Molecular vibration as a novel explanatory mechanism for the expression of animal colouration

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KEYWORDS: Animal colouration, melanins, molecular vibration, pigmentation, Raman spectroscopy

Abstract

Animal colouration is characterized by the concentration of pigments in integumentary structures and by the nanoscale arrangement of constitutive elements. However, the influence of molecular vibration on colour expression has been overlooked in biology. Molecular vibration occurs in the infrared spectral region, but vibrational and electronic properties can influence each other. Thus, the vibration of pigment molecules may also affect their absorption properties and the resulting colours. We calculated for the first time the relative contribution of molecular vibration (by means of Raman spectroscopy) and concentration (by means of HPLC) of melanin polymers, the most common animal pigments, to generate diversity in plumage colour in 47 species of birds. Vibrational characteristics explained >9 times more variance in colour expression than the concentration of melanins. Additionally, we modelled melanin Raman spectra on the basis of the chemical structure of their constituent monomers and calculated the Huang-Rhys factors for each vibrational mode, which indicate the contribution of these modes to the electronic spectra responsible for the resulting colours. High Huang-Rhys factors frequently coincided with the vibrational modes of melanin monomers. Our results can be explained by the influence of molecular vibration on the absorption properties of melanins. The colour of organisms may thus mainly result from the vibrational properties of their molecules and only residually from their concentration. As a given melanin concentration can give rise to different colours because different structural melanin conformations can present different vibrational characteristics, vibrational effects may favour phenotypic plasticity and thus constitute an important evolutionary force.

53 **Introduction**

54 Visual traits are the most important and widespread mechanisms of animal
55 communication¹. In many cases, as exemplified by bird plumage colouration, these
56 represent the most diverse phenotypic traits that can be observed in nature, and as a
57 consequence research on visual traits has played a crucial role in the establishment and
58 development of sexual selection and speciation theories². Animals get the optical
59 properties associated to visual traits by two main mechanisms: the use of chemical
60 compounds (pigments) and the development of specialized integumentary structures able
61 to generate distinctive optical properties³, although pigments and specialized structures
62 often interact. Thus, the colours expressed in the integument of animals are characterized
63 by the pigment or structure that it contains. This characterization is key for understanding
64 the evolution of visual traits, because it provides an indication of the constraints associated
65 to the acquisition of pigments or specialized structures. These constraints, in turn,
66 determine the intensity at which individual animals can express visual traits and thus
67 obtain fitness benefits⁴⁻⁶, thus affecting the evolvability of traits.

68 However, biologists have traditionally overlooked a fundamental concept in physics,
69 which states that molecules exhibit constant movement because of vibrations of their
70 covalent bond network (see however, e.g. ref. 7). These vibrations can occur at different
71 intensities and following different modes, including synchronous and asynchronous bond
72 stretching, angle bending or dihedral torsions⁸. Molecular vibration produces Raman
73 scattering of light⁹. Although molecules vibrate at frequencies in the infrared (IR) spectral
74 region while the visible absorption spectra of molecules (i.e., colour) derive from the
75 energy states of their constitutive electrons, vibrational and electronic properties of
76 molecules influence each other (i.e., vibronic coupling). Indeed, the broadening of
77 electronic spectral stems from the nuclear dynamics of the solute and the environment¹⁰.
78 Concretely, in the case of the solute, the vibrational progressions are mainly responsible

79 for the shape of the band, and their extension is related to structural differences between
80 the minima at the initial and final electronic states and on the frequency of the most
81 displaced modes. Therefore, the knowledge of the vibrational pattern is a prerequisite to
82 properly model the nuclear contributions to the lineshape and, moreover, this makes a
83 potential connection between vibrational spectroscopies (e.g., Raman) and
84 the broadening of the final electronic spectra. This means that colour traits are not only
85 the result of changes in the energetic states of electrons in the constitutive molecules of
86 pigments or specialized structures, but they are also tuned by the effect of molecular
87 vibrations.

88 Thus, in pigments that appear with different structural conformations (e.g.,
89 molecular assemblies as in polymers), these will be associated to variation in the
90 frequency, intensity or mode of molecular vibration¹¹. However, these structural changes
91 should not necessarily be associated to pigment concentration. If this was the case,
92 pigment molecular vibration would be a better predictor of the optical properties (i.e.,
93 colours) than pigment concentration. This possibility, however, has never been
94 contemplated in evolutionary biology. Nevertheless, there is evidence that vibronic
95 coupling predicts the visible absorption spectra of biomolecules, including those of many
96 biological pigments like flavins¹² and carotenoids¹³, and even the perceived colours
97 produced by anthraquinones¹⁴. If such vibrational effects would be present in pigments
98 that are synthesized by animals, this would have deep evolutionary implications, as the
99 genetic and physiological basis of synthesizing pigments with different vibrational
100 characteristics during development may be under selection.

101 Here we explore this new field in biology to determine the magnitude of the
102 contribution of molecular vibration to explain optical properties of pigments in animal visual
103 traits. Our results show that the vibrational characteristics of pigments are more relevant
104 than their concentration for explaining the observed diversity in animal colouration.

105 Melanins are biopolