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DIETARY CALCIUM, BUT NOT A GLUTATHIONE INHIBITOR, AFFECTS BIB SIZE IN JUVENILE MALE HOUSE SPARROWS

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Abstract. We tested whether the expression of melanin-based plumage traits reflects their bearer's levels of circulating antioxidants by repeatedly injected molting juvenile male House Sparrows (*Passer domesticus*) with a low or high dose of a substance (DL-buthionine-S,R-sulfoximine, hereafter BSO) that reduces the levels of cellular glutathione, a key antioxidant that inhibits melanin production. We predicted that birds with progressively lower glutathione levels would produce a progressively larger bib, a melanin-based trait present in males and related to their competitive ability. These injections were combined with dietary calcium supplements because we had found in a previous study that dietary calcium had a negative effect upon bib size in this species, perhaps because calcium level and glutathione level covary. We found that bib size was negatively related to dietary calcium but was unaffected by BSO injections. We also found no effect of BSO injections on the reflectance of bib feathers, although there was an interactive effect with dietary calcium, and no effect upon the size or reflectance of structurally based plumage traits. Bib size was not related to body condition at the time the bib was first being produced, nor was it related to the levels of circulating glutathione or total antioxidants. Evidence suggested that BSO injections reduced glutathione but that the effects were short-lived, possibly explaining why we found no effect on bib size. In sum, we found no evidence that bib size in House Sparrows is related to glutathione levels but confirmed that it is negatively related to dietary calcium.

Key words: *melanin,* Passer domesticus*, calcium, glutathione, signal, diet, antioxidant.*

El Calcio de la Dieta, pero no un Inhibidor Glutatión, Afecta el Tamaño del Babero en el Macho Juvenil de *Passer domesticus*

Resumen. Evaluamos si la expresión de los rasgos del plumaje basados en la melanina reflejan los niveles del portador de antioxidantes circulantes inyectando de manera repetida en machos juveniles mudando de *Passer domesticus* una dosis baja y alta de una sustancia (DL-butionina-S,R-sulfoximina, de aquí en más BSO) que reduce el nivel de glutatión celular, un antioxidante clave que inhibe la producción de melanina. Predijimos que las aves con niveles progresivamente menores de glutatión producirían un babero progresivamente más grande, un rasgo basado en la melanina presente en los machos y relacionado con su habilidad competitiva. Estas inyecciones fueron combinadas con suplementos de calcio en la dieta debido a que habíamos encontrado en un estudio previo que el calcio de la dieta tenía un efecto negativo sobre el tamaño del babero en esta especie, tal vez debido a que el nivel de calcio y el nivel de glutatión covarían. Encontramos que el tamaño del babero estuvo negativamente relacionado con el calcio de la dieta pero no estuvo afectado por las inyecciones de BSO. Tampoco encontramos un efecto de las inyecciones de BSO sobre la reflectancia de las plumas del babero, aunque hubo un efecto interactivo con el calcio de la dieta y no hubo un efecto sobre el tamaño o la reflectancia de los rasgos estructurales del plumaje. El tamaño del babero no estuvo relacionado con la condición corporal en el momento en que el babero estuvo siendo producido por primera vez, y tampoco estuvo relacionado con los niveles de glutatión circulante o antioxidantes totales. La evidencia sugiere que las inyecciones de BSO redujeron el glutatión pero que los efectos fueron de corto plazo, posiblemente explicando por qué no encontramos un efecto en el tamaño del babero. En resumen, no encontramos evidencia de que el tamaño del babero en *Passer domesticus* esté relacionado con los niveles de glutatión pero confirmamos que está negativamente relacionado al calcio de la dieta.

INTRODUCTION

Males of many bird species possess distinct patches of black feathers on their throat or breast, the size of which is often positively correlated with their success in attracting mates or dominating conspecifics in contests over food or nest sites (Searcy and Nowicki 2005). Given these potential benefits, what prevents all males from producing a large plumage patch and thereby reaping the associated benefits? The leading

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hypothesis is that the production of these feather patches depends upon one or more limiting resources (see Jawor and Breitwisch 2003, McGraw 2006 for reviews), and this constraint means that only those individuals that can acquire or afford to allocate sufficient quantities of these resources can produce a large patch.

The coloration of these plumage signals is due to the presence of melanin pigments (phaeomelanins in brown feathers and eumelanins in black feathers). Birds synthesize melanins through a complex pathway beginning with the amino acid tyrosine (Lerner and Fitzpatrick 1950, Prota 1992), which has led some to speculate that any variation in the size of melanin-enriched patches of black feathers is more likely genetic in origin (Roulin and Djikstra 2003). However, several studies have found that the expression of melanin-based traits can be affected by the level of dietary minerals (McGraw 2007) or circulating testosterone (Buchanan et al. 2003) and consequently may indicate some aspect of their bearer's condition (Griffith et al. 2006).

One recent and intriguing hypothesis is that these colored patches of feathers reveal their bearer's ability to cope with oxidative stress (Alonso-Alvarez et al. 2007), which is the imbalance between the presence of reactive oxygen species automatically generated by cellular chemical reactions and the availability of antioxidant compounds. The development of melanocytes, the epidermal cells where melanin production takes place, is very sensitive to oxidative stress (McGraw 2005). Thus, the expression of a melanin-based trait could advertise the effectiveness with which their bearer's antioxidants have quenched the damaging free radicals and consequently prevented them from impairing melanocyte development.

This hypothesis is particularly attractive because there is a direct and well-established link between a key antioxidant and melanin synthesis. The most prevalent and important cellular antioxidant is glutathione (hereafter GSH), a tripeptide thiol found in almost all animal cells that performs multiple functions designed to protect somatic health, including scavenging free radicals (Anderson 1998). Intriguingly, however, it also negatively influences the activity of the enzyme tyrosinase, which is essential for the initial conversion of tyrosine into dopaquinone (McGraw 2006), and also binds to the intermediate quinone L-DOPA such that high levels of GSH inhibit melanin production. Hence, molting male birds face a trade-off between signal production and health, since suppressing their cellular GSH level would result in them producing a large black plumage patch but would simultaneously increase their vulnerability to damaging oxidative stress and disease (McGraw 2005). However, high-quality individuals may be able to reduce GSH levels without risking oxidative stress by increasing the levels of alternative antioxidants that do not affect melanin production, or by acquiring other resources that modulate the effects of GSH. Hence, oxidative stress may enforce the honesty of melanin-based feather patches because low-quality males are unable to mobilize these alternative antioxidants and must therefore maintain a high level of GSH, which prevents them from developing a large patch.

Galván and Alonso-Alvarez (2008) tested this hypothesis by repeatedly injecting nestling Great Tits (*Parus major*) with a nontoxic, specific inhibitor of GSH (DL-buthionine-S, R-sulfoximine, hereafter BSO). Great Tits treated with BSO developed larger patches of melanin-based plumage than untreated control males, and the extent of melanistic plumage increased with the concentration of BSO used. More recently, Hõrak et al. (2010) used repeated BSO injections to reduce GSH levels in erythrocytes of European Greenfinches (*Chloris chloris*) after plucking their outer tail feathers and found that the black feather tips of the regrown feathers had lower reflectance than those produced by greenfinches injected with a saline control.

We performed a similar experiment in the House Sparrow (*Passer domesticus*), a common urban and suburban bird nearly worldwide (Anderson 2006). The females are plain brown while the males have a suite of striking black, brown, and white plumage characters, the most prominent of which is a black throat patch or "bib." Males with large bibs are dominant over males with small bibs in both captive and free-living populations, are preferred by females, and, in some populations, have greater lifetime reproductive success (see Nakagawa et al. 2007 for a meta-analysis).

The House Sparrow's bib has been the focus of several studies germane to the current experiment. Manipulations of diet quality or food availability have failed to affect bib size (e.g., Gonzalez et al. 1999, McGraw et al. 2002, Buchanan et al. 2003), although males provided with diets deficient in tyrosine, the amino acid precursor to melanin, produced bib feathers that were lighter in color (Poston et al. 2005). More recently, we provided molting juvenile male sparrows with artificial diets varying in their level of calcium, a mineral previously implicated as an important component of the melanin pathway (McGraw 2003, 2008), and found that birds on diets containing low concentrations of calcium produced larger bibs than did those on medium or high calcium diets (Stewart and Westneat 2010).

This result was initially paradoxical because inorganic elements such as calcium, zinc, and copper are thought to be limiting factors in melanin synthesis (McGraw 2003, 2006) and thus we expected calcium supplements would enhance the size of bib in the same way that they enhanced the black breast patch of male Zebra Finches (*Taeniopygia guttata*) (McGraw 2007). However, the result is intriguing in the context of antioxidant-inhibition of melanin production because Staal et al. (1994) found an unexplained but strong positive correlation between levels of intracellular GSH and calcium in chickens, and De Talamoni et al. (1996) injected chicks with BSO and found that the levels of both GSH and calcium decreased. This suggests that there may be a direct

connection between cellular levels of GSH and calcium, an element essential for many physiological interactions and cell-signaling processes (Klasing 1998), and would explain why House Sparrows maintained on high-calcium diets produced small bibs (Stewart and Westneat 2010), since their GSH levels would also have been high and consequently melanin production would have been inhibited.

We therefore hypothesized that calcium and GSH are both key components of a complex set of pathways that link antioxidants, dietary elements, and the development of melanin-based plumage. We tested this idea by simultaneously manipulating the levels of both dietary calcium and GSH in juvenile House Sparrows during their molt. We predicted that bib size would decrease as both dietary calcium and cellular GSH increased, and that the effect would be exacerbated when both were increased simultaneously. We also examined the spectral reflectance of the eumelanin-containing bib feathers and also that of the phaeomelanin-containing chestnut lesser and median wing coverts to test whether GSH and calcium have the same effect upon both types of melanin. We also examined the size and reflectance of the white wing bar feathers, which do not contain melanin and thus should not be affected by our manipulations. Finally, we also measured the width of growth bars on their tail feather to test whether any plumage differences between the birds in each group could be due to differences in their molt rate.

METHODS

We used mist nets to capture 84 juvenile male House Sparrows at the University of Kentucky's Coldstream Farm on the outskirts of Lexington, Kentucky, from mid-July to mid-August 2010. They were transferred to the university's Ecological Research Facility 5 km away where they were fitted with a numbered metal leg band (L and M Bird Bands, Inc., San Bernardino, CA) and weighed to the nearest 0.1g. We measured the length of their right tarsus to the nearest 0.1 mm then used calipers to measure the length and width of their "proto-bib," which is a small, approximately rectangular patch of dusky feathers below their bill, and calculated its area as simply the product of its length and width.

Birds were placed into individual metal cages (25 × 25 × 40 cm) within three large outdoor aviaries. Each cage was outfitted with perches and a capped plastic tube for roosting, and food was provided ad libitum via two hoppers of millet attached to the front of the cage. The hoppers had a high back and sides so that the birds fed out of an opening at the front, and were filled to only two-thirds of their capacity so that spillage was negligible. Each cage also included one fountain containing tap water and one fountain containing tap water plus a commercial vitamin mix and 0.26 g L^{-1} of the anticoccidial drug sulfadimethoxine (Sigma, St Louis, MO) (after Hill and Brawner 1998).

DIET TREATMENT

In our previous experiment (Stewart and Westneat 2010), individually housed juvenile males were maintained on one of four artificial diets (Murphy and King 1982) containing variable amounts of calcium in the form of calcium carbonate ($CaCO_3$). One of these diets contained 0.1% calcium, another 1.2%. We also used these two levels in the current experiment. We chose the former level because it is the concentration of calcium in the types of grain sparrows commonly consume (Klasing 1998) and therefore presumably representative of the level they obtain in the wild, and the latter level because it was the same as that in the calcium-supplemented diet provided to Zebra Finches that enhanced the size of their melanin-based breast patch (McGraw 2007).

We achieved the target levels of calcium by using either millet or millet supplemented with $CaCO_3$, following the protocol of McGraw (2007). Half of the birds were maintained on diets of pure millet and thus received a level of calcium similar to that received by birds on the 0.1% Ca diet in the previous experiment, while the other half were maintained on seed supplemented with 30 g kg^{-1} of $CaCO_3$ (Sigma) and thus received a level of calcium similar to that received by birds on the 1.2% Ca diet in the previous experiment ("Ca-supplemented"). Powdered $CaCO_3$ and millet were shaken vigorously in a plastic container so that the millet was thoroughly coated (McGraw 2007), then this was added to the feed hoppers. To test whether birds on different diets were consuming different amounts we measured food consumption in 21 birds (9 seed only, 12 Ca-supplemented) during a two-week period in the middle of the trial. Every two days we weighed the amount of food left in the hopper and calculated how much had been consumed by subtracting it from the amount provided during the previous check.

During the first two weeks of the experiment the birds were initially provided with pure millet and not given the calcium-supplemented millet until after a week in captivity in case the unusual appearance of this diet impaired their ability to adapt to captivity. However, we found that the birds readily ate the supplemented diet and thus the rest of the experimental birds were placed on the supplemented diet on the day they were caught. We pooled all the birds for the analyses since there were no differences in post-molt measurements between those given the supplemented diet after a short delay and those given it immediately, probably because they did not begin to molt until after several weeks in captivity.

GLUTATHIONE INHIBITOR TREATMENT

Our experiment had a full factorial design that combined two levels of dietary calcium (low vs. high) with three levels of BSO (none, low, high). Birds were placed on their treatments in 14 groups, with each group containing 6 birds (= 84 birds), 1 from each combination of diet and BSO. Note that the birds were placed into the experiment in 8 blocks

representing the date when injections began, since in some instances more than one group was at the same molt stage. Body mass did not vary by block ($F_{7,76} = 0.97$, $P = 0.46$), suggesting that there were no broad differences in initial condition between birds that started the experiment at different times. Because we could not know the exact date when the subjects would begin to molt their bib, we tried to anticipate it on the basis of our knowledge of the sequence of molt in the House Sparrow, then scheduled the series of injections so that they would coincide with at least the early stage of bib molt. In juvenile House Sparrows, the first feathers to be molted are usually the innermost primaries, closely followed by the chestnut wing coverts. The appearance of the first bib feathers usually coincides with the molt of the 4th or 5th primaries, although this varies somewhat from bird to bird (I. R. K. Stewart, pers. obs.). We caught and examined each of our captive birds at approximately weekly intervals and identified any that had started to molt either their 2nd or 3rd primaries or chestnut coverts. Once we had accumulated six such birds we allocated them to a group, then took a blood sample (~50 µL) from the brachial vein of each bird and placed it in an Eppendorf tube, which was kept it on ice for <4 hr then centrifuged for 10 min at 10 000 revolutions min^{-1}. The plasma was removed and transferred to a new tube, and both the plasma and the pellet of red blood cells were frozen at –80 °C until analysis (within 4 months).

BSO was dissolved in sterile phosphate-buffered saline (PBS, Sigma) at a level of either 1.3 mg mL^{-1} (low dose) or 55 mg mL^{-1} (high dose), and aliquots were frozen at –20 °C until required. Birds were injected subcutaneously in their back with 0.1 mL of either a low dose of BSO (= 0.13 mg), a high dose of BSO (= 5.5 mg), or PBS as a control. These levels were similar to those used with nestling Great Tits by Galván and Alonso-Alvarez (2008) but were increased proportionately to account for the fact that House Sparrows are significantly larger (mean body mass ~25 g vs. ~18 g). The interval between the date of capture and the date the bird received its first injection was 15.7 ± 8.9 days (mean ± SD), and all of birds had been consuming the calcium-supplemented diet for at least one day (11.9 ± 9.2 days, mean ± SD) before they received their first injection. Birds were injected once every three days over three weeks (a total of seven injections), and before each injection we weighed them to the nearest 0.1 g and recorded the progress of their molt, particularly that of the bib. Immediately before the final injection we took a blood sample and centrifuged it to obtain plasma and a red blood cell pellet which were frozen at –80 °C.

POST-MOLT PLUMAGE MEASUREMENTS

Birds were measured once their molt was completed, or at least sufficiently advanced that the relevant plumage traits could be measured. The bib is molted upward from its lower margin (i.e., the black feathers in the middle of the breast are the first to appear and those below the bill are the last to appear; I. R. K. Stewart, pers. obs.), so its maximum length and breadth can still be measured even if the upper region is composed of old, unmolted feathers. Birds were measured in four blocks between October and December since they had started and thus finished their molt at different times. We weighed each bird to the nearest 0.1 g then measured the size of its bib by holding it with its bill perpendicular to its body and using calipers to measure its length and width at their maximum points. We calculated bib size by the regression equation derived by Møller (1987): bib size (mm^2) = 167 + (0.45 × length × width). We measured the length of the white tip of the second and third median coverts. These are the feathers responsible for the white wing bar (Poston et al. 2005, Bókony et al. 2006) and have a white tip but a dark brown basal region. Finally, we plucked one of the outer tail feathers and used calipers to measure the distance between at least six of the growth bars (after Grubb 1989), then calculated the bars' mean width.

We measured the reflectance of feathers composing the black bib, white wing bar, and chestnut coverts in situ. We obtained reflectance spectra from 300 to 700 nm with a USB2000 spectrometer (Ocean Optics, Inc., Dunedin, FL) standardized to both white and dark standards, with the probe/light source at 45° to the plane of the feather. Each feather's reflectance was measured twice. The two values of mean reflectance between 300 and 700 nm were significantly repeatable (Lessells and Boag 1987; bib ANOVA: $r_{IC} = 0.57$, $F_{54,55} = 3.6$, $P < 0.001$; white wing bar ANOVA: $r_{IC} = 0.77$, $F_{52,53} = 7.6$, $P < 0.001$; chestnut coverts ANOVA: $r_{IC} = 0.73$, $F_{55,56} = 6.5$, $P < 0.001$) and so were averaged for the analyses.

MEASUREMENTS OF FEATHER CALCIUM LEVELS AND ANTIOXIDANTS

We measured the amount of calcium in a single weighed bib feather taken from 30 birds, 5 from each of the 6 combinations of diet and BSO, using inductively coupled plasma emission spectrometry and following the same procedure used previously (Stewart and Westneat 2010).

We assessed GSH levels in the erythrocytes obtained from the blood samples taken just before the first and final BSO injections with a commercial kit (703002, Cayman Chemicals, Inc., Ann Arbor, MI). Briefly, the red blood cell pellet was weighed, lysed in ultrapure water, and centrifuged, and then the supernatant was deproteinized in an equal volume of 0.1 g mL^{-1} of metaphosphoric acid before being combined with several other reagents in a Costar 96-well plate. The results of the subsequent reaction were read at 405 nm in a Tecan Genios plate reader (Tecan USA, Durham, NC). We used Magellan 4 software (Tecan USA) to calculate GSH concentrations in each well in reference to a standard curve. Samples were assayed in duplicate, and the two readings were averaged for the analysis. The samples

obtained before the final injection were run in two plates, for which the mean intra-assay coefficient was 6.6%. The mean concentration in the two plates differed significantly ($t_{73} = 3.95$, $P < 0.001$), so we standardized the data from each plate so that the overall mean was 0 and the standard deviation was 1 before we pooled the data for the analysis. However, the results were qualitatively unchanged if each plate was analyzed separately.

We assayed the total antioxidant status in the plasma from the final blood sample with a commercial kit (709001, Cayman). Briefly, 5 μL of plasma was diluted 1:7.5 in assay buffer then combined with other reagents in a 96-well plate. The absorbance in each well was measured with a Tecan Genios plate reader at 405 nm, then the total antioxidants in each well were calculated in reference to a Trolox standard curve with Magellan 4 software. Most samples were assayed in duplicate, and the readings were averaged for the analysis. The samples were analyzed in three batches but the results were pooled for the analysis since there was no difference in mean total antioxidant status between assays (ANOVA: $F_{2,87} = 0.24$, $P = 0.79$). The intra-assay coefficient was 12.4% and the inter-assay coefficient was 20.1%

STATISTICAL ANALYSES

The data were analyzed in MYSTAT 12 (SPSS, Chicago). We provide means ± standard error throughout.

RESULTS

We started the experiment with 84 birds. Of these, 26 (31%) died during the 5-month experiment, another escaped during handling, and another lost all of its body feathers several weeks after the injections and was removed from the experiment, although it subsequently molted as normal. This left a sample size of 56 birds for most of the analyses. Moreover, for six of these birds, the three-week injection regime had ended before they began to molt their bib. Mortality was approximately evenly spread across diets and treatments (16 seed vs. 10 Ca-supplemented; 11 PBS, 8 low BSO, 7 high BSO).

MORPHOLOGY

The diet or BSO treatment produced few discernable physiological effects on the subjects during the injections. A repeated-measures ANOVA revealed no effect of diet or treatment on body mass changes during the 3-week injection period (diet $F_{1,52} = 0.21$, $P = 0.65$; treatment $F_{2,52} = 0.37$, $P = 0.69$; diet × treatment $F_{2,52} = 0.93$, $P = 0.40$), although birds on the Ca-supplemented diet tended to lose more mass during the course of the experiment (diet $F_{1,48} = 3.54$, $P = 0.07$; date of final measurement $F_{3,48} = 0.47$, $P = 0.70$; diet × date $F_{3,48} = 0.66$, $P = 0.58$). However, there was no difference in food consumption between birds on the seed-only diet and those on the Ca-supplemented diet (repeated-measures ANOVA: $F_{1,18} = 1.35$, $P = 0.26$, and $F_{1,18} = 1.04$, $P = 0.32$ after controlling for the fact that 3% of the supplemented diet was $CaCO_3$). The average width of growth bars on the tail feathers was not related to diet or treatment (ANOVA: diet $F_{1,36} = 0.02$, $P = 0.88$; treatment $F_{2,36} = 1.70$, $P = 0.20$; diet × treatment $F_{2,36} = 0.15$, $P = 0.86$), although it was positively correlated with body condition, calculated as the residual of the regression of mass on tarsus length, on the day of the final injection ($r = 0.27$, $n = 66$, $P = 0.03$), which supports the assumption that growth-bar width is an index of condition during molt (Grubb 1989).

Calcium supplementation had a nonsignificant negative effect upon bib size (ANOVA: $F_{1,50} = 3.51$, $P = 0.07$, Fig. 1A), although the effect became significant after the removal of a single low outlier from the seed-only treatment ($t_{53} = 2.30$, $P = 0.03$). However, there was no effect of BSO treatment (ANOVA: $F_{2,50} = 0.41$, $P = 0.67$, Fig. 1B) or the interaction between the two (ANOVA: $F_{2,50} = 0.45$, $P = 0.64$). When we repeated the analysis after excluding the six birds that did not begin to molt their bib until after the injection regime had ended, the effect of diet became significant (ANOVA: $F_{1,44} = 11.66$, $P = 0.001$) but there was still no effect of either BSO treatment (ANOVA: $F_{2,44} = 0.70$, $P = 0.50$) or its interaction with calcium (ANOVA: $F_{2,44} = 0.92$, $P = 0.41$). We did not control for block in the analysis as it had no effect on bib size (ANOVA: $F_{7,48} = 1.65$, $P = 0.14$). The size of the proto-bib that males possessed as juveniles was significantly positively correlated with their eventual bib size ($r = 0.42$, $n = 56$, $P < 0.001$, Fig. 2; $r = 0.32$, $n = 52$, $P = 0.019$ after omission of four birds that did not have discernable proto-bibs). Bib size was not related to physical size, as estimated by tarsus length ($r = 0.08$, $n = 56$, $P = 0.54$), or body condition at the mid-point of the injections ($r = –0.06$, $n = 56$, $P = 0.69$). There was no effect of diet or

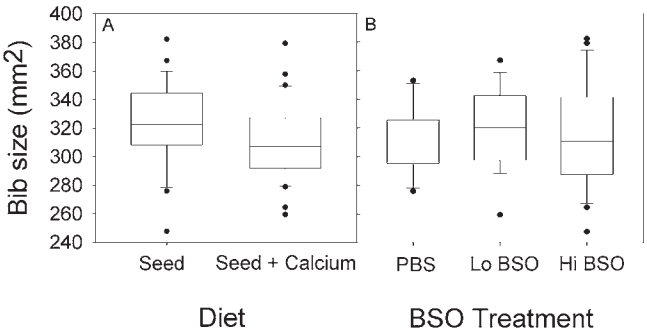

FIGURE 1. Boxplot illustrating the mean size (mm^2) of the bib produced by juvenile male House Sparrows (A) maintained on a diet of either pure millet or millet supplemented with 30 g kg^{-1} of calcium carbonate or (B) given repeated injections of either phosphate-buffered saline (PBS), 0.13 mg of buthionine-S, R-sulfoximine (Lo BSO), or 5.5 mg of buthionine-S, R-sulfoximine (Hi BSO) during the start of their bib molt. Horizontal bars in the plot indicate the 10^{th}, 25^{th}, 50^{th}, 75^{th}, and 90^{th} percentiles, and filled circles represent values outside these ranges.

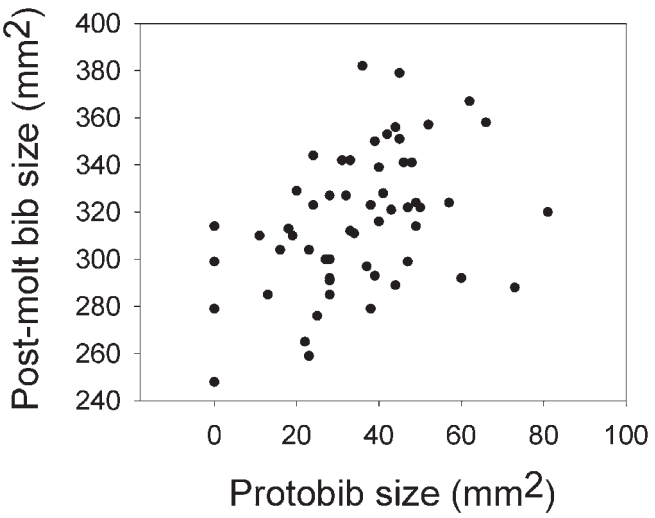

FIGURE 2. Scatterplot of the relationship between the size of juvenile male House Sparrows' proto-bib and the size of their bib after molt. The relationship is significantly positive ($r = 0.42$, $n = 56$, $P < 0.001$).

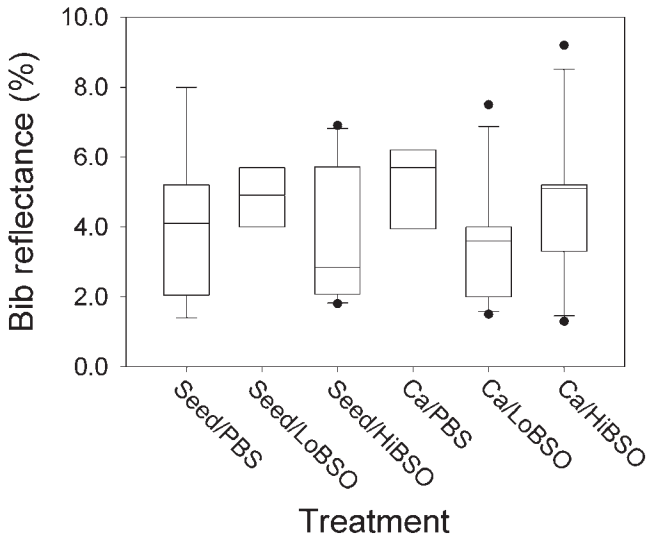

FIGURE 3. Boxplot illustrating the mean reflectance (%) of bib feathers produced by juvenile male House Sparrows that received repeated injections with either phosphate-buffered saline (PBS), 0.13 mg of buthionine-S, R-sulfoximine (LoBSO), or 5.5 mg of buthionine-S,R-sulfoximine (HiBSO) during their molt in combination with a dietary regime of either pure millet (Seed) or seed supplemented with 30g kg^{-1} of calcium carbonate (Ca). Horizontal bars in the plot indicate the 10th, 25th, 50th, 75th, and 90th percentiles, and filled circles represent values outside these ranges.

treatment on the width of the white wing bar (ANOVA: diet $F_{1,47} = 0.20$, $P = 0.66$; treatment $F_{2,47} = 0.15$, $P = 0.61$; diet × treatment $F_{2,47} = 0.5$, $P = 0.61$).

Neither diet nor treatment affected the reflectance of the black bib feathers (ANOVA: diet $F_{1,50} = 0.21$, $P = 0.65$; treatment $F_{2,50} = 0.46$, $P = 0.64$) though there was a significant interaction between the two (ANOVA: $F_{2,50} = 3.56$, $P = 0.04$, Fig. 3) with birds in the low BSO treatment having relatively high reflectance when on the seed diet but relatively low reflectance when on the Ca-supplemented diet. Bib reflectance was not correlated with bib size ($r = –0.06$, $n = 56$, $P = 0.67$). There was no effect of diet or treatment on the reflectance of the chestnut coverts (ANOVA: diet $F_{1,50} = 0.01$, $P = 0.93$; treatment $F_{2,50} = 0.28$, $P = 0.76$; diet × treatment $F_{2,50} = 0.75$, $P = 0.48$) or on the reflectance of the white wing bar (ANOVA: diet $F_{1,56} = 0.15$, $P = 0.70$; treatment $F_{2,56} = 1.25$, $P = 0.29$; diet × treatment $F_{2,56} = 0.61$, $P = 0.55$), although wing bar reflectance was positively correlated with mean bar size ($r = 0.28$, $n = 55$, $P = 0.045$). Reflectance of the bib was correlated with that of the chestnut coverts ($r = 0.35$, $n = 56$, $P = 0.03$) but not that of the wing bar ($r = 0.06$, $n = 54$, $P = 1.00$). Reflectance of the chestnut coverts was positively correlated with that of the wing bar ($r = 0.54$, $n = 54$, $P < 0.001$).

FEATHER CALCIUM ANALYSES

Levels of calcium in feathers were strongly affected by diet (ANOVA: $F_{1,24} = 47.2$, $P < 0.001$) but not by BSO treatment (ANOVA: $F_{2,24} = 0.35$, $P = 0.71$) or the interaction between the two (ANOVA: $F_{2,24} = 0.56$, $P = 0.58$). The calcium concentration in bib feathers from birds on the Ca-supplemented diet was approximately four times higher than in bib feathers from birds on the seed-only diet (Appendix available at http://dx.doi.org/10.1525/cond.2013.130006).

ANTIOXIDANT ASSAYS

We had data on pre-experimental GSH levels from only 25 birds because we had diluted the blood pellet by a greater amount and so the levels from a further 10 birds were below the detection limit. Nevertheless, pre-experimental GSH levels did not vary by treatment ($F_{2,20} = 0.77$, $P = 0.48$). GSH levels measured at the end of the series of injections may have been influenced by diet; calcium-supplemented birds tended to have lower levels of GSH, although the difference was not significant (ANOVA: $F_{1,47} = 3.74$, $P = 0.06$). However, GSH levels were not affected by treatment (ANOVA: $F_{2,47} = 0.43$, $P = 0.66$, Fig. 4), and there was no interaction between diet and treatment (ANOVA: $F_{2,47} = 0.65$, $P = 0.53$). Standardized GSH levels did decline in the expected manner (PBS 0.19 ± 0.33, low BSO –0.04 ± 0.17, high BSO –0.11 ± 0.23), but the difference was not significant because of wide variation in each category. Bib size was not correlated with GSH level ($r = 0.24$, $n = 44$, $P = 0.11$), and there was no difference in GSH level at the time of the final blood sample between birds that survived the experiment and those that died ($t_{51} = 0.03$, $P = 0.98$).

Total antioxidant status was not related to diet or treatment (ANOVA: diet $F_{1,74} = 0.02$, $P = 0.90$; treatment $F_{2,74} = 0.04$, $P = 0.96$; diet × treatment $F_{2,74} = 1.39$, $P = 0.26$) and was not correlated with GSH level ($r = 0.14$, $n = 73$, $P = 0.24$) or bib size ($r = 0.05$, $n = 55$, $P = 0.70$). However, birds that survived

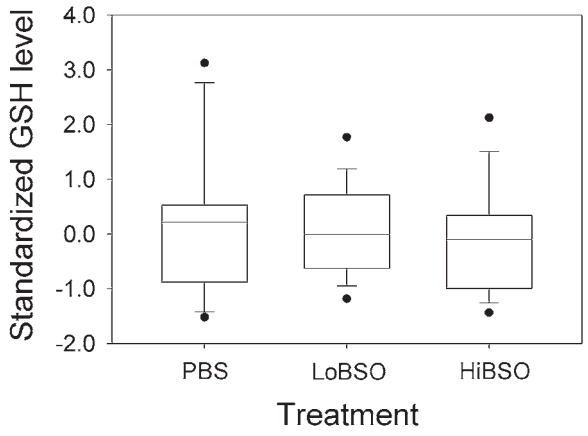

FIGURE 4. Boxplot illustrating the standardized glutathione (GSH) level in erythrocytes of juvenile male House Sparrows after being injected 6 times at 3-day intervals with either phosphate-buffered saline (PBS), 0.13 mg of buthionine-S, R-sulfoximine (LoBSO), or 5.5 mg of buthionine-S,R-sulfoximine (HiBSO). Horizontal bars in the plot indicate the 10th, 25th, 50th, 75th, and 90th percentiles, and filled circles represent values outside these ranges.

the experiment had significantly higher levels of total antioxidants at the time of the final blood sample than those which died ($t_{78} = 3.58$, $P = 0.001$).

We performed a short experiment in 2011 to test the duration of the effect of BSO upon GSH levels in House Sparrows. We captured 13 adult House Sparrows and housed them in two large outdoor aviaries with ad libitum seed and water at the same research facility. We took a blood sample from each bird, then injected it with a freshly prepared solution of either PBS ($n = 4$), low BSO ($n = 4$), or high BSO ($n = 5$), using the same concentrations and methods as before. We then recaptured and bled the birds after 24 hr, 48 hr, and 72 hr (i.e., each bird was bled on 4 successive days after being injected with BSO on the first day). We processed the blood and then estimated GSH levels in erythrocytes with the same kit and equipment as before. There was no difference between the GSH level of birds in each treatment immediately before the BSO injections (day 1, ANOVA: $F_{2,10} = 0.26$, $P = 0.78$), though there was on day 2 (ANOVA: $F_{2,10} = 5.9$, $P = 0.02$), with the mean level declining with increasing BSO levels. However, there was no difference on day 3 (ANOVA: $F_{2,9} = 2.0$, $P = 0.19$, after omission of one high outlier from the PBS treatment) or on day 4 (ANOVA: $F_{2,9} = 0.51$, $P = 0.62$, after omission of one high outlier from the low-BSO treatment). An individual's GSH level was not repeatable across the four sampling periods (ANOVA: $r_{IC} = 0.15$, $F_{12,39} = 1.70$, $P = 0.10$).

DISCUSSION

We repeatedly injected molting juvenile male House Sparrows with a substance (BSO) that inhibits the levels of the antioxidant GSH, which itself inhibits the production of melanin, and predicted that birds with progressively lower GSH levels would produce progressively larger bibs. These injections were combined with dietary calcium supplements because we had found in a previous study that dietary calcium had a negative effect upon bib size. Since we hypothesized that this result arose because calcium level and GSH level covary, we predicted that bib size would decrease as both cellular GSH and dietary calcium increased, and that the effect would be exacerbated when both were increased simultaneously. However, we found that BSO treatment had no effect upon bib size.

There are two possible explanations for this. The first is that GSH does not inhibit melanin production in the House Sparrow as strongly as it does in the two other species of bird studied thus far. It is not clear why the relationship between GSH and melanin would vary by species since both compounds are conserved, although it may be significant that both the Great Tit and European Greenfinch also possess extensive carotenoid-based plumage, which the House Sparrow does not. Nevertheless, until data are available from a greater number of taxonomically diverse species it should not be assumed that there is an inherent link between GSH and melanin in birds. The second explanation is that our BSO injections did not have a sufficiently negative effect upon GSH levels. This possibility is difficult to reject. Although the mean GSH level in erythrocytes did decline with increasing levels of BSO, the variation within each group was considerable, and consequently there was no significant difference between groups. It is possible that we failed to detect any difference in GSH levels because of the relatively long interval between the BSO injection and bleeding, such that GSH levels were initially reduced by the BSO injection but had regained their original level by the time of blood sampling three days later. For instance, BSO injections in mice depressed GSH levels in the liver within 2 hr, but these returned to their initial level within 25 hr (Griffith 1982). However, in both European Greenfinches (Hõrak et al. 2010) and nestling Great Tits (Galván and Alonso-Alvarez 2008) GSH levels were still depressed compared to controls 1 day and 2 days, respectively, after BSO injection, which is why we had still expected to see an effect, albeit diminished, of BSO upon GSH levels in House Sparrows 3 days after injection. We subsequently performed a short experiment where we resampled birds daily following an injection of BSO, which indicated that BSO injections did depress GSH levels for at least 24 hr but they were replenished within 48 hr. Thus the repeated BSO injections may not have depressed GSH levels for long enough to affect bib development. We recommend that future studies deliver BSO continuously via subcutaneous implants or slow-release pellets. This should negate the birds' ability to regain GSH levels rapidly following each injection, and would also minimize any confounding effects of the stress associated with repeated capture and injection.

We found that juvenile male House Sparrows maintained throughout their molt on diets high in calcium produced smaller bibs than those on diets low in calcium, confirming the finding of Stewart and Westneat (2010). These data suggest that dietary calcium inhibits rather than enhances melanin production, perhaps because high levels of dietary calcium decrease the absorption of trace elements such as zinc and copper (Klasing 1998), which are essential for melanin synthesis (McGraw 2003, 2007). Carsberg et al. (1995) found evidence of an inhibitory effect of elevated intracellular calcium levels upon melanin production in cultured human melanocytes, and Fuller (1987) indirectly demonstrated a similar phenomenon, in which application of the calcium ionophore A23187 reduced tyrosinase levels in mouse cell cultures by 50%, likely because it mobilized calcium out of intracellular organelles. We did find a significant interaction between treatment and diet upon the reflectance of bib feathers, with birds in the intermediate BSO treatment producing lighter-colored bib feathers when maintained on pure seed but darker feathers when maintained on calcium-supplemented seed, suggesting that an intermediate level of GSH may affect melanin production but the direction of that effect is contingent upon intracellular calcium level. Several studies of human cells have shown a relationship between existing melanization and calcium level. For example, Hoogduijn et al. (2003) raised extracellular calcium levels in a culture of human melanocytes and found that cytoplasmic calcium in cells with high melanin concentrations increased less than in those with low melanin concentrations. Similarly, birds may adjust the influx or efflux of calcium into their melanocytes in response to GSH levels in order to optimize melanin synthesis.

