	2.50
ZF_16	Orange	27,468,906	96.64	3.34	2.48
ZF_19	Orange	14,665,642	95.60	1.78	1.29
ZF_22	Orange	16,110,316	95.71	1.96	1.39
ZF_28	Orange	16,255,120	96.04	1.98	1.45
ZF_29	Orange	15,020,976	95.80	1.83	1.33
ZF_31	Orange	19,225,650	96.06	2.34	1.70
ZF_32	Orange	20,129,150	95.93	2.45	1.77
ZF_33	Orange	13,013,334	96.07	1.58	1.15
ZF_34	Orange	28,986,970	96.30	3.53	2.52
ZF_35	Orange	14,124,670	94.43	1.72	1.18
ZF_36	Orange	45,815,296	96.31	5.57	4.06
ZF_15	White	13,804,498	96.02	1.68	1.26
ZF_24	White	22,261,030	95.85	2.71	1.96
ZF_27	White	31,828,386	95.69	3.87	2.79
ZF_49	White	61,430,224	96.84	7.47	5.39
ZF_50	White	28,092,896	96.60	3.42	2.41
ZF_51	White	59,697,864	96.70	7.26	5.18
ZF_52	White	53,653,130	96.45	6.53	4.64

ZF_53	White	47,928,862	96.59	5.83	4.21
ZF_54	White	46,700,730	96.70	5.68	4.07
ZF_37	Wildtype	15,110,930	96.07	1.84	1.32
ZF_38	Wildtype	31,827,880	96.29	3.87	2.72
ZF_39	Wildtype	19,251,732	95.65	2.34	1.69
ZF_40	Wildtype	15,432,512	96.45	1.88	1.33
ZF_41	Wildtype	12,758,132	95.45	1.55	1.12
ZF_42	Wildtype	25,999,918	95.95	3.16	2.23
ZF_43	Wildtype	40,746,220	96.60	4.96	3.53
ZF_44	Wildtype	35,941,316	96.46	4.37	3.15
ZF_45	Wildtype	22,933,554	95.89	2.79	1.99
ZF_46	Wildtype	47,931,638	96.52	5.83	4.09
ZF_47	Wildtype	23,296,404	96.23	2.83	2.01

Figure S1. Sequence alignment of multiple bird species around the candidate splice-site mutation in ZNRF3. The candidate position is highlighted in orange. Strong sequence conservation at this position is shown, except for the orange breasted zebra finch. A scheme of the partial amino acid content of exon 2 and the location of the 5-prime mutated splice-site are shown, relative to the first intron. Directionality is given by the white arrow.

Reference

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CHAPTER V

Slc7a11 downregulation is rapidly reversed after cessation of competitive social stress in zebra finches

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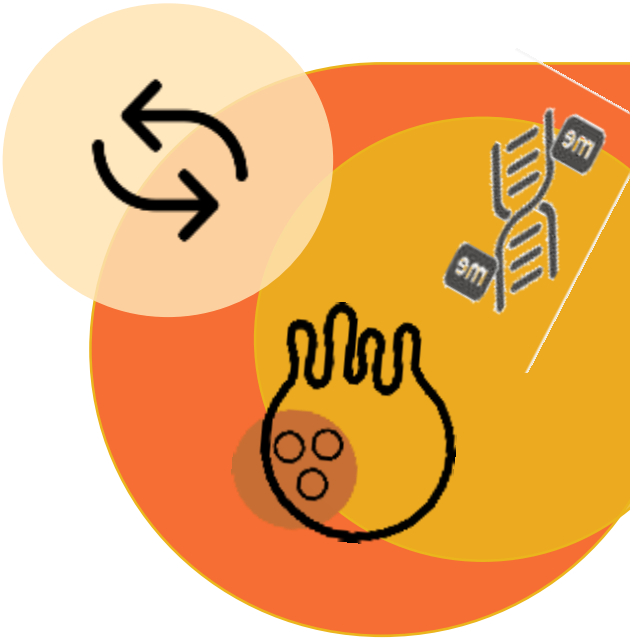

ABSTRACT

Background: Gene expression can be modulated by epigenetic modifications, which may lead to a rapid adaptation to environmental stress. After stress cessation, changes in gene expression could be reversed, which would allow organisms to maintain their phenotype under transient environments, but this mechanism is poorly understood. Social stress downregulates a gene directly involved in pheomelanin synthesis (*Slc7a11*) by changing DNA m^5C levels, avoiding cellular damage caused by stress. We thus investigated if *Slc7a11* expression is reversed in melanocytes of growing flank feathers to avoid changes in the pigmentation phenotype.

Methods and Results: We measured the expression level of *Slc7a11* at three time points: before stress exposure, immediately after stress exposure and six weeks after stress cessation in 37 male zebra finches (*Taeniopygia guttata*). No differences in *Slc7a11* expression were detected between birds exposed to stress and controls six weeks after stress elimination, indicating that stress removal led to a cessation of *Slc7a11* downregulation.

Conclusions: Reversibility in *Slc7a11* expression, probably mediated by reversible changes in DNA methylation, may thus avoid altering the pigmentation phenotype during transient stressful conditions. This is one of the few studies in vertebrates supporting the idea that reversible gene expression responses allow organisms adapting to changing environmental conditions.

KEYWORDS

Competitive social environment, environmental stress, gene expression, pheomelanin synthesis, DNA methylation reversibility, stress cessation.

INTRODUCTION

Environments constantly change, thus the ability to rapidly respond to fluctuations allows organisms to adapt to new conditions (Weake & Workman 2010) and avoid stress consequences (Storey, 1996). Otherwise, environmental changes may induce oxidative stress (Schröder & Krutmann 2005), i.e. the imbalance between reactive oxygen species (ROS) and antioxidant compounds. A powerful mechanism of adaptation to environmental stress implies changes in gene expression (López-Maury et al., 2008) thought the action of epigentic mechanisms (Champagne, 2010; Weaver et al., 2010; Zhang & Meaney, 2010) that can be rapidly returned to the basal state after stress disappearance (Sørensen et al., 2005; Yale & Bohnert, 2001), leading to changes in the phenotype (Gabriel, 1999). However, much work is still required to understand the mechanisms by which gene expression regulation acts as an environmental stress adaptation under changing conditions (Hawkins & Storey, 2020). In particular, the study of gene regulatory changes associated with social environmental factors is limited to a few species and, furthermore, the reversibility of gene expression after cessation of social stress is still poorly unexplored (Tung & Gilad, 2013).

The expression of pheomelanin-based coloration seems to be a physiological mechanism that allows organisms to deal with environmental stress. Pheomelanin is an orange-reddish pigment that is synthesized by melanocytes through the incorporation of the semi-essential amino acid cysteine, a constituent of the main cellular antioxidant glutathione (GSH). Thus, the downregulation of genes involved in pheomelanin synthesis induced by epigenetic mechanisms helps to avoid the damaging consequences of oxidative stress (Rodríguez-Martínez & Galván, 2019; Rodríguez-Martínez et al., 2019). Specifically, the downregulation of the gene *Slc7a11* (solute carrier family 7 member 11) has been shown to trigger physiological responses that allow some bird species to deal with stressful conditions (Rodríguez-Martínez & Galván, 2019; Rodríguez-Martínez et al., 2019; Galván et al., 2017; Galván, 2018). In some bird species, *Slc7a11* epigenetic lability may have evolved as an adaptive mechanism sensitive to different sources of environmental stress to adjust pheomelanin synthesis to the prevailing conditions and thus protect cells

against oxidative stress, actually representing a source of phenotypic plasticity (Galván, 2018).

As the regulation of gene expression is often reversible (Hawkins & Storey, 2020) and the regulation of genes involved in pheomelanin synthesis seem to be physiological advantageous, it is expected that the expression of *Slc7a11* can be reversed after stress disappearance if this mechanism can actually be considered an adaptive plastic response. Here we investigate if *Slc7a11* expression in melanocytes of growing feather is reversed after 6 weeks from the disappearance of an experimental inducement of environmental stress in male zebra finches (*Taeniopygia guttata*). We previously showed that a competitive environment downregulated *Slc7a11*, possibly allowing birds to avoid the expected oxidative damage from social interactions (Rodríguez-Martínez & Galván, 2019). Thus, we analyzed *Slc7a11* expression level in the same birds after the removal of the competitive environment to evaluate if gene expression is reversed, which would represent an avoidance of changes in the pigmentation phenotype under transient environments.

MATERIAL AND METHODS

Experimental design

The study was carried out at Estación Biológica de Doñana (Seville, Spain) with 37 captive adult male zebra finches. Birds were housed in cages (60 × 40 × 25 cm) with controlled temperature (average: 24 ± 0.5°C) and light-dark daily cycle (13L:11D) during the experiment. 23 of these birds had previously been exposed to environmental stress mediated by competitive social interactions, keeping them in groups of six birds during feather growth. These conditions led to a decrease of the levels of 5-methylcytosine (m^5C) in DNA of the *Slc7a11* gene and to a decrease of its expression level, which was significantly lower than that of control birds that had been kept alone (Rodríguez-Martínez & Galván, 2019). Fourteen of the birds used here had been controls during the experiment. After this procedure, all birds,

including the birds that had been in groups, were kept alone again. Six weeks under these conditions without competitive social interactions, pheomelanin-pigmented flank feathers were plucked again to quantify *Slc7a11* expression in follicular feather melanocytes (see Supplementary Material). *Slc7a11* expression levels at the end of exposure to social stress (Rodríguez-Martínez & Galván, 2019) are used here as "initial" gene expression levels, while expression levels after six weeks of stress removal are used as "final" levels (see Supplementary material 2).

Statistical analyses

We employed general linear models (GLMs) to evaluate if gene expression is reversed after stress cessation. We performed three GLMs considering the expression level of *Slc7a11* (ΔC_t) at three time points: before stress exposure, immediately after stress exposure and six weeks after stress cessation. ΔC_t was the response variable in each model. The previous experimental condition (social stress vs. control) was included in the models as a fixed factor, thus testing if ΔC_t levels differed between stressed and control birds in each time point.

All variables were $\log_{10}$-transformed prior to analyses to normalize their distributions. Inspections of residuals confirmed that the normality assumption was fulfilled. All statistical analyses were made with STATISTICA 13 (StatSoft, Tulsa, OK, USA).

RESULTS

Before social stress exposure, the expression of *Slc7a11* in the melanocytes of growing flank feathers of male zabra finches did not differ between the groups ($F_{1,31}$ = 1.87, P = 0.18). Social stress had a significant effect on *Slc7a11* expression level ($F_{1,34}$ = 5.63, P = 0.023), as already shown in our previous study. *Slc7a11* expression level in birds exposed to social stress (mean ± SE: 1.204 ± 0.005) was lower than that of control birds (mean ± SE: 1.185 ± 0.005). However, after 6 weeks from stress

elimination, the expression of *Slc7a11* did not differ between the birds that had been exposed to stress and control birds ($F_{1,33}$ = 1.47, P = 0.23), indicating that stress elimination made that birds stopped downregulating *Slc7a11* (Figure 1).

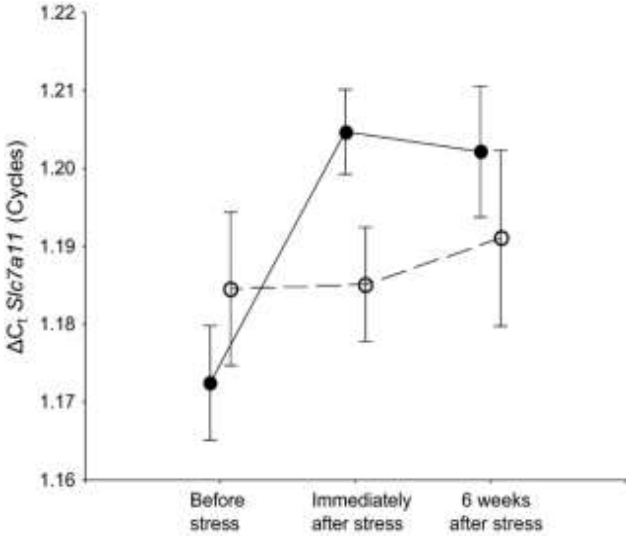

Figure 1. Normalized expression levels (ΔC_t) of the gene *Slc7a11* (mean ± SE) in melanocytes of growing flank feathers of male zebra finches exposed to competitive social stress (black circles) and control birds (open circles). Values before the beginning of the experiment, immediately after the experiment and six weeks after the experiment are shown. Higher ΔC_t values indicate lower gene expression.

DISCUSSION

Organisms can rapidly respond to environmental stress by regulating gene expression (De Nadal et al., 2014). Such response may be reversible to allow adapting to transient environmental stress without consequences in the phenotype (Gabriel, 2005). In male zebra finches, the downregulation of the gene *Slc7a11* in feather melanocytes mediated by decreases in m^5C levels in DNA seems to be a physiologically advantageous response against the oxidative damage that pheomelanin synthesis may induce under stress produced by a competitive social environment (Rodríguez-Martínez & Galván, 2019). Here, in the same experimental birds, we detected a change in *Slc7a11* expression after the termination of social stress. This suggests that the expression of *Slc7a11* in birds can be reversibly

regulated to adjust pheomelanin synthesis to the prevailing environmental conditions.

Slc7a11 is the main gene directly regulating pheomelanin synthesis in melanocytes (Rodríguez-Martínez et al., 2019). As a consequence of its regulation mechanism, *Slc7a11* expression affects cellular oxidative stress, as cysteine transported inside melanocytes and used to synthesize pheomelanin cannot be used for GSH synthesis and antioxidant protection. In male zebra finches, *Slc7a11* downregulation helps to avoid oxidative stress triggered by a competitive social environment (Rodríguez-Martínez & Galván, 2019) and exposure to a pro-oxidant substance (Galván et al., 2017). Furthermore, *Slc7a11* downregulation has also been found in feather melanocytes of Eurasian nuthatches *Sitta europaea* exposed to a natural source of environmental stress (predation risk) during development, leading to a decrease in the intensity of pheomelanin-based plumage coloration (Galván, 2018). Thus, environmental stress can determine the pigmentation phenotype of birds by inducing *Slc7a11* downregulation, which may lead to consequences for behavioural processes such as those involved in sexual selection (Galván & Rodríguez-Martínez, 2018). However, this phenotypic plasticity should be dependent on the duration of stress, as reversible changes in gene expression are expected under short stress periods (Gabriel, 2005). Our findings support this, as a short exposure (15 days) of male zebra finches to a competitive social environment led to *Slc7a11* downregulation (Rodríguez-Martínez & Galván, 2019), and a reversed state was then detected.

Our study shows that this reversion in gene expression can be fast, as the disappearance of differences in gene expression between experimental and control birds was observed only six weeks after the termination of social stress. Furthermore, we previously showed that social stress did not affect the pigmentation phenotype of birds despite changes in *Slc7a11* DNA methylation and expression, indicating transient cellular changes that help avoiding oxidative stress of pheomelanin production without phenotypic consequences (Rodríguez-Martínez & Galván, 2019). The present study shows that such transient cellular changes are also rapidly reversed, which is probably essential to avoid evolutionary consequences of changes in the external phenotype, like effects on sexual selection

(Galván & Rodríguez-Martínez, 2018), under rapid environmental changes. This finding is thus relevant to understand the adaptiveness of gene expression changes produced by stress (Gabriel, 2005).

After stress elimination, less cysteine is required for antioxidant protection, and excess cysteine is toxic to cells. Pheomelanin synthesis actually contributes to cysteine homeostasis (Rodríguez-Martínez et al., 2019). Thus, it may be physiologically advantageous for male zebra finches to stop downregulating *Slc7a11* in feather melanocyes after the termination of exposure to social stress. This may explain the reversibility in gene expression observed in this study. As the previous downregulation of *Slc7a11* in these birds under exposure to social competition occurred as a consequence of a decrease in DNA m^5C levels (Rodríguez-Martínez & Galván, 2019), it is likely that the reversibility of *Slc7a11* expression after stress cessation is also mediated by an epigenetic modification related to DNA m^5C. Adjusting the expression of genes involved in pheomelanin synthesis to stress conditions by reversible changes in DNA methylation may thus allow zebra finches to rapidly adapt to changing environments.

Reversible changes in gene expression mediated by epigenetic mechanisms are a known short-term strategy to deal with transient environmental conditions in plants (Boyko & Kovalchuk, 2008). In fact, however, gene expression in virtually any organism seems to be able to be rapidly reversed following stress elimination and return to baseline levels in time periods ranging from minutes (Sørensen et al., 2005; Yale & Bohnert, 2001) to weeks after stress cessation (Hijaz et al., 2019). In particular, the social environment affects gene expression, thus avoiding physiological consequences of environmental stress (Tung et al, 2012). However, a reversibility of gene expression together with a reversibility of the corresponding phenotype has only been reported regarding social status in honey bees (Herb et al., 2012) and cichlid fish (Burmeister et l., 2005). Our study supports the idea that reversible changes in gene expression are an adaptive mechanism to deal with transient environmental conditions, here exemplified by an episode of social competition, without phenotypic consequences. In particular, adjusting pheomelanin synthesis to changing environmental conditions seems to be a phenotypical plastic response to protect cells against oxidative stress. This, in turn,

makes the regulation of pheomelanin synthesis an adaptive strategy to avoid the evolutionary consequences of external phenotypic changes under rapid environmental changes. To our knowledge, this is the first study in vertebrates that shows gene expression reversibility after stress disappearance allowing organisms to adapt to transient environmental stress without consequences in their external phenotype.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare no conflicts of interest. This study was approved by the Bioethics Subcommittee of Consejo Superior de Investigaciones Cientificas (CSIC; ref. 651/2018) and local authorities (Consejería de Agricultura, Pesca y Medio Ambiente, Junta de Andalucía; ref. 23/02/2018/016).

