The expression of melanin-based plumage is separately modulated by exogenous oxidative stress and a melanocortin

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Melanin-based traits involved in animal communication have been traditionally viewed as occurring under strict genetic control. However, it is generally accepted that both genetic and environmental factors influence melanin production. Medical studies suggest that, among environmental factors influencing melanization, oxidative stress could play a relevant role. On the other hand, genetic control would be exerted by the melanocortin system, and particularly by the alpha-melanocyte-stimulating hormone (α-MSH), which triggers the production of eumelanins (black pigments). To determine how the melanocortin system and an exogenous source of oxidative stress interact in the expression of melanin-based plumage, developing red-legged partridges (*Alectoris rufa*) were manipulated. Some partridges were injected with α-MSH, while other birds received a pro-oxidant molecule (diquat) in drinking water. Controls and birds receiving both treatments were also studied. Both α-MSH- and diquat-treated individuals presented larger eumelanin-based traits than controls, but α-MSH+diquat-treated birds showed the largest traits, suggesting that oxidative stress and melanocortins promote additive but independent effects. Diquat also induced a decline in the level of a key intracellular antioxidant (glutathione), which is associated with high expression of eumelanin-based signals in other bird species. Some scenarios for the evolution of melanin-based traits in relation to oxidative stress are proposed.

Keywords: melanocortin system; eumelanin; glutathione; oxidative stress; pheomelanin

1. INTRODUCTION

Melanins and carotenoids are the main pigments conferring colour to the integuments of vertebrates, the former being responsible for most black and brown colours and the latter for many reds, yellows and oranges (Brush 1978). The reliability of the information transmitted by those coloured traits acting as signals in animal communication has frequently been associated with the costs of producing or acquiring pigments. In recent years, many studies have centred on the costs of producing carotenoid-based signals (Møller *et al.* 2000). Carotenoids may act as antioxidants and immunostimulants and are obtained exclusively from the diet (Møller *et al.* 2000). Therefore, trade-offs might arise between allocating carotenoids to these physiological functions or to signalling functions (that is, conferring colour to integuments, where they no longer fulfil their physiological roles; Olson & Owens 1998; von Schantz *et al.* 1999; Møller *et al.* 2000). These trade-offs would limit the expression of carotenoid-based signals to those individuals with a high antioxidant or immune capacity, thus able to allocate a high amount of carotenoid resources to the integuments (Alonso-Alvarez *et al.* 2004, 2008). In the case of melanin-based signals, however, a similar explanation has

only recently arisen (Galván & Alonso-Alvarez 2008; Galván & Solano 2009). This disparity may be due to the fact that melanins can be synthesized endogenously by the organism (i.e. they may not be limited resources; e.g. Prota 1992) and because it has been believed that their production is mostly under tight genetic control, precluding the possibility of condition-dependent effects and environmental influences on their production (Griffith *et al.* 2006).

The genetic control in the expression of melanic traits is relatively well known (e.g. Mundy 2006; Lin & Fisher 2007). In vertebrates, this control depends on the pro-opiomelanocortin (POMC) gene, which encodes different peptides that are part of the melanocortin system: α-, β- and γ-melanocyte-stimulating hormones (MSH) and adrenocorticotropic hormone (ACTH). The other elements comprising the melanocortin system are five melanocortin receptors (MCRs) and their antagonists, agouti-signalling (ASIP) and agouti-related (AGRP) proteins (Boswell & Takeuchi 2005). In the context of the melanocortin system, the most important elements explaining melanization are α-MSH and ASIP (Boswell & Takeuchi 2005; Nadeau *et al.* 2008). In birds, the function of the latter may also be fulfilled by AGRP (Boswell & Takeuchi 2005). α-MSH and ASIP/AGRP bind to the melanocortin 1 receptor (MC1R) in the membrane of melanin-producing cells of vertebrates (i.e. melanocytes; see figure 1), leading to an increase and a decrease, respectively, in intracellular cyclic adenosine monophosphate
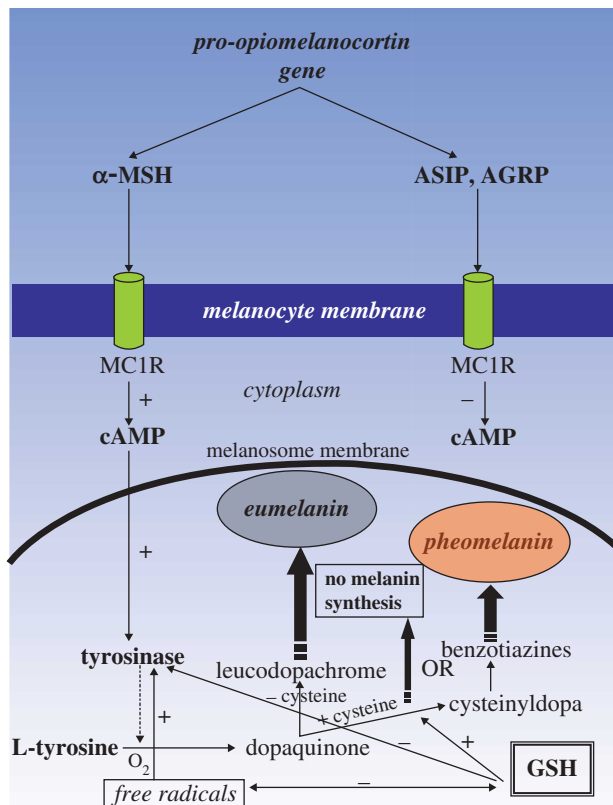
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Figure 1. Schematic representation of the melanogenesis pathway in vertebrate melanocytes that generates eumelanic and pheomelanic traits. ASIP: agouti signalling protein; AGRP: agouti-related protein; MSH: alpha-melanocyte-stimulating hormone; MC1R: melanocortin 1 receptor; cAMP: cyclic adenosine monophosphate; GSH: glutathione.

