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RESEARCH ARTICLE

Individual quality via sensitivity to cysteine availability in a melanin-based honest signaling system

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ABSTRACT

The evolution of honest animal communication is mostly understood through the handicap principle, which is intrinsically dependent on the concept of individual quality: low-quality individuals are prevented from producing high-quality signals because, if they did so, they would pay greater production costs than high-quality individuals. We tested an alternative explanation for the black bib size of male house sparrows, *Passer domesticus*, an honest signal of quality the expression of which is negatively related to levels of the pigment pheomelanin in the constituent feathers. We previously showed that experimental depletion of cysteine, which participates in pheomelanogenesis, improves the phenotype (bibs larger than in controls) of high-quality males (birds with largest bibs initially) only. Here, we conducted an experiment under opposite conditions, increasing the availability of dietary cysteine, and obtained opposite results: deteriorated phenotypes (bibs smaller than in controls) were only expressed by high-quality birds. Some birds were also treated with the pro-oxidant diquat dibromide, and we found that the cellular resistance to free radicals of high-quality birds benefited more from the antioxidant activity of cysteine against diquat than that of low-quality birds. These findings support the existence of a mechanism uncoupling cysteine and pheomelanin in low-quality birds that confers on them a low sensitivity to variations in cysteine availability. This constitutes an explanation for the evolution of signal honesty that overcomes the limitations of the handicap principle, because it provides a specific definition of individual quality and because costs are no longer required to prevent low-quality individuals from producing large signals.

KEY WORDS: House sparrow, Handicap principle, Honest communication, Signaling costs, Pheomelanin

INTRODUCTION

Biological communication is mainly honest communication, as most biological signals are honest (Searcy and Nowicki, 2005). This means that the information conveyed by signals increases the fitness of the signals' recipients, which leads to the evolution of these traits (Hasson, 1994). Central to signal honesty is the concept of genotypic quality, particularly because the most studied mechanism by which organisms achieve signal honesty (i.e. the handicap principle) states that a high signal expression is limited to high-quality signalers (Zahavi, 1975). Thus, the benefit that recipients obtain from assessing signal expression in others is the capacity to determine their individual genotypic quality, hence allowing fitness outcomes to be maximized by mating according to the quality of signalers (Hasson, 1997). Despite the importance of this concept in evolutionary biology, however, the meaning of individual quality is not clear because its genetic basis has not been explicitly defined (Wilson and Nussey, 2010). As a result, individual quality is still an elusive concept that is used with different meanings in different contexts, often causing confusion (Bergeron et al., 2011; Lailvaux and Kasumovic, 2011).

The handicap principle assumes that individual quality is the capacity of signalers to afford the costs derived from signal production or maintenance, so that low-quality signalers pay greater relative fitness costs or are less efficient at converting signaling into fitness for a given level of signal expression than high-quality signalers (Grafen, 1990; Getty, 2006). However, this definition of individual quality suffers the same problems mentioned above, as the capacity to afford signaling costs is very ambiguous and lacks a defined genetic and physiological basis. In fact, this basis, which is assumed to exist, is often termed the 'black box' in the process of signal evolution (Dale, 2006). Most importantly, the existence of the costs predicted by the handicap principle, which should prevent low-quality signalers from producing signals as large as those produced by high-quality individuals and thus the appearance of bluffs (Hasson, 1994), is not fully accepted in evolutionary biology because natural selection is not expected to favor the evolution of signals when it implies incurring substantive costs and because such costs are frequently not found in empirical studies (Zollman et al., 2013; Huttegger et al., 2015; Kane and Zollman, 2015; Számadó, 2011; Számadó and Penn, 2015). It is then imperative to construct a specific basis that can be tested to understand the evolution of honest signaling (Számadó and Penn, 2015).

We have previously proposed that handicap costs are not necessary to explain the evolution of the honest signaling system of the house sparrow, *Passer domesticus* (a classical model species), if the basic physiological mechanisms controlling the expression of its signal (i.e. the size of the black chest bib in males) are fully understood (Galván et al., 2015). This trait has been intensively studied (Anderson, 2006) and it is known that its expression positively affects fitness and reflects overall genotypic quality, as males displaying larger bibs are dominant over other males, have better body condition and, in some populations, achieve higher lifetime reproductive success (reviewed in Nakagawa et al., 2007). Thus, male house sparrows with larger bibs are considered to be high-quality birds while males with smaller bibs are low-quality birds (Galván et al., 2015).

The black chest bib is a plumage patch formed by the deposition of the pigment melanin in feathers, and the size of the patch is negatively related to the feather concentration of one of the two chemical forms of melanins (i.e. pheomelanin) and unrelated to the concentration of the other chemical form (i.e. eumelanin; Galván

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et al., 2014). Thus, only a low deposition of pheomelanin in feathers allows male house sparrows to develop large black bibs. Pheomelanin is synthesized in the melanocytes of birds when the thiol compounds glutathione (GSH) and cysteine are above a certain concentration, because the sulfhydryl groups of these compounds are incorporated into the pigment structure (García-Borrón and Olivares Sánchez, 2011; Riley et al., 2011). We were therefore able to create the physiological conditions necessary to induce the expression of large bibs by treating male house sparrows with DL-buthionine-(*S*,*R*)-sulfoximine (BSO), which specifically enhances cysteine catabolism and inhibits γ-glutamylcysteine synthetase, the enzyme that catalyzes the rate-limiting step in GSH synthesis (i.e. Griffith, 1982; Dizdar et al., 1997), during bib development. BSO therefore reduced cysteine and GSH levels in all birds, but an increase in bib size was only observed in high-quality birds, i.e. those with the largest bibs initially (Galván et al., 2015). Cysteine levels in erythrocytes were negatively related to pheomelanin levels in the bib feathers as expected from the fact that pheomelanogenesis requires a constant supply of cysteine and therefore consumes this amino acid, but in high-quality birds only. As a consequence, cysteine levels were negatively related to bib size in high-quality birds only (Galván et al., 2015). These findings led us to predict the existence of a physiological mechanism that uncouples pheomelanin and cysteine levels in low-quality birds, which would make them insensitive to variations in cysteine levels. These variations may occur as a consequence of changes in endogenous or exogenous oxidative stress, as cysteine is one of the three constituent amino acids of GSH, the most important intracellular antioxidant (Wu et al., 2004). Therefore, it may be the existence of this physiological mechanism, and not the expectation of paying physiological costs, that prevents low-quality male house sparrows from producing bibs of a size inconsistent with their quality (Galván et al., 2015).

