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RESEARCH ARTICLE

Oxidative Challenges Do Not Impact Pheomelanin-Dependent Coloration in Male Japanese Quails

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ABSTRACT

Colorful traits play an important role in animal communication. Melanin-based colorations are the most extended color traits in animals and are produced by two types of endogenous melanic pigments: eumelanins and pheomelanins, the last ones being the least studied in the context of communication. The production of pheomelanin requires a semi-essential amino acid, cysteine, which is also used for the synthesis of an important endogenous antioxidant, glutathione. Hence, it has been proposed that the synthesis of pheomelanin and glutathione may compete for the cysteine available in the organism. In that case, pheomelanic colorations are predicted to be less intense when the individual is facing an oxidative challenge, and therefore, they would provide information on the oxidative status of the bearer. Here, we experimentally evaluated this hypothesis using male Japanese quails (*Coturnix japonica*) as a model of study, a species with pheomelanin-based plumage in the breast and cheeks. During feather growth, individuals were exposed to one of three possible conditions: Control (saline), an endogenous oxidative challenge (*Escherichia coli* lipopolysaccharide injections), or an exogenous oxidative challenge (paraquat injections). Contrary to predictions, we found that: (1) Birds from the three groups exhibited less intense pheomelanic colorations in feathers after the experimental manipulation, and the magnitude of this change did not differ among groups. (2) There was no effect of the experimental treatments on the proportion reduced/oxidized glutathione, an index of oxidative status. (3) Lipid peroxidation was lower after the experimental manipulation, with birds exposed to the paraquat challenge experiencing a stronger decline than other groups. (4) Cysteine and total glutathione levels decreased after the experimental manipulation, with no differences per group in the magnitude of the decline. Taken together the results do not support the hypothesis that oxidative status plays a key role at determining the variation in the intensity of pheomelanic colorations.

1 | Introduction

Animal interactions frequently require communication among individuals. Colorful traits play an important role as social signals because they can provide information about the signaler's condition among other characteristics (Hill and McGraw 2006; Delhey et al. 2010). Underlying mechanisms of production of those colored traits importantly determine their potential to communicate reliable information about the individual's condition (Maynard-Smith and Harper 2003). There are mainly three mechanisms of production of colored traits: structural arrangement, dietary pigments, and endogenous

Research Highlights

- Melanin-based colorations are the most extended color traits in animals.
- The physiological mechanisms that underpin the potential link between pheomelanic colored traits and individual condition remains elusive.
- The syntheses of pheomelanin and glutathione (endogenous antioxidant) require cysteine available in the organism.
- Increased demand for antioxidants was proposed to compromise the synthesis of pheomelanin.
- No such a compromise in pheomelanic colorations was found after two different oxidative challenges.

pigments. Melanins are endogenous pigments that confer colors to teguments, hair and feathers in vertebrates (Galván and Alonso-Alvarez 2009) and have two principal forms: eumelanin and pheomelanin. Eumelanin is responsible for black–gray colorations, whereas pheomelanin produces yellow–brown–red colorations (Galván and Solano 2016). Eumelanic colorations have received far more attention but pheomelanic traits are also common and have the potential to produce conspicuous patterns with signaling potential (McGraw 2006). However, the physiological mechanism that underpins the potential link between pheomelanic colored traits and individual condition remains elusive.

Pheomelanin production depends on the availability of cysteine, a semi-essential amino acid mainly obtained from diet but that can also be endogenously synthesized (Rodríguez-Martínez and Galván 2019). Interestingly, cysteine surplus can be toxic when accumulated in the organism, producing health disorders such as metabolic acidosis, growth deficiencies and—in oviparous organisms—eggshell thinning (Galván et al. 2019; Rodríguez-Martínez and Galván 2019). Hence, pheomelanic colorations have been proposed as a sink of cysteine surplus that prevents organism from suffering noxious consequences of holding elevated levels of this amino acid (Galván, Ghanem, and Møller 2012). This potential role of pheomelanic colorations as cysteine sink would be particularly advantageous when cysteine is not required for other physiological functions, such as antioxidant protection (Galván, Ghanem, and Møller 2012; Galván and Alonso-Alvarez 2017; Rodríguez-Martínez and Galván 2019, 2020; Galván and Sanz 2020).

Oxidative stress is an imbalance between the level of reactive oxygen species and the organism's capacity to neutralize them (Finkel and Holbrook 2000). Although reactive oxygen species play an important role in cell signaling processes (Di Meo et al. 2016), a sustained overproduction that overtakes the antioxidant system can exert damage to biomolecules. Reactive oxygen species are endogenously produced mainly during the process of energy production by the mitochondria and the defense response against pathogen agents, but they can also stem from environmental exposure to exogenous sources such as ionizing and ultraviolet radiation, pesticides, and heavy metals (Klaunig 2018). Organisms counter with an endogenous barrier defense against pro-oxidant agents, and one of the most important endogenous antioxidants is glutathione (Wu et al. 2004). Glutathione and pheomelanin depend on the cysteine available in the organism for their synthesis, and thereby, it has been proposed that production pathways of both molecules compete for the use of this amino acid (Galván and Alonso-Alvarez 2009). Hence, it has been predicted that under pro-oxidant conditions, the use of cysteine for the synthesis of glutathione is privileged at expenses of pheomelanin production for colored traits (Galván and Alonso-Alvarez 2009; Galván and Møller 2013). This competitive allocation of cysteine to glutathione (for antioxidant defense) or pheomelanin (for colored traits) would provide a potential underlying mechanism for pheomelanic colorations to be a reliable signal of the oxidative status of an individual (Galván and Sanz 2020). Consistently with this hypothesis, an observational study showed that in Eurasian nuthatch (*S. europaea*) nestlings, higher levels of oxidative stress derived from elevated predation risk were associated with lower pheomelanic colorations in feathers and lower glutathione levels in erythrocytes (Galván and Sanz 2020).

