1 **Black bib size is associated with feather content of pheomelanin in male**
2 **house sparrows**

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24 Dear Editor,

25 Pigments constitute the basis of most visual signals involved in animal communication. Melanins are the
 26 most common pigments, often representing the basis of signals that are honest (i.e. signalers transfer
 27 reliable information about their genotypic quality to receivers; McGraw, 2008). The black chest bib of male
 28 house sparrows *Passer domesticus* is a melanin-based plumage patch (figure 1) that constitutes one of the
 29 most intensively studied animal signals (Anderson, 2006). Birds develop the bib not only during the breeding
 30 season but all year round, larger bibs being likely associated with higher melanocyte numbers. Male house
 31 sparrows displaying larger bibs are dominant in aggressive interactions with other males, have better body
 32 condition, and, in some populations, achieve higher lifetime reproductive success (reviewed in Nakagawa et
 33 al., 2007). This has been explained through the handicap principle, i.e. low-quality house sparrows do not
 34 display large bibs because their production represents costs that these birds cannot afford. In particular, bib
 35 expression depends on testosterone levels which in turn suppresses the immune system, and
 36 immunosuppression costs would be only affordable by high-quality males (i.e., the immunocompetence
 37 handicap hypothesis; González et al., 1999).

38 However, the signal content of house sparrows' bibs is assumed to be intimately related to their
 39 constitutive pigments (i.e. melanins), so signal expression (i.e. bib size) is likely to depend more primarily on
 40 the mechanisms that control overall melanin biosynthesis than on testosterone level, although testosterone
 41 clearly affects the deposition of melanins in feathers (e.g., Haase et al., 1995). Two main chemically distinct
 42 forms of melanin are identified in vertebrate animals: eumelanin, a polymer of 5,6-dihydroxyindole (DHI) and
 43 5,6-dihydroxyindole-2-carboxylic acid (DHICA) units, and pheomelanin, a polymer of benzothiazine and
 44 benzothiazole derivatives (García-Borrón and Olivares Sánchez, 2011). The environmental conditions under
 45 which producing pheomelanin and eumelanin is costly differ for each pigment (Galván and Alonso-Alvarez,
 46 2009), and the genetic and physiological bases of melanogenesis are relatively well known (García-Borrón
 47 and Olivares Sánchez, 2011). Thus, understanding how the melanin composition of the house sparrow's bib
 48 affects its size might be very useful to understand why this signal is honest.

49 To our knowledge, a chemical analysis of house sparrow bib melanins has never been done, and it
 50 has been assumed that variation in bib characteristics depends on its content of 'melanin' in general terms or
 51 mostly on eumelanin content (McGraw, 2008). Thus, it has been assumed that the honesty of the signal
 52 should lie in producing more melanin (which would correspond to more melanocytes, assuming that melanin
 53 production per melanocyte is independent of bib size), but here we aim at determining the content of

54 eumelanin and pheomelanin in bibs separately, considering that the mechanisms of synthesis of the two
 55 chemical forms differ and that this may help understanding the honesty of bib size. We therefore analyse the
 56 associations between the size of the bib of male house sparrows (figure 1) kept in captivity during the course
 57 of a experiment (in which the levels of the antioxidant glutathione, GSH, were manipulated in some birds
 58 while others were controls) and the content of pheomelanin and eumelanin in their feathers. To measure
 59 pheomelanin, we used the specific markers 4-amino-3-hydroxyphenylalanine (4-AHP) and thiazole-2,4,5-
 60 tricarboxylic acid (TTCA), and to analyse eumelanin, we used the specific marker pyrrole-2,3,5-tricarboxylic
 61 acid (PTCA) (see Online Supporting Information for experimental and analytical procedures).