We found that GSH levels in blood samples taken from sparrows in the early stages of molting their bib were positively, though not significantly, correlated with their eventual bib size. We expected the two to be negatively correlated because of the inhibitory effect of GSH upon melanin synthesis. We also found that birds on calcium-supplemented diets had lower GSH levels than those on seed-only diets, which is also inconsistent with GSH inhibiting melanin synthesis because these birds produced smaller bibs, rather than the larger bibs we expected. This result also rejects our supposition that the reason sparrows maintained on high-calcium diets in the previous experiment (Stewart and Westneat 2010) developed smaller bibs was because their GSH level increased because calcium and GSH covaried, and it was the latter that was directly responsible for their smaller bib sizes. Instead, the negative effect of calcium appears to act through a different mechanism.

These data contradict the result of a very similar experiment with the Zebra Finch (McGraw 2007), in which calcium supplementation enhanced the size, though not the reflectance, of the male's black breast patch. We reiterate our earlier argument (Stewart and Westneat 2010) that the difference between species may be due to differences in how they assimilate calcium, and that calcium likely functions within a complex suite of physiological interactions which affect melanin synthesis in such a way that an increase in a single component may not have a consistently predictable result across species. More studies are needed to test whether inorganic elements affect melanin production, yet in some species the visual evidence suggests that this is unlikely. For example, crows (*Corvus*) are relatively common, large birds (~300 g), many of which have entirely black plumage that presumably requires a proportionately large amount of dietary calcium to produce. However, intraspecific plumage variation in most species of crows is negligible, at least to the human eye, and it is difficult to believe that House Sparrows struggle to find enough calcium with which to melanize the distal end of the ~100 small feathers that compose the bib while crows feeding in similar suburban and agricultural settings all find enough calcium with which to melanize their entire plumage. Moreover, there are several species of medium-sized passerines (e.g., Icteridae) that have predominantly black plumage despite a diet consisting primarily of seeds, fruit, or insects, all of which are low in calcium (Klasing 1998).

Plasma antioxidant levels, as estimated by individual total antioxidant status, did not differ by treatment, and there was no correlation between an individual's GSH and total antioxidant levels. Total antioxidant levels measured in blood samples collected during molt were much higher in sparrows that survived the experiment than in those that died, while there was no difference in their GSH levels. This contrast may have arisen because GSH is specifically involved in protection against reactive oxygen species (Anderson 1998) and thus may affect survival only over a relatively long period, whereas several of the other antioxidants included in the assay may assist with the immune response to parasites (e.g., McGraw and Ardia 2004) and are thus more likely to affect survival in the short term.

The experiment produced relatively few effects on the bib, the main sexual character in the House Sparrow and the primary focus of the study. Bib size was not related to body condition when birds were in the early stages of producing their bib, which fails to support previous studies demonstrating or implying that the size of the House Sparrow's bib is condition-dependent (Veiga and Puerta 1996, Gonzalez et al. 1999, Griffith et al. 1999), although we caution that our study involved individually caged birds provided with food ad libitum. Intriguingly, however, the size of the bib that birds possessed after their molt was positively correlated with the size of their proto-bib, the small rectangle of dusky feathers on the throat of juvenile males that first appears around the time that they leave the nest (~18 days old; D. F. Westneat, unpubl. data). This suggests that the size of the melanin-based plumage trait that male sparrows exhibit as adults is partly determined at a relatively early ontogenetic stage, either because

it has a significant genetic component or because their initial body condition is influenced by maternal effects such as yolk testosterone levels or maternal phenotype (Strasser and Schwabl 2004, Jensen et al. 2006). A nestling's body condition may have a positive and persistent effect upon its condition as a juvenile during molt, such that nestlings in good condition produce larger proto-bibs and also larger bibs as adults. In support of this idea, in a nest-box population at a nearby farm the body mass of House Sparrow nestlings banded at 10 days of age was strongly correlated with their mass when they were recaptured up to several months later as independent juveniles ($r = 0.53$, $n = 120$, $P < 0.001$). Alternatively, juveniles with large proto-bibs may be dominant over those with small proto-bibs in contests over food and thus in better condition at the time of molt, which could also result in them producing larger bibs.

In conclusion, our experiment produced several apparently conflicting results. Repeatedly injecting juvenile male House Sparrows with a substance known to reduce the level of GSH, a cellular antioxidant that inhibits melanin production, did not affect the expression of a melanin-based trait, although it is not certain that the levels of GSH were reduced sufficiently. Moreover, levels of GSH measured during the period when the trait was first produced were not inversely correlated with trait size; indeed, the relationship trended positive. Furthermore, birds provided with supplemental calcium, a mineral hypothesized to be necessary for the production of melanin-based traits, actually produced significantly smaller bibs and had lower levels of GSH. There was also no relationship between trait size and the circulating level of either GSH or plasma antioxidants. Overall, we failed to support the hypothesis that male birds' sexual ornaments indicate their bearer's level of circulating antioxidants.

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