Experimental studies evaluating this hypothesis have manipulated either the antioxidant capacity of the individuals or the exposure to pro-oxidant compounds resulting in inconsistent findings. Interestingly, those experimental manipulations have not always succeeded at modifying the oxidative status of the individuals in the targeted direction, and results obtained regarding pheomelanic colorations have provided mixed support for the hypothesis proposed. For example, one study conducted on zebra finches (*Taeniopygia guttata*) failed at experimentally increasing oxidative stress markers in birds treated with an inhibitor of glutathione synthesis, but pheomelanic coloration in feathers was reduced (Rodríguez-Martínez and Galván 2019). By contrast, in a second study on the same species, an exogenous oxidative challenge unexpectedly alleviated oxidative stress levels, although consistently with the apparently improved oxidative status, pheomelanic coloration in feathers was enhanced (Rodríguez-Martínez and Galván 2020). Finally, results obtained in an experimental study using house sparrows (*Passer domesticus*) add to the inconclusive scenario: although the exogenous administration of a pro-oxidant agent (diquat) increased oxidative damage, it did neither affect the levels of glutathione nor cysteine, nor the size of a feather patch that is negatively linked to pheomelanin deposition (Galván and Alonso-Alvarez 2017).

Here, we used male Japanese quails to evaluate whether, under pro-oxidant conditions, the competitive allocation of cysteine to synthesis of glutathione versus pheomelanin underpins the variation in pheomelanic colorations. Japanese quails exhibit sexual dimorphism in body size and feather coloration. Unlike females, males exhibit rusty coloration covering the throat and chest (hereafter ventral patch) and in each side of the face, covering the cheeks (Hiyama et al. 2018). Intensity of the cheek patch coloration is positively associated with female preferences (Hiyama et al. 2018), and due to its link with the expression of the tyrosinase gene, it is assumed to be pheomelanin dependent (Wakamatsu, Ito, and Rees 2002; Hiyama et al. 2018). Accordingly, chestnut and brown colors have been considered, in the literature of the area, to be predominantly produced by pheomelanin (Galván and Møller 2011; Galván, Mousseau, and

Møller 2011; Galván and Møller 2013; Galván and Wakamatsu 2016). To manipulate the oxidative status of individuals, two groups of birds were exposed to one of two types of oxidative challenges whereas a third group received a control treatment. If the above mentioned hypothesis is correct, we expect that individuals exposed to pro-oxidant conditions would exhibit impaired pheomelanic colorations and diminished circulating cysteine as compared to controls.

2 | Methods

Seventy-six adult male Japanese quails obtained through a local commercial supplier were included in the study (12 for the pilot study, 64 for the final study). At the beginning of the experiment, birds were 30 weeks of age and were individually housed in 50 × 29 × 20 cm (length × wide × height) cages, located in an outdoors aviary at CTBC. They were exposed to a 14:10 h light:dark cycle, 18.8 (±0.06)°C ambient temperature, ad libitum access to water and 30 g daily (Woodard et al. 1973) of commercial food for quails (Purina, Codor reproductina HP, Nestlé—21% protein, 2% lipids, 3.4% calcium, and 5% fiber). Each individual was identified with a numbered ring. Four months before the experiment started, birds received an ocular dose of the Newcastle vaccine (B1, MAVER) to prevent deaths from this cause.

2.1 | Pilot Study

Before the experiment started, a pilot study was conducted aiming at determining doses of lipopolysaccharide (LPS) and paraquat to be administrated, peak of the febrile response triggered by LPS, time required for fully feather regrown and time for pheomelanic coloration to be detected in the growing feathers (see Supporting Information).