(cAMP) levels (Bowers et al. 1997). High cAMP levels stimulate the activity of tyrosinase, an enzyme catalysing the first step of melanogenesis, which produces dopaquinone through oxidation of the amino acid tyrosine (Boswell & Takeuchi 2005; figure 1). Cysteine is another amino acid influencing this process, leading to the formation of cysteinyldopas and thus to the production of pheomelanins when its level is higher than the level of dopaquinone being produced, while eumelanins are produced when the level of dopaquinone is higher than the level of cysteine (Ozeki et al. 1997). This means that high tyrosinase activity promotes a switch from pheo- to eumelanin production (Ozeki et al. 1997; figure 1). Eumelanin is mostly a black pigment, whereas pheomelanin produces yellow–reddish colours (Toral et al. 2008). Eumelanin and pheomelanin are often synthesized simultaneously in the melanocyte, but the amount of one pigment generally prevails, which is translated into a different colour gamut (Ozeki et al. 1997).

In spite of strong support for genetic control in melanin production, melanin-based traits are frequently related to the condition of the bearer, which often fluctuates with environmental conditions (reviewed in Griffith et al. 2006; McGraw 2008). But what kind of environmental factors might influence the expression of these traits? Medical bibliography suggests that, among environmental factors, oxidative stress could play a prominent role (e.g. Chakraborty et al. 1996; Meyskens et al. 2001; Wittgen et al. 2007). Oxidative stress is commonly defined as the imbalance between the production of reactive oxygen species (ROS) and the state

of the antioxidant and repair machinery, with the balance tipped towards the former (Finkel & Holbrook 2000). In this sense, low levels of a key cysteine-containing intracellular antioxidant found in virtually all animal cells (i.e. glutathione; GSH) have often been related to the deposition of melanin in the skin of humans and other mammals (e.g. Halprin & Okhawara 1966; Benedetto et al. 1981; del Marmol et al. 1993). High GSH levels in fact inhibit eumelanin production through different molecular pathways (Galván & Alonso-Alvarez 2008; figure 1). First, the thiol groups of the amino acid cysteine and GSH promote the formation of cysteinyldopas and thus a switch from eu- to pheomelanogenesis (reviewed in Galván & Solano 2009; figure 1). Second, these thiol groups interact with the tyrosinase active site, thus inhibiting its activity and the production of dopaquinone (del Marmol et al. 1993). Finally, since certain levels of free radicals (i.e. peroxide radicals) are apparently able to stimulate tyrosinase activity, the antioxidant action of GSH could ultimately decrease dopaquinone production (Karg et al. 1993; figure 1). Accordingly, it has been proposed that eumelanin-based traits may signal the ability to cope with oxidative stress because those individuals with low enough levels of GSH, as required for melanogenesis, must also have enough alternative antioxidant resources to counteract the paucity of GSH (Galván & Alonso-Alvarez 2008). Additionally, free radicals could also deplete GSH levels (e.g. Sewalk et al. 2001; figure 1), leading to higher eumelanin production (see also §4c).

Although it appears that both the melanocortin system and environmentally induced oxidative stress could simultaneously influence melanin production, how these two factors interact in the expression of those melanin-based traits potentially involved in animal communication remains unknown. Here, we aimed to address this question by manipulating captive red-legged partridges (*Alectoris rufa*) during development. These birds develop conspicuous eumelanin- and pheomelanin-based plumage traits in the first few weeks of life. Some partridges were injected with α-MSH, while other birds received a certain dose of a pro-oxidant substance (diquat) in drinking water. Controls and birds receiving both treatments were also studied (i.e. a two-way design). Since diquat is an agent that generates oxidative stress and decreases GSH levels (Sewalk et al. 2001), and α-MSH stimulates tyrosinase and enhances eumelanin production (Höhn & Braun 1980; Bowers et al. 1997), we predicted that partridges grown under both treatments would present a higher expression of eumelanin-based traits than controls. If the effect of these factors is independent, we would expect an additive effect on the size of eumelanic traits (no interaction). In support of this, we also expect that diquat would induce a decrease in GSH levels, whereas α-MSH should not. With regard to the expression of pheomelanic traits, inhibition would be expected since both types of melanins are competitively produced (Galván & Solano 2009). Blood samples were taken before and after the treatment to assess GSH levels and several oxidative stress markers. Thus, the total antioxidant status (TAS) of plasma (i.e. a measure of antioxidant capacity) and the level of lipid peroxidation in erythrocytes (i.e. a measure of oxidative damage in cell membranes) were assessed. Lower TAS values in diquat-treated birds