62 The general linear model (GLM) exploring the relationship between final bib size (see Online
 63 Supporting Information) and TTCA concentration showed a negative and significant effect of TTCA levels (b
 64 $= -6.02$, $t = -2.73$, $F_{1,36} = 7.47$, $p = 0.009$; figure 2), in addition to the effects of experimental treatment ($F_{1,36} =$
 65 3.75 , $p = 0.061$) and tarsus length ($F_{1,36} = 0.01$, $p = 0.933$). The model with 4-AHP showed a marginally non-
 66 significant effect of this variable on bib size ($b = -2.09$, $t = -1.84$, $F_{1,37} = 3.27$, $p = 0.079$). The relationship
 67 between bib size and PTCA levels was not significant ($b = 1.13$, $F_{1,37} = 0.16$, $p = 0.686$). Therefore, the bib
 68 size of male house sparrows was negatively correlated with the levels of pheomelanin (TTCA) in its feathers,
 69 and unrelated to the levels of eumelanin. The total content of eumelanin is considerably greater than the total
 70 content of pheomelanin but the latter is more variable among individual birds than the content of eumelanin
 71 (see Online Supporting Information).

72 Our results suggest that, in male house sparrows, developing large bibs means producing small
 73 amounts of pheomelanin. This may seem counterintuitive because some authors have assumed, but never
 74 proved, that bib size variability depends on eumelanin levels in the bib feathers as the black colour of the bib
 75 may be conferred by eumelanin and not by pheomelanin, which produces lighter colours than eumelanin
 76 (McGraw, 2008). However, pheomelanin and eumelanin are produced under different conditions, and thiols
 77 that promote pheomelanin production inhibit the activity of the enzyme tyrosinase, hence reducing eumelanin
 78 production (del Marmol et al., 1993). Therefore, bib size may be either positively related to eumelanin levels
 79 or negatively related to pheomelanin levels. Our study confirms the latter. It also confirms previous
 80 observations suggesting that pheomelanin is biologically relevant even if present at low proportions in
 81 feathers whose appearance is given by the darker colors conferred by eumelanin (Galván et al., 2010).
 82 Recently, the size of a brown plumage patch in masked boobies *Sula dactylatra* has also been reported to
 83 be negatively associated to the levels of pheomelanin (4-AHP) but not related to the levels of eumelanin
 84 (PTCA) in the patch's feathers (Fargallo et al., 2014). Thus, the honesty in the signaling content of the black

85 bib of male house sparrows should be based on the mechanisms that lead to the production of small
86 amounts of one of its constitutive pigments, i.e. pheomelanin (see also Online Supporting Information).

87 This finding is relevant for understanding the honesty in the bib size of male house sparrows. The
88 control of dishonesty might be mediated by the production costs of large bibs, and the only information on
89 production costs so far proposed for this signaling system (although non-physiological costs are also
90 possible for large plumage patches; Senar, 2006) is provided by the immunocompetence handicap
91 hypothesis (González et al., 1999). However, to our knowledge there is no empirical demonstration that the
92 physiological costs derived from testosterone-mediated immunosuppression are so high that the production
93 of large bibs is not possible for low-quality birds (i.e., those with smaller bibs), just that males with large bibs
94 have higher levels of immunosuppression (González et al., 1999). Therefore, production costs are not
95 enough to explain why the signaling system is not invaded by cheaters producing bluffs, i.e. males producing
96 bibs disproportionally larger relative to their genotypic quality, and the evolutionary logic of the honesty of
97 house sparrows' bib remains unclear. Pheomelanin is produced when thiols (cysteine or GSH) are present,
98 while eumelanin is produced when thiols are absent or below a threshold level in melanocytes (García-
99 Borrón and Olivares Sánchez, 2011). Therefore, to achieve a low production of pheomelanin that allows the
100 development of a large bib it is necessary that thiol levels in melanocytes, where melanogenesis takes place,
101 are low. In experiments not shown, we found that induced reductions of the levels of GSH led to the
102 development of better phenotypes (i.e., larger bibs) in high-quality birds only (i.e., those which already
103 displayed larger bibs before the experimental manipulation), suggesting that a physiological mechanism may
104 have evolved in low-quality birds to avoid producing bibs of size not corresponding to their quality. Given the
105 existence of specific transporters of cystine (the dimer of cysteine) that takes it into melanocytes (Chintala et
106 al., 2005) or pump it out of melanosomes (Chiaverini et al., 2012), this may constitute a mechanism of
107 control of signal expression. If the genetic basis of variation in the quality of male house sparrows was
108 actually variation in the expression of genes coding for those transporters (*Slc7a11* and *CTNS*; Chintala et
109 al., 2005 and Chiaverini et al., 2012), then birds could develop bibs of size according to their quality and
110 production costs would not be necessary to explain the honesty of the signal. That is, the expression of
111 these genes may be linked to the quality of the birds so that the physiological state of low-quality birds may
112 prevent them from producing large bibs even if those birds would decide to invest in the production of large
113 bibs. Indeed, this opens a new perspective for the evolution of one of the most intensively studied systems of
114 honest communication.