2.2 | Experimental Treatment

A chronogram of the experimental design can be found at Figure 1. Briefly, a 200 μL blood sample was obtained from the brachial vein for measuring oxidative damage to lipids (lipid peroxidation, hereafter), cysteine and glutathione at the beginning of the experiment. Once blood collection was completed, cheek and chest feathers color were measured (see below), and 24 h after that a feather patch area of 1 × 1 cm (right cheek) and 3 × 4 cm (chest) was extracted to promote feather regrowth. Six days after feather extraction, quails were randomly assigned to one of three groups: control ($n = 21$), LPS oxidative challenge ($n = 21$), or paraquat oxidative challenge ($n = 22$). In the *control group*, individuals received five intraperitoneal injections of 1 mL/kg saline solution (PiSA). In the *LPS oxidative challenge group*, birds received five intraperitoneal injections of 1 mg/kg LPS (Sigma L2630) (De Boever et al. 2008; Bertani and Ruiz 2018). In the *paraquat oxidative challenge group*, quails received five intraperitoneal injections of 10 mg/kg paraquat (Dragocson; 1,1′-dimethyl-4,4′ bipyridium). As in controls, the volume of injection for LPS and paraquat treatments was 1 mL/kg. LPS mimics a bacterial infection by promoting the release of cytokines and inducing an inflammatory response that involves the production of reactive oxygen species and increased oxidative stress (Lynn and Golenbock 1992). Paraquat is a bipyridilium herbicide that generates reactive oxygen species via redox cycling, increasing oxidative damage (Koch and Hill 2017). Both treatments have been shown to induce cytotoxicity in quail and other galliformes (e.g., Galvani et al. 2000; Grolleau 2000; Khan et al. 2014; Lian-Mei et al. 2015; Zheng et al. 2016; Baylor and Butler 2019; Soni, Haldar, and Chaturvedi 2019; Armour et al. 2020; Han et al. 2020; Sun et al. 2020). In all groups, intraperitoneal injections were administrated every other day (starting 6 days after feather extraction, see Figure S1). Blood sampling was repeated 24 h after the third (for measuring glutathione and cysteine) and 24 h after the fifth injections (for measuring oxidative damage to lipids). At these sampling points, target feathers were still growing and therefore pheomelanin-based coloration generated was exposed to the physiological conditions captured by our blood variables. Within 1 h after blood collection, samples were centrifuged at 10,000 × *g* for 10 min, red blood cells were separated from plasma, and both were stored at −80°C up to laboratory analyses. Plumage color was measured again 21 days after first color measurement (see below), when the new feathers were fully regrown. In addition, body mass was

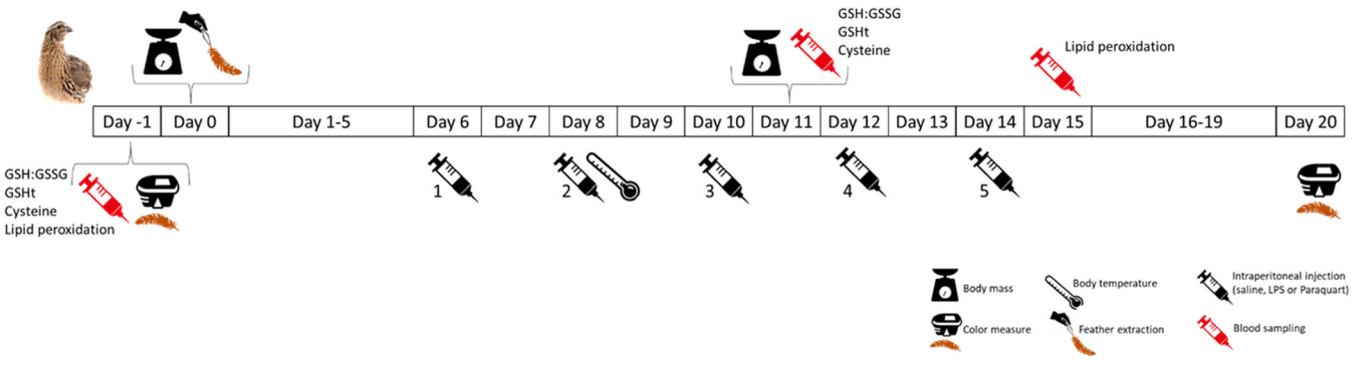

FIGURE 1 | Timing of the experimental procedure. Feathers were removed 1 day after the first blood sampling (to quantify pre-treatment levels of GSH:GSSG, GSHt, cysteine, and lipid peroxidation) and color measuring. Five intra-peritoneal injections were administrated every other day, starting 6 days after feather removal. Six days after the last intra-peritoneal injection, a final color measurement was performed on the regrown feathers. Consecutive blood sampling was performed 24 h after third (to quantify post-treatment levels of GSH:GSSG, GSHt and cysteine) and 24 h after the fifth intra-peritoneal injections (to quantify post-treatment levels of lipid peroxidation).

measured the day of feather extraction and 24 h after the third injection. Internal (cloacal) body temperature, 10 h after the second injection was recorded (this is still within the peak identified in the pilot study) using a digital thermometer (Fluke 52- II, 60 Hz).

2.3 | Color

Plumage color was measured, 7 days before starting the injections and 21 days after the first measure, using a portable spectrophotometer (Minolta CM-2600d) that records the reflectance at 10-nm intervals from 360 to 700 nm using a pulsed xenon lamp as light source. We took nine color measurements distributed along the ventral pheomelanic patch and three covering the right cheek patch, within the areas to be plucked. The same measurements were collected after these plumage patches were totally regrown, at the end of the experiment. Reflectance spectra measured from each patch at each sampling time were averaged for further calculation of the total brightness (summation of the percentage of reflectance at each wavelength measured) and the slope of the whole reflectance spectrum of the ventral and the cheek patch (i.e., the slope of the linear regression of reflectance against wavelength) (Galván 2018; Galván and Rodríguez-Martínez 2018). Higher values of total brightness and slope correspond to less intense pheomelanic colorations.