AUTHOR CONTRIBUTIONS

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

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SUPPLEMENTARY MATERIAL

Measurement of gene expression in melanocytes

15-20 feathers were immersed in RNAlater solution (Ambion, Thermo Fisher Scientific) to stabilize and protect RNA, and stored at −80 °C. Melanocytes were isolated from the dermal papillae of feathers, which 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 samples. Total RNA was extracted using TRI Reagent (Ambion, Thermo Fisher Scientific) and quantifed with a Qubit 4 Fluorometer (Invitrogen, Termo Fisher Scientifc). Genomic DNA carry-over was removed with a TURBO DNA-free kit (Ambion). After extracting total RNA, complementary DNA (cDNA) was prepared from total RNA using RevertAid Reverse Transcriptase provided in the RevertAid First Stand cDNA Synthesis kit (Thermo Scientific, Thermo Fisher Scientific). Quantitative polymerase chain reaction (qPCR) was performed on cDNA of *Slc7a11*.

Quantitative polymerase chain 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. 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 primer was 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 for *Slc7a11* were 5'-GCTGGGGAATTGCTGCTTTC-3' and 5'-TGCAACGAAGAACATCCTGGA-3'.

Cycle threshold (C_t) levels were used to measure gene expression, with lower C_t values indicating more mRNA and higher gene expression levels. Normalization was made by subtracting C_t values for *GAPDH* from C_t values for *Slc7a11* (ΔC_t).

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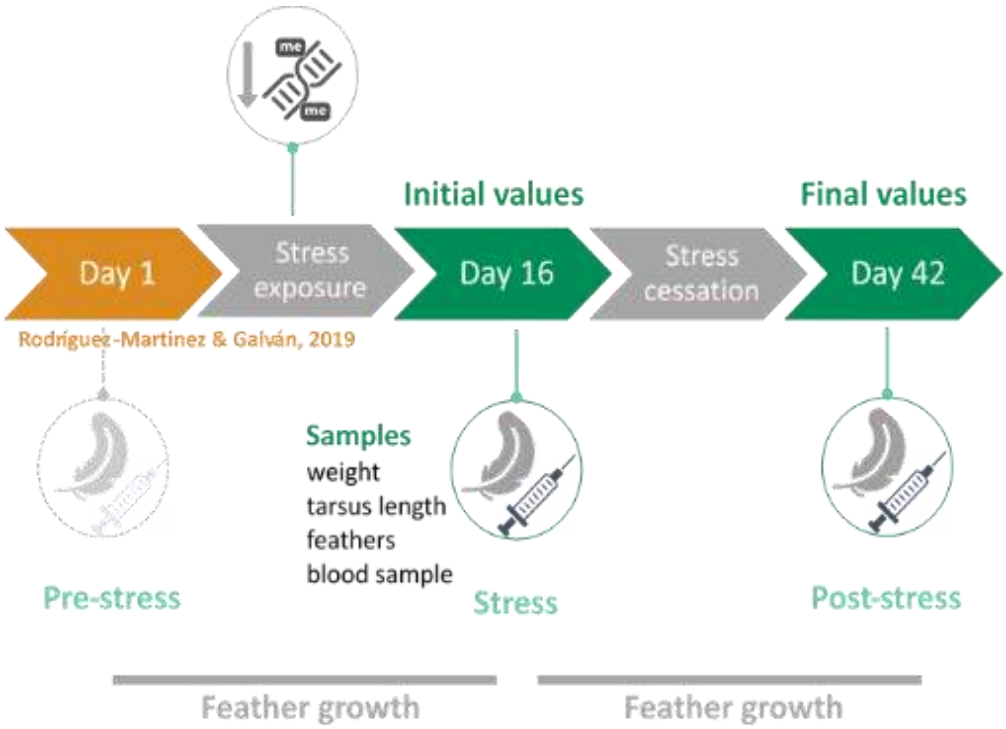
SUPPLEMENTARY MATERIAL 2



CHAPTER VI

Females mate with males with diminished pheomelanin-based coloration in the Eurasian nuthatch *Sitta europaea*

Galván, I., & **Rodríguez-Martínez, S.**

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ABSTRACT

Sexual selection can drive the evolution of phenotypic traits because of female preferences for exaggerated trait expression in males. Sexual selection can also lead to the evolutionary loss of traits, a process to which female preferences for diminished male trait expression are hypothesized to contribute. However, empirical evidence of female preferences for diminished male traits is virtually lacking. Eurasian nuthatches *Sitta europaea* provide an opportunity to test this possibility, as a chestnut flank patch produced by the pigment pheomelanin is present since the first plumage of these birds and its color is more intense in nestlings in poor condition in our study population. It has been proposed that developing birds in poor condition may increase their production of pheomelanin as a detoxifying strategy. Female nuthatches may thus prefer mating with males showing flank feathers of diminished color, as this could indicate that males experienced good conditions early in development, which can positively affect the fitness of future generations. Here we show results according with this prediction in a wild population of Eurasian nuthatches, as adult males with lighter chestnut feathers paired earlier in the season, while chestnut coloration had no effect on female mating success. Chestnut color expression was not affected by the body condition of birds, suggesting that females obtain information on the body condition in early life of their potential mates and not on their current body condition. This constitutes one of the few examples of females mating with males showing diminished traits and provides the only explanation so far by which this process can occur.

KEYWORDS

Early life effects, female preference for diminished traits, nuthatch, plumage coloration, pheomelanin, sexual selection

INTRODUCTION

Understanding variation in female preferences for male traits helps explain how phenotypes evolve by sexual selection (Jennions & Petrie, 1997). Some models of sexual selection predict that this occurs as an endless process in which periods of female preference for exaggerated phenotypic traits (e.g., large morphological or intense color characters) alternate with periods of preference for diminished traits (i.e., perpetual evolution; Mead and Arnold 2004). Indeed, phylogenetic comparisons between species show frequent losses of sexually selected traits (Wiens et al., 2011), a phenomenon to which female preferences for diminished male traits may contribute (Wiens, 2001). However, almost two decades after the proposal of this mechanism, the empirical evidence of female preferences for diminished male traits not associated to heterospecific mating avoidance or predation risk remains limited to one example already echoed by Wiens (2001): a population of the house sparrow *Passer domesticus* in which females, in contrast to females in other populations (Nakagawa et al., 2007), prefer mating with males displaying smaller black bib plumage patches (Griffith et al., 1999). This may suggest that the theoretical possibility of female preference for diminished male traits has not been assumed by researchers, as findings in this direction may be disregarded as incoherent evidence of sexual selection.

Although female preferences can drive the evolution of male traits by an arbitrary attractive-only effect (Fukamachi et al., 2009), empirical studies suggest that in most cases females obtain adaptive benefits from their choices, either directly by getting genetic pools that increase the viability of their offspring or indirectly by getting resources that help raising the offspring (e.g., Jaquiéry et al., 2010). In this regard, understanding the mechanisms that regulate the expression of male traits is essential to get insight into female preferences (Folstad & Karter, 1992), as these preferences are mostly understood through the information that male traits honestly signal about their ability to produce the traits (Zahavi, 1975). This information is in turn necessary to evaluate the likelihood that female preferences for diminished male traits actually operates at any time.

Recent findings in the Eurasian nuthatch *Sitta europaea* provide a unique opportunity to test a possibility of female preference for a diminished male trait. These birds are sexually dichromatic regarding a chestnut (dark orange) flank plumage patch produced by the deposition of the pigment pheomelanin in feathers, whose color intensity is higher in males than in females (Figure 1). This color patch is already present in the first plumage developed by nestlings, which are virtually identical to adults (Figure 1). However, the color intensity of this trait depends on the physical condition, a predictor of survival prospects (Matthysen, 1989), in nestlings but not in adults: nestling nuthatches in poorer body condition develop more intense flank feathers than nestlings in better condition in at least one population (Galván, 2017). As the synthesis of pheomelanin and its deposition in inert integumentary structures like feathers can help removing the amino acid cysteine, which is toxic if in excess and this excess is more likely to arise during the nesting stage (i.e., under low relative stress levels), it has been proposed that nestling nuthatches in poor condition increase their production of pheomelanin to pigment their plumage as an adaptive detoxifying strategy (Galván, 2017). Thus, plumage coloration in adult nuthatches may be a developmental consequence of selection operating in juveniles.

However, the plumage of nuthatches is sexually dichromatic as mentioned above, indicating that sexual selection operates on this trait (Heinsohn et al. ,2005). The absence of condition-dependence in adult nuthatches suggests that preferences of adult females for male flank coloration might be related to information different from the current body condition of males. Instead, adult females may obtain information about the condition experienced in early life by their potential mates by assessing the color intensity of their flank patches, as suggested for some morphological traits in other birds with marked sexual dimorphism (Carranza & Hidalgo de Trucios, 1993). This would only be possible if plumage color expression was correlated between years in individual males, and this is indeed likely, as bird color traits involved in sexual selection uses to be consistent across years (Galván & Møller, 2009; Gladbach et al., 2010; Chaine & Lyon, 2015). This information may be highly relevant for the fitness of female nuthatches, as conditions experienced in early life can have transgenerational effects (Burton & Metcalfe, 2014). In birds

specifically, it is known that stress experienced early during development can lead to poor body condition in the affected offspring and negative fitness-related effects in subsequent generations (Naguib et al., 2006; Goerlich et al., 2012). Therefore, female nuthatches would benefit from mating with males with a diminished expression of flank coloration, as this would be indicative of good physical condition during development (Galván, 2017). We thus tested this prediction in a wild population of Eurasian nuthatches using laying date as a measure of mating success, as laying date reflects pairing date (Potti &Montalvo, 1991; Johnsen et al., 1998).

MATERIAL AND METHODS

Field methods

The study was carried out during three consecutive breeding seasons (April-May 2015-2017) in the Natural Park of Sierra Norte de Sevilla, southern Spain (37°47’ N, 06°04' W). Frequent checks of wood nest boxes placed in the study area provided data on dates of clutch initiation (laying date) for all breeding pairs.

12-15 days after hatching, adults were captured and banded with numbered rings. The adults were weighed with a portable electronic balance to the nearest 0.1 g and their tarsus length was measured to the nearest 0.01 mm with a digital calliper. Sex was determined on the basis of the color intensity of the flank plumage patch, which is darker in males (Galván, 2017). We plucked 5-6 chestnut flank body feathers and stored them in the dark until reflectance measurements were made (see below). In total, 37 Eurasian nuthatches (17 males and 20 females) belonging to 27 breeding pairs were captured and examined for flank coloration. Flank feathers could not be taken in one female, and body mass could not be measured in other two females.

Analysis of plumage color expression

To analyze the color expression of flank feathers, we used an Ocean Optics Jaz spectrophotometer (range 220-1000 nm) with ultraviolet (deuterium) and visible (tungsten-halogen) lamps and a bifurcated 400 micrometer ber optic probe. The fiber optic probe both provided illumination and obtained light reflected from the sample, with a reading area of ca. 1 mm^2. 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 sample. All measurements were relative to a diffuse reflectance standard tablet (WS-1, Ocean Optics, Dunedin, FL), and reference measurements were frequently made. An average spectrum of five readings on different points of the feathers was obtained for each bird, removing the probe after each measurement. Reflectance curves were determined by calculating the median of the percent reflectance in 10 nm intervals. Spectral data was summarized as the summed reflectance across the 300-700 nm range. As pheomelanin reflectance spectra are approximately linear and spectral variation largely correspond to variation in the slope of these lines (Galván & Wakamatsu, 2016), we also calculated the slope of percentage reflectance regressed against wavelength as an alternative measure of pheomelanin-based color expression. In both parameters, summed reflectance and reflectance slope, lower values denote darker colors and higher color intensity. Then we determined which parameter was a better indicator of the pheomelanin content of feathers (see below). To minimize observer bias, reflectance measurements were made by a technician blindly from the aims of the study.

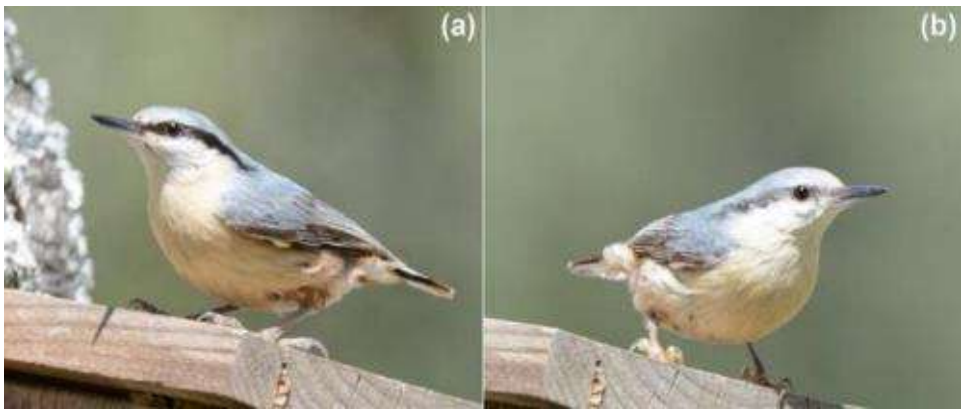

Figure 1. Male (a) and female (b) Eurasian nuthatches from the study area showing chestnut flank feathers (credits: Ismael Galván).

Analysis of feather pheomelanin content

The flank feathers of 16 nuthatch nestlings from 2017 were analyzed by micro-Raman spectroscopy to determine their relative content of melanins, as 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 et al., 2013; Galván & Jorge, 2015). We used 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 using a NIR excitation laser of 780 nm. 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 nestling. In each feather, we obtained five Raman spectra from different barbs and barbules. 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 et al., 2017). In the nuthatch feathers, we only detected Raman signal from pheomelanin, together with bands that can be assigned to keratin (Hsu et al., 1976) (Figure 2). Therefore, we used the three diagnostic bands of pheomelanin and four diagnostic bands of keratin (Figure 2a) to fit Voigt deconvolution functions to the Raman curves to obtain spectral parameters derived from each spectrum. An average Raman spectrum was calculated for the spectra obtained from the five feathers of each nestling.

In the analyses, we used the intensity (maximum value at the vertical axis) of the band at 2000 cm-1 calculated from the deconvolution functions as an index of pheomelanin content (Figure 2a), as this is a good predictor of pheomelanin concentration in feathers (Galván et al. 2013) and the 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 thus regressed the slope and the summed reflectance of feathers against the intensity of the 2000 cm-1 Raman band to determine which color trait better reflects the pheomelanin content of feathers. The slope of reflectance spectra was significantly correlated with the intensity of the 2000 cm-1 Raman band ($r = -0.51$, $n = 16$, $P = 0.042$; Figure 2b), while the summed reflectance was not ($r = -0.35$, $n = 16$, $P = 0.177$; Figure 2c). This indicates that the slope of the regression of reflectance values against wavelength decreases as the pheomelanin content of feathers increases (Figure 2b). Although the effect size is not large, its statistical significance with a sample size as low as 16 birds suggests that the association might be biologically relevant. We therefore used reflectance slope as an index of pheomelanin-based color expression in the analyses.

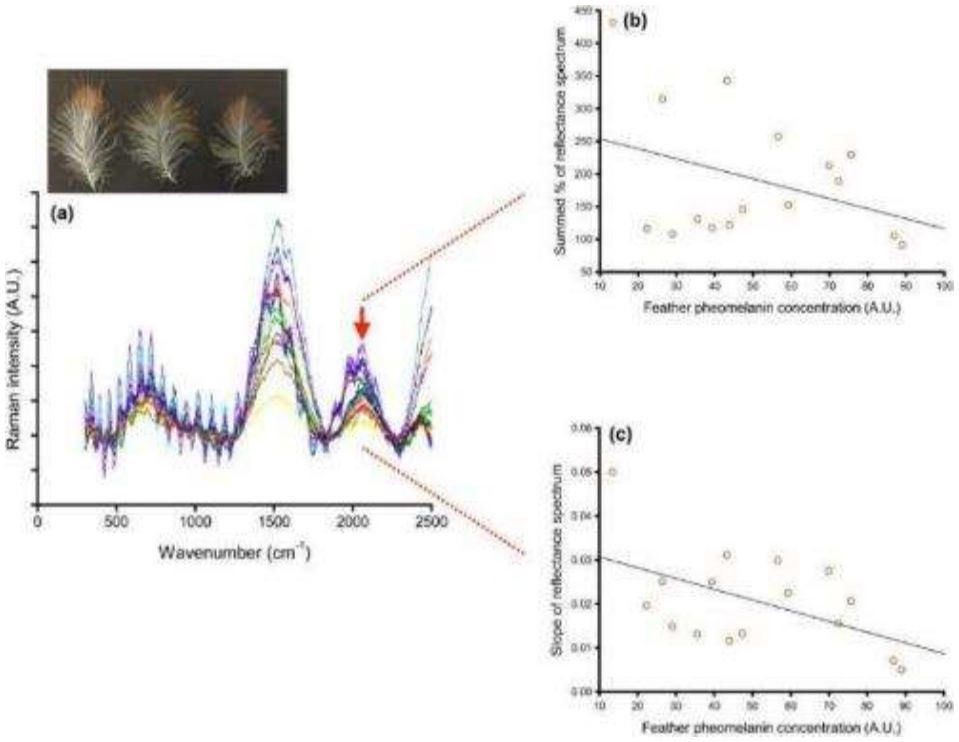

Figure 2. a: Raman spectra of flank covert feathers (shown in the insert photograph) of 16 Eurasian nuthatch nestlings. Each color corresponds to a different bird. The spectra show the three diagnostic Raman bands of pheomelanin at about 500, 1500

and 2000 cm^{-1}. The intensity of the band at 2000 cm^{-1} (marked with a red arrow) was used as an index of pheomelanin concentration in feathers. Other bands at about 1350, 1430, 1600 and 1700 cm^{-1}, corresponding to keratin, were also used to deconvolute Raman spectra. b: relationship between pheomelanin concentration and the summed percentage of reflectance across the 300-700 nm in the same feathers. The line is the best-fit line. c: relationship between pheomelanin concentration and the slope of reflectance spectra of the same feathers. The line is the best-fit line.