A physiological constraint leading to insensitivity to variations in cysteine levels thus constitutes an explanation for the evolution of honesty in the house sparrow signaling system that is alternative to the handicap costs that have been suggested (but not found) for this system (González et al., 1999; Buchanan et al., 2003; Laucht et al., 2011; Laucht and Dale, 2012). Indeed, we did not find greater costs in terms of cellular oxidative stress in low-quality males (Galván et al., 2015). The handicap principle also has an intrinsic undiscussed assumption, i.e. animals have the ability to (consciously) decide whether to express a large signal as costs only appear after signals have begun to be produced (Zollman et al., 2013; Dessalles, 2014), and the cysteine–pheomelanin mechanism may also explain the honesty of the bib size of house sparrows without the need to make this assumption. Furthermore, different genes controlling the transport of cysteine into and out of melanocytes to influence pheomelanogenesis are known, so the cysteine–pheomelanin mechanism also provides a hypothetical specific genetic basis for the evolution of honesty (Galván et al., 2014), thus overcoming the lack of a testable background in current definitions of individual quality (see above).

Our aim here was to further test the cysteine–pheomelanin mechanism of honesty in male house sparrows. To obtain empirical evidence that allows us to fully demonstrate the existence of a mechanism uncoupling cysteine and pheomelanin levels in low-quality birds that explains the honesty of the bib size, it is necessary to demonstrate that the impossibility of inducing the development of bibs of a size inconsistent with their quality in low-quality males also exists when cysteine availability is high; that is, when the physiological conditions created are opposite to those following the previous experimental manipulation (i.e. Galván et al., 2015). We therefore induced the development of new bibs in male house sparrows during a period in which some birds were treated with dietary cysteine whereas others served as controls. As cysteine enhances pheomelanogenesis and bib size is negatively affected by pheomelanin levels (Galván et al., 2014), treatment with cysteine should induce the development of small bibs compared with those of controls, thus producing a deterioration of the phenotype. If the proposed mechanism uncoupling cysteine and pheomelanin levels has evolved in low-quality birds, these predictions should be fulfilled in high-quality birds only.

As cysteine exerts antioxidant activity by being part of GSH, a proper experimental demonstration of our hypothesis should include treatment with another exogenous substance that exerts the opposite effect, i.e. a pro-oxidant substance. We therefore treated another group of house sparrows with diquat dibromide, a bipyridylium aquatic herbicide that produces intracellular superoxide anion (e.g. Sewalk et al., 2000; Zeman et al., 2005; Xu et al., 2007), while cysteine was simultaneously provided. The predicted deteriorating effect of cysteine on the phenotype of male house sparrows should be neutralized by the effect of diquat, as birds should be forced to make use of cysteine for combating the free radicals produced by diquat, which would make cysteine not fully available for pheomelanogenesis, thus allowing birds to produce larger bibs. Birds receiving both cysteine and diquat should therefore develop bibs of a similar size to those of controls (assuming similar but opposite effect sizes). The phenotype deterioration should be more marked in birds receiving cysteine only, a prediction only valid for high-quality birds (see above). Birds receiving only diquat should experience a decrease in cysteine, GSH and pheomelanin levels, which should lead to the development of larger bibs than in controls, again in high-quality birds only. A precedent for this in other bird species (the red-legged partridge, *Alectoris rufa*) supports the last prediction as developing animals receiving diquat in water produced larger black birds in adulthood while enduring lower erythrocyte GSH values (Galván and Alonso-Alvarez, 2009).

We tested these predictions by measuring bib size in male house sparrows before (at capture, initial bib size) and after the experimental treatments (final bib size), considering initial bib size a measure of intrinsic overall quality (Møller, 1992; Galván et al., 2015). We also measured size-corrected body mass (commonly known as body condition) of birds and cysteine and GSH levels in erythrocytes before (initial levels) and after the experimental treatments (final levels). Additionally, we investigated the effects of the treatments on measures of antioxidant capacity [hydrosoluble antioxidant capacity in plasma (AOX) and erythrocyte resistance to hemolysis derived from oxidative stress] and oxidative damage [malondialdehyde (MDA) in plasma] in all birds.

MATERIALS AND METHODS

This experiment received approval from the Bioethics Subcommittee of the Spanish National Research Council (CSIC) on 9 March 2011, and was conducted with authorization by local authorities (Consejería de Agricultura y Medio Ambiente, Junta de Comunidades de Castilla-La Mancha; authorization reference: 186620).

Pilot study

The toxicity of any substance depends on the dose at which it is administered in relation to the specific characteristics of the animal

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model (Stine and Brown, 2015). Diquat is a pro-oxidant substance and therefore must be administered at the optimal dose to cause the desired effect (i.e. oxidative damage) without significantly affecting the physiology of the birds (Koch and Hill, 2017).

In contrast, cysteine exerts an antioxidant effect by being part of GSH, but it can also cause toxicity because of its oxidation to the dimer cystine, which decreases GSH levels (Viña et al., 1983; Munday, 1989). Thus, the cysteine dose must also be adjusted to cause the desired effect (i.e. increased sulfhydryl group availability) and avoid toxicity.

We conducted a pilot study to estimate the optimal doses of cysteine and diquat. In June–July 2011, 26 free-ranging adult male house sparrows were captured (see 'Experimental design', below) and kept in eight cages (six cages with three birds and two cages with four birds), each containing six water dispensers and four troughs with *ad libitum* food, for 24 days. In three of these cages, cysteine (Sigma-Aldrich, St Louis, MO, USA) was provided in the drinking water at a dose of 0.01, 0.1 or 1 g l^{-1}. In three other cages, diquat (Syngenta, Madrid, Spain) was provided in the drinking water at a dose of 0.125, 0.25 or 0.50 ml l^{-1}. In another cage, cysteine was provided in three water dispensers at a dose of 1 g l^{-1} and diquat was provided in the other three water dispensers at a dose of 0.50 ml l^{-1}. Birds in the final cage received water only and served as controls. The number of cages and birds was chosen to reduce potential damage to the birds following the advice of the Bioethics Subcommittee of the Spanish National Research Council (CSIC). At the end of the pilot study, the mean percentage change in body mass (there were no significant differences in tarsus length between treatments; one-way ANOVA: $F_{7,25}$=1.10, P=0.403) of control birds was −11.80%, while the mean total GSH (tGSH) level in erythrocytes (measured as explained below) at the end of the study was 3.89 mmol g^{-1}. The mean change in body mass and tGSH levels at the end of the study of birds receiving cysteine were, respectively: −17.40% and 2.44 mmol g^{-1} for 0.01 g l^{-1} cysteine (one bird died during the study); −9.21% and 3.88 mmol g^{-1} for 0.1 g l^{-1} cysteine; and −11.24% and 3.49 mmol g^{-1} for 1 g l^{-1} cysteine. As birds receiving the intermediate dose of cysteine showed the weakest decrease in body mass and final tGSH levels very similar to those of control birds, we used the dose of 0.1 g l^{-1} cysteine in the subsequent experiment.