2.4 | Glutathione

Reduced and oxidized glutathione (GSH and GSSG, respectively) were measured in erythrocytes, before feather extraction and 24 h after the third injection (to have an overview during the critical time of colored feather growth), using a commercial kit (CAS 38185; Sigma-Aldrich). Briefly, 1 mg of RBCs was weighted and diluted in 11 μL of Milli-Q water, after that, 48 μL 5% sulfosalicylic acid were added, and the mix was centrifuged 8000 × *g* for 10 min at 4°C. Supernatant was recovered and 385 μL of Milli-Q water were added, two aliquots of 200 μL were obtained from this dilution, one was used for estimating GSH levels, and the other for quantifying GSSG levels. In the aliquot to estimate GSSG levels 4 μL of masking solution were added. Absorbance of the reaction was measured by duplicate at 405 nm using a microplate reader Multiskan Fisher (Thermo Scientific). Final values are expressed as ng of GSH and GSSG per mg of erythrocytes. Also, we calculated the ratio GSH:GSSG as an index of oxidative status, with lower values indicating higher oxidative stress. Intra- and inter-plate coefficients of variation were 7.6% and 5.9%, respectively. In addition, levels of total glutathione (GHSt) were estimated as the sum of GSH and GSSG levels.

2.5 | Cysteine

Plasma cysteine levels were estimated in samples collected before feather extraction and 24 h after the third injection to capture the levels of this key substrate during the critical time of feather growth and pigmentation. We used a commercial kit (Mak255; Sigma-Aldrich) and followed manufacturer instructions. Briefly, plasma samples were centrifuged 10,000 × *g* for 5 min at 4°C, and 5 μL of the supernatant recovered were placed in a microplate by duplicate. Similarly, 5 μL of each point of the standard curve were added to the microplate by duplicate. After this, 5 μL of buffer were added to each well of the plate to reach a final volume of 10 μL, and then, 200 μL of master mix (193 μL buffer + 5 μL enzyme mix I + 1 μL reducing agent + 1 μL blocking agent) were added and mixed by pipetting. The plate was incubated at 37°C for 30 min, and after this time, 30 μL enzyme mix II was added and mixed by pipetting. The plate was incubated again at 37°C for 5 min, after which 5 μL probe buffer were added. Fluorescence was measured using a kinetic mode during 30 min at λ excitation = 365 and λ emission = 450 nm in a microplate reader Synergy H1F (Biotek). Cysteine levels are expressed as nm of cysteine per μL of plasma. Intra- and inter-plate coefficients of variation were 3.5% and 3.7%, respectively.

2.6 | Lipid Peroxidation

Lipid peroxidation was estimated through the thiobarbituric acid reactive substances assay before feather extraction and 24 h after the fifth injection to have an overview of the impact of the whole manipulation. Briefly, 100 μL of trichloroacetic acid (10%, p/v) were added to 100 μL of plasma, the solution was mixed by inversion three times, and then, it was centrifuged 10,000 × *g* for 10 min at 4°C. Approximately 170 μL of supernatant were recovered and 100 μL of 0.375% thiobarbituric acid pH 4.2 were added. A standard curve of MDA (1,1,3,3-tetramethoxypropane) was prepared and treated with trichloroacetic and thiobarbituric acid following the same procedure described above for the samples. Samples and points of the standard curve were incubated at 92°C for 45 min, in a water bath. After incubation, preparations were maintained at −20°C for 5 min to stop the reaction. Absorbance was read at 530 nm using a microplate reader Multiskan Fisher (Thermo Scientific). Final values are expressed as μmol of MDA equivalents per mL of plasma. Intra- and inter-plate coefficients of variation were 2.9% and 1.4%, respectively.

2.7 | Statistical Analyses

We fitted independent linear mixed models to evaluate the effect of the experimental treatment on feather pheomelanic coloration, glutathione (GSH:GSSG, GSHt), cysteine, lipid peroxidation, and body mass. All models included treatment (i.e., control, LPS, or paraquat) and time (i.e., pre-treatment or post-treatment) as fixed factors, the interaction between them, and the individual identity as a random intercept to account for repeated measures within individuals. GSHt, cysteine and lipid peroxidation levels were log transformed before statistical analyses. The effect of the experimental treatment on body temperature 10 h after the second LPS injection was tested using a linear model that included experimental treatment as factor.

All analyses were performed using R (ver. 4.1.1; R Core Team 2021), packages lme4 (Bates et al. 2015), LMERConvenienceFunctions

(Tremblay and Ransijn 2020), and lmerTest (Kuznetsova, Brockhoff, and Christensen 2017). Models satisfied statistical assumptions, and stepwise backward deletion of nonsignificant terms ($p > 0.05$) was used for model selection.

3 | Results

3.1 | Body Mass and Temperature

There was no effect of the experimental treatment on body mass, 24 h after the third injection (time: $F_{1,\ 56.27} = 1.13$, $p = 0.29$; treatment: $F_{2,\ 55.01} = 0.04$, $p = 0.96$; time × treatment: $F_{2,\ 54.24} = 0.44$, $p = 0.65$). However, birds from the LPS group had higher body temperature, 10 h after the second injection, than the other two groups (treatment: $F_{2,\ 49} = 4.31$, $p = 0.019$).

3.2 | Color

Both total brightness and color slope of the ventral patch were higher in birds from the three groups after the treatment than before, but there were not differences among groups in the magnitude of this change (Table 1; Figure 2A,B). Total brightness and color slope of the cheek patch were not affected by the experimental manipulation, time or the interaction between them (Table 1; Figure 2C,D).

3.3 | Glutathione

Twenty-four hours after the third injection, there was no effect of the experimental treatment, time or its interaction on GSH:GSSG ratio (Table 2; Figure 3A). Similarly, GSHt levels were not affected by the experimental treatment, 24 h after the third injection. In all groups, GSHt levels were lower, 24 h after the third injection, but the magnitude of the change did not differ among groups (Table 2; Figure 3B).

3.4 | Lipid Peroxidation

Lipid peroxidation levels in plasma, 24 h after the fifth injection, were lower in all birds as compared to pre-treatment levels (Table 2; Figure 3C), and this decline was apparent only in birds from the paraquat group (post hoc: control vs. LPS $\beta = -0.135 \pm 0.82$, $t_{34} = -0.738$, $p = 0.743$; control vs. paraquat $\beta = -0.438 \pm 0.206$, $t_{34} = -2.127$, $p = 0.041$; LPS vs. paraquat $\beta = -0.34 \pm 0.206$, $t_{34} = -1.474$, $p = 0.316$).

3.5 | Cysteine

Cysteine levels, 24 h after the third injection, were lower after treatment in all the three groups when compared with pre-treatment levels, and the magnitude of this change did not differ among groups (Table 2; Figure 3D).

4 | Discussion

Here, we evaluated whether, under pro-oxidant conditions, the competitive allocation of cysteine to glutathione versus pheomelanin synthesis underpins the variation in pheomelanic coloration. To this aim, we experimentally exposed male Japanese quails to one of two pro-oxidant treatments (LPS or paraquat injections) to evaluate its consequences on pheomelanic colorations, glutathione, lipid peroxidation and cysteine levels in comparison to a control group. Overall, we found a decline in the intensity of pheomelanic coloration in feathers of the ventral patch in all three groups. This was accompanied by a decrease in cysteine and GSHt levels in all birds included in the experiment, regardless of the specific experimental treatment they received (either LPS, paraquat, or control). There was no change in the GSH:GSSG ratio throughout the experiment. However, lipid peroxidation was also lower in all three groups after the experimental manipulation, 24 h after the fifth injection (when feathers were still growing), and this decline was more pronounced in the birds that received the oxidative challenges than in controls. These

TABLE 1 | Effect of an oxidative challenge (i.e., control, LPS, or paraquat) on male Japanese quail's ventral and cheek patch feather color.

	Ventral patch					
	Brightness			Slope		
Variables	***F***	**df**	***p***	***F***	**df**	***p***
Treatment	0.98	2, 54.26	0.38	0.13	2, 55.02	0.88
Time	**82.54**	**1, 54.95**	**< 0.001**	**11.93**	**1, 55.69**	**0.001**
Time × treatment	1.54	2, 52.73	0.22	0.92	2, 53.56	0.41
	Cheek patch					
	Brightness			**Slope**		
Variables	***F***	**df**	***p***	***F***	**df**	***p***
Treatment	1.64	2, 54.14	0.20	1.66	2, 55.07	0.20
Time	0.004	1, 55.26	0.95	0.17	1, 56.75	0.68
Time × treatment	0.11	2, 53.45	0.89	0.56	2, 54.80	0.58

Note: Color was estimated as total brightness and color slope in feathers before being removed and 20 days after its removal, when new feathers were fully grown (5 days after the last injection).