Statistical analyses

We tested for a potential dependence of laying date on the flank color expression of males and females separately by using general linear models (GLM). We standarized laying date (mean = 0, s.d. = 1) to compare values between the three years of study. Standardized laying date values thus represent deviations from the yearly means. We also tested for an association between flank color expression and body condition, the latter being calculated as the residuals of body mass regressed against tarsus length (Galván, 2017). Males and females were pooled in these models for body condition, and the sex of birds was added as a covariate to account for the effect of sexual dichromatism. Inspections of residuals from the models confirmed that normality assumptions were fulfilled. Lastly, we tested for a correlation between the color expression of males and females in 10 pairs in which we could capture both sexes.

RESULTS

Flank color expression explained 32.8 % of variance in the laying date of male nuthatches, exerting a significant and negative effect (b = -68.88, $F_{1,15}$ = 7.31, P = 0.016). This means that males displaying lighter chestnut plumage patches paired earlier in the season (Figure 3). In contrast, flank color expression in females only explained 21.8 % of variance in their laying date, and the effect was not significant (b = 15.98, $F_{1,17}$ = 1.64, P = 0.218; Fig. 3).

In the model testing for an effect of body condition on flank color expression, neither the interaction between body condition and sex ($F_{1,30}$ = 0.16, P = 0.688) nor body condition ($F_{1,31}$ = 0.88, P = 0.356) had a significant effect. Only the sex of birds

had a significant effect ($F_{1,34}$ = 10.7, P = 0.002), indicating darker flank plumage patches in males (mean ± s.e.: 0.024 ± 0.003) than in females (0.038 ± 0.003).

The correlation between the flank color expression of paired males and females was not significant (r = -0.40, n = 10, P = 0.250). This suggests that the flank color of males was unrelated to that of their mates.

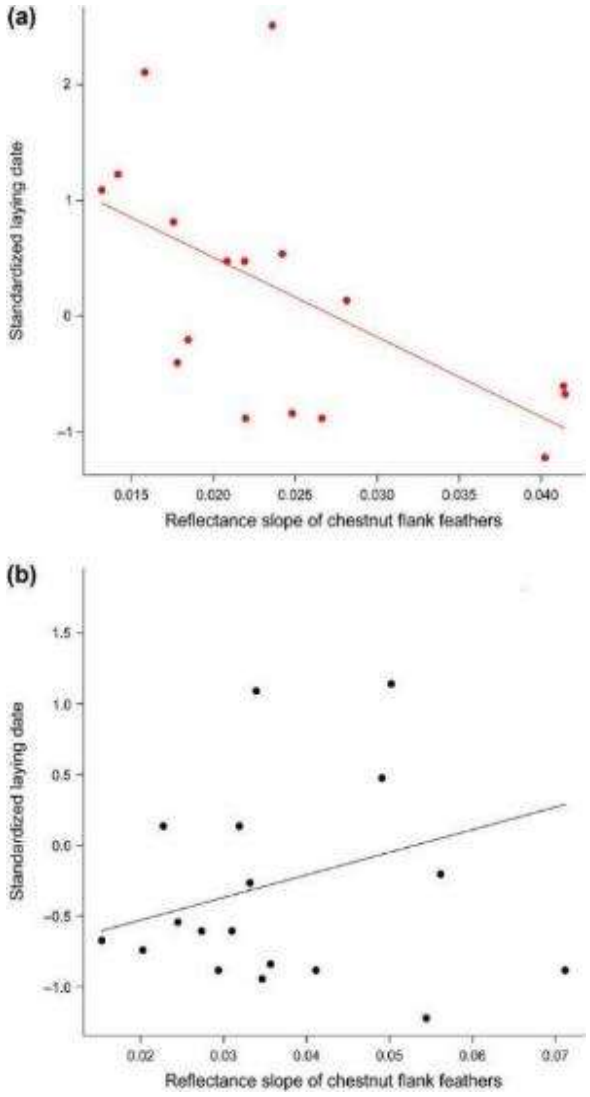

Figure 3. Relationship between laying date (standardized to reflect deviations from yearly means) and color expression level of chestnut flank feathers (measured as summed reflectance in the 300-700 nm spectral range) in male (red symbols and line) and female (black symbols and line) Eurasian nuthatches. Lower reflectance levels indicate darker (i.e., more intense) coloration. Lines are best-fit lines.

DISCUSSION

Our results suggest that female Eurasian nuthatches tend to mate with males with a diminished expression of chestnut flank coloration, as predicted on the basis that the intensity of chestnut coloration is higher in nestling nuthatches in poor body condition (Galván, 2017). In contrast, the flank coloration of female nuthatches does not affect their mating behavior, suggesting the existence of preferences of females to males but not the opposite. Accordingly, there was no assortative mating regarding flank color, as the color expression values of paired males and females were not correlated. In male birds, color traits that are involved in sexual selection tend to be consistent across years (Galván & Møller, 2009; Chaine & Lyon, 2015). Thus, and although we could not test this in nuthatches, it is likely that the intensity of flank color expression in male nuthatches is correlated across their multiple plumage molts throughout their lifespans, and therefore females could obtain information on the conditions experienced during early life by assessing the flank color expression of their potential mates. As birds reared under stressful conditions that lead to poor body condition (Krause et al., 2017) can exert a negative effect on the fitness of birds of subsequent generations (Naguib et al., 2006), the tendency of female nuthatches to mate with males with lighter flank coloration may be adaptive, as they may potentially avoid mating with males that provide a negative effect on their offspring. These effects can be exerted through the heritability of epigenetic mechanisms that influence the expression of genes that regulate stress responses (Goerlich et al., 2012).

As already noted by Galván (2017), the expression of the chestnut flank coloration does not depend on the current body condition of adult nuthatches, and thus, females cannot obtain information on the current body condition of their potential mates by assessing their flank coloration. This may thus be an exception to the observation that sexually selected traits often, but not always, show heightened condition-dependence and thus signal the capacity to afford the costs of producing the traits (Cotton et al., 2004). In this case, the reason for the absence of condition-dependence in adult nuthatches may be the proposed detoxifying function of pheomelanin, the pigment that produces the flank coloration and whose synthesis

increases in developing nuthatches in poor body condition (Galván, 2017). Therefore, in our study population the chestnut flank coloration is not a trait costly to produce whose expression is limited to individuals of high genotypic quality as predicted by the handicap principle (Zahavi, 1975), but is more intensely produced by individuals in poor condition probably as an adaptive detoxifying strategy. This is in accordance with recent proposals of alternatives to the handicap principle that are based on the knowledge of the physiological mechanisms regulating trait expression (Számadó & Penn, 2015; Galván & Alonso-Alvarez, 2017). This is also in accordance with the suggestion that the chestnut flank coloration of adult nuthatches is a developmental consequence of natural selection operating in nestlings, while sexual dichromatism in the chestnut flank coloration of nestling nuthatches is a developmental consequence of sexual selection operating in adults (Galván, 2017).

It must be noted that, in other passerine bird (the barn swallow *Hirundo rustica*), the expression of pheomelanin-based plumage coloration is positively related to body condition in nestlings (Arai et al., 2017) and to annual survival rate in adults (Galván & Møller, 2013), and, accordingly, females show a preference for males with intense plumage coloration in at least some populations (Scordato & Safran, 2014). Between-species comparisons indicate that the production of large amounts of pheomelanin to pigment plumage constrains physiological processes that require antioxidant protection by glutathione (GSH), the most important intracellular antioxidant, such as the development of large brains (Galván & Møller, 2011) or performance under exposure to ionizing radiation (Galván et al., 2014). This is because the sulfhydryl group of cysteine, a constituent amino acid of GSH, enters the pheomelanin synthesis pathway, thus pheomelanin production reduces the availability of cysteine for GSH synthesis and this can cause chronic oxidative stress (Napolitano et al., 2014). However, only an adaptive benefit of pheomelanin can explain its evolution, and such benefit may be the detoxifying capacity of pheomelanin by helping to avoid excess cysteine, which is toxic (Galván et al., 2012). Therefore, it is environmental conditions what determine whether pheomelanin production is physiologically advantageous or detrimental (Galván et al., 2012), which may explain differences in the condition-dependence and signaling content of

pheomelanin-based coloration between species. In fact, we have evidence that the expression of color of flank feathers in Eurasian nuthatch nestlings reared in a population different from the population of the present study, with different levels of environmental oxidative stress, shows the reversed pattern to that found here (i.e., color intensity is positively associated to body condition; Galván & Sanz, unpublished results). Future studies should thus consider environmental gradients of oxidative stress when explaining the signaling content of pheomelanin-based coloration.

To our knowledge, our study is the only finding suggesting that females may show preferences for a diminished male trait not associated to heterospecific mating avoidance or predation risk since the results provided by Griffith et al. (1999) in a population of house sparrows. This possibility, however, theoretically exists in certain models of sexual selection (Mead & Arnold, 2004) and may actually be responsible for the evolutionary loss of sexually selected traits observed in some clades (Wiens, 2001). The potential detoxifying function of pheomelanin-based pigmentation provides a mechanism by which females tend to mate with males with a diminished expression of pheomelanin-based coloration as we found here, thus representing the first mechanistic justification for such female mating behavior. Indeed, the evolution of signals can be understood only by considering the physiological mechanisms that regulate their expression (Galván & Alonso-Alvarez, 2017).

The question now arises why the flank plumage patch of nuthatches does not become unpigmented, if females tend to mate with males with lighter flanks and this potentially provide them with higher fitness benefits. The answer might be in the stressful factors that create differences in the body condition of nuthatches during development. Nuthatch populations may differ in the expression of flank coloration, which would suggest that populations may differ in the intensity of stressful factors and maybe also in the intensity of sexual selection (Galván & Sanz, unpublished results). Thus, similar to the black bib of male house sparrows (Griffith et al., 1999; Nkagawa et al., 2007), preferences of female nuthatches for the flank coloration of males may differ between populations with different intensity of stressful factors affecting body condition in early life. This should be investigated by future studies,

as it may help understanding seemingly paradoxical findings in sexual selection and obtaining a more complete view of the influence of this process in phenotypic evolution.

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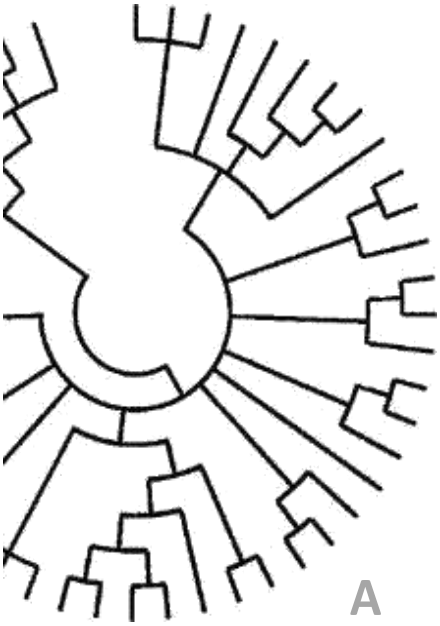
SECTION 2

Adaptive value of expressing pheomelanin-based pigmentation phenotypes

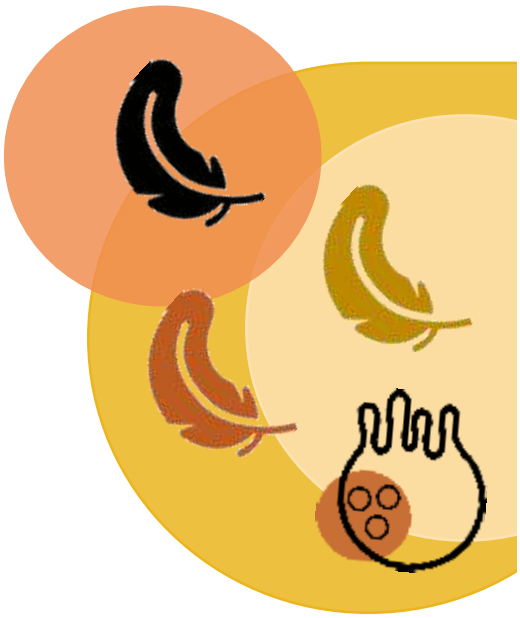


CHAPTER VII

A negative association between melanin-based plumage color heterogeneity and intensity in birds

Galván, I., & **Rodríguez-Martínez, S.**

Physiological and Biochemical Zoology, 92(3), 266-273. (2019)



ABSTRACT

Even though plumage diversity is one of the most diverse phenotypic traits in nature, the reasons why some species exhibit more distinctive colors than others are poorly known. In the case of melanins, the most abundant pigments in birds, different chemical forms lead to different plumage colors and different amounts of those forms lead to different color intensities. However, the synhesis of some melanin forms is more physiologically limited than others. We hypothesize that an evolutionary solution to this scenario may consist in a negative association between melanin-based color heterogeneity and intensity. Here we confirm this prediction after analyzing the diversity and expression level of melanin-based plumage colors in 96 species of birds breeding in the Iberian Peninsula. After controlling for phylogenetic effects, the intensity of the plumage colors of birds decreased with the number of different colors, suggesting that the physiological mechanism of melanin synthesis does not favor the production of both a heterogeneity of melanin forms and large amounts of these forms. These findings contribute to a better understanding of bird phenotypic diversity.

KEYWORDS

Birds; color heterogeneity; melanin-based pigmentation; phylogeny; plumage coloration

INTRODUCTION

Understanding the mechanics of plumage coloration helps to explain the evolution of bird phenotypic diversity (Hubbard et al., 2010). Some comparative studies on plumage coloration suggest that differences in body size can explain the observed interspecific variability in color heterogeneity (i.e., number of colors; Galván et al., 2013). However, species do not only differ in color heterogeneity, but also in the level of expression (i.e., intensity) of these colors (Gomez & Théry, 2004). In some

cases, interspecific differences in plumage color intensity respond to the adaptive benefits of avoiding hybridization between closely related species (Sætre et al., 1997), or are associated with different light environments (McNaught & Owens, 2002). Hybridization avoidance, however, cannot represent a general explanation for the interspecific variation in bird plumage color intensity (McNaught & Owens, 2002), and it is likely that environmental light is neither a general explanation as derived from its weak association with bird color heterogeneity (Galván et al., 2013).

Instead of, or in addition to the ecological variables mentioned above, the physiology of plumage color production may provide an explanation for the interspecific variation in color expression levels. A detailed knowledge of these physiological aspects, however, has never been explored in a comparative context of plumage coloration.

Melanins are the most common pigments responsible for the plumage color of birds, and are synthesized in specialized cells called melanocytes in a process that comprises physiological limitations, particularly regarding the synthesis of certain chemical forms (Galván & Solano, 2016). Therefore, it is likely that producing several forms of melanins, which is required to generate several colors (Galván & Wakamatsu, 2016), is more constraining than producing a single or few melanin forms (and a single or few colors) to pigment the plumage. An evolutionary solution to this physiological limitation may consist in producing low amounts of different melanin forms. An economy of pigments in fact represents an explanation for the evolution of melanin-based color traits (Galván & Solano, 2009). Such adaptive economy should lead to a negative association between the production of different colors and the level of expression (i.e., intensity) of each color, as variations in the concentration of melanins in feathers are reflected by variations in plumage color intensity (McGraw et al., 2005; Galván & Wakamatsu, 2016). We tested this prediction in 96 species of birds breeding from the Iberian Peninsula showing a large variability in melanin-based plumage color patterns.

METHODS

Bird Species Selection

Color data of plumage pigmentation corresponding to 96 birds collected in peninsular Spain (table S1), was obtained from museum skins deposited in the bird collection of Doñana Biological Station, Consejo Superior de Investigaciones Científicas (EBD, Sevilla, Spain). In these birds, we observed up to five clearly distinguishable colors (hues) (Table S1). Only birds with plumage pigmented by melanins (Galván & Wakamatsu, 2016) were considered, excluding those with plumage coloration produced by other pigments such as carotenoids, or structural colors. One of the selected species (*Petronia petronia*) has a small yellow patch produced by carotenoids, but its small size makes that its contribution to overall plumage reflectance is negligible. Sexually dichromatic species were also excluded because specific values of the analyzed traits cannot be assigned to species in which sexes differ in pigmentation patterns.

Plumage Color Data

To characterize the pigmentation phenotype of birds, we used two adult specimens of each species (Table S1) in which we determined the different color hues that we could perceive, and obtained the reflectance spectrum of each of them to quantify the intensity of color expression. Unpigmented plumage areas, bill and legs were excluded from the analysis. Thus, reflectance measurements were only indicative of melanin-based colored plumage areas.

To obtain the reflectance spectra of the different colors observed in the plumage of birds, we used 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 provided both illumination and obtained light reflected from the sample, with a reading area of ca. 1 mm^2. All measurements were taken at a 90° angle to the sample and reference

measurements on reflectance standard tablet (WS-1, Ocean Optics) were frequently made. For each specimen, five randomly readings were obtained for each color hue, removing the probe after each measurement. Reflectance curves were determined by calculating the median of the percent reflectance in 10 nm intervals. For each color hue, color intensity was summarized as the summed reflectance across the 300-700 nm range, with lower values (i.e. darker colors) denoting higher color intensity.