Among birds receiving diquat, those receiving the highest dose (0.50 ml l^{-1}) died during the study. The mean percentage change in body mass and final tGSH levels of birds receiving diquat at the other two doses were, respectively: −24.15% and 2.81 mmol g^{-1} for 0.125 ml l^{-1} diquat; and −20.14% and 3.48 mmol g^{-1} for 0.25 ml l^{-1} diquat. As birds receiving the intermediate dose of diquat showed the weakest decrease in body condition and total GSH levels very similar to those of control birds, we used the dose of 0.25 ml l^{-1} diquat in the definitive experiment.

Lastly, the birds receiving both cysteine and diquat at the highest doses showed a large decrease in body mass (−26.30%) but tGSH levels were not markedly lower than in control birds (3.12 mmol g^{-1}). Furthermore, these birds survived the pilot study despite receiving diquat at a dose of 0.50 ml l^{-1}, suggesting that the antioxidant effect of cysteine protected them from the pro-oxidant effect of diquat. Birds used in the pilot study were not used in the subsequent experiment; once fully recovered, they were released at the site of capture.

Experimental design

The study was carried out in August–October 2011 on 76 captive adult male house sparrows that were captured with mist nets in the surroundings of Dehesa Galiana experimental facility (Diputación Provincial de Ciudad Real, Ciudad Real, Spain). All birds were marked with a numbered metal ring and randomly assigned to one of four different indoor aviaries (4×3×3 m), such that the body condition of birds (i.e. size-independent body mass) was equally distributed among aviaries. A window in each aviary produced a natural light regime. The birds were provided with *ad libitum* food consisting of a commercial mixture of seeds for canaries (Kiki, Callosa de Segura, Spain) and cuttlefish bone to ensure coverage of calcium needs. After capture, the birds were left to acclimate in the aviaries for 2 weeks.

On 5 August, blood samples were taken with a syringe from the jugular vein. A maximum volume of 200 µl of blood was taken following Diehl et al. (2001). The blood was immediately stored at 4°C and maintained for a maximum of 6 h until centrifugation at 4°C and 3500 ***g*** for 5 min. After centrifugation, the plasma was separated from the cell portion and both were stored at −80°C until biochemical analysis. The same day, a photograph of the bib of birds was taken with a digital camera (Olympus E-50). The birds were held in the same posture, at a fixed distance from the camera, under standardized illumination, following Galván et al. (2015). Bib size was measured by selecting the total black area on the throat and chest with Adobe Photoshop and converting pixels to cm^2 (McGraw et al., 2003). The analysis of bib size was made by a technician blinded to the aims of the study. In the house sparrow, the molting period occurs at the time our experiment was conducted (i.e. July–September; Anderson, 2006); therefore, to avoid interindividual differences in the phenology of plumage molt, 3 days after samples were taken (8 August) we plucked the feathers of the bib patch (see Galván et al., 2015), thus inducing the growth of new bib feathers. The neighboring feathers that surround the black bib patch were also plucked at this time (Galván et al., 2015). We measured the mass of the feathers that were plucked in all birds and found no difference between treatments (one-way ANOVA: $F_{3,71}$=0.98, P=0.408; mean±s.e.m. for all birds: 195.74±4.79 mg), indicating that the number of feathers plucked was very similar in birds from all treatments. All measurements taken before the beginning of the experiment on 5–8 August are referred to as 'initial values'.

The experimental treatment started the same day that the bib feathers were plucked (8 August). Each aviary was exposed to one of four experimental treatments (i.e. cysteine, diquat, cysteine +diquat or control) by placing two, 1 liter water dispensers where the focus substance (i.e. cysteine or diquat) was dissolved (the aviary with the cysteine+diquat treatment was provided with one water dispenser containing cysteine and one water dispenser containing diquat). The water was replaced 2 times per week. We transferred all birds and the experimental water dispensers from one aviary to another 3 times (every 15 days) during the course of the experiment, so that all birds were exposed to their corresponding treatments in the four aviaries during the same period.

The duration of the experimental treatment was 67 days. Before the end of the experiment, on 29 September, we took an intermediate blood sample from the birds in order to check their health. On 13 October, the birds were captured and the final bib size was measured as explained above. Blood samples and body mass measurements were also taken on the same day. All measurements taken after the end of the experiment on 13 October are referred to as 'final values'. Final values could not be obtained for 15 birds in total (7 birds escaped and 8 died during the experiment). There was no bias between treatments in birds that died during the experiment (χ^2_3=0.80, P=0.850). The birds were released at the site where they were captured. Data obtained during the experiment are provided as part of Table S1.

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Measurement of cysteine levels in erythrocytes

We measured cysteine levels following the method developed by Švagera et al. (2012) with some modifications (see Galván et al., 2015). Cysteine levels are expressed as micromoles per gram of pellet.

Measurement of GSH levels in erythrocytes

tGSH levels were determined by following the method described by Tietze (1969) and Griffith (1980) with some modifications. Details of the use of this technique with bird samples are published elsewhere (Galván and Alonso-Alvarez, 2008). Concentration is presented as micromoles of GSH per gram of pellet. tGSH is the sum of reduced (GSH) and oxidized (GSSG) glutathione, but we could not measure GSSG levels. Thus, it must be considered that variation in tGSH levels is partly due to variation in GSSG, although tGSH is indicative of overall intracellular antioxidant capacity (Galván and Alonso-Alvarez, 2008).