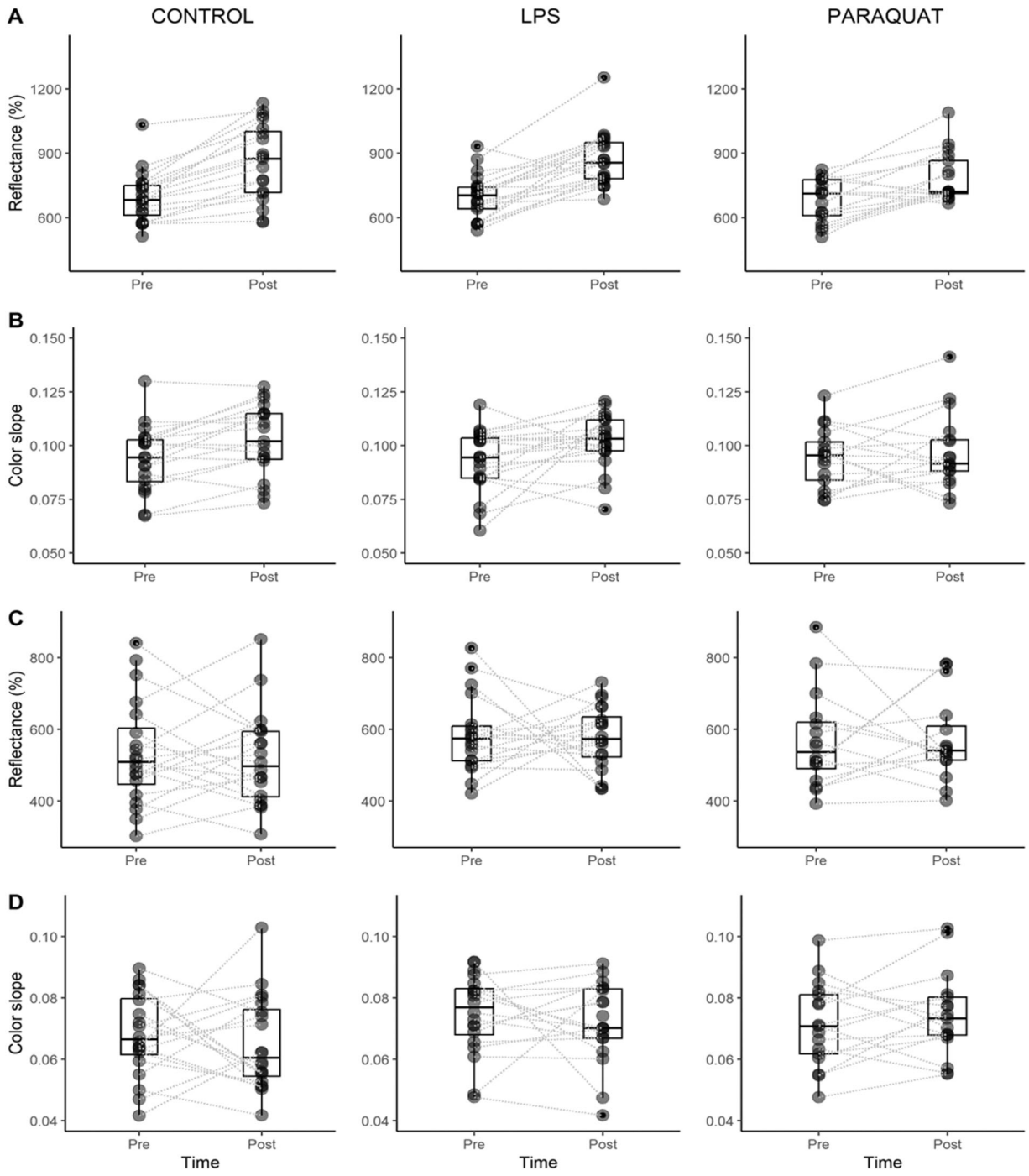

FIGURE 2 | Male Japanese quails' ventral (A, B) and cheek (C, D) patch feathers color before and after injections (i.e., control, LPS, or paraquat). Color was estimated as total brightness (A, C) and reflectance spectra slope (B, D) in the plumage before feathers were removed (pre-treatment) and 20 days later, when feathers were fully regrown (post-treatment, 5 days after the last injection). Higher values of total reflectance or color slope indicate less intense pheomelanin-based colorations. Boxes represent interquartile range, central line correspond to median value, circles show each individual measured, and gray dotted lines connect pre-treatment and post-treatment values for each individual.

results suggest that pro-oxidant conditions do not strengthen the competition between glutathione and pheomelanin for the use of cysteine available in the organism, and hence, oxidative stress may not play an important role at underpinning variation in pheomelanic colorations as proposed. The three previous studies that experimentally tested the same hypothesis evaluated here, provided mixed support with a decline in the intensity of pheomelanic colorations in absence of the expected increase in oxidative stress markers (Rodríguez-Martínez and Galván 2019), an increase in the intensity of pheomelanic colorations accompanied by an augment in the antioxidant capacity (Rodríguez-Martínez and Galván 2020), and an increase in oxidative damage in absence of an effect on glutathione and cysteine levels, or on the size of the color patch (Galván and Alonso-Alvarez 2017). This previous evidence, together with results obtained here, provide weak support for the hypothesized link between pheomelanic coloration and oxidative stress.

TABLE 2 | Effect of an oxidative challenge (i.e., control, LPS, or paraquat) on male Japanese quail's red blood cells glutathione levels, lipid peroxidation, and plasma cysteine.

	GSH:GSSG			GSHt			Lipid peroxidation			Cysteine		
	24 h after the 3rd injection			24 h after the 3rd injection			24 h after the 5th injection			24 h after the 3rd injection		
	F	df	*p*	*F*	df	*p*	*F*	df	*p*	*F*	df	*p*
Treatment	0.44	2, 53.80	0.65	0.09	52.01	0.91	0.77	2, 34.75	0.47	1.49	2, 28.35	0.24
Time	0.002	1, 54.45	0.97	**5.45**	**53.41**	**0.02**	441.9	1, 34.02	<0.0001	**5.87**	**1, 29.48**	**0.006**
Time × treatment	0.16	2, 52.51	0.85	0.60	51.26	0.55	4.54	**2, 34.08**	**0.018**	1.06	2, 27.24	0.36

Note: Glutathione was estimated as reduced:oxidized glutathione ratio (GSH:GSSG ratio) and total glutathione (GSHt) in erythrocytes, whereas lipid peroxidation (MDA equivalents) and cysteine were measured in plasma samples. Pre-treatment values for all variables were analyzed before the first injection, whereas post-treatment values were measured 24 h after the third injection for glutathione and cysteine, and 24 h after the fifth injection for lipid peroxidation.