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124 References

- 125 Anderson, T.R. 2006. Biology of the ubiquitous house sparrow (New York: Oxford University Press).
- 126 Chiaverini, C., Sillard, L., Flori, E., Ito, S., Briganti, S., Wakamatsu, K., Fontas, E., Berard, E., Cailliez, M.,
 127 Cochat, P. et al. (2012). Cystinosin is a melanosomal protein that regulates melanin synthesis.
 128 FASEB J. 26, 3779-3789.
- 129 Chintala, S., Li, W., Lamoreux, M.L., Ito, S., Wakamatsu, K., Sviderskaya, E.V., Bennett, D.C., Park, Y.-M.,
 130 Gahl, W.A., Huizing, M. et al. (2005). *Slc7a11* gene controls production of pheomelanin pigment and
 131 proliferation of cultured cells. Proc. Natl. Acad. Sci. USA 102, 10964-10969.
- 132 del Marmol, V., Solano, F., Sels, A., Huez, G., Libert, A., Lejeune, F. and Ghanem, G. (1993). Glutathione
 133 depletion increases tyrosinase activity in human melanoma cells. J. Invest. Dermatol. 101, 871-874.
- 134 Fargallo, J.A., Velando, A., López-Rull, I., Gañán, N., Lifshitz, N., Wakamatsu, K. and Torres, R. (2014). Sex-
 135 specific phenotypic integration: endocrine profiles, coloration, and behavior in fledgling boobies.
 136 Behav. Ecol. 25, 76-87.
- 137 Galván, I. and Alonso-Alvarez, C. (2009). The expression of melanin-based plumage is separately
 138 modulated by exogenous oxidative stress and a melanocortin. Proc. R. Soc. B 276, 3089-3097.
- 139 Galván, I., Bijlsma, R.G., Negro, J.J., Jarén, M. and Garrido-Fernández, J. (2010). Environmental constraints
 140 for plumage melanization in the northern goshawk *Accipiter gentilis*. J. Avian Biol. 41, 523-531.

- 141 García-Borrón, J.C. and Olivares Sánchez, M.C. (2011). Biosynthesis of melanins. In Melanins and
142 melanosomes: biosynthesis, biogenesis, physiological, and pathological functions, J. Borovanský
143 and P.A. Riley, eds. (Weinheim: Wiley-Blackwell), pp. 87-116.
- 144 González, G., Sorci, G. and de Lope, F. (1999). Seasonal variation in the relationship between cellular
145 immune response and badge size in male house sparrows (*Passer domesticus*). Behav. Ecol.
146 Sociobiol. 46, 117-122.
- 147 Haase, E., Ito, S. and Wakamatsu, K. (1995). Influences of sex, castration, and androgens on the eumelanin
148 and pheomelanin contents of different feathers in wild mallards. Pigment Cell Res. 8, 164-170.
- 149 McGraw, K.J. (2008). An update on the honesty of melanin-based color signals in birds. Pigment Cell
150 Melanoma Res. 21, 133-138.
- 151 Nakagawa, S., Ockendon, N., Gillespie, D.O.S., Hatchwell, B.J. and Burke, T. (2007). Assessing the function
152 of house sparrows' bib size using a flexible meta-analysis method. Behav. Ecol. 18, 831-840.
- 153 Senar, J.C. (2006). Color displays as intrasexual signals of aggression and dominance. In Bird Coloration,
154 volume 2: function and evolution, G.E. Hill and K.J. McGraw, eds. (Cambridge, MA: Harvard
155 University Press), pp. 87-136.
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Figure 1. Male house sparrows used in the experiment showing (A) the initial bib just before the beginning of the experiment, (B) the bib after all its feathers and neighboring feathers were plucked in the beginning of the experiment, and (C) the final bib with newly grown feathers at the end of the experiment.