Measurement of uric acid levels and antioxidant capacity in plasma

The uricase/peroxidase method was used to assess uric acid concentration in plasma (kits from Biosystems, Barcelona, Spain). AOX of plasma was determined through a colorimetric assay based upon the oxidation of 2,2V-azinobis(3-ethylbenzo-thiazoline-6-sulfonate) (Erel, 2004).

Measurement of MDA levels in plasma

The protocol of Agarwal and Chase (2002) with modifications by Nussey et al. (2009) was followed to quantify MDA in plasma. Samples and standards were injected into an Agilent 1100 Series HPLC system (Agilent, Waldbronn, Germany) fitted with a fluorescence detector set and a 5 µm ODS-2 C-18 4.0×250 mm column maintained at 37°C. The mobile phase was MeOH:KH_2PO_4 (50 mmol l^{-1}; 40:60 v/v), running isocratically for 10 min at a flow rate of 1 ml min^{-1}. Data were collected at 515 nm (excitation) and 553 nm (emission).

Measurement of resistance to hemolysis under free radical exposure

Whole blood was exposed to a thermo-controlled free radical aggression by adding 2,2-azobis-(amidinopropane) hydrochloride (AAPH) (Rojas Wahl et al., 1998). Immediately after blood collection, a 10 µl sample was diluted and mixed in 365 µl of buffer. Within 6 h of blood collection, 80 µl of buffer-diluted blood was incubated at 40°C with 136 µl of a 150 mmol l^{-1} solution of AAPH. The lysis of erythrocytes was assessed with a microplate reader device (PowerWave XS2) measuring absorbance at 540 nm every few seconds. We quantified the time (in s) needed to hemolyze 50% of erythrocytes, which is indicated by a sharp decline in absorbance. For logistic reasons, we could not perform these analyses with the initial samples, so we analyzed the change in this variable using intermediate measurements as initial values (see below).

Statistical analyses

Similar to Galván et al. (2015), we divided birds into two groups regarding their 'intrinsic' quality: those with initial bib size equal to or lower than the median initial bib size of all birds were considered as 'low quality', while those with initial bib size larger than the median initial bib size were considered as 'high quality'. There were 36 low-quality birds (11 cysteine only, nine diquat only, nine cysteine+diquat, and seven controls) and 36 high-quality birds (five cysteine only, nine diquat only, 11 cysteine+diquat, and 11 controls). Three birds escaped and one bird died before we could perform the first measure of their bib.

In our previous experiment, which led to the proposal of the cysteine–pheomelanin mechanism of honesty, we found that the final bib size of male house sparrows was negatively related to final cysteine and marginally to GSH levels (Galván et al., 2015), so to corroborate the evidence for this mechanism and determine that the physiological conditions were similar in this new experiment, we tested again for the association between final bib size and cysteine and GSH levels using general linear models (GLM). Variation in body size was controlled for by adding tarsus length as a covariate. Treatment was added to the models as a fixed factor with four levels (cysteine only, diquat only, cysteine+diquat and control). As birds of different quality may respond differently to the different treatments (see Introduction), bird quality was added as another fixed factor. We also included the interactions between cysteine or GSH level and quality, between cysteine or tGSH level and treatment, and between treatment and quality. Final levels of cysteine and tGSH were negatively correlated (r=−0.44, N=43, P=0.003), so cysteine and tGSH were separately considered in different GLMs.

There were statistically significant differences between birds exposed to the different treatments in the initial levels of some of the variables (one-way ANCOVA: body condition: $F_{3,71}$=3.98, P=0.011; one-way ANOVA: cysteine: $F_{3,68}$=4.51, P=0.006; tGSH: $F_{3,49}$=0.72, P=0.542; AOX: $F_{3,59}$=3.28, P=0.027; MDA: $F_{3,72}$=3.02, P=0.035; bib size: $F_{3,68}$=0.92, P=0.228). Mean±s.e.m. initial values of the different significant variables were as follows for control, cysteine, diquat and cysteine+diquat treatments, respectively: body condition: 1.19±0.01, 1.22±0.01, 1.20±0.01 and 1.21±0.01; cysteine: 3.70±0.07, 3.58±0.04, 3.41±0.05 and 3.54±0.05; AOX: 0.34±0.02, 0.42±0.03, 0.34±0.02 and 0.36±0.02; MDA: 0.59±0.03, 0.46±0.03, 0.53±0.03 and 0.49±0.03. Therefore, to analyze the effects of treatment and quality on the change in the levels of these variables during the course of the experiment, we used GLMs with final values as response variables and initial values as covariates instead of including repeated-measures effects (Galván et al., 2015). Treatment, quality (low versus high) and their interaction were added as fixed factors. In the model for AOX, we also controlled by uric acid levels (e.g. Galván et al., 2010) by adding the final levels of this variable (there were no significant differences between treatments: $F_{3,57}$=2.47, P=0.070) and the residuals of initial AOX levels regressed against initial uric acid levels as covariates. In the model for resistance to hemolysis, initial values could not be measured, so we considered intermediate values instead, as well as the lag time to control for variation in the time at which the decline in absorbance started. Thus, the model for resistance to hemolysis included the residuals of intermediate time needed to hemolyze 50% of erythrocytes regressed against intermediate lag time, and final lag time as covariates. In the models for body condition and bib size, tarsus length was a covariate to control for body size.

In all models, a backwards stepwise procedure was used to remove non-significant terms, using P=0.1 as a threshold to abandon the model. Continuous variables were $\log_{10}$ transformed. When the effect of treatment or the interaction between quality and treatment was significant, differences between factor levels were analyzed by means of Fisher least significant difference (LSD) *post hoc* tests.