Lipid peroxidation decreased in all groups, 24 h after the fifth injection. Interestingly, this decline was more pronounced in paraquat injected birds as compared to controls and LPS, which is contrary to our predictions. This may have resulted from a transitory increase in antioxidants mobilization stimulated by the chronic treatment administrated here, as has been suggested previously (Galván and Alonso-Alvarez 2008; Radak, Chung, and Goto 2008; Costantini and Verhulst 2009). Other dietary or non-measured enzymatic antioxidants (Costantini and Verhulst 2009), perhaps obtained through the diet supplied, may be responsible for the fact that the total amount of glutathione decreased regardless of the experimental group, in absence of an increase in the GSH:GSSG ratio. We expected lower GSH:GSSG ratio in birds treated with LPS and paraquat as compared with controls, indicating increased levels of oxidative stress. However, we found no changes in GSH:GSSG during the experiment in any group. It should be noted, however, that GSH:GSSG was measured after the third experimental injection whereas lipid peroxidation was measured after the fifth injection. It is therefore plausible that the different time of exposure to oxidative challenges, before the blood sampling, may explain contrasted patterns for GSH:GSSG ratio and lipid peroxidation. Finally, cysteine levels were predicted to be lower in birds from the LPS and paraquat treated groups as compared to controls, yet we found a general decrease in all birds irrespective of the experimental treatment, this may be linked to its constitutive role in β-keratin, the cysteine-rich protein present in nails, skin, hair, and feathers (Strasser et al. 2015), as birds in all three groups were regrowing feathers trough the experiment. Hence, cysteine could have been allocated to other physiological functions, but apparently not to the synthesis of glutathione that could have potentially been elicited by our oxidative challenges.

It may appear that our LPS and paraquat injections did not produce the expected clear increase in pro-oxidant conditions aimed in our design, given the lack of effects on GSH:GSSG and the above discussed increase in lipid peroxidation. However, LPS administration triggered a febrile response, as revealed by the higher temperature recorded in LPS injected birds, indicating that the immune challenged did occur (Maloney and Gray 1998; De Boever et al. 2008). Also, previous studies have shown that LPS doses of 1 mg/kg like those used here resulted in febrile responses and increased markers of oxidative stress in chicken (Han et al. 2020). In addition, in Japanese quails, paraquat administrated in the same doses used here, but daily during 7 days, resulted in a significant increase in oxidative stress estimated through lipid peroxidation in lungs (but not plasma) and plasma levels of GSH:GSSG (Galvani et al. 2000). Moreover, in our pilot study, one extra injection of paraquat at the same concentration used in this study increased the risk of dead (see Supporting Information). Hence, the apparent absence of an effect of the experimental treatment on markers of oxidative stress may be attributable to a suboptimal sampling strategy that limited our capacity to detect it, rather than to the actual magnitude of the oxidative challenge (Meitern et al. 2013). Although measuring oxidative markers in blood is considered an acceptable strategy to estimate current systemic levels of oxidative stress (Selman et al. 2012), a positive correlation between blood and internal organs' estimates is not always found for all the markers (Margaritelis et al. 2015;