compared to control birds are expected, as antioxidant resources are exhausted in combating the oxidative challenge. Similarly, a greater level of oxidative damage in these birds is expected. Nonetheless, since the oxidative challenge was maintained for several weeks, we cannot *a priori* discard the presence of compensatory mechanisms increasing TAS levels and buffering oxidative damage at the end of the study (e.g. Galván & Alonso-Alvarez 2008; Monaghan *et al.* 2009). To conclude, the development of the eumelanic black-spotted bib and the eumelanic–pheomelanic flank bands (description below) was determined from pictures taken before and after the treatment.

2. MATERIAL AND METHODS

(a) *Study species*

The red-legged partridge is a galliform bird with a distribution range that covers southwestern Europe. Most pairs are monogamous with long-term bonds, but multiple mates have also been recorded (del Hoyo *et al.* 1994). Like other *Alectoris* species, red-legged partridges present conspicuous plumage patterns and only a modest degree of sexual dichromatism. Therefore, the *Alectoris* genus is atypical among Galliformes, which are a mostly cryptic or dichromatic species. Apart from red integuments in bill, eye ring and legs (del Hoyo *et al.* 1994), the plumage of red-legged partridges also presents a complex design. The most striking features are a black-spotted bib extending from the throat to the breast, and 8–9 lateral bands composed of coloured flank feathers (see Bortolotti *et al.* 2006; figure 2). Each of these flank feathers presents, from proximal to distal, a 3–5 mm wide white bar, a 2 mm wide black bar and 5 mm wide brown bar (Bortolotti *et al.* 2006). The black bar of each feather is aligned with contiguous black bars, thus creating continuous lateral black bands (Bortolotti *et al.* 2006; figure 2). The black bands mainly contain eumelanin, while the brown bands mainly contain pheomelanin, as deduced from biochemical analyses of pigments (Toral *et al.* 2008; J. J. Negro 2008, personal communication) and analysis of the shape of reflectance spectral curves (McGraw 2006). It must be noted that eumelanin and pheomelanin normally occur simultaneously in the tissues (Ozeki *et al.* 1997), but the darker colour conferred by eumelanin (Toral *et al.* 2008) makes evident the lower content of this pigment in the brown bands of partridges as compared to the black bands. For the sake of simplicity, we refer to black bands as 'eumelanic bands' and to brown bands as 'pheomelanic bands'.

During aggressive and intersexual encounters, red-legged partridges display these flank bands by turning laterally and thus exhibit them to their opponents or potential mates (Bortolotti *et al.* 2006). The size of the black bands is positively related to size-corrected body mass (commonly 'body condition'; Hayes & Shonkwiler 2001) in males and negatively to the ratio of heterophils : lymphocytes in females (a proxy of physiological stress), suggesting that this trait acts as a signal of quality in both sexes (Bortolotti *et al.* 2006). Along the same lines, positive correlations between the shape of the bib, size-corrected body mass and cell-mediated immunity have been detected (L. Pérez-Rodríguez and F. R. Mougeot 2008, unpublished data).

(b) *Experimental design*

The study was carried out in July–October 2008 on 78 captive partridges at the Dehesa Galiana experimental facility
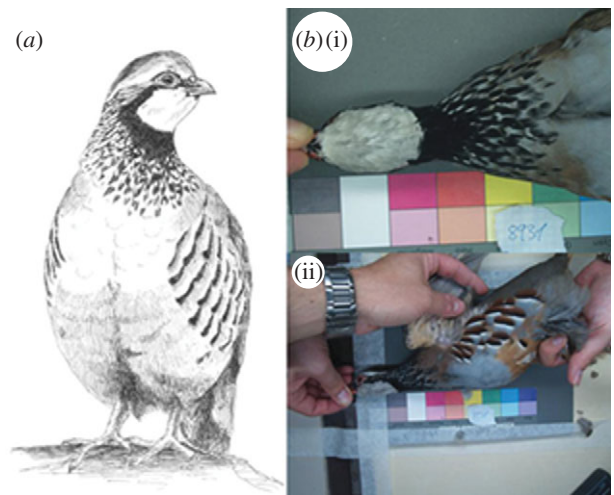

Figure 2. (*a*) Illustration of an adult red-legged partridge, showing the melanic black-spotted bib and the flank bands (courtesy of L. Pérez-Rodríguez). (*b*(i)) Picture taken to measure the extension of the bib after the end of the experiment. (*b*(ii)) Picture taken to measure the size of the eumelanic (black) and pheomelanic (brown) flank bands.