RESULTS

Relationship between final bib size and cysteine and GSH levels

The interaction between cysteine levels and quality was not significant ($F_{1,43}$=1.39, P=0.244; cysteine: $F_{1,43}$=3.77, P=0.059;

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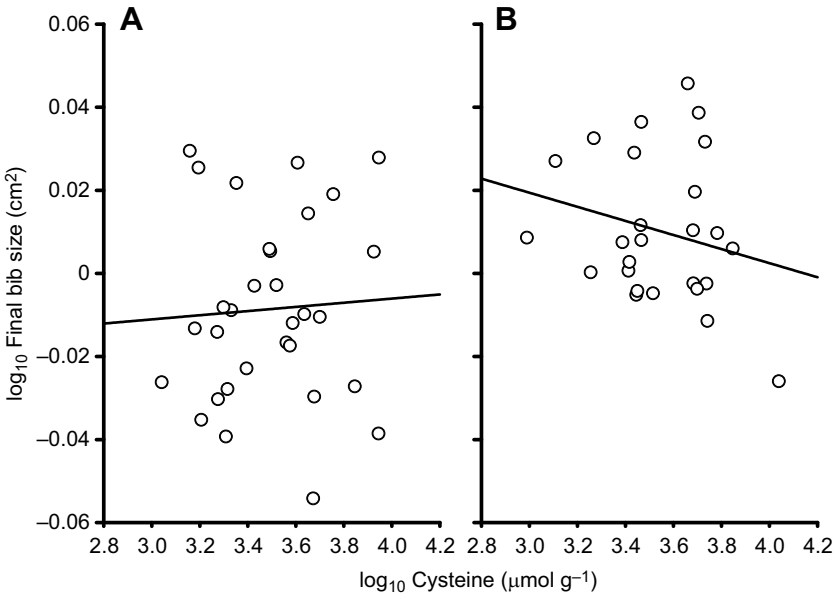

Fig . 1. Relationship between final bib size and cysteine levels in erythrocytes of male house sparrows. (A) Low-quality males. (B) High-quality males. Final bib size values are partial residuals of a general linear model (GLM) excluding cysteine levels and quality. Regression lines are shown.

quality: $F_{1,43}$=1.90, P=0.175), but the correlation between final bib size and cysteine levels was marginally non-significant in high-quality birds (b=−0.07, t=−1.98, P=0.053) while there was a lack of correlation in low-quality birds (b=−0.04, t=−1.20, P=0.237; Fig. 1). The same model also included a significant interaction between treatment and quality ($F_{3,43}$=3.12, P=0.036; treatment: $F_{3,43}$=2.17, P=0.105; tarsus length: $F_{1,43}$=2.47, P=0.123).

A different model resulted in a non-significant interaction between tGSH levels and quality ($F_{1,31}$=0.02, P=0.884; tGSH: $F_{1,31}$=0.28, P=0.600; quality: $F_{1,31}$=0.06, P=0.802), with a lack of tendency for the relationship between final bib size and tGSH levels in both high-quality (b=0.01, t=0.17, P=0.863) and low-quality birds (b=3.07×10^{-3}, t=0.05, P=0.961). The interaction between treatment and quality was also significant in this model ($F_{3,31}$=4.59, P=0.009; treatment: $F_{3,31}$=0.09, P=0.964; tarsus length: $F_{1,31}$=0.01, P=0.909).

Thus, these results are similar to our previous findings (Galván et al., 2015) in that there was a clear tendency for final bib size to be negatively related to cysteine levels, but not to tGSH levels, in high-quality males only.

Effect of treatment on the change in body condition, cysteine and GSH levels

The change in the body condition of birds during the course of the experiment did not depend on their quality ($F_{1,54}$=2.05, P=0.158) or on the interaction between quality and treatment ($F_{3,51}$=0.73, P=0.537). The final model included a significant effect of treatment ($F_{3,55}$=3.50, P=0.021), which was due to a decrease in body condition in diquat-treated birds compared with controls (P=0.011) that was not observed in birds treated with both diquat and cysteine (P=0.114). The body condition of birds treated with cysteine+diquat did not differ from that of birds in the diquat-only group (P=0.283). Birds treated with cysteine only also decreased in body condition compared with controls (P=0.005), showing body condition levels not different from those of birds treated with diquat alone (P=0.675) (Fig. 2A).

Neither treatment ($F_{3,48}$=0.40, P=0.752) nor quality ($F_{1,48}$=0.46, P=0.502), or their interaction ($F_{3,45}$=0.47, P=0.703), affected the change in cysteine levels of birds (Fig. 2B). Similarly, there were no effects on the change in tGSH levels of treatment either alone ($F_{3,30}$=0.23, P=0.873) or in interaction with quality ($F_{3,27}$=0.51, P=0.678; Fig. 2B).

Effect of treatment on plasma antioxidant capacity

The experimental treatment significantly affected the change in AOX levels in birds ($F_{3,33}$=3.33, P=0.031; uric acid: $F_{1,33}$=37.19, P<0.0001), but showed no interaction with quality ($F_{3,30}$=0.90, P=0.452; quality: $F_{1,33}$=3.05, P=0.090). Final AOX levels in birds treated with cysteine alone were lower than those of controls (P=0.005) and also tended to be lower than those in birds in the diquat-only group (P=0.077; Fig. 3). There was also a non-significant tendency for AOX levels to decrease in diquat-treated birds compared with controls (P=0.177) that was clearly absent among birds treated with both diquat and cysteine (P=0.761). AOX levels in birds treated with cysteine+diquat did not differ from those of birds treated with diquat alone (P=0.292).

Effect of treatment on erythrocyte resistance to free radicals

There was a significant effect of treatment on change in the time needed to hemolyze 50% of erythrocytes ($F_{3,51}$=7.61, P<0.001), but this effect was not independent of the quality of birds as there was a marginally non-significant interaction between treatment and quality ($F_{3,51}$=2.75, P=0.052). Among low-quality birds, those treated with diquat alone showed a lower final capacity to combat free radicals than those in the other groups (control: P=0.029, cysteine: P<0.001, cysteine+diquat: P=0.005). Among high-quality birds, those treated with both cysteine and diquat showed a greater final capacity to combat free radicals than those in the other groups (control: P=0.052, cysteine: P=0.003, diquat: P<0.001) and birds treated with diquat alone showed a lower capacity than controls (P=0.044). There was a tendency for high-quality birds supplemented with cysteine alone to show a decreased capacity to combat free radicals compared with controls (P=0.114; least squares mean±s.e.m. log time to 50% lysis of erythrocytes: control: 1.53±0.04, cysteine: 1.39±0.07; Fig. 4).

Effect of treatment on oxidative damage

The change in MDA levels was significantly affected by treatment ($F_{3,55}$=6.73, P<0.001) and marginally by quality ($F_{1,55}$=3.05, P=0.086; least squares mean±s.e.m. log MDA values: low

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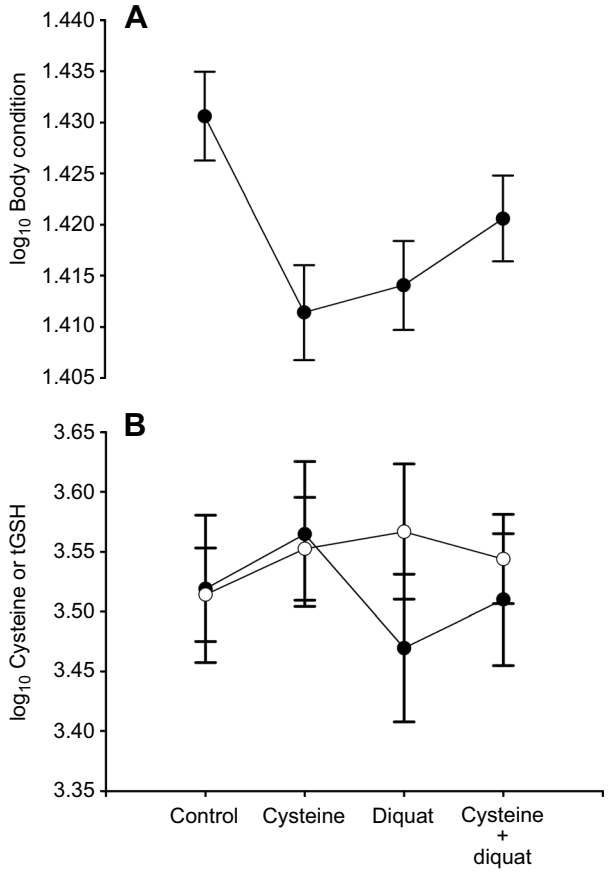

Fig. 2. Body condition and levels of cysteine and total glutathione (tGSH) in erythrocytes of male house sparrows exposed to different experimental treatments. (A) Body condition (size-corrected body mass, g). (B) Cysteine (filled circles) and tGSH (open circles) levels (μmol g^{-1}). The final values shown are least squares means (±s.e.m.) from GLMs controlling for initial values, so they reflect changes in the measured variables during the course of the experiment.

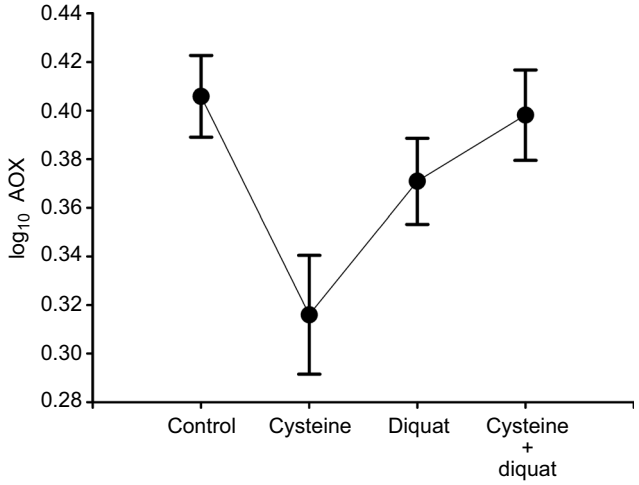

Fig. 3. Hydrosoluble antioxidant capacity (AOX) in plasma of male house sparrows exposed to different experimental treatments. The final levels (mmol l^{-1} Trolox) shown are least squares means (±s.e.m.) from a GLM controlling for initial AOX and uric acid levels, so they reflect changes in AOX during the course of the experiment.

quality: 0.61±0.02, high quality: 0.56±0.02), but not by their interaction ($F_{3,50}$=0.24, P=0.869). The treatment effect was due to birds in the diquat-only group having higher final MDA levels than those in the other groups (control: P<0.0001, cysteine: P=0.013, cysteine+diquat: P=0.057) and cysteine+diquat-treated birds having higher levels than controls (P=0.015). There was no difference in the change in MDA levels between controls and birds treated with cysteine alone (P=0.153) or between birds in the cysteine-only and cysteine+diquat groups (P=0.444) (Fig. 5).

Effect of treatment on bib expression

There were no significant effects of treatment ($F_{3,51}$=1.50, P=0.226) or quality ($F_{1,51}$=0.41, P=0.523) on the change in bib size, but their interaction was only marginally non-significant ($F_{3,51}$=2.36, P=0.082). If effects on final bib size, rather than the change on bib size, are analyzed by removing initial bib size from the model, the interaction between treatment and quality was only marginally non-significant ($F_{3,52}$=2.73, P=0.053). The effect of the interaction on the change in bib size was due to a lack of differences between treatments among low-quality birds (0.177<P<0.966) and to significant differences among high-quality birds treated with cysteine alone, which developed smaller bibs than controls (P=0.006) but similar-sized bibs to birds in the diquat-only (P=0.108) and cysteine+diquat groups (P=0.225). Among high-quality birds, the final bib size of controls did not differ from that of birds treated with diquat alone (P=0.239) but was larger than that in cysteine+diquat-treated birds (P=0.047; Fig. 6).

DISCUSSION

Our findings indicate that dietary cysteine supplements deteriorate the phenotype of male house sparrows only in birds of high intrinsic quality, while low-quality birds are insensitive to cysteine treatment and do not experience any influence on bib expression. This corroborates the results of our previous experiment in which cysteine was experimentally depleted and bib expression increased in high-quality males only (Galván et al., 2015). The effect of cysteine on bib expression is due to the participation of this amino acid in the synthesis of the pigment pheomelanin, levels of which are negatively related to bib size in male house sparrows (Galván et al., 2014). As cysteine depletion decreases pheomelanin levels

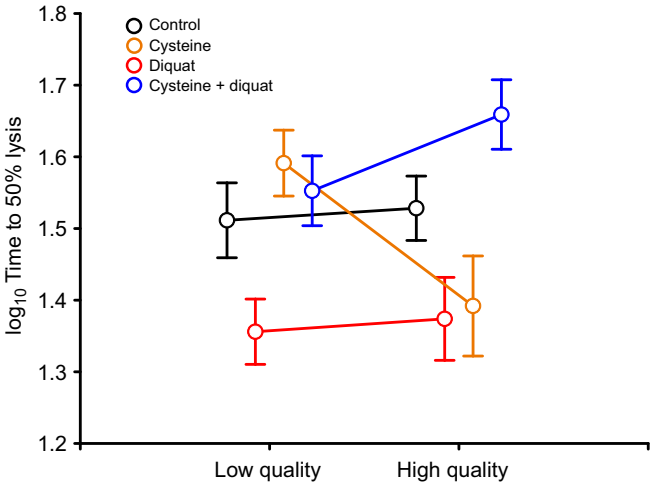

Fig. 4. Time to lyse 50% of erythrocytes under free radical attack in low- and high-quality male house sparrows exposed to different experimental treatments. The final time (in s) to 50% lysis values shown are least squares means (±s.e.m.) from a GLM controlling for initial values, so they reflect changes in resistance to hemolysis during the course of the experiment.

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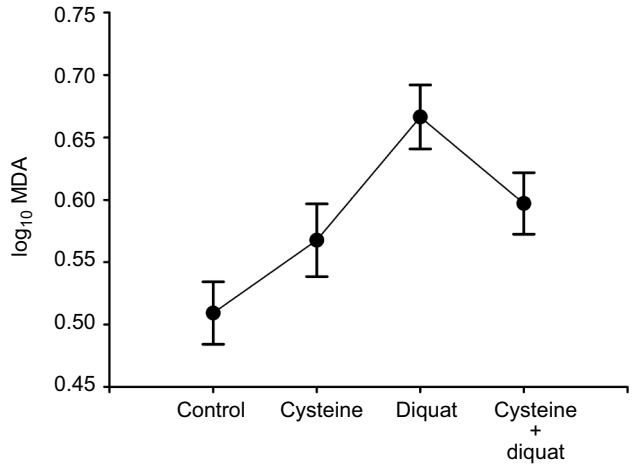

Fig. 5. Oxidative damage levels in plasma of male house sparrows exposed to different experimental treatments. The final malondialdehyde (MDA) levels (μmol l^{-1}) shown are least squares means (±s.e.m.) from a GLM controlling for initial MDA levels, so they reflect changes in oxidative damage during the course of the experiment.

(Galván et al., 2015), it is likely that cysteine supplementation increases pheomelanin levels, hence the negative effect on bib size found here.

We did not find significant increments in cysteine levels in erythrocytes of birds supplemented with cysteine compared with controls, but this may be because the effect of cysteine supplementation was not strong enough to exert an influence on systemic cysteine levels. However, our results show that cysteine supplementation might have increased cysteine levels at least in melanocytes of the pigmentary units of bib feathers and also in other tissues, for two reasons. (1) Cysteine supplementation produced the predicted effect on the bib size of high-quality birds, and this effect was neutralized by the simultaneous administration of diquat, which produced smaller bibs but less markedly so (i.e. marginally non-significant effect), as predicted. This suggests that some of the cysteine is used to combat the pro-oxidant effects of diquat and as a consequence less cysteine is available for pheomelanogenesis. (2) Cysteine supplementation produced antioxidant effects that

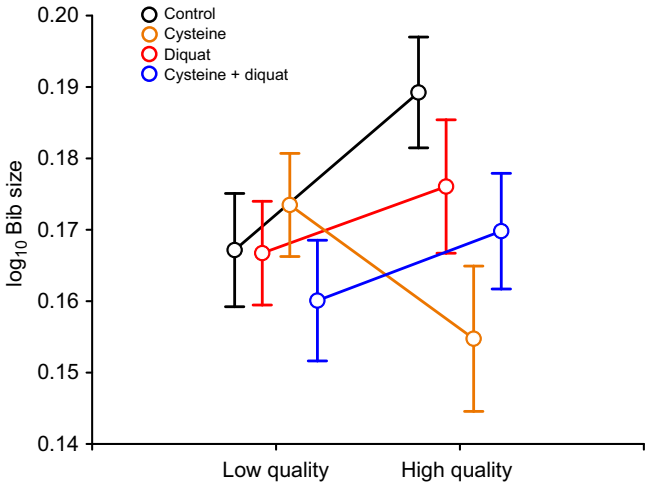

Fig. 6. Final bib size of male house sparrows exposed to different experimental treatments. Final bib size values shown are least squares means (±s.e.m.) from a GLM controlling for initial bib size, so they reflect changes in bib size during the course of the experiment.

counteracted the pro-oxidant nature of diquat. This can be inferred from the fact that body condition, hydrosoluble AOX in plasma and the resistance of erythrocytes to free radical attacks decreased in birds treated with diquat, whereas birds treated with both diquat and cysteine apparently avoided these effects. This also applies to oxidative damage because, although it increased in both diquat- and cysteine+diquat-treated birds compared with controls, the increase in the latter was less marked than in birds treated with diquat alone. Furthermore, this was also observed during the pilot study, where house sparrows treated with a high dose of diquat only survived if they were simultaneously treated with cysteine. Our study thus supports a direct role for cysteine in determining bib expression in male house sparrows, with experimental increases and decreases of cysteine availability producing opposing effects on bib development.

Another result from our experiment is the detrimental effect of cysteine supplements alone on the health status of birds. We found evidence of this from the effects on body condition and on AOX levels. While diquat reduced these parameters compared with controls and the effects of diquat were counteracted by supplementation with cysteine, there was a clear tendency of cysteine supplements alone to also produce them. Furthermore, high-quality birds treated with cysteine alone showed a clear decrease in erythrocyte resistance to free radicals compared with high-quality birds supplemented with both cysteine and diquat, which also showed a trend towards reduced values compared with controls. All these findings might be related to the toxic effects of excess cysteine, which in birds is associated with multiple physiological alterations (Klasing, 1998). We did not find evidence of detrimental effects on the health of birds of the dose of cysteine used in the experiment during the pilot study. However, we must consider that the final experiment lasted longer. Chronic exposure to the same dose could have led to oxidative stress as a result of cysteine formation (e.g. Viña et al., 1983; Munday, 1989). Thus, male house sparrows exposed to cysteine suffered some oxidative stress that was counteracted by the simultaneous exposure to diquat, as the use of cysteine to combat the pro-oxidant effect of diquat probably prevented a pro-oxidant effect of cysteine itself. This supports the double-side effect of cysteine, which is relevant to understanding the adaptive benefits of expressing pheomelanin-based traits under environmental conditions producing different levels of oxidative stress (Galván et al., 2012).

Our experiment also confirms that male house sparrows of intrinsic low quality are insensitive to variations in cysteine availability, so variations in cysteine levels do not lead to either increases (Galván et al., 2015) or decreases (this study) in bib size as opposed to effects in high-quality males. This lends support to the proposal that a physiological mechanism uncoupling cysteine and pheomelanin levels has evolved in low-quality birds, which would block the transport of cysteine to melanocytes to synthesize pheomelanin beyond a certain threshold in cysteine levels (i.e. the cysteine–pheomelanin mechanism of honesty). While future studies will be necessary to reveal the nature of this mechanism, it is likely that it is based on genes that control the transport of cysteine into and out of melanocytes such as *Slc7a11* and *CTNS* (Galván et al., 2014), and thus low-quality birds may differ from high-quality birds in the level of expression of these genes and maybe also in the sensitivity of their expression to environmental oxidative stress which is expected to affect cysteine levels. We believe that these possibilities are highly plausible in view of the key physiological role that cysteine is known to play in acting as a sensor of proteins to environmental oxidative stress, which may

facilitate the perception of this agent to cells (Limón-Pacheco and Gonsebatt, 2009).

Importantly, the cysteine–pheomelanin mechanism explains the honesty in the bib size system of male house sparrows (Nakagawa et al., 2007), overcoming the limitations of the handicap principle. First, the handicap principle suffers from the lack of a specific theoretical framework that can be tested, as derived from its loose definition of genotypic quality (Számadó and Penn, 2015). In contrast, the cysteine–pheomelanin mechanism offers a concrete definition: individual quality is equal to sensitivity to cysteine availability. Furthermore, with this mechanism it is not necessary to have greater relative costs for low-quality birds, and it is known that natural selection should not promote the evolution of signals under the high costs required by the handicap principle to explain why low-quality individuals do not produce high-quality signals (Zollman et al., 2013; Huttegger et al., 2015; Kane and Zollman, 2015; Számadó, 2011; Számadó and Penn, 2015). Lastly, another consequence of the handicap principle is the need for animals to decide whether to express a high-quality signal (Zollman et al., 2013; Dessalles, 2014), while the cysteine–pheomelanin mechanism represents a mechanistic explanation for the impossibility of modifying the phenotype of low-quality individuals so that signal expression depends on the presence/absence of a physiological mechanism and not on individuals' decisions. This eliminates the possibility of the appearance of cheaters (Hasson, 1994). Although we have obtained evidence of the existence of the cysteine mechanism of honesty only in the house sparrow, melanins are the most common pigments in animals, being present from bacteria to humans (Hill, 1992), so the validity of this mechanism for understanding the evolution of honesty is likely to be wide.

However, we analyzed the effect of treatment on measures of antioxidant capacity and oxidative damage and found evidence of differential effects according to the quality of birds in one case. The resistance of erythrocytes to a free radical attack in low-quality birds decreased under exposure to diquat compared with the other treatments, and birds receiving both cysteine and diquat showed a similar resistance to control birds. High-quality birds receiving both cysteine and diquat showed a resistance to free radicals that was higher than that of high-quality birds receiving diquat alone and of control birds. This means that low-quality birds benefited from the antioxidant role of cysteine but to a lesser extent than high-quality birds, which may be in accordance with the proposed low sensitivity of low-quality birds to variations in cysteine availability. Indeed, high-quality birds may actually have mounted an adaptive response via cysteine, counteracting the pro-oxidant effect of diquat, which would resemble hormesis mechanisms, frequently observed in oxidative processes (Vázquez-Medina et al., 2011; Costantini, 2014). Additionally, a tendency to show a decreased resistance to a free radical attack in birds supplemented with cysteine alone compared with controls was observed in high-quality but not low-quality birds. If the transport of cysteine to melanocytes is blocked beyond a certain threshold cysteine level as we propose here, this would not only leave pheomelanin synthesis unaffected but also have a smaller influence on the cysteine-related antioxidant capacity of melanocytes because, inside the cells, the majority of cysteine is incorporated into GSH to constitute the most important intracellular antioxidant (Lu, 1999). This may explain why we did not observe differential effects for low- and high-quality birds in either AOX or MDA levels, as these were determined in the plasma and not in cells. These results thus support a relatively low sensitivity of low-quality birds to variations in cysteine availability.

It must be noted that treatment with diquat did not produce any effect on the bib size of house sparrows, contrary to what we previously found in developing red-legged partridges, where diquat produced a decrease in the size of a pheomelanin-based plumage trait (Galván and Alonso-Alvarez, 2009). In our previous study, however, diquat also produced a clear depletion of tGSH levels in erythrocytes that was not found here. While cysteine affects the expression of melanin-based traits by directly participating in the melanogenesis pathway (García-Borrón and Olivares Sánchez, 2011; Galván et al., 2015), diquat affects such expression indirectly by producing oxidative stress and depleting cysteine and GSH levels (Galván and Alonso-Alvarez, 2009). In this experiment, diquat produced oxidative stress and damage but did not affect cysteine and GSH at a systemic level, which may be the reason why it did not affect the bib size of house sparrows. The direct role of cysteine in melanogenesis, in contrast, may have allowed treatment with cysteine to affect bib expression despite it not affecting cysteine and GSH at a systemic level.

In conclusion, our experiment supports the existence of a physiological mechanism uncoupling cysteine and pheomelanin in low-quality male house sparrows that makes them fairly insensitive to variations in cysteine availability. This prevents them from increasing or decreasing bib size in response to prevailing conditions of oxidative stress and related availability of cysteine and also precludes them from benefiting from the antioxidant role of cysteine, in contrast to the higher physiological lability observed in high-quality birds. This mechanism may explain the evolution of honesty in the bib system of male house sparrows, and probably other species with melanin-based signals, which could overcome the limitations of the handicap principle about greater relative costs for low-quality individuals, as the existence of such costs is no longer a requirement. Our findings indicate that, at least in the honest signaling system of house sparrows, individual quality can be defined as sensitivity to variations in cysteine availability. This may represent a change of paradigm in animal communication.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: I.G.; Methodology: I.G., C.A.-A.; Validation: I.G., C.A.-A.; Formal analysis: I.G.; Investigation: I.G., C.A.-A.; Resources: C.A.-A.; Writing - original draft: I.G.; Funding acquisition: I.G., C.A.-A.

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Supplementary information
Supplementary information available online at
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