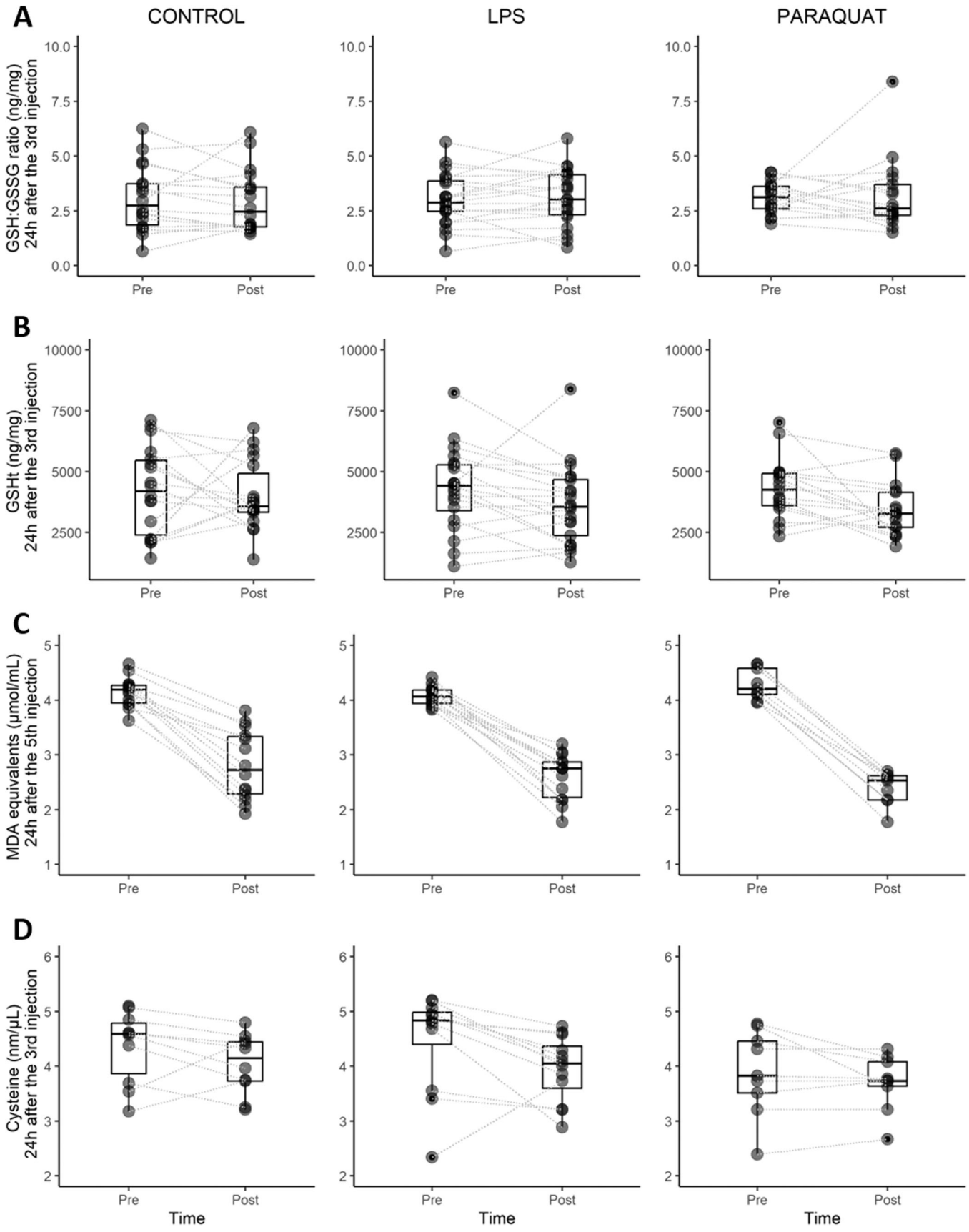

FIGURE 3 | Legend on next page.

Speakman et al. 2015; Costantini 2022). Indeed, a recent study on Japanese quails reported mostly nonsignificant associations between oxidative stress biomarkers among different tissues, including blood (Romero-Haro, Pérez-Rodríguez, and Tschirren 2023). Also, experimental administration of paraquat (and equivalent compounds) doses near to the DL50 do not always result in detectable changes in blood oxidative stress markers despite the increased mortality recorded (Meitern et al. 2013). Moreover, numerous studies have found that consequences of an oxidative challenge are not equally detectable through different markers, even when the same biological substrate is analyzed (Hoffman 2002; Simitzis et al. 2012; Henry et al. 2015; Han et al. 2020; Montoya et al. 2020; Romero-Haro, Pérez-Rodríguez, and Tschirren 2023). Similarly, previous studies testing the hypothesis evaluated here were also inconsistent at detecting an increase in oxidative stress after an experimental oxidative challenge, as described above (Galván and Alonso-Alvarez 2017; Rodríguez-Martínez and Galván 2019, 2020). Thus, it is reasonable to assume that our endogenous and exogenous oxidative challenges actually altered the oxidative balance of individuals during the experiment.

Finally, it is interesting to highlight the general pattern exhibited by the birds of the study irrespective of their experimental group: when comparing pre-treatment and post-treatment measures, all birds exhibited a decrease in plasmatic cysteine and in GSHt in erythrocytes that was concomitant to a decline in the intensity of pheomelanic coloration in feathers of the ventral patch, yet the feathers in the cheek patch resulted unaffected. It should be noted the implication of the color of this feather patch on female mate choice in this species has not been demonstrated so far. However, this pattern of covariation is indeed consistent with the existence of a functional connection between cysteine availability and glutathione and pheomelanin production. However, the fact that experimental oxidative challenges did not alter any of these variables indicates that oxidative stress may not play a key role at modulating a potential trade-off between cysteine used for glutathione versus pheomelanin syntheses. Thus, the hypothetical value of pheomelanin-based traits as honest signals of the oxidative status of the bearer is not supported by our results. The general pattern found in the responses exhibited by birds in the three groups of the experiment may be result of a response to one (or more) of the variables that all the birds experienced through the study, such as ad libitum access to food, diet composition, water, temperature, captivity conditions, and/or handling (vehicle substance used for all injections, repeated sampling, stress). Further studies are required to explore potential factors that may underpin the covariation between cysteine levels and pheomelanic colorations (i.e., dietary cysteine or other physiological processes that involve this amino acid), to evaluate the potential of pheomelanic colorations as condition-dependent signals.

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Ethics Statement

This research complies with the current laws of Mexico and the Animal Behaviour Guidelines for experimentation with animals. The Research Ethics Committee from UATx approved the experimental procedures according to Mexican Guidelines for Animal Care, based on recommendations by The Association for Assessment and Accreditation of Laboratory Animal Care International (Norma Oficial Mexicana NOM-062-200-1999, México).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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FIGURE 3 | Male Japanese quails' levels of reduced:oxidized glutathione ratio (GSH:GSSG ratio) in erythrocytes (A), total glutathione (GSHt) in erythrocytes (B), lipid peroxidation in plasma (C), and cysteine in plasma (D) before and after injections (i.e., control, LPS, or paraquat). GSH:GSSG ratio is calculated from the concentration of each compound in ng/mg of erythrocytes. Plasma lipid peroxidation levels were estimated as MDA equivalents (μmol/mL). Reported values were measured before the first injection (pre-treatment), 24 h after the third injection for GSH:GSSG ratio, GSHt and cysteine (post-treatment), and 24 h after the fifth injection for lipid peroxidation (post-treatment). Boxes represent interquartile range, central line correspond to median value, circles show each individual measured, and gray dotted lines connect pre-treatment and post-treatment values for each individual.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.