Figure 2. Relationship between bib size and pheomelanin levels in bib feathers (TTCA levels) of male house sparrows. The ordinate axis depicts the residual size of the bib patch, calculated as the residuals of a GLM with bib size as the response variable, treatment as a fixed factor and tarsus length as a covariate, but not including TTCA levels. Thus, the ordinate axis values shows the variation in bib patch size that is not explained by neither treatment nor tarsus length so that the association with TTCA levels is not affected by those confounding factors. The line is the regression line.

Figure 1

Figure 2

234 Supporting information for:

235 **Black bib size is associated with feather content of pheomelanin in male**
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238

239 **Experimental procedure**

240 In June-July 2010, 53 free ranging adult male house sparrows were captured with mist nests in the
 241 surroundings of the Dehesa Galiana experimental facility (Ciudad Real, Spain), identified with a metal ring
 242 and housed alone in individual cages (0.6 m long x 0.4 m wide x 0.4 m tall; Italgabbie, Caltrano, Italy) in an
 243 indoor aviary (7.4 x 3.3 x 2.5 m). The birds were provided with ad libitum water and food for canaries and
 244 cuttlefish bone. On 13 July, tarsus length was measured with digital calipers. This process was made for the
 245 aims of an experiment in which the levels of glutathione (GSH), a key intracellular antioxidant, were
 246 manipulated in the birds.

247 In the house sparrow, the moulting period takes place at the time our experiment was conducted (i.e.
 248 July-September; Anderson, 2006), but to avoid inter-individual differences in the phenology of plumage
 249 moult, on 20 July we plucked the feathers of the bib patch, thus inducing the growth of all the bib feathers.
 250 The bib area of male house sparrows is fixed at moult and does not change until the next molt (Anderson,
 251 2006), but bib feathers lose their grey tips during the course of the season so that bibs at the end of the
 252 season have lost part of feather barbules. Thus, to analyse the association between bib size and melanin
 253 content we only considered the size of newly developed bibs (figure 1). With this aim, on 27 September the
 254 birds were captured and a photograph of the bib of birds was taken with a digital camera (Olympus E-50).
 255 The birds were held in the same posture, at a fixed distance from the camera, under standardized
 256 illumination (figure 1). Bib size was measured by selecting the total black area on throat and chest with
 257 Adobe Photoshop and converting pixels to cm^2 with the reference of a scale that was included as
 258 background in all photographs. The analysis of bib size was made by a technician blinded to the aims of the
 259 study. The body mass of birds was measured with a digital balance. We also plucked 15-20 feathers at
 260 random from the inner part of the bib of all birds, which were stored in plastic bags and kept at dark until

261 biochemical analyses were carried out. The birds were released back to nature at the same site where they
 262 were captured after all measurements were taken.

263

264 **Analysis of melanins**

265 The analysis of melanins was based on the formation and detection by HPLC of specific degradation
 266 products, 4-amino-3-hydroxyphenylalanine (4-AHP) by reductive hydrolysis of pheomelanin with hydriodic
 267 acid (HI) and pyrrole-2,3,5-tricarboxylic acid (PTCA) and thiazole-2,4,5-tricarboxylic acid (TTCA) by alkaline
 268 H₂O₂ oxidation of eumelanin and pheomelanin, respectively. Thus, 4-AHP and TTCA are specific to
 269 pheomelanin and PTCA is specific to eumelanin. We used two alternative markers for pheomelanin because
 270 TTCA and 4-AHP are specific of the two different moieties of the pigment, benzothiazole and benzothiazine,
 271 respectively (Wakamatsu et al., 2009). Details of sample analyses have been published elsewhere (e.g.,
 272 Galván et al. 2012).