(Ciudad Real, Spain). We studied developing chicks obtained from eggs laid by adult partridges housed in outdoor cages ($1 \times 0.5 \times 0.4$ m). Breeding cages were inspected daily, and the eggs removed, identified and immediately stored at 15°C. At this temperature, embryo development is arrested (Thear 1987). Stored eggs were transferred to incubators (38°C) every 15 days. Hatchlings from two successive 'incubation series' were available, hatching dates being 8 June and 23 June. The chicks used in this study were obtained from 25 different breeding couples. Birds were identified with a numbered plastic ring (extensible) and kept in indoor aviaries with *ad libitum* food. Just before the first set of birds began to develop the plumage traits (i.e. about 20 days of age), all of them were randomly assigned to one of the four different treatments (see below), marked with a numbered metal ring and transferred to one of four different indoor aviaries ($4 \times 3 \times 3$ m; light regime: 13 h : 11 h, L : D; *ad libitum* pelleted food). Birds from the second incubation series were kept in four other aviaries under the same conditions. Birds received diquat, α-MSH or both, while a fourth group served as a control. The identity of the parents of each bird was randomized among experimental groups.

Just before the birds were randomly assigned to the treatments and placed in the corresponding aviaries (20 days of age), they were sampled for blood and weighed, and their tarsus length was measured with a digital calliper ('initial values'). The same was done at the end of the experiment, 33 days later ('final values'). Blood samples were stored at 4°C and centrifuged within 5 h after extraction. Plasma and cell fractions were immediately stored at -80°C.

(i) *α-MSH treatment*

α-MSH (Sigma, St. Louis, USA) was administered by means of intramuscular injections in the left pectoral muscle at a dose of 0.04 mg of α-MSH dissolved in 2 ml of phosphate buffered saline (PBS) per bird. Control birds received 2 ml of PBS only. The injection site was sterilized with alcohol. Injections were administered every two days during a 15-day period (i.e. 8 injections per bird). Hence, each bird

received a total amount of 0.32 mg of α-MSH. This dose is equivalent to that used by Höhn & Braun (1980), who reported increased levels of feather melanization in other gallinacean species (i.e. ptarmigans, genus *Lagopus*), but carried out over a longer time period (they used a one-week period with daily injections). Here, the total α-MSH amount was corrected for mean body mass of growing partridges (ptarmigans are heavier).

(ii) *Diquat treatment*

Diquat dibromide is commonly used as an aquatic herbicide for which the mechanism of action is the production of ROS, particularly intracellular superoxide anion (e.g. Sewalk *et al.* 2001; Zeman *et al.* 2005; Xu *et al.* 2007). In our study, diquat was supplied in drinking water. The commercial product (Reglone, Syngenta, Madrid) consisted of 20 per cent w/v of diquat dibromide in water (consulted with the company). The treatment with diquat in water lasted 33 days, overlapping with the greater part of the feather development. Each aviary contained a tank with 4 l of water, which was replaced every 4 days to avoid the disintegration of the diquat molecule (i.e. following product properties; Syngenta, Madrid). Therefore, each aviary contained a single drinking treatment (i.e. diquat plus water or only water), whereas both α-MSH- and PBS-injected birds were present in each aviary.

To serve as an orientation for the diquat dose, a pilot study was previously performed on another group of 20-day old partridges ($n = 36$). These birds were randomly assigned to one of four doses of diquat (0.25, 0.50, 1 and 2 ml diquat per litre of water; $n = 7$ birds per group), or received water only ($n = 8$). Each group was kept in a separate aviary (conditions described above). Body masses were recorded at the beginning and at the end of the pilot study (a 15-day interval). To know whether water intake was influenced by diquat, daily water consumption was estimated during four days. A Spearman correlation test showed that diquat doses were unrelated to water intake ($r_s = -0.53$, $n = 5$, $p = 0.36$). Mean values were: 16, 14, 14, 16 and 11 ml of water in control, 0.25-, 0.50-, 1- and 2-ml l^{-1} groups, respectively. The 0.50 ml l^{-1} dose was chosen for use in the definitive experiment because it induced a weak decline in mass gain, but far from the strong body mass loss exhibited by birds in the highest dose (no bird died during the pilot study or in the subsequent experiment; see below).

(c) *Measurement of plumage melanization*

When the birds reached an age of 93 days, photographs of the breast and of the left flank of birds were taken with a digital camera (Olympus E-500; figure 2). The birds were held in the same posture, at a fixed distance from the camera and under standardized illumination (figure 2). The black and brown areas were measured with the 'magic wand' tool of Adobe Photoshop (units in pixels). To control for a subtle change in technical parameters of the camera between the two incubation series of birds, all measurements were standardized (mean=0, s.d.=1). The repeatability (estimated following Lessells & Boag 1987, here and thereafter) of the measurements taken twice was very high ($r = 0.93$, $F_{72,73} = 27.74$; $r = 0.89$, $F_{70,71} = 18.04$; and $r = 0.93$, $F_{70,71} = 108.02$, for black-striped bib, eumelanic and pheomelanic flank bands, respectively; all $p < 0.0001$). Therefore, mean values were used.