As each color patch covers a different proportion of plumage, the pigmentation of a species cannot be characterized by equally treating the reflectance of different colors. Thus, we weighted the summed reflectance of each hue by the proportion of plumage covered by the corresponding color patch. The total number of pixels of each perceived hue was calculated, and then divided by the total number of pixels of the entire plumage, to thus obtain the proportion of plumage covered by each color patch. To calculate the pixels covered by each hue, we obtained two digital photographs of each bird specimen, one of the ventral side and other of the dorsal side. The photographs were taken from a fixed distance and under standardized light conditions, and analyzed with GIMP software.

Statistical Analyses

Considering that species are evolutionarily related through a phylogeny, they cannot be treated as independent sample units in statistical analyses (Felsenstein, 1985). We obtained 1,000 probable phylogenies for the 96 species used in the study from www.birdtree.org, where branch lengths are related to the proportion of nucleotide substitutions among taxa (Figure S1). We obtained the consensus tree of the 1,000 probable phylogenies using the R package *phytools* (Revell, 2012).

However, controlling for phylogenetic effects in the analyses may not be correct under the absence of phylogenetic signal in the studied traits and particularly in the residuals of the model being tested (Symonds & Blomberg, 2014). We thus estimated the phylogenetic signal in the studied traits and in the residuals of the model (see below) using Blomberg's K statistic (Blomberg et al., 2003). K = 1 indicates

that a strong phylogenetic signal exists and that trait evolution follows Brownian motion. If K > 1, then close relatives are more similar than expected under Brownian motion, while, if K < 1, closely related species are less similar than expected under Brownian motion due to a loss of phylogenetic signal (Blomberg et al., 2003). We estimated Blomberg's K using the *phylosig* command of *phytools* (Revell, 2012).

We used phylogenetic generalized least squares (PGLS) models to analyze the relationship between plumage reflectance (in ln) (response variable) and number of colors (in ln) (continuous predictor), using the pgls command of the caper package (Orme et al., 2013). Body mass could be a confounding variable, as this can explain interspecific variability in plumage color heterogeneity (Galván et al., 2013). However, body mass could not be included as an additional predictor in the PGLS model because it was strongly positively related to the number of colors ($F_{1,94} = 4.79$, $P = 0.03$) and, furthermore, it was not related to plumage reflectance ($F_{2,93} = 10.57$, $P = 0.51$). This indicates the absence of confounding effects by body mass. In our PGLS model, we assume different patterns of trait variation throughout the phylogeny using branch length modifiers that we calculated through the maximum likelihood estimation of the Pagel's δ parameter (Pagel, 1999).

RESULTS

The number of colors and the intensity of colors of the 96 species of birds analyzed had a low phylogenetic signal, with K-values < 1 indicating a loss of phylogenetic signal (Table 1). However, the K-value for the number of colors was significantly different from zero (Table 1), discarding an absence of phylogenetic signal. The phylogenetic signal of the residuals of the PGLS model was also low (K = 0.35), but its associated p-value was not excessively high (P = 0.185). Overall, this indicates the necessity of controlling for phylogenetic effects in the analysis of the relationship between plumage reflectance and number of colors.

Table1. Phylogenetic signal of plumage reflectance (i.e., color intensity), number of colors and body mass for the 96 species of birds included in the study. The Blomberg's K statistic and the p-value for H_0: K = 0 are shown.

	K	P
Plumage reflectance (in ln)	0.34	0.19
Number of colors (in ln)	0.52	< 0.001
Body mass (in ln)	2.61	< 0.001

The PGLS model showed that the relationship between plumage reflectance and number of colors was significant ($P < 0.001$, $\delta = 1.13$). As predicted, the number of colors had a positive effect on plumage reflectance ($b = 0.90$, $P < 0.001$), indicating that the intensity of colors decreases with their heterogeneity and thus that the colors of birds with more colors are lighter than the colors of birds with fewer colors (Figure 1).

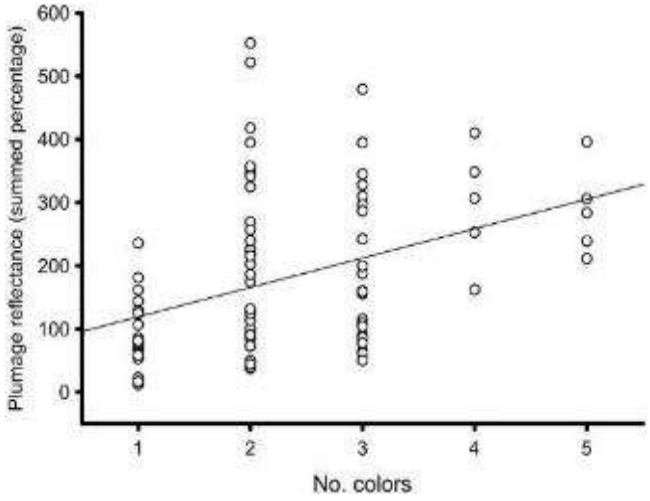


Figure 1. Relationship between melanin-based plumage reflectance and number of different colors in 96 species of birds. Higher reflectance values indicate less intense (i.e., lighter) colors. The line is the regression line.

DISCUSSION

Birds synthesize melanins in melanocytes located in the skin, which control the type and amounts of melanins that are deposited in the growing feathers (Lin et al., 2013). The diversity of colors that melanins confer to feathers is given by the chemical

heterogeneity of these pigments, with the sulphurated forms termed pheomelanins generating lighter colors (yellowish to chestnut) and the non-sulphurated forms termed eumelanins generating darker colors (black, brown and grey) (Galván & Wakamatsu, 2016). The synthesis of pheomelanin only occurs, however, when sulfhydryls in melanocytes are above a threshold level (Ito & Wakamatsu, 2008). As these sulfhydryls are mainly composed by a tripeptide that acts as the main intracellular antioxidant (i.e., glutathione, GSH) and the free form of its constituent amino acid cysteine, the synthesis of pheomelanin represents the consumption of a valuable antioxidant resource and thus represents a constraining process when antioxidants are required for other vital processes (Napolitano et al., 2014). The production of pheomelanins is therefore more limited in physiological terms than that of eumelanins, and thus not all melanin-based colors are equally favored during the process of melanin synthesis. The existence of a physiological mechanism that maintains cysteine homeostasis in cells (Stipanuk et al., 2009) makes that pheomelanin synthesis is only favored under certain physiological and environmental conditions (Galván et al., 2012), as cysteine depletions are not likely to occur.

Given these conditions, a production of large amounts of melanins (which leads to a high color expression) that includes pheomelanins is not expected to be physiologically favored. An evolutionary solution to this unviable physiological scenario may consist in producing small amounts of each melanin form when their chemical heterogeneity has been selected to be large (i.e., when the diversity of pheomelanins is included), which may be possible given the independent control of pigmentation exerted by melanocytes (Lin et al., 2013). We hypothesize that this evolutionary solution, which may be favored by natural selection, explains the negative association between melanin-based color heterogeneity and expression that we found in the plumage of 96 species of birds. A similar negative association between the expression of phenotypic traits was reported for song elaboration and plumage ornamentation in a group of birds (Badyaev et al., 2002). However, these authors interpreted their results as the consequence of trade-offs, resolved by birds, between producing different costly traits. We suggest a more mechanistic interpretation of these negative associations: instead of the result of 'conscious'

trades by birds, plumage patterns consisting in colors that are both heterogeneous and intense are not produced because they are not favored by the physiological conditions under which melanins are synthesized.

The plumage of birds is one of the most diverse phenotypic traits found in nature, and considering the physiological mechanisms that control the production of pigments has begun to provide a context on which some general rules can be applied to understand how such diversity has evolved (Galván et al., 2017). This study adds a new element to that context, namely that heterogeneous coloration is incompatible with intense coloration. Although we focused on melanins, these are the most abundant pigments in birds (Delhey, 2015), and therefore these findings are of general validity to understand the evolution of bird phenotypic diversity.

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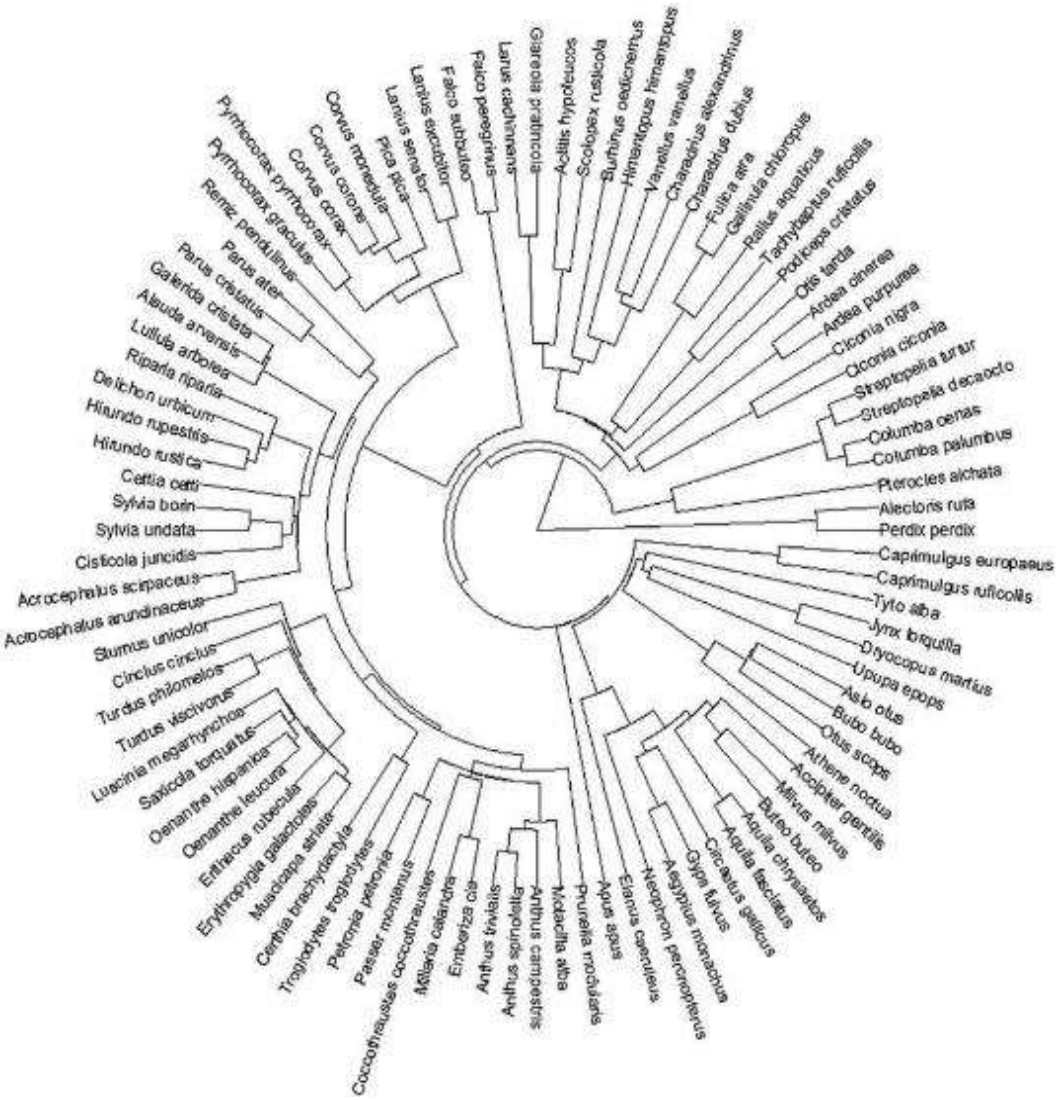
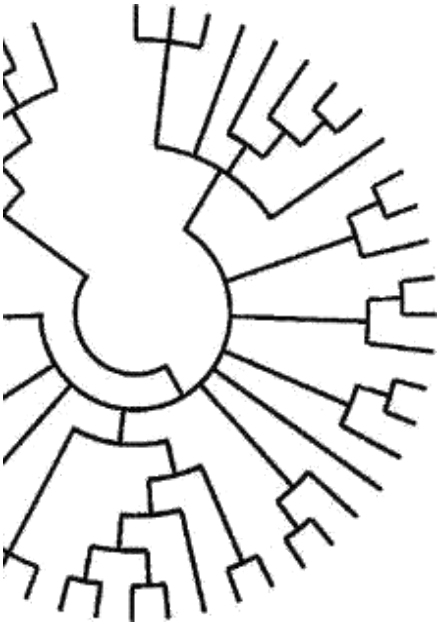
SUPPLEMENTARY MATERIAL

Table S1. Species names, plumage reflectance values, number of colors and body mass for the 96 species of birds included in the study.

Species	No. colors	Reflectance (summed percentage)	Body mass (g)
Accipiter gentilis	1	143.68	800
Acrocephalus arundinaceus	2	552.40	31
Acrocephalus scirpaceus	2	394.72	13
Actitis hypoleucos	2	95.59	46
Aegypius monachus	1	129.45	10,000
Alauda arvensis	2	348.91	45
Alectoris rufa	5	283.58	425
Anthus campestris	2	91.61	24
Anthus spinoletta	1	66.22	22
Anthus trivialis	2	86.42	23
Apus apus	1	69.67	42
Aquila chrysaetos	1	78.23	4,775
Aquila fasciatus	1	53.21	2,050
Ardea cinerea	2	220.07	900
Ardea purpurea	3	155.97	1,000
Asio otus	3	242.41	328
Athene noctua	1	77.37	170
Bubo bubo	3	328.19	2,250
Burhinus oedicnemus	2	341.82	410
Buteo buteo	1	235.69	875
Caprimulgus europaeus	3	105.19	88
Caprimulgus ruficollis	3	115.00	75
Certhia brachydactyla	2	84.66	10
Cettia cetti	2	226.73	14
Charadrius alexandrinus	3	159.95	50
Charadrius dubius	2	204.06	40
Ciconia ciconia	1	124.79	3,250
Ciconia nigra	1	180.94	3,000
Cinclus cinclus	2	123.31	65
Circaetus gallicus	1	161.40	1,750
Cisticola juncidis	2	356.15	10
Coccothraustes coccothraustes	5	305.86	55
Columba oenas	4	410.18	295
Columba palumbus	4	252.67	485
Corvus corax	1	73.82	1,145
Corvus corone	1	85.82	518
Corvus monedula	2	72.52	245
Delichon urbicum	1	58.41	18
Dryocopus martius	1	106.74	325
Elanus caeruleus	2	202.32	233
Emberiza cia	5	211.36	25
Erithacus rubecula	2	125.73	19
Erythropygia galactotes	3	479.27	21
Falco peregrinus	3	345.09	950
Falco subbuteo	2	225.46	235
Fulica atra	2	100.11	775
Galerida cristata	2	417.70	40

Figure S1. Consensus phylogenetic tree for the 96 species of birds included in the study obtained from www.birdtree.org. Branch lengths are proportional to nucleotide substitutions.



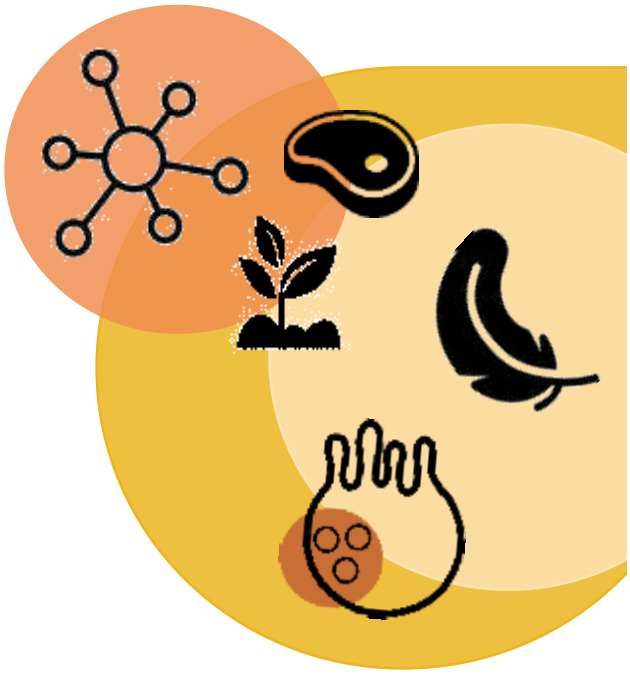


CHAPTER VIII

Juvenile pheomelanin-based plumage coloration has evolved more frequently in carnivorous species

Sol Rodríguez-Martínez & Ismael Galván

Ibis, 162(1), 238-244.



ABSTRACT

Distinctive pheomelanin-based plumage coloration in juvenile birds has been proposed as a signal of immaturity to avoid aggression by older conspecifics, but recent findings suggest a detoxifying strategy. Pheomelanin synthesis implies the consumption of cysteine, a semi-essential amino acid that is necessary for the synthesis of the antioxidant glutathione (GSH) but that may be toxic if in excess in the diet. As the nestling stage probably represents a low-stress period with limited requirement for GSH protection, the synthesis of pheomelanin in developing birds may help to maintain cysteine homeostasis, particularly in species with a high content of protein in the diet (i.e. carnivores). Here we confirm this hypothesis showing that, among 53 species of Western Palaearctic birds, juvenile pheomelanin-based coloration has evolved more frequently in strictly carnivorous species than in species with other diets.

KEYWORDS

Cysteine excess, diet, juvenile plumage, pheomelanin.

INTRODUCTION

Bird plumage coloration usually changes with age (Booth, 1990): plumage coloration becomes less dull in adult birds (Kilner, 2006). However, some species not only differ in colour intensity but also in the pattern of pigmentation (Moreno & Soler, 2011). The reasons that have favoured the evolution of these age-related changes are unclear, but understanding the mechanisms that regulate colour production may help to provide insight into how pigmentation affects the way animals interact with their environment. This is because different forms of melanins, the most common pigments produced, change with age, implying different physiological constraints between juvenile and adult birds (Galván & Solano, 2009).

Some comparative studies suggest that distinctive juvenile plumage has evolved as a signal of sexual immaturity to avoid aggression between conspecifics (Ligon & Hill, 2013), and it has been assumed that the expression of juvenile plumage involves lower physiological costs than the expression of adult plumage (Moreno & Soler, 2011). Alternatively, instead of a signalling function, the plumage displayed by juvenile birds may have evolved as a result of a detoxifying strategy (Galván, 2017). This is because the reddish-chestnut coloration that is usually characteristic of the plumage of juvenile birds (del Hoyo et al., 1992–2002; 2003–2010) is the result of the presence of pheomelanin in feathers (Galván, 2017) and the synthesis of this pigment implies the consumption of the main intracellular antioxidant and physiological reservoir of cysteine (i.e. glutathione, GSH). Using this antioxidant to synthesize pigments removes it from circulation, which may induce chronic oxidative stress in the individual (Napolitano et al., 2014).

Cysteine is a semi-essential amino acid that birds largely obtain from the diet, but it causes problems such as metabolic acidosis, thinning of eggshells and poor growth when levels in the individual are higher than needed for protein and GSH synthesis (Klasing, 1998). As feathers are inert structures in which cysteine cannot exert any physiological effect, the incorporation of cysteine in feathers during the synthesis of pheomelanin may help to avoid toxicity due to excess cysteine. Juvenile birds may generally suffer less physiological stress than adults (Galván et al., 2017) due to the reduction of physical exercise resulting from not having to find resources on their own, given they are being fed by their parents (Vaanholt et al., 2007, 2008). These relative low stress levels may make juvenile birds more prone to the accumulation of excess cysteine, as they may have lower requirements of GSH for antioxidant protection (Galván, 2017). In particular, animal food sources tend to have higher protein contents than plant-based food sources, and the latter are harder to digest (Millward, 1999; Mariotti, 2017). This suggests that, even at similar cysteine contents, cysteine in carnivorous diets is more physiologically available than cysteine in non-carnivorous diets. Thus, the distinctive pheomelanin-based plumage of juvenile birds may have evolved because of its potential benefits of removing excess cysteine, especially in species with a high protein content in the diet (i.e. carnivores; see Bertagnolli & Wedding, 1977; Nikiforova et al., 2002). For instance,

it has recently been shown that developing Gyrfalcons *Falco rusticolus*, a strictly carnivorous bird, upregulate the expression of *CTNS*, a gene that pumps cystine (i.e. the dimer of cysteine) out of the organelles where melanin synthesis takes place in melanocytes (melanosomes), in response to a high food abundance in their rearing territories (Galván et al., 2017). *CTNS* expression thus avoids cysteine accumulation in melanosomes, which may in turn inhibit pheomelanin synthesis (Chiaverini et al., 2012). However, not all species may be able to adjust *CTNS* expression to protein (and cysteine; Stipanuk et al., 2009) intake (Galván et al., 2017) and in the absence of *CTNS* environmental lability the synthesis of pheomelanin may constitute an alternative mechanism to help avoid cysteine accumulation and toxicity. Nevertheless, the detoxifying hypothesis for juvenile plumage has never been explored in a comparative context across species of birds. We therefore predict that juvenile plumage produced by pheomelanin-based colours and that is distinctively different from that of adults should have evolved more frequently in carnivorous species than in non-carnivorous species. We test this prediction in 53 species of birds from the Western Palaearctic that exhibit different diet habits and melanin-based plumage colour differences between adult and juvenile birds.

METHODS

Data collection

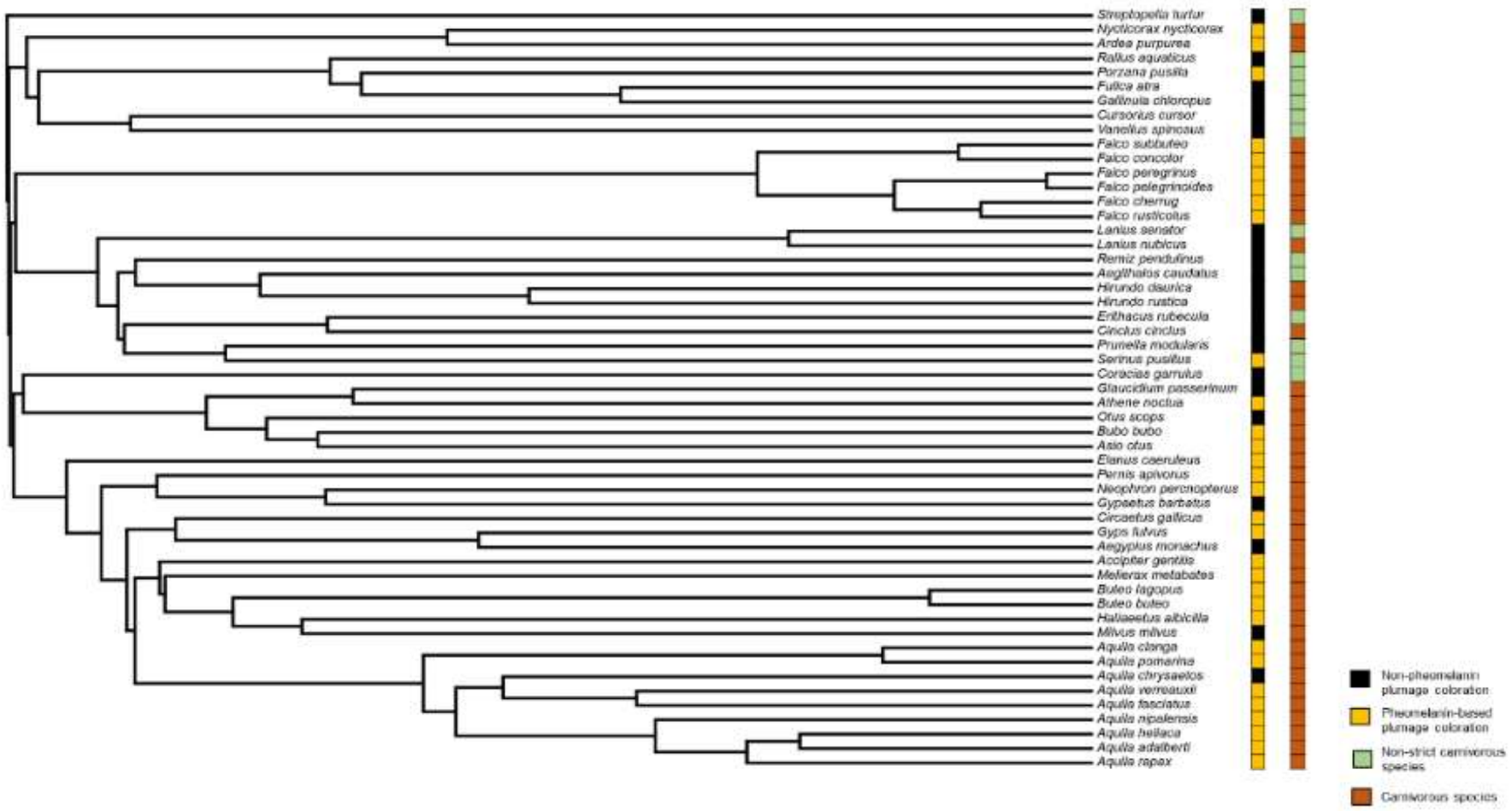
Information on plumage differences between juvenile and adult birds corresponding to 53 species from the Western Palaearctic was obtained by examining colour plates in a bird identification guide (Svensson et al., 2000). Only birds with melanin-based plumage were selected (Galván & Wakamatsu, 2016), excluding birds with plumage coloration resulting from other pigments, such as carotenoids, or feather structures, because these are produced by mechanisms different from those that regulate the synthesis of the characteristic pigment of the plumage of juvenile birds, pheomelanin. We also excluded species in which diet changes between seasons, as seasonal variation may affect the protein content of the diet and plumage coloration, hence confounding the results. Birds with marked sexual dichromatism were also

excluded, as sex-related effects may be difficult to control for and sexual selection could have a stronger effect on the expression of juvenile plumage than diet. Therefore, including birds with dichromatic or seasonal plumage would prevent us from conducting comparisons in similar conditions because there may be other evolutionary pressures at work. Changes in plumage coloration between juveniles and adults were scored into two categories. A score of 1 was assigned to species in which the juvenile plumage displays pheomelanin-based coloration, i.e. exhibits any of the colours that are produced by pheomelanin in feathers (orange or light brown; Galván & Wakamatsu, 2016), and the adult plumage does not (Figure 1). A score of 0 was assigned when the juvenile plumage displays any of the colours that are produced by the other main form of melanin, termed eumelanin, whose production does not consume cysteine-GSH (black, grey or dark brown; Galván & Wakamatsu, 2016), and the adult plumage does not. A score of 0 was also assigned to species in which the juvenile plumage only differs from that of adults in intensity, not in type, of pigmentation (Figure 1, Table S1).

Information on the diet of species was obtained from specific sources for each group of species (Cramp & Simmons, 1988; del Hoyo et al., 1992–2002; Ferguson-Lees & Christie, 2001; Lefranc & Worfolk, 1997). Only species whose diet does not change between breeding and non-breeding seasons were considered, as variation in the protein content of diet may affect plumage coloration. Each species was categorized as a strict carnivore (1) when it consumes only animal protein, a non-strict carnivore (0) when it has an omnivorous diet or as non-carnivorous when animal protein is never present in its diet (Figure 2, Table S1). As only one species was categorized as non-carnivorous (the European Turtle Dove *Streptopelia turtur*), we considered it non-strict carnivorous.

Figure 1. Images of two congeneric species of strictly carnivorous birds with different age-related plumage pigmentation changes. black colours represent eumelanin-based plumage colouration while grey colours correspond to pheomelanin-based colouration. (a): juvenile (left) and adult (right) Spanish Imperial Eagles *Aquila adalberti*. Figure taken from Negro et al. 2018 (CC BY 4.0 license: https://creativecommons.org/licenses/by/4.0/). (b): juvenile (above; photograph courtesy of Carlos Pacheco) and adult (below; photograph by David Illig: https://flic.kr/p/9usE42; license CC BY-NC 2.0: https://creativecommons.org/licenses/by-nc/2.0/) Golden Eagles *Aquila chrysaetos*. (c): juvenile (left) and adult (right) Eastern Imperial Eagles *Aquila heliaca*. (right; photograph by Andi Li: https:// www.flickr.com/photos/andy_li/; CC BY-NC 2.0 license: https://creativecommons.org/licenses/by-nd/2.0/).

Figure 2. Consensus phylogenetic tree for the 53 species of birds included in the study obtained from www.birdtree.org. Branch lengths are proportional to nucleotide substitutions. Juvenile colour score values (black: non-pheomelanin plumage coloration; yellow: pheomelanin-based plumage coloration) and diet habitat (green: non-strict carnivorous species; orange: carnivorous species) for the 53 species of birds included in the study.



Phylogenetic analyses

To test whether distinctive juvenile plumage coloration produced by pheomelanin depends on the type of diet, a phylogenetic logistic regression model (Ives & Garland, 2010) with the age-related plumage colour change score as a response variable and diet score as a fixed predictor was performed using the algorithm implemented by Ho and Ané (2014) in the R package *phylolm*. We obtained 1000 alternative phylogenetic trees for the 53 species of birds from www.birdtree.org (Jetz et al., 2012; Hackett SJ et al., 2008), then constructed a consensus tree using the package *phytools* (Revell, 2012) (see Figure 2) and ultrametrized it with a value of 0.1 for the smoothing parameter d, using the R packages *ape* (Paradis et al., 2004) and *phangorn* (Schliep, 2011). As the response variable was binary, phylogenetic signal was measured with the parameter D developed by Fritz and Purvis (2010). D and P-values for H_o (D = 0) were calculated with the package caper (Orme et al., 2013): D = 1 is indicative of a random mode of evolution (i.e. no phylogenetic signal), whereas D = 0 is indicative of a Brownian motion mode of evolution, in which changes in the trait along the phylogeny are proportional to the degree of relatedness among species. D can also be <0 in highly clustered traits and >1 when phylogenetic overdispersion exists (Fritz & Purvis, 2010).

RESULTS

Pheomelanin-based plumage pigmentation during the juvenile stage was observed in 29 of 39 (74%) strictly carnivorous species and only two of 14 (14%) non-strictly carnivorous species (Figure 3). Accordingly, the phylogenetic logistic regression model recovered a significant (b = 2.32, P = 0.003; Figure 3) relationship between diet and juvenile plumage coloration. This result indicates that the presence of juvenile plumage coloration produced by pheomelanin is higher among strictly carnivorous than among non-carnivorous birds. The phylogenetic signal for plumage colour score (D = –0.40) was not statistically different from zero (P = 0.746), suggesting that the presence of pheomelanin-based plumage coloration in the juvenile stage of birds is a conserved trait.

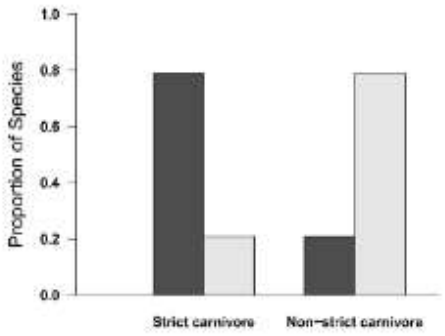


Figure 3. Proportion of species exhibiting pheomelanin-based juvenile plumage coloration (black) relative to the proportion of species in which juveniles exhibit other plumage coloration (grey) for strictly carnivorous (n = 39) and non-strictly carnivorous species (n = 14).

DISCUSSION

When the plumage of juvenile birds is distinctively different from that of adults, it commonly exhibits orange and light brown colours, as can be seen from an examination of bird illustrations (Svensson et al., 2000). These colours are produced by the presence of pheomelanin in feathers (Galván & Wakamatsu, 2016). The type of juvenile plumage coloration (i.e. greater or less presence of pheomelanin in juveniles than in adults) exhibits phylogenetic conservatism, suggesting that juvenile plumage may provide similar functional benefits to all species and thus selection pressures are constant along the phylogeny. This is because, when some but not all species in a phylogeny achieve a particular character-state in relation to a trait, this results in a loss of phylogenetic signal (i.e. the signal is reduced relative to that expected from a Brownian motion model of evolution; Blomberg et al., 2003).

Our study also suggests that pheomelanin-based plumage in juvenile birds has evolved more frequently in strictly carnivorous species than in species that include food sources other than animal proteins in their diet. We propose that this is due to the potential adaptive benefit that the synthesis of pheomelanin for pigmentation could offer, as it may help to avoid the toxicity of excess cysteine with

high protein content in the diet. The production of pheomelanin may thus contribute to cysteine homeostasis when the expression of the gene *CTNS* cannot be adjusted to protein intake, as *CTNS* lability is not expected to have evolved in every species (Galván et al., 2017). This potential benefit may be particularly advantageous for birds developing feathers during the nestling stage, a period of low physical activity in which they are fed by their parents. Exercise can increase oxidative stress (Vaanholt et al., 2007; 2008), and thus the absence of parental and feeding effort results in the nestling stage constituting a period of relatively low stress as compared with post-fledging stages, when juveniles must find food on their own with a high expenditure of energetic resources (Weimerskirch et al., 2003). This may explain the abundance of pheomelanin- based plumage in juvenile birds, which probably have a relatively low requirement of antioxidant resources. Recent findings in birds suggest a positive association between plasma protein concentration and the expression of pheomelanin-based plumage coloration (Minias et al., 2018). As total plasma protein levels increase with the intake of dietary proteins (Leveille & Sauberlich, 1961), this result could be in agreement with the detoxifying hypothesis of pheomelanin-based pigmentation as articulated here.

Our results suggest that pheomelanin-based pigmentation has evolved more frequently in strictly carnivorous species, but variability still exists across species and, accordingly, the phylogenetic signal for the type of juvenile plumage coloration was significant (i.e. D is not statistically different from 0) but not as strong as it could be if the estimated value for D was near 0. Thus, there are closely related species that have a strict carnivorous diet but differ in juvenile plumage pigmentation.

An example of this variability is represented by the Spanish Imperial Eagle *Aquila adalberti* and the Golden Eagle *Aquila chrysaetos*, two congeneric raptor species that differ in juvenile pigmentation. The first plumage of the Spanish Imperial Eagle is full orange, a colour conferred by the presence of pheomelanin in feathers (Galván & Wakamatsu, 2016), and it changes to a fully black plumage, a colour conferred by eumelanin, in adulthood (Figure 1a). The Golden Eagle presents a reverse pattern, as the first plumage is almost fully black, subsequently changing to a lighter brown–orange plumage in adulthood (Figure 1b). It could thus be said that juvenile Spanish Eagles are pheomelanic, whereas juvenile Golden Eagles are

eumelanic. As with all raptors, both species are strictly carnivorous. We suggest that the different pigmentation patterns may be related to the different trophic ecology of the species. The Spanish Imperial Eagle is a specialist that preys preferentially on rabbits (Ferrer & Negro, 2004), whereas Golden Eagles prey on a much wider diversity of species (Watson, 2010). The predictability of food resources is higher for specialists than for generalists (Overington et al., 2008) and increases with food abundance in raptors (Millon et al., 2008). Pheomelanin synthesis may help to avoid excess dietary cysteine but, if cysteine is not in excess, it may cause oxidative stress by consuming GSH (Napolitano et al., 2014). If food availability and predictability are higher for Spanish Imperial Eagles than for Golden Eagles, it is then likely that natural selection has favoured the evolution of pheomelanin-based pigmentation in the former but not the latter. Indeed, the sister species of the Spanish Imperial Eagle is the Eastern Imperial Eagle *Aquila heliaca*, which consumes a more diverse diet (Katzner et al., 2005) and whose juvenile plumage is less intensely orange (Figure 1c). A non-excluding alternative hypothesis is that these species differ in the permeability of *CTNS* expression to food abundance (Galván et al., 2017). Future studies should test these possibilities.

Understanding the physiological mechanisms of colour production helps to explain the evolution of plumage diversity (Delhey, 2015). This study seeks to provide an explanation for differences between the plumage pigmentation of juvenile and adult birds as a physiological response to diet. Although we focused our study on birds, melanins are the most abundant pigments in animals (McGraw, 2006), and thus these findings will contribute to our understanding of the evolution of pigmentation phenotypes in other taxa.

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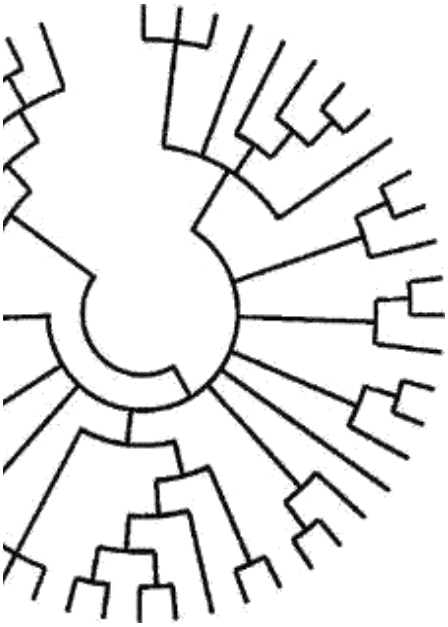
SUPPLEMENTARY MATERIAL

Table S1. Species names, juvenile colour score values (0: non-pheomelanin plumage coloration; 1: pheomelanin-based plumage coloration) and diet habit (0: non-strictly carnivorous species; 1: carnivorous species) for the 53 species of birds included in the study.

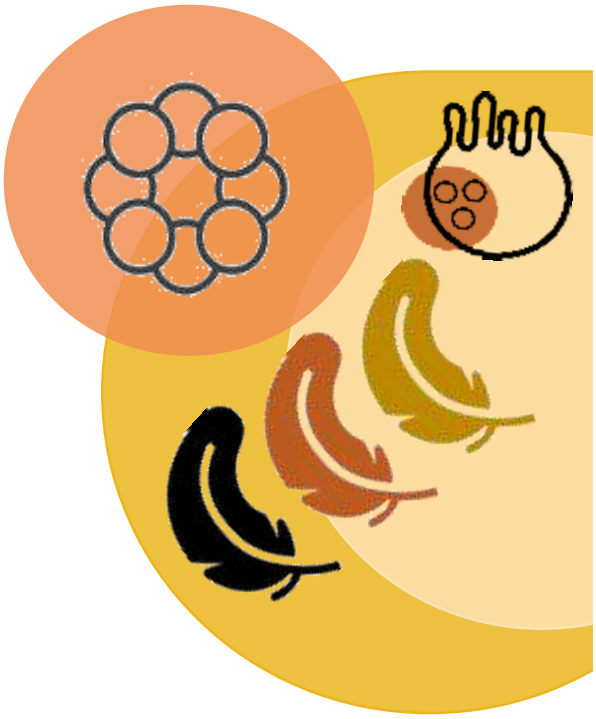
Species	Color Score	Diet
Aquila rapax	1	1
Aquila adalberti	1	1
Aquila heliaca	1	1
Aquila nipalensis	1	1
Aquila fasciatus	1	1
Aquila verreauxii	1	1
Aquila chrysaetos	0	1
Aquila pomarina	1	1
Aquila clanga	1	1
Milvus milvus	0	0
Haliaeetus albicilla	1	1
Buteo buteo	1	1
Buteo lagopus	1	1
Melierax metabates	1	1
Accipiter gentilis	1	1
Aegypius monachus	0	1
Gyps fulvus	1	1
Circaetus gallicus	1	1
Gypaetus barbatus	0	1
Neophron percnopterus	1	1
Pernis apivorus	1	1
Elanus caeruleus	1	1
Asio otus	1	1
Bubo bubo	1	1
Otus scops	0	0
Athene noctua	1	1
Glaucidium passerinum	0	1
Coracias garrulous	0	0
Serinus pusillus	1	0
Prunella modularis	0	0
Cinclus cinclus	0	1
Erithacus rubecula	0	0
Hirundo rustica	0	1
Hirundo daurica	0	1
Aegithalos caudatus	0	0
Remiz pendulinus	0	0
Lanius nubicus	0	0
Lanius senator	0	0
Falco rusticolus	1	1
Falco cherrug	1	1
Falco pelegrinoides	1	1
Falco peregrinus	1	1
Falco concolor	1	1
Falco subbuteo	1	1
Vanellus spinosus	0	0
Cursorius cursor	0	0
Gallinula chloropus	0	0
Fulica atra	0	0
Porzana pusilla	1	0
Rallus aquaticus	0	0
Ardea purpurea	1	0
Nycticorax nycticorax	1	0
Streptopelia turtur	0	0



CHAPTER IX

Exposure to sulfur in soil explains pigmentation by pheomelanin in birds inhabiting Iceland

Sol Rodríguez-Martínez, Ólafur Arnalds, Jón Guðmundsson, María Svavarsdóttir, Fanney Ó. Gísladóttir, Ólafur K. Nielsen & Ismael Galván



Under review

ABSTRACT

Melanins are the most common pigments in birds and mammals. The synthesis of the sulfurated form of melanin, termed pheomelanin, is promoted by high levels of thiols in melanocytes. Exposure to elemental sulfur in the environment can potentially influence intracellular thiol levels in terrestrial animals, but this has never been investigated in the wild. This is particularly relevant in areas with high volcanic activity that facilitate sulfur exposure. We explored for an association between the pheomelanin content of feathers in 35 breeding birds from 13 phylogenetically diverse species and 29 locations in Iceland, and the sulfur content of nearby soil samples. After controlling for the effect of phylogenetic relatedness between species, we found that the pheomelanin content of feathers was significantly correlated with the sulfur content of soil, indicating that pheomelanin production increases with sulfur exposure. Our results suggest that environmental exposure to relatively high levels of elemental sulfur promotes the expression of pheomelanin-based pigmentation phenotypes in birds, likely by an influence on intracellular thiols during development. This is the first evidence that soil chemistry can affect the phenotype of animals. Highly volcanic areas such as Iceland are thus potential sources of phenotypic diversity by promoting sulfur exposure in animals.

KEYWORDS

Bird coloration, pheomelanin, pigmentation phenotype, soil chemistry, sulfur exposure, volcanic activity.

INTRODUCTION

With few exceptions, the body surface of all organisms is coated with pigments that play a diversity of vital functions. Pigments determine the appearance of organisms to a large extent, and organic evolution is mediated in a significant part by this

appearance. External coloration greatly determines the capacity to adapt to the environment and reinforces the differentiation of incipient species (Seehausen et al., 2008; Manceau et al. 2011). Highly diversified animal clades are indeed often associated to the evolution of conspicuous color traits, because sexual selection, which plays an important role in the generation of isolating mechanisms by adaptive radiation, usually favors conspicuous phenotypes (Maan & Seehausen, 2011).

Melanins are the most common pigments in birds and mammals (Galván & Wakamatsu, 2016). These pigments are synthesized within lysosome-like organelles called melanosomes in melanocytes. These cells extend dendrites to transfer the melanin-containing melanosomes to the target cells, mainly keratinocytes in the hypodermis and the pigmentary units of feather or hair follicles, via a shedding vesicle system (Ando et al., 2012). This confers pigmentation to the skin and associated structures such as feathers and hairs.

The synthesis of melanins in melanosomes consists in the oxidation of the amino acid tyrosine and the subsequent polymerization of the products that are formed. In the absence or under certain threshold concentration of thiol compounds (i.e., compounds that can transfer sulfhydryl groups, -SH) in melanosomes, the resulting pigment is called eumelanin, a dark polymer of indole units. However, when thiol compounds, mainly the amino acid cysteine, are under certain threshold concentration in melanosomes, sulfhydryl groups are incorporated into the structure of the polymer. The resulting pigment is then called pheomelanin, oligomers of sulfur-containing heterocycles (García-Borrón & Olivares Sánchez, 2011). With the exception of psittaciform birds, the only organisms in which an evolutionary loss of mixed melanogenesis has been reported (Neves et al., 2020), eumelanin and pheomelanin are present at different proportions in skin, feathers and hairs, largely contributing to the diversity in pigmentation phenotypes observed in birds and mammals. While eumelanin is a dark pigment producing black, grey and dark brown colors, pheomelanin produces reddish, orange and yellowish colors (Galván & Wakamatsu, 2016).

As both tyrosine and cysteine are semi-essential amino acids, meaning that they are partly acquired in the diet, there is a potential environmental effect on the ability to synthesize melanin pigments through the availability of food resources.

This potential effect, however, has been investigated only in a few studies. Dietary amino acids have been shown to affect hair eumelanin production in cats (Anderson et al., 2002). Fargallo et al. (2007) showed that the proportion of male Eurasian kestrel *Falco tinnunculus* nestlings that display the greyest plumage patches, which are produced by a low pheomelanin:eumelanin ratio in feathers, was higher in years of high prey abundance. In nestlings of another raptor species, the Northern goshawk *Accipiter gentilis*, the abundance of available prey has been shown to predict plumage pheomelanin content (Galván et al., 2019).

These dietary effects are particularly relevant to pheomelanin, because the amino acid precursor that is limiting for pheomelanin synthesis (cysteine) plays essential biological roles but also causes oxidative stress if in excess (Janaky et al., 2000; Dilger & Baker, 2008). Once incorporated to the process of pheomelanin synthesis, sulfhydryl groups from cysteine are not released back to melanocytes and keep being part of the pigment structure, meaning that pheomelanin production can contribute to cysteine homeostasis (Galván et al., 2012). This has experimentally been demonstrated in developing Eurasian nuthatches *Sitta europaea*, in which dietary excess cysteine induces changes in DNA and RNA methylation in some genes that regulate cysteine metabolism in feather follicular melanocytes, favoring pheomelanin-based feather pigmentation and avoiding oxidative stress (Rodríguez-Martínez et al., 2019). The need for cysteine homeostasis, determined by the level of dietary intake of cysteine, may therefore constitute an evolutionary pressure affecting the expression of pheomelanin-based pigmentation in birds and mammals. Indeed, juvenile plumage expressing pheomelanin-based coloration has evolved more frequently in species of birds that have strict carnivorous diets, which have more abundant and physiologically available proteins, than in species with other diets (Rodríguez-Martínez & Galván, 2020). Therefore, kinetic conditions in melanocytes promote pheomelanin production under high thiol levels (Ito & Wakamatsu, 2008), and this may then be favored by selection due to the benefits of cysteine homeostasis.

However, environmental effects on thiol levels in melanocytes that influence pheomelanin synthesis and pigmentation may not be exerted only through an effect of dietary cysteine. Exposure to elemental sulfur can also affect intracellular thiols.

Biological effects of exposure to elemental sulfur are poorly studied, but it is known that terrestrial animals can be exposed to environmental sulfur via sulfur-containing ingested food, through inhalation of sulfur dust, by skin contact, and from exposure to sulfur in the surrounding environment (Kuklińska et al., 2013). Thus, the exposure to high levels of environmental sulfur can potentially exert physiological effects on birds and mammals. These effects influence intracellular thiol levels, which can produce toxicity as observed in rats inhaling sulfur particles and in guinea pigs ingesting sulfur-containing food (Kuklińska et al., 2013). These effects can also influence the development of integumentary structures that contain sulfur, as observed in sheep fed with sulfur-containing food and experiencing an increase in wool growth (Starks et al., 1954). A high increase in intracellular thiol levels can thus be toxic. From a kinetic perspective, this promotes pheomelanin synthesis in melanocytes (Ito & Wakamatsu, 2008). Additionally, the use of thiols for pheomelanin synthesis can avoid the toxicity of high thiol levels (Rodríguez-Martínez et al., 2019). Therefore, exposure to high levels of environmental sulfur chemically promotes the production of large amounts of pheomelanin, and this may also be adaptive for birds and mammals. This would mean that exposure to environmental sulfur may contribute to the evolution of pheomelanin-based pigmentation, as pheomelanin-based color phenotypes should evolve more frequently in species inhabiting environments exposed to high sulfur levels. This possibility has never been contemplated.

Iceland is an ideal natural model to test this hypothesis. This 103,000 km^2 North Atlantic island is one of the most volcanically active areas in the world, with about 30 active volcanic systems and volcanic eruptions occurring every 3-5 years on average (Schmidt et al., 2014). As sulfur dioxide (SO_2) is typically the third most emitted volcanic gas after water and carbon dioxide, the daily emission of sulfur in the form of SO_2 into the atmosphere during volcanic eruptions in Iceland can exceed by several orders of magnitude daily SO_2 emissions from all anthropogenic sources in Europe (Schmidt et al., 2015). Volcanic SO_2 is then deposited on soils, which thus act as SO_2 sinks (Garland, 1977). Additionally, there is intense aeolian activity in Iceland due to the suspension of enormous amounts of volcanic dust particles in the atmosphere, which adsorb SO_2 on their surface and thus contribute, when

depositing on soil, to spreading SO_2 and to transform it into sulfites and sulfates (Urupina et al., 2019). Although both volcanic SO_2 gas and dust particles can be transported over long distances before being settled on soil, most SO_2 deposition occurs around the volcanic cloud and causes extreme loading of the local ecosystems (Parnell & Burke, 1990; Delmelle et al., 2001), mainly on waters, soils and vegetation (Gíslason et al., 2015). Also, SO_2 adsorption on dust surface most likely occurs in the part of the cloud closest to the vent where ash concentration is the highest (Urupina et al., 2019). Lastly, natural hydrothermal waters, which contain high levels of sulfates, thiosulfates, polythionates and sulfide (Kaasalainen & Stefánsson, 2011), represent an important input source of sulfur for hydrological systems in Iceland (Robinson et al., 2009). Animals inhabiting Iceland might consequently experience a high exposure to environmental sulfur.

However, sulfur distribution in the soil of Iceland is not homogeneous, hence sulfur exposure is expected to spatially vary along the island. This is reflected by the distribution of soil types. Icelandic soil types are categorized, on a decreasing level of carbon content, into Histosols and Andosols, in addition to Vitrisols and Leptosols (rock/scree) which have near absence of organic matter and Cryosols (permafrost) (Arnalds, 2015). Carbon content differences in Icelandic soils roughly provides information about sulfur distribution, because the availability of sulfur is generally related to the organic content of soils (e.g., Tabatabai et al., 2005).

The aim of this study is to test for an association between the intensity of pheomelanin-based pigmentation in birds and sulfur content of soil in Iceland. For this, we sampled for feathers 35 specimens from 13 species deposited in the bird collection of the Icelandic Institute of Natural History that had been collected during the breeding season in 29 locations in Iceland, and obtained soil samples from nearby locations. We predicted that, if exposure to environmental sulfur affects the evolution of pheomelanin-based pigmentation, the relative content of pheomelanin in feathers should increase with the relative content of sulfur in the soil where birds developed the feathers, after controlling for phylogenetic effects. This is the first study investigating a possible effect of environmental sulfur exposure on the evolution of pigmentation.

MATERIAL AND METHODS

Species selection and sampling of feathers

Colors produced by pheomelanin can readily been distinguished from those produced by the other form of melanin (eumelanin), as the former produces dull yellow and orange colors while the latter produces black, grey and dark brown colors (Galván and Wakamatsu, 2016). On this basis, we assessed the plumage pigmentation phenotype of the species of birds that breed in Iceland regarding the presence of pheomelanin pigment in feathers (Hilmarsson, 2011), and identified 17 species that show plumage patches expressing colors indicative of highest pheomelanin concentrations in feathers (i.e., color categories 4-6 in Galván & Wakamatsu, 2016). Any possible effect of soil sulfur content on pheomelanin-based plumage pigmentation may therefore most likely to be detected in these species. In the bird collection of the Icelandic Institute of Natural History, we searched for specimens collected during the breeding season across Iceland for which we could also obtain soil samples near the bird sampling locations, and that covered the diversity of soil types in Iceland (Arnalds, 2015).

We finally selected 13 species, and we could sample for feathers a total of 35 bird specimens. The selected species and numbers of sampled specimens were the Eurasian wren *Troglodytes troglodytes* (1 specimen), the redwing *Turdus iliacus* (1 specimen), the red-throated loon *Gavia stellata* (5 specimens), the horned grebe *Podiceps auritus* (2 specimens), the European golden plover *Pluvialis apricaria* (5 specimens), the black-tailed godwit *Limosa limosa* (4 specimens), the dunlin *Calidris alpina* (1 specimen), the red-necked phalarope *Phalaropus lobatus* (3 specimens), the common snipe *Gallinago gallinago* (2 specimens), the harlequin duck *Histrionicus histrionicus* (2 specimens), the Eurasian teal *Anas crecca* (3 specimens), the gadwall *Anas strepera* (3 specimens) and the Eurasian wigeon *Anas penelope* (3 specimens). The species thus belong to seven different families, covering a wide phylogenetic spectrum. All specimens corresponded to adult birds in breeding plumage, excepting two European golden plover specimens that were downy chicks. We included these chicks because their downy feathers contain the same yellow

color produced by pheomelanin that is observed in adult feathers (Galván & Wakamatsu, 2016; this study).

Although eight of these species are migratory and/or conduct the pre-breeding molt that leads to the breeding plumage on the wintering grounds or during migration (i.e. the red-throated loon, the horned grebe, the European golden plover, the black-tailed godwit, the dunlin, the red-necked phalarope, the common snipe and the harlequin duck; Cramp et al. 1978, 1983), it is expected that chronic exposure to soil sulfur in the breeding grounds (approximately half a year) affects their physiology all year round. Although little is known about the duration of physiological effects of elemental sulfur exposure in animals (Kuklińska et al., 2013), studies on humans show long-term effects after exposure to toxic sulfurated agents (Jafari & Ghanei, 2010) and after early diets with sulfur containing amino acids (Rees, 2002). Additionally, studies in Iceland and abroad show a high interyear fidelity to the breeding sites in several of these species such as the horned grebe (Konter and Konter, 2006), the golden plover and the dunlin (Klima & Johnson, 2005), the black-tailed godwit (Kruk et al., 1998), the red-necked phalarope (Schamel & Tracy, 1991) and the harlequin duck (Bengtson, 1972). Thus, differences are expected to arise when comparing the plumage pigmentation of birds sampled in sites with different sulfur contents in soil.

We collected body feathers from the 35 bird specimens. The target plumage patches were those displaying yellow and orange colors produced by the presence of pheomelanin in feathers (Figure 1). These plumage patches were previously analyzed by Galván and Wakamatsu (2016) in three out of the 13 species included in this study, showing high pheomelanin concentrations as compared with other species. Considering the levels of thiazole-2,4,5-tricarboxylic acid (TTCA), a specific marker of the pheomelanin moiety (benzothiazole) whose levels are predicted by color intensity (Galván & Wakamatsu, 2016), pheomelanin concentrations in these three species were as follows (in µg/mg feather): gadwall (55.05), red-throated loon (42.60) and black-tailed godwit (12.82).

Figure 1. Images of species of birds included in the study. a: Eurasian wren *Troglodytes troglodytes*; b: redwing *Turdus iliacus*; c: red-throated loon *Gavia stellata*; d: horned grebe *Podiceps auritus*; e: European golden plover *Pluvialis apricaria*; f: black-tailed godwit *Limosa limosa*; g: dunlin *Calidris alpina*; h: red-necked phalarope *Phalaropus lobatus*; i: common snipe *Gallinago gallinago*; j: harlequin duck *Histrionicus histrionicus*; k: Eurasian teal *Anas crecca*; l: gadwall *Anas strepera*; m: Eurasian wigeon *Anas penelope*. The reddish/chestnut plumage patch observed in the image of each species corresponds to the plumage patches pigmented by pheomelanin that were sampled from the bird specimens. All images were taken during the breeding season of birds. Photo credits and locations: a, i, k and l: Rafael Palomo Santana (Spain); b, d, e, f and m: Ólafur K. Nielsen (Iceland); c: Sindri Skúlason (Iceland); g, h and j: Greg Barsh (Alaska).

Measurement of pheomelanin content of feathers

The feathers collected from bird specimens were analyzed by micro-Raman spectroscopy to determine their relative content of pheomelanin, which exhibits distinctive Raman signal that can be used for its non-destructive identification and quantification (Galván et al., 2013a; Galván & Jorge, 2015; Galván & Rodríguez-Martínez, 2018; Galván et al., 2018). We used 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 using a near-infrared excitation laser of 780 nm. We analyzed two barbs and two barbules chosen

at random for each feather. Barbs were analyzed using a 50x confocal objective and setting laser power at 7 mW, while barbules were analyzed using a 100x objective and setting laser power at 2.5 mW. Integration time was 3 s, and the number of accumulations was 12. The system was operated with Thermo Fisher OMNIC 8.1 software. Calibration and alignment of the spectrograph were checked using pure polystyrene.

We calculated the average Raman spectrum for each bird specimen, and fitted it to an amplitude Gaussian deconvolution function using Origin v.9.1 (OriginLab Corporation, Northampton, MA, USA). We considered the three diagnostic Raman bands of pheomelanin, at about 500, 1500 and 2000 cm^{-1} (Galván et al., 2013a,b). The mean (± SE) adjusted R^2 of fitting these functions to the spectra was 0.91 ± 0.01. From the deconvolution functions, we calculated the position, area, intensity and width of the three Raman bands. As it was not possible to include the band at about 2000 cm^{-1} in all deconvolution functions due to low intensity, we used the area of the band at about 1500 cm^{-1} for the relative quantification of pheomelanin in feathers, as this is the most important and repeatable predictor of pheomelanin concentration in both bird feathers and mammalian hairs (Galván et al., 2013a).

Sampling of soil

We used the geographical coordinates associated to the bird specimens to obtain information on soil types for the bird sampling sites in Iceland. We obtained this information from the *Nytjaland* database, a GIS Icelandic Farmland Database created by the Agricultural University of Iceland. This database includes a good resolution supervised classification of soils and vegetation classes based on satellite images (Arnalds, 2015), and allowed us to classify bird sampling sites into the following soil categories: Brown Andosols (differentiating those in grassland, richly vegetated heathland, poorly vegetated heathland, birch shrubland and forestry areas), Brown Andosols and Leptosols (in mossland), Gleyic and Histic Andosols (differentiating those in cultivated land and semi-wetland), Gleyic and Histic Andosols and Histosols

(in wetland), Vitrisols (differentiating those in partially vegetated land and sparsely vegetated land), and absence of soil or poorly developed soil (lakes and rivers, glaciers and others) (Arnalds, 2015).

We then searched for sites within a 10 km-radius circular area around bird sampling sites that had the same soil category and for which there were soil samples available at the Agricultural University of Iceland. We chose the soil sampling site that was closer to the bird sampling site, within the 10 km area, if more than one was available. The geographical correspondence between bird and soil sampling sites is shown in Figure 2.

We collected about 10 g of soil from each soil sampling site. We dried the soil samples in a stove and sieved them to <2 mm particle size before sulfur analyses.

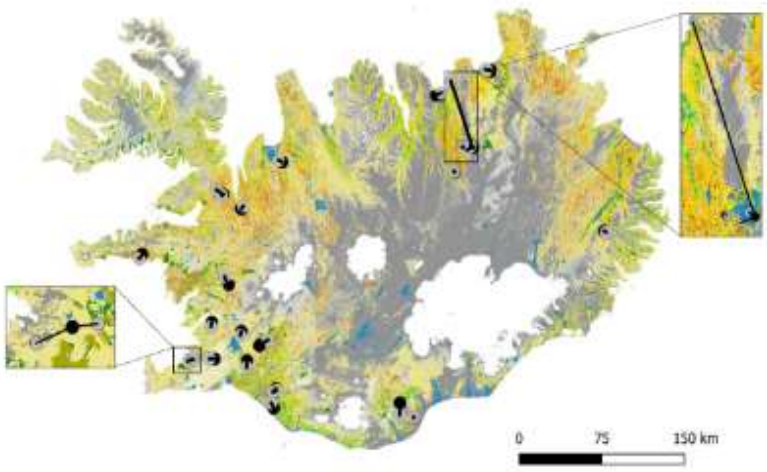


Figure 2. Distribution of bird sampling points (grey circles) and soil sampling points (black circles) across Iceland. Lines show the correspondence between both sampling points. Each color in the map represents a category of soil, excepting blue and light grey patches that represent lakes and rivers, and white patches that represent glaciers.

Measurement of sulfur content of soil

A semi-quantitative analysis of elemental sulfur in the soil samples was made by dispersive X-ray irradiation in an Inspect-S environmental scanning electron microscope (ESEM) (FEI Company, Hillsboro, OR, USA). ESEM spectra of soil samples were obtained with an acceleration voltage, an acquisition time of 20 s, and under a pressure of 0.40 Torr in vacuum. For each sample, measurements were taken at four points chosen at random on the soil surface, and the average value was then calculated. The apparent concentration of sulfur provided by the equipment, calibrated with an iron disulfide (FeS_2) standard, was used as semi-quantitative measurement of total sulfur in the soil samples. The repeatability of these analyses, considering the four measurements taken per sample, was high ($r = 0.86$, $F_{34,105} = 25.94$, $P < 0.001$).

Phylogenetic analyses for testing feather pheomelanin-soil sulfur covariation

Species are evolutionarily related through a phylogeny, thus they cannot be treated as independent sample units in statistical analyses (Felsenstein, 1985). Therefore, to test whether the pheomelanin content of the feathers of birds breeding in Iceland depends on the sulfur content of soil, we conducted phylogenetic linear regression models pooling the data from all species. In the models, the pheomelanin content of feathers (in $\log_{10}$) was the response variable and the sulfur soil content (in $\log_{10}$) was a covariate. As we had multiple observations per species because we were interested in exploring intraspecific variation in pheomelanin-based pigmentation in different species of birds, we used the *Rphylopars* package in R environment to estimate the phylogenetic covariance of the two traits across species while considering within-species covariance (Goolsby et al., 2017). We fitted the models to data considering different modes of evolution (Brownian motion (BM), Ornstein-Uhlenbeck (OU) and early burst (EB)), in addition to the model without phylogeny (i.e., considering

independent sample units), and used the Akaike information criterion (AIC) to choose the best model.

To incorporate in the models information about the phylogenetic relationships between the 13 species of birds used in the study, we obtained 1,000 probable phylogenies with the *Phylogeny subsets* tool of www.birdtree.org, and then constructed a consensus tree using the R package *phytools* (Revell, 2012) (see Figure 3). We ultrametrized the consensus tree by using a value of 0.1 for the smoothing parameter δ in the R packages *ape* (Paradis et al., 2004) and *phangorn* (Schliep, 2011). We measured the amount of phylogenetic signal in the response variable using the *fast.SSC* tool in *Rphylopars* (Goolsby et al., 2017). This tool calculates the scaled sum of squared changes (SSC) between ancestral and descendant nodes as described in Klingenberg and Gidaszewski (2010), using a fast ancestral state reconstruction algorithm. We also used this tool to calculate the corresponding P-value for H_0: SSC = 1, using 1000 phylogenetic permutations. SSC = 1 is indicative of a Brownian motion model of evolution in which changes in the trait along the phylogeny are proportional to the degree of relatedness among species, while SSC <1 and >1 are indicative of less or more phylogenetic signal, respectively, than expected under Brownian motion. SSC was calculated considering the average pheomelanin content of feathers per species.

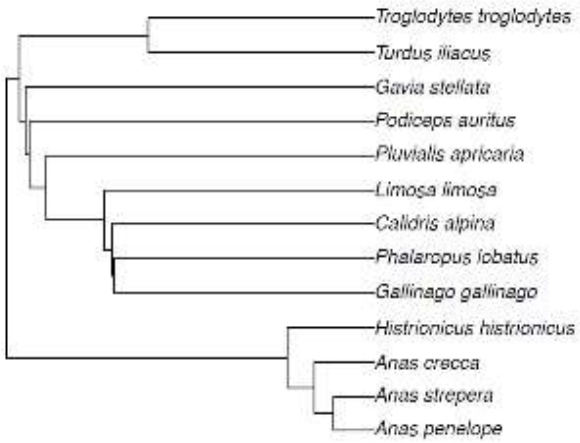


Figure 3. Consensus phylogenetic tree for the 13 species of birds breeding in Iceland included in the study. Branch lengths are proportional to nucleotide substitutions.

RESULTS

The model assuming a Brownian motion mode of evolution was the best model at explaining the covariation between the pheomelanin content of feathers and the sulfur content of soil, and it was significantly better (ΔAIC = 7.50) than the second model in importance, represented by that assuming an early burst mode of evolution (Table 1). The Brownian motion model revealed a significant and positive relationship between the pheomelanin content of feathers and the sulfur content of soil where the birds were collected (b = 6.21, $F_{1,11}$ = 5.18, P = 0.044). This indicates that the amount of pheomelanin pigment produced by birds in Iceland depends on the level of elemental sulfur in soil to which they were exposed during breeding (Figure 4).

The value of SSC for the pheomelanin content of feathers was >1 (SSC = 1.89), but not statistically different from 1 (P = 0.395). This is indicative of a strong phylogenetic signal, but still expected under Brownian motion, i.e. changes in the pheomelanin content of feathers along the phylogeny are proportional to the degree of relatedness among species. The expression of pheomelanin-based pigmentation phenotypes in birds that breed in Iceland is thus an evolutionarily conserved trait.

Table 1. Summary of Akaike Information Criterion (AIC) results for models relating feathers pheomelanin content (pheomel.cont) and soil sulphur content (sulphur.cont). Regression were performed under Brownian Motion (BM), early burst (EB) and Ornstein-Uhlenbeck (OU) models of evolution. AIC values (AIC), estimate (β), degrees of freedom (DF), F-statistic (F) and p-value were shown.

	AIC	β	DF	F	p
pheomel.cont ~ sulphur.cont, model=BM	7.593	6.213	11	5.178	0.044
pheomel.cont ~ sulphur.cont, model=EB	15.095	6.505	11	5.678	0.036
pheomel.cont ~ sulphur.cont	41.118	2.676	33	4.463	0.042
pheomel.cont ~ sulphur.cont, model=OU	80.883	6.213	11	5.176	0.044

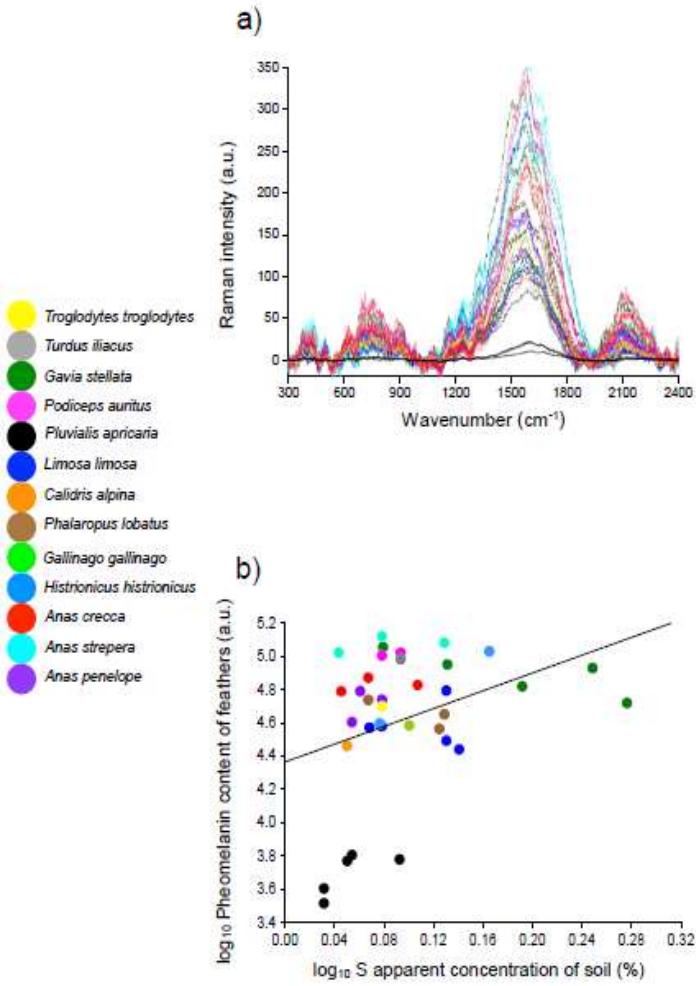


Figure 4. a) Raw Raman spectra of pheomelanin obtained from feathers of 35 bird specimens from 13 species collected during breeding in Iceland. The feathers were taken from the reddish/chestnut plumage patches observed in Figure 1. Each spectrum represents the average spectrum from two feathers (laser beam focused on four barbs and four barbules) of a single specimen. b) Relationship between the pheomelanin content of feathers and the total content of sulfur in the soil of the sites where bird specimens were collected in Iceland. Pheomelanin content values were obtained by deconvoluting the Raman spectra in a) and calculating the area of the main band at about 1500 cm^{-1} from the deconvolution function. Sulfur content values were obtained by dispersive X-ray irradiation of soil samples in an environmental scanning electron microscope (ESEM) at vacuum. The line is the best fit line.

DISCUSSION

Environmental exposure to sulfur can affect intracellular thiol levels in organisms (Kuklińska et al., 2013). Pheomelanin synthesis in melanocytes is promoted by high thiol levels (Ito & Wakamatsu, 2008). Accordingly, our results show that birds with phenotypes profusely pigmented by pheomelanin breeding in Iceland develop their plumage in sites with soils with higher sulfur contents than sites of birds with phenotypes produced by lower amounts of pheomelanin. This is observed considering both intraspecific and interspecific covariation between pheomelanin content of feathers and soil sulfur content, at least across the 13 species of birds included in this study. This suggests that exposure to high sulfur soil levels in Iceland has favored the expression of pheomelanin-based plumage phenotypes, likely through an increase in thiol levels during development. Selection may have favored the maintenance of such phenotypes due to the benefits of pheomelanin production to avoid sulfur/thiol toxicity (Rodríguez-Martínez et al., 2019), thus explaining the spatial pattern presently observed in birds inhabiting Iceland. This is also supported by our results, as we found that the plumage pigmentation phenotype of Icelandic birds is a phylogenetically conserved trait, suggesting that it has strongly been favored by natural selection. It is then likely that the susceptibility of the pigmentary system of birds to be affected by sulfur exposure leads to phenotypic plasticity, producing intense pheomelanin-based pigmentation phenotypes when the degree of exposure to sulfur in soil is high, and natural selection benefits such phenotypes due to their adaptive benefits under high sulfur exposure.

None of the species studied here have a breeding distribution range restricted to Iceland. It is thus expected that the association between the expression of pheomelanin-based pigmentation and soil sulfur content has arisen as a consequence of chemical and selective pressures acting on the phenotype of birds along their whole breeding ranges, and can be observed outside Iceland. Future studies should test this hypothesis in other regions to determine its capacity to explain bird phenotypic diversity globally, but the association may be particularly likely to arise in Iceland due to the high levels of environmental sulfur to which organisms might be exposed (see Introduction). The effect of soil characteristics on

the expression of pigmentation phenotypes in birds is thus likely to be specially strong in places with high volcanic activity such as Iceland. Indeed, 5 out of the 13 species studied here present subspecies whose breeding distribution is restricted to Iceland or nearby islands and Scandinavia (*Calidris alpina schinzii*, *Gallinago gallinago faeroeensis*, *Limosa limosa islandica*, *Turdus iliacus coburni* and *Troglodytes troglodytes islandicus*) and whose plumage pigmentation is more intensely reddish/chestnut, which is indicative of higher pheomelanin production (Galván & Wakamatsu, 2016), than that of other subspecies (Cramp et al., 1983; 1988).

Although some studies have related bird species distribution with some characteristics of soils (e.g., Kålås et al., 1997; Gunnarsson et al., 2015; Wilson et al., 2005), the implications of developing in certain soil types on the external phenotype remained unexplored in birds and any other vertebrates. Our study therefore unveils an effect that soil sulfur content can exert on the pigmentation phenotype of animals, likely by affecting the amount of pheomelanin produced by melanocytes of feather follicles during development.

The activity of wild vertebrates has a known influence on soil formation and chemistry (Bancroft et al., 2005; Eldridge & James, 2009; Platt et al., 2016), but this is the first study showing an opposite effect, i.e. an influence of soil chemistry on phenotypic characteristics of vertebrates. The expression of the pigmentation phenotype, in particular that generated by melanins which are the most common pigments in birds and mammals, can be influenced by environmental factors such as food availability (Galván et al., 2019), solar and terrestrial radiations (Galván et al., 2018), predation risk (Galván, 2018), social interactions (McGraw et al., 2003), parasites (Jacquin et al., 2011) and several conditions during development that can potentially affect melanin synthesis (Hubbard et al., 2015). Our study shows that soil characteristics represents an additional environmental factor affecting the expression of melanin-based pigmentation in birds, likely by influencing exposure to sulfur and intracellular thiol levels, meaning that soil arises as a novel source of vertebrate phenotypic diversity. This is particularly relevant for regions with high volcanic activity such as Iceland, which may thus exert a significant contribution to

animal pigmentation diversity by enhancing phenotypic plasticity through effects on sulfur exposure.

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GENERAL DISCUSSION

Organisms have the ability to respond and adapt to environmental changes by adjusting their physiological responses through changes in gene expression (López-Maury et al., 2008). The activation of this mechanism leads to a short and/or long-term plastic response, which may constrain the evolution of phenotypic traits (Gabriel, 2005). In particular, an adaptive phenotype depends on the interaction of the genotype with the environment both during the expression of the trait and during its selection (Nager & van Noordwijk, 1995). For this reason, the study of phenotypic trait evolution requires an integrative approach as the one adopted in this thesis. By combining experimental procedures performed in captivity as well as in natural populations under varying environmental conditions, measurements of changes in gene expression, epigenetic modifications, and phylogenetic analyses, a comprehensive view of the evolution of melanin-based plumage was obtained.

Environmental conditions can affect gene expression patterns by epigenetic modifications and lead to phenotypic changes (Tammen et al., 2013). Pheomelanin synthesis seems to be a physiological mechanism dependent on environmental conditions. Therefore, if pigmentation phenotypes evolve in accordance with the environmental conditions, opposite effects on pigmentation of two different environmental factors that are known to affect pheomelanin synthesis are expected: increased environmental oxidative stress should reduce pheomelanin synthesis and pigmentation, while increased systemic cysteine levels should favor pheomelanin synthesis and pigmentation. For this reason, it was essential to determine if melanocytes from growing pheomelanin pigmented feathers show epigenetic lability and respond to an increase in cysteine availability by favoring pheomelanin synthesis (**Chapter I**) and if an increase in environmental oxidative stress reduces pheomelanin synthesis to avoid the expected oxidative damage (**Chapter II** and **III**). The experimental supplementation with dietary cysteine in birds (**Chapter I**) led to increased cysteine levels in erythrocytes but did not induce systemic oxidative stress nor negative effects on body condition as expected. Cysteine supplementation induced the downregulation of genes involved in intracellular cysteine metabolism (*GCLC*), cysteine transport to the cytosol from the extracellular medium (*Slc7a11*)

and from melanosomes (*CTNS*), and regulation of tyrosinase activity (*MC1R* and *ASIP*), in feather melanocytes. These changes were mediated by epigenetic modifications, as revealed by increases in DNA 5-methylcytosine (m^5C) in all genes except *Slc7a11*, which experienced RNA N6-methyladenosine (m^6A) depletion. Consequently, more pheomelanin was synthesized in feather melanocytes leading to more intense plumage coloration. These results suggest that excess cysteine induces epigenetic modifications that regulate the expression of genes involved in cysteine metabolism and pheomelanin synthesis in feather melanocytes, favoring pheomelanin production and avoiding the expected oxidative stress caused by excessive levels of cysteine. Therefore, pheomelanin synthesis seems to be a physiological mechanism that protects cells against oxidative stress. However, this regulatory mechanism is not expected to be functional in all species (Galván & Alonso-Alvarez, 2017), but in those that already have a genetic basis leading to the production of large amounts of pheomelanin, which is reflected in pigmentation phenotypes consisting of light brown or orange colors (Galván & Wakamatsu, 2016). In that sense, to get a global view on the evolution of pheomelanin-based pigmentation, it was necessary to determine if pheomelanin synthesis occurs as a detoxifying strategy in a phylogenetic context (**Chapter VIII**).

Once the influence of excess cysteine in pheomelanin synthesis was determined, the next step was to analyze if genes involved in pheomelanin synthesis are also regulated when available cysteine is required for antioxidant protection against environmental stress (**Chapters II** and **III**). In particular, the social environment is an important factor that induces a physiological stress response (Creel et al., 2013) and is intimately related to melanin synthesis (Slominski et al., 2004). A competitive social environment experimentally induced in birds did not lead to systemic oxidative stress or damage nor had a negative effect on the body condition of birds, suggesting that a protective mechanism avoids the expected oxidative damage of social interactions (**Chapter II**). In fact, epigenetic modifications mediated by changes in DNA methylation levels have induced changes in the expression of the main gene involved in pheomelanin synthesis (*Slc7a11)*. The downregulation of *Slc7a11* limited pheomelanin synthesis which helps to avoid the expected oxidative stress of social interactions. However, no effects on the intensity

of pheomelanin-based plumage coloration were detected. This may be explained by the short duration of stress exposure and/or the low stress that social interactions may have caused to birds, which may be sufficient to activate the protective epigenetic mechanism but not to induce changes in pigmentation phenotype. Thus, the results suggest that *Slc7a11* epigenetic lability may have evolved in some bird species as a mechanism sensitive to environmental stress (Galván et al., 2017; **Chapter I** and **II**). In that sense, it seems that animals have the capacity to adjust their pigmentation phenotype by limiting pheomelanin synthesis under environmental stress conditions. Such response is expected in birds expose to any exogenous source of oxidative stress. In contrast, an improvement in the antioxidant status of birds exposed to oxidative stress experimentally induced by the administration of a chemical stressor was found (**Chapter III**). Birds increased the production of pheomelanin, which consequently led to a darker plumage phenotype mediated by the regulation of the expression of *AGRP*, a gene that decreases the activity of tyrosinase and consequently promotes pheomelanin synthesis (Ozeki et al., 1997). In that sense, oxidative stress probably activated a hormetic response that did not only enhance the oxidative status of birds but also favored the expression of genes that promotes pheomelanin synthesis. This suggests that the genes involved in pheomelanin synthesis differentially respond to different environmental sources of stress to maintain homeostasis (**Chapters I**, **II**, and **III**). However, to infer the physiological implications of pheomelanin synthesis it was also necessary to evaluate if the synthesis of pheomelanin-based plumage coloration promotes oxidative damage (**Chapter IV**). Birds that profusely pigment their plumage with pheomelanin showed lower physical condition and higher oxidative damage in DNA than male zebra finches with unpigmented plumage or pigmented with eumelanin, supporting the idea that a constant and abundant production of pheomelanin may lead to chronic oxidative stress and damage (Napolitano et al., 2014; Mitra et al., 2012; Galván et al., 2014; Galván et al., 2017). These results suggest that pheomelanin synthesis constitutes a physiological limitation for organisms expressing pheomelanin-based color phenotypes. However, birds of the pheomelanin-based (orange) color morph not only steeply reduced their DNA damage levels during the treatment but also decreased plumage flank color expression. The genotypic characterization of the distinct color morphs (i.e., orange, black, and white) showed

mutations in genes that mainly affect pigmentation and do not have known effects on the antioxidant metabolism, thus it seems that the physiological effects are a consequence of pheomelanin synthesis. Therefore, pheomelanin-based plumage coloration reveals dual physiological properties in organisms: oxidative status and condition seem to be weaker than animals that do not produce pheomelanin but also reveals phenotypic plasticity that allows them to adjust pheomelanin production that animals not producing pheomelanin do not possess.

A powerful mechanism of adaptation to environmental stress is modulated by epigenetic modifications that can be rapidly returned to the basal state after stress disappearance (Sørensen et al., 2005; Yale, 2001) to avoid consequences in phenotype (Gabriel, 2005). Otherwise, the adjustment of a trait expression to changes in environmental conditions may affect the adaptive value of sexual traits (Greenfield & Rodriguez, 2004; Kokko & Heubel, 2008) and hence, influence sexual selection. To avoid consequences in external phenotype, phenotypic plasticity should be dependent on the duration of stress, as reversible changes in gene expression are expected under short stress periods (Gabriel, 2005). In a bird species, a short exposure (15 days) to environmental stress (i.e., competitive social environment) led to *Slc7a11* downregulation (**Chapter II**), and a reversed state was detected shortly after that (**Chapter V**). Reversibility in *Slc7a11* expression, probably mediated by epigenetic modifications, may thus avoid changes in the pigmentation phenotype during transient stressful conditions. In that sense, pheomelanin synthesis is adjusted to the prevailing environmental conditions to avoid oxidative stress without consequences in the phenotype. This, in turn, makes the regulation of pheomelanin synthesis an adaptive strategy to avoid the evolutionary consequences of external phenotypic changes, like effects on sexual selection, under rapid environmental changes. In fact, recent findings reveal that pheomelanin-based plumage phenotype is a signaling trait under strong selection that depends on the physical condition of an individual during their development (Galván, 2017). In particular, the intensity of chestnut coloration seems to be higher in individuals with poor body conditions (Galván, 2017) hence females have shown a preference for males with a diminished expression of chestnut flank coloration (**Chapter VI**). Female preferences for males with lighter flank coloration seem to be an adaptive strategy,

as stress experienced early during development can lead to poor body condition in the affected offspring and negative fitness-related effects in subsequent generations (Naguib et al., 2006; Goerlich et al., 2012). Therefore, mating decisions based on plumage coloration allow females to avoid males with poor physical condition and, thus, a negative effect on the physical condition of their offspring. In that sense, pheomelanin synthesis not only seems to be a physiological mechanism regulated by epigenetic modifications to maintain cellular homeostasis (**Chapters I**, **II**, **III**, **IV**, and **IV**) but also seems to be involved in phenotypic traits evolution (**Chapter VI**). Overall, the results of these six chapters (**Section 1**) show for the first time that animals (birds) have an epigenetic lability that allows them to adjust their pigmentation phenotype to the prevailing environmental conditions, and thus avoid stress consequences. In that sense, pheomelanin synthesis seems to be an adaptive physiological mechanism that helps to maintain cellular homeostasis and is involved in the evolution of pigmentation phenotypes. However, to get a global view of the evolution of bird pigmentation phenotypic diversity, it was essential to focus on the interspecific differences in plumage coloration.

Species do not only differ in color heterogeneity but also in the level of expression (i.e., intensity) of these colors (Gomez & Théry, 2004). Interspecific variation in color expression levels may depend on the physiology of plumage color production. In particular, producing several forms of melanin, which is required to generate several colors (Galván & Wakamatsu, 2016), is more constraining than producing a single or few melanin forms to pigment the plumage. In a phylogenetic context, a negative association between the production of different colors and the intensity of each color was found after analyzing the diversity and expression level of melanin-based plumage coloration (**Chapter VII**). The intensity of the plumage colors of birds decreased with the number of different colors, suggesting that the physiological mechanism of melanin synthesis does not favor the production of both heterogeneity of melanin forms and large amounts of these forms. As melanin is the most abundant pigment in birds (Delhey, 2015), this evolutionary solution to a physiological limitation may be of general validity to understand the evolution of bird phenotypic diversity. However, differences in plumage coloration do not only occur between bird species, but also through different life stages within the same species

(Booth, 1990). This is because different forms of melanin, the most common pigments involved in changes with age, imply different physiological constraints (Galván & Solano, 2009). In particular, juvenile birds - generally suffering less physiological stress than adults- are more prone to experience systemic excess cysteine, as they may have lower requirements of GSH for antioxidant protection (Galván, 2017). To avoid excess cysteine, juvenile plumage expressing pheomelanin-based coloration has evolved more frequently in species of birds with strictly protein diets (i.e., carnivores) that imply high cysteine intakes (**Chapter VIII**). Pheomelanin-based coloration in juvenile plumage could represent a potential adaptive benefit that evolved as a result of a detoxifying strategy. Therefore, the need for cysteine homeostasis constitutes an evolutionary pressure that affects the expression of pheomelanin-based pigmentation in birds. However, environmental effects on thiol levels in melanocytes that influence pheomelanin synthesis may not be exerted only through the effect of dietary cysteine but also by the exposure to other environmental factors. In particular, exposure to elemental sulfur can affect intracellular thiols in organisms (Kuklińska et al., 2013) and consequently pheomelanin synthesis. The exposure to high levels of elemental sulfur in the environment, in areas with high volcanic activity like Iceland, favored the production of pheomelanin in the plumage of different species of birds, likely by an influence of increased intracellular thiols during development (**Chapter IX**). In particular, feathers profusely pigmented by pheomelanin were sampled in sites with higher sulfur content of soil than sites where feathers with lower amounts of pheomelanin were collected, suggesting that soil characteristics represent an additional environmental factor affecting the expression of melanin-based pigmentation in birds. Selection may have favored the maintenance of such phenotypes due to the benefits of pheomelanin production to avoid sulfur/thiol toxicity (**Chapters VIII** and **IX**) but also due to the benefits of dealing with the physiological constraints of melanin-based plumage coloration (**Chapter VII**). Together, these three final chapters (**Section 2**) provides a great understanding of the evolution of plumage pigmentation diversity. Although this thesis is focused on bird plumage, the results obtained here also contribute to the understanding of the evolution of pigmentation phenotypes in other taxa, as melanin is the most abundant pigment in animals and one of the most abundant pigments in nature.

To summarize, pheomelanin synthesis seems to have evolved as a physiological mechanism that provides phenotypic plasticity to birds, allowing to adjust the pigmentation phenotype to the prevailing environmental conditions through epigenetic modifications and changes in the expression of genes involved in melanin synthesis. This, in turn, allows avoiding oxidative damage under stressful conditions. By understanding both the proximate mechanisms of pheomelanin synthesis and the ultimate causes of the expression of pheomelanin-based plumage phenotypes, I was able to reveal an integrative view of the evolution of melanin-based traits. Therefore, this thesis contributes to a better understanding of bird phenotypic diversity by a multidisciplinary approach that integrates phylogenetic analyses and experimental procedures performed in natural and experimental populations.

CONCLUSIONS

1. Pheomelanin synthesis is a physiological mechanism that allows animals (birds) to avoid environmental oxidative stress. Regulating the expression of pheomelanin-based coloration helps to maintain cellular homeostasis in accordance with environmental conditions.

2. Feather melanocytes show epigenetic lability under environmental stress that leads to phenotypic plasticity in pigmentation. The epigenetic modifications are mediated by changes in DNA and/or RNA methylation levels that regulate the expression of genes involved in pheomelanin synthesis. This adaptive response is physiologically advantageous by producing opposing effects on pigmentation in accordance with the prevailing environmental conditions: increased environmental oxidative stress reduces pheomelanin synthesis and pigmentation, and increased dietary cysteine favors pheomelanin synthesis and pigmentation. In both cases, epigenetic modifications are physiologically advantageous as the expected oxidative stress and damage are avoided.

3. Changes in the expression of the main gene involved in pheomelanin synthesis are reversed after stress cessation. Reversibility in the expression of genes involved in pheomelanin synthesis allows birds to adapt to changing environmental conditions and thus avoid consequences in the pigmentation phenotype. This, in turn, makes the epigenetic regulation of pheomelanin synthesis an adaptive strategy to avoid the evolutionary consequences on external phenotype under rapid environmental changes.

4. The regulatory activity of genotypes leading to the production of large amounts of pheomelanin creates weak physiological conditions but is more labile than other pigmentation genotypes, making pheomelanin-based plumage coloration phenotypically plastic. This gives pheomelanin-pigmented organisms a greater physiologically adaptive potential.

5. Sexual selection drives the evolution of pheomelanin-based plumage coloration. In accordance with the detoxifying strategy, females prefer to

mate with males displaying feathers of reduced pheomelanin-based color intensity. Thus, the evolution of plumage coloration seems to be dependent on the physiological mechanisms that regulate pheomelanin synthesis.

6. Pheomelanin synthesis evolved as a physiological mechanism to adjust pigmentation phenotype to the prevailing environmental conditions. This adaptive mechanism is mediated by epigenetic modifications that regulate the expression of genes involved in melanin synthesis to maintain cellular homeostasis. Therefore, the expression of pheomelanin-based plumage depends on environmental conditions that promote oxidative stress.

7. The evolution of melanin-based plumage depends both on the interaction of the genotype with environmental conditions during the expression of the trait and during trait selection. In that sense, plumage phenotypic diversity depends on physiological constraints during its synthesis under different environmental conditions. As melanin is the most abundant pigment in nature the results obtained here also contribute to the understanding of the evolution of pigmentation phenotype diversity in other taxa.

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