273

274 **Statistical analyses**

275 General linear models (GLM) were used to analyse the association between bib size and the levels of
 276 pheomelanin and eumelanin in feathers. Bib size was the response variable, and pheomelanin (TTCA or 4-
 277 AHP) or eumelanin (PTCA) levels were added as covariates (log₁₀-transformed). Given the level of
 278 correlation between the different melanin markers (Pearson correlation tests: PTCA-TTCA: $r = 0.34$, $p =$
 279 0.033 ; PTCA-4-AHP: $r = -0.04$, $p = 0.827$; TTCA-4-AHP: $r = 0.68$, $p < 0.0001$, all $n = 40$), the effect of each
 280 melanin marker on bib size was tested on separate models. Variation in body size was controlled for by
 281 adding tarsus length as an additional covariate. As birds were exposed to an experimental treatment, bib
 282 size may be affected by this potential source of variation, so treatment (control vs. manipulated) was added
 283 to the model as a fixed factor as well as its interaction with melanin level. A backward stepwise procedure
 284 was used to remove non-significant terms, using a p -value of 0.1 as a threshold to abandon the model.

285 We also calculated the total content of eumelanin and pheomelanin of the bib of house sparrows by
 286 multiplying the concentrations of TTCA, 4-AHP and PTCA by bib size, as well as the coefficient of variation
 287 (CV, calculated with values in log₁₀) to determine the variability among individual birds in the total content of
 288 the different melanin forms.

289

290 **Results**

291 The mean ($\pm$ SE) concentration of TTCA (the benzothiazole unit marker for pheomelanin) in the bib feathers
 292 of male house sparrows was 65.69 ± 7.77 ng/mg feather, while the mean concentration of 4-AHP (the
 293 benzothiazine unit marker for pheomelanin) was 23.66 ± 2.70 ng/mg feather. The mean concentration of
 294 PTCA (eumelanin marker) was 1072.75 ± 35.57 ng/mg feather.

295 The mean ($\pm$ SE) total content of pheomelanin was 665.51 ± 38.60 ng with a CV of 5.85 for TTCA,
 296 and 324.23 ± 51.65 ng with a CV of 10.85 for 4-AHP. Regarding eumelanin, the mean total content of PTCA
 297 was 6570.31 ± 405.11 ng and its CV was 4.81.

298 The association between bib size and TTCA levels did not change when only control birds were
 299 considered ($b = -8.08$, $t = -2.19$, $F_{1,17} = 4.81$, $p = 0.042$). All results kept unchanged if tarsus length was
 300 removed from the models.

301

302 **Discussion**

303 The dispersion of data around the mean was higher for the total content of pheomelanin in the bib than in the
 304 total content of eumelanin. Thus, although the total content of eumelanin in the bib was considerably greater
 305 than the total content of pheomelanin, the content of pheomelanin was more variable among individual birds
 306 than the content of eumelanin, up to more than twice in the case of 4-AHP. This supports the idea that
 307 pheomelanin, even at relative low concentrations in the bib of house sparrows, may have a significant role
 308 mediating the honesty in the bib size of male house sparrows. This is because honest signals are traits
 309 whose expression depends on the physical condition of the bearers and are sexually selected (which applies
 310 to the bib of male house sparrows; Nakagawa et al., 2007), and this makes that these traits are a large target
 311 for genetic variation (Tomkins et al., 2004).

312

313 **References**

314 Anderson, T.R. 2006. Biology of the ubiquitous house sparrow (New York: Oxford University Press).

- 315 Galván, I., Erritzøe, J., Wakamatsu, K. and Møller, A.P. (2012). High prevalence of cataracts in birds with
316 pheomelanin-based colouration. *Comp. Biochem. Physiol. A* **162**, 259-264.
- 317 Nakagawa, S., Ockendon, N., Gillespie, D.O.S., Hatchwell, B.J. and Burke, T. (2007). Assessing the function
318 of house sparrows' bib size using a flexible meta-analysis method. *Behav. Ecol.* **18**, 831-840.
- 319 Tomkins, J.L., Radwan, J., Kotiaho, J.S. and Tregenza, T. (2004). Genic capture and resolving the lek
320 paradox. *Trends. Ecol. Evol.* **19**, 323-328.
- 321 Wakamatsu, K., Ohtara, K. and Ito, S. (2009). Chemical analysis of late stages of pheomelanogenesis:
322 conversion of dihydrobenzothiazine to a benzothiazole structure. *Pigment Cell Melanoma Res.* **22**, 474-486.