(d) *Total glutathione*

Total glutathione (tGSH hereafter) levels in red blood cells were determined by following the method described by Tietze (1969) and Griffith (1980) with some particular modifications (see electronic supplementary material for further details).

(e) *Antioxidant status*

TAS of plasma was assessed by means of commercial kits (Randox Laboratories Ltd, Crumlin, UK) adapted to an automated spectrophotometer (A25-Autoanalyzer; Biosystems SA, Barcelona; see Alonso-Alvarez *et al.* 2008 and electronic supplementary material for further details).

(f) *Uric acid*

Plasma uric acid levels were determined by spectrophotometry, using commercial kits (Biosystems SA, Barcelona) that followed the uricase/peroxidase method.

(g) *Lipid peroxidation in erythrocytes*

Lipid peroxidation was assessed following Aust (1985; see Alonso-Alvarez *et al.* 2008 and electronic supplementary material for further details).

(h) *Molecular sexing*

To avoid any potential bias owing to sex, partridges were molecularly sexed from an aliquot of the blood cell fraction. DNA from the sex chromosomes (Z and W) was amplified by PCR using the primers 2550F and 2718R and the procedure described by Griffiths *et al.* (1998).

(i) *Statistical analyses*

Generalized linear mixed models (GLMMs) were performed to investigate the effects of diquat, α-MSH and their interaction on size-corrected body mass, growth rate and plumage melanization. All of these variables were dependent variables in single GLMMs, and diquat (presence vs. absence) and α-MSH (presence vs. absence) treatments were added as fixed factors together with their interaction. Other terms in the models were the sex of the bird and its interaction with diquat and α-MSH treatments. The identity of the parents (i.e. the cage where the egg was laid) and the aviary in which the birds were placed during the experiment were tested as random factors, although they never showed a significant influence (always $p > 0.10$, except in the model for size-corrected body mass; see §3), and were hence removed to increase the degrees of freedom of the models. In the analyses of plumage melanization, tarsus length recorded at the end of the experiment was introduced as a covariate to control for the effect of body size variability. Nonetheless, models including random effects showed the same findings. Since initial and final values of blood variables showed relatively high coefficients of correlation among them (Pearson correlation: tGSH: $r = 0.79$, $p < 0.0001$; TAS: $r = 0.14$, $p = 0.220$; TBARS: $r = 0.18$, $p = 0.132$), changes in tGSH, TAS and TBARS were analysed by means of repeated-measures ANCOVAs, including the moment at which the measures were taken (initial vs. final values) as a within-subjects factor, and other explanatory variables as in the models explained above. Since the concentration and, hence, the effect of any substance on an organism depend on the body mass through which it is distributed, the mean body mass during the experiment (calculated from initial

and final body masses) was tested as a covariate in the analyses of change in tGSH, TAS and TBARS. In the analysis of TAS change, the change in uric acid levels was controlled for by including this variable (final minus initial values) as a covariate in the model (see Cohen *et al.* 2007). In the analyses of plumage melanization, tarsus length recorded at the end of the experiment was introduced as a covariate to control for the effect of body size variability. The aviary in which the birds were placed during the experiment and the identity of the parents could not be considered as factors in the analyses of change in tGSH, TAS and TBARS because it was not possible to perform repeated-measures ANCOVAs with random factors, but GLMMs with the difference between final and initial values of these variables as a dependent variable showed that they had no significant effects ($p > 0.10$), also reporting the same findings.

Starting with the saturated model, a backward stepwise procedure was used to remove non-significant terms (here $p > 0.1$) and to obtain the best fitted model. Non-significant terms included in significant interactions were not removed. Tarsus length was always maintained in the models performed for plumage melanization variables in order to control for body size. Pair-wise LSD tests were used to investigate significant differences between levels of interacting factors. The distribution of residuals obtained from the models showed that normality assumption was fulfilled. Only the results of the models obtained after the stepwise procedure are shown.

3. RESULTS

(a) *Effects of diquat, α-MSH and their interaction on growth and size-corrected body mass*

The size-corrected body mass of partridges, assessed by a model with body mass at the end of the experiment as the dependent variable and tarsus length as a covariate (tarsus length effect: $F_{1,67} = 55.90$, $p < 0.0001$) was only influenced by the sex of the bird ($F_{1,67} = 2.95$, $p = 0.091$) and the aviary in which they were placed ($F_{1,67} = 2.89$, $p = 0.011$). Similarly, growing rate, measured through the change in tarsus length from the beginning to the end of the experiment, and body mass gain were only predicted by the sex of the bird (growing rate: $F_{1,74} = 9.90$, $p = 0.002$; body mass gain: $F_{1,74} = 6.12$, $p = 0.016$), with males (mean $\pm$ s.e., growing rate: 1.21 ± 0.13 cm; body mass gain: 78.13 ± 4.95 g) growing more and gaining more mass than females (growing rate: 0.64 ± 0.13 cm; body mass gain: 63.31 ± 3.37 g). The treatments and their interactions did not show significant effects in either model ($p > 0.1$). Furthermore, no bird died during the course of the experiment.

(b) *Effects of diquat and α-MSH on total glutathione and plasma antioxidants*

There were no significant differences in the initial levels of tGSH between controls and birds treated with diquat ($F_{1,70} = 1.50$, $p = 0.224$) or α-MSH ($F_{1,70} = 1.43$, $p = 0.236$), and the same applied to TAS (diquat treatment: $F_{1,73} = 0.13$, $p = 0.714$; α-MSH treatment: $F_{1,73} = 0.05$, $p = 0.829$). In the case of the change in tGSH levels in red blood cells, the diquat treatment in interaction with the within-subjects factor was the only factor remaining in the model ($F_{1,70} = 8.74$, $p = 0.004$). Whereas control birds showed a positive
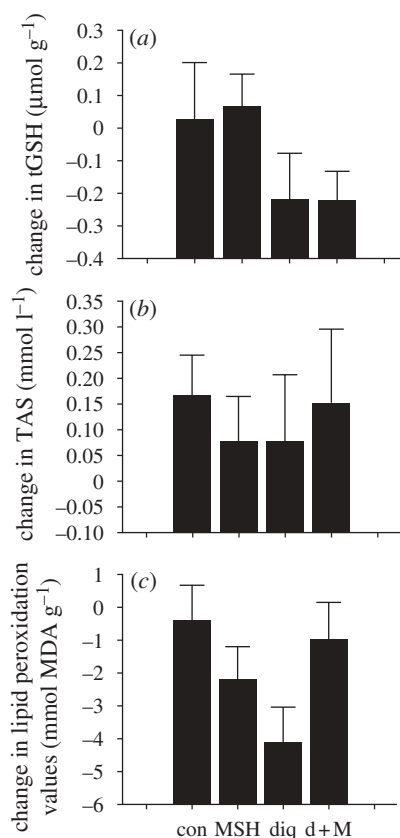
Figure 3. Change in total glutathione (tGSH) levels of erythrocytes (*a*), antioxidants in plasma (TAS; *b*) and lipid peroxidation of erythrocytes (TBARS, *c*) in the different treatments to which red-legged partridges were exposed during the experiment. Means+s.e. are shown. con, control; diq, diquat; d+M, diquat+MSH.

change in tGSH values (i.e. $+0.29 \pm 0.16$ μmol g^{-1}; pair-wise LSD test: $p = 0.044$), diquat-treated birds presented a decrease (-0.28 ± 0.15 μmol g^{-1}; $p = 0.037$). Both treatments including diquat (i.e. alone or combined with α-MSH) showed similar success in decreasing tGSH levels (figure 3*a*), suggesting that the effect was only mediated by oxidative stress.

In contrast, change in TAS was only predicted by the change in uric acid levels in interaction with the within-subjects factor ($\beta = 0.45$, $F_{1,71} = 23.65$, $p < 0.0001$; figure 3*b*).

(c) *Effects of diquat and α-MSH on the lipid peroxidation of erythrocytes*

There were no significant differences in the initial levels of TBARS between controls and birds treated with diquat ($F_{1,72} = 0.03$, $p = 0.872$) or α-MSH ($F_{1,72} = 1.90$, $p = 0.173$). The model analysing variability in the change of lipid peroxidation in erythrocytes included the interaction of the within-subjects factor with diquat ($F_{1,65} = 1.49$, $p = 0.226$) and α-MSH treatments ($F_{1,65} = 0.05$, $p = 0.816$) and with both treatments ($F_{1,65} = 5.67$, $p = 0.020$). The α-MSH treatment in birds also exposed to diquat apparently inhibited the decline in TBARS levels shown by those birds only treated with diquat (figure 3*c*), as there were differences between initial and final TBARS levels in the latter group (pair-wise LSD test: $p = 0.055$; figure 3*c*) but not in the former ($p = 0.455$; figure 3*c*). Meanwhile,
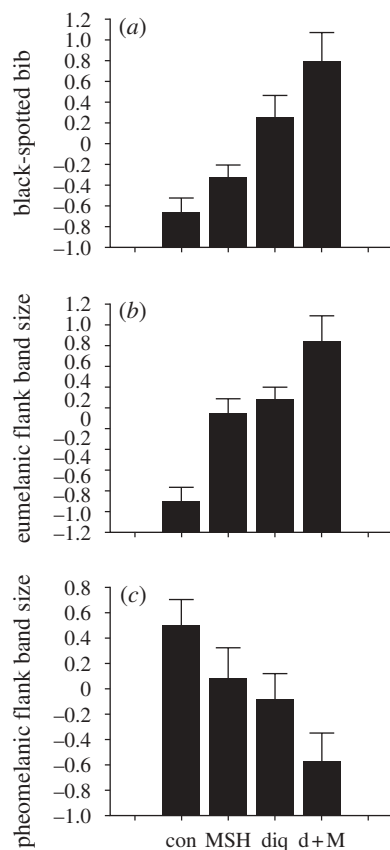
Figure 4. Size of (a) the eumelanic black-spotted bib pattern, (b) eumelanic flank bands and (c) pheomelanic flank bands of fully grown partridges at the end of the experiment. Note that all values were standardized between measurement series (mean=0, s.d.=1; see methods). Means+s.e. are shown. con, control; diq, diquat; d+M, diquat+MSH.

the change shown by control birds did not significantly differ with respect to the change shown by birds treated with α-MSH (both *p*-values>0.2), but the magnitude of the change experienced by birds treated with diquat only was lower (P = 0.055; figure 3c).

(d) Effects of diquat and α-MSH on plumage melanization

In the black-spotted bib, the extension of eumelanic feathers was explained by diquat ($F_{1,69} = 25.45$, $p < 0.0001$) and α-MSH treatments ($F_{1,69} = 5.10$, $p = 0.027$), while the effect of tarsus length was not significant ($F_{1,69} = 0.30$, $p = 0.584$). The obtained model explained 31.7 per cent of variance. All experimental birds developed larger black-spotted bibs than controls (figure 4a). The impact of both treatments apparently induced an additive effect, as α-MSH+diquat-treated birds showed the largest traits, their values being approximately the result of the sum of areas shown by both treatments separately (figure 4a). Furthermore, diquat-treated birds showed larger bibs than α-MSH-treated birds (figure 4a).

The model analysing the variability in the area of eumelanic flank bands explained 41.2 per cent of variance, and included diquat ($F_{1,67} = 23.77$, $p < 0.0001$) and α-MSH ($F_{1,67} = 17.94$, $p < 0.0001$) treatments and tarsus length as a covariate ($F_{1,67} = 1.82$, $p = 0.182$).

Again, all treated birds developed larger eumelanic bands than controls (figure 4b). Similarly, an additive effect between both treatments seems to be present, though areas shown by birds treated separately with α-MSH or diquat did not differ (figure 4b).

Finally, the model testing variability in the area of pheomelanic flank bands explained 16.4 per cent of variance, again including diquat ($F_{1,67} = 8.19$, $p = 0.006$) and α-MSH ($F_{1,67} = 3.92$, $p = 0.052$) treatments and tarsus length as a covariate ($F_{1,67} = 0.68$, $p = 0.412$). In contrast to eumelanic traits, all experimental birds presented smaller bands than controls (figure 4c), while the effect was marginally significant in the case of birds treated with α-MSH only ($p = 0.052$). The combined effect of diquat and α-MSH was again stronger than the effect of any of them acting separately (figure 4c).

4. DISCUSSION

Our results show that exogenous oxidative stress may have an important role in the expression of melanin-based plumage irrespective of the activity of the melanocortin system. This also indicates that those environmental factors influencing the level of oxidative stress of organisms may be mirrored in the expression of melanin-based traits used in animal communication.

(a) Influence of diquat on oxidative stress and plumage melanization

The presence of oxidative stress in diquat-treated birds was suggested by the decline in the level of a key intracellular antioxidant: glutathione. It has been amply reported that glutathione concentration in different tissues decreases as a consequence of oxidative challenges (e.g. Cho *et al*. 1981; Lautier *et al*. 1992; Ohtsuka *et al*. 1994; Luperchio *et al*. 1996; Zhang *et al*. 1999; Sewalk *et al*. 2001; Noriega *et al*. 2002). In birds in particular, decreases in glutathione levels have been observed in the brain of mallards (*Anas platyrhynchos*) hatched from eggs exposed to diquat (Sewalk *et al*. 2001).

More surprising, however, is the result obtained from the analysis of lipid peroxidation. Although it is broadly recognized that diquat promotes lipid peroxidation (e.g. Burk *et al*. 1980; Yumino *et al*. 2002), here birds treated with diquat presented a clear decrease in TBARS levels. A potential explanation could be that developing birds mounted an adaptive response (e.g. Dimova *et al*. 2008) during the weeks of treatment, overcoming the initial challenge and reaching in fact higher levels of lipid integrity than those found in controls. However, we must be cautious in our conclusions because the specificity of the TBARS method has been questioned in respect of changes in lipid metabolism potentially interfering with their values (Monaghan *et al*. 2009). Interestingly, birds treated with both diquat and α-MSH did not show such a decline. In this case, the absence of an effect could be associated with changes in lipid metabolism, as α-MSH levels have been positively related to plasma fatty acid levels in birds (Tachibana *et al*. 2007).

Similarly, the lack of diquat-induced effects in TAS change could also be the result of compensatory mechanisms. In great tit (*Parus major*) nestlings in which erythrocyte tGSH levels were experimentally reduced, TAS increased (Galván & Alonso-Alvarez

2008). However, whereas in that study tGSH levels were manipulated by an inhibitor of glutathione synthesis (buthionine sulfoximine; BSO), here birds were exposed to an agent (i.e. diquat) that generates ROS (Sewalk *et al.* 2001; Zeman *et al.* 2005; Xu *et al.* 2007). Therefore, the effect on the antioxidant machinery should have been generalized, perhaps compromising an effective mobilization of antioxidants.

With regard to the production of melanin-based traits, diquat-treated birds showed larger black-spotted bibs and eumelanic flank bands than controls. This agrees with the depletion of GSH, given that this antioxidant may interfere with eumelanin synthesis by different routes (see figure 1; Galván & Alonso-Alvarez 2008). In contrast, the extension of the pheomelanic brown flank bands was lower in diquat-treated partridges, consistent with the idea that GSH probably serves as a substrate for pheomelanin production (Galván & Solano 2009). In the first step of the melanogenesis pathway, the hydroxylation of the amino acid L-tyrosine by tyrosinase produces dopaquinone, which reacts with thiol groups to form pheomelanin precursors if cysteine levels are higher than those of dopaquinone (Ozeki *et al.* 1997; see figure 1). Whether thiol groups used during pheomelanogenesis are obtained from intracellular GSH or from free cysteine is still under debate, some authors defending cysteine as the most probable resource on the basis of an active transport of free cysteine from cytosol to melanosomes through the cell membrane (Potterf *et al.* 1999). In any case, GSH is the main physiological reservoir of cysteine, and thus it influences cysteine levels and potentially the process of pheomelanogenesis (Benedetto *et al.* 1981). These considerations support the view that the presence of GSH favours pheomelanogenesis as opposed to eumelanogenesis (Galván & Solano 2009), and hence support the opposing effects of diquat on eumelanic and pheomelanic plumage traits of partridges.

(b) *Influence of α-MSH on plumage melanization*
As expected, α-MSH promoted an increase in the expression of eumelanin-based plumage, but independent of changes in glutathione levels. This was expected because, instead of decreasing GSH levels as oxidative stress does (e.g. Ohtsuka *et al.* 1994; Luperchio *et al.* 1996; Zhang *et al.* 1999), α-MSH affects melanization levels by specifically activating the MC1R, which subsequently enhances the activity of tyrosinase (Ozeki *et al.* 1997; Boswell & Takeuchi 2005). The lack of influence of α-MSH on oxidative stress markers is supported by findings showing that, in contrast to mammals, α-MSH does not enhance heat production in birds (Tachibana *et al.* 2007), which in turn is associated with oxidative stress (e.g. Feder & Hofmann 1999). Along the same line, feeding rate, which is also positively related to free radical production (Yu 1996), is decreased by α-MSH in birds (Cline & Smith 2007). Therefore, while any effect on melanization caused by exogenous oxidative stress would be mediated by changes in GSH levels, the effect promoted by α-MSH would directly act on a specific receptor (MC1R), which affects tyrosinase activity (Boswell & Takeuchi 2005). This is also supported by the additive effect of the treatments on the plumage (i.e. no interaction). Thus, for instance, a multiplicative or

an inhibitory effect was not present when diquat and α-MSH acted simultaneously (figure 4*a–c*).

(c) *Evolutionary model*
The implications derived from the previously overlooked relevance of oxidative stress as an agent shaping the expression of plumage melanization are diverse and should be explored to understand the origin and maintenance of melanic traits. This should not be limited to melanin-based traits involved in animal communication (i.e. signals). In fact, melanin-based traits are widespread among vertebrates because they are involved in crypsis and protection against ultraviolet radiation and mechanical damage (Majerus 1998; McGraw 2006; Schreiber *et al.* 2006). We propose that melanic traits may evolve under two different scenarios, which would determine the type of melanic trait favoured by selection (i.e. eu- vs. pheomelanic traits).

The first of these scenarios assumes that adaptation acts through a process of parsimony in which the easiest way prevails (Stewart 1993). We suggest that, under high environmental oxidative stress (i.e. stress generated by exogenous factors acting on all individuals of a population), eumelanic traits may be favoured as a consequence of the economy of antioxidants. In these conditions, free radicals would stimulate tyrosinase activity and decrease GSH levels (figure 1), with the maintenance of high GSH levels being particularly costly. Under less stressful conditions, GSH levels might be higher and then pheomelanogenesis should prevail (Galván & Solano 2009).

The second evolutionary scenario considers melanic traits under a context of honest signalling of quality (e.g. Hoi & Griggio 2008; Kingma *et al.* 2008; McGraw 2008; Galván & Møller 2009). The honesty of these signals would be mediated by the relative cost that signallers experience during the trait production or during its maintenance (Hasson 1997; Alonso-Alvarez *et al.* 2009). This, however, should also depend on environmental conditions. Under high environmental oxidative stress favouring eumelanogenesis (as described above), maintaining high GSH levels necessary for pheomelanin production should be costly, which implies that pheomelanic traits should be favoured as honest signals of individual quality. In contrast, under less stressful environments in which high levels of GSH are less critical to self-maintenance, pheomelanic signals would be less reliable. In these conditions, eumelanin-based traits would be favoured because only high-quality individuals might be able to afford the decrease of GSH that allows eumelanization by mobilizing alternative antioxidant resources (Galván & Alonso-Alvarez 2008; Galván & Solano 2009). Future studies should address these possibilities in order to obtain a general framework for understanding the evolutionary origin and maintenance of melanin-based traits in a wide taxonomic spectrum of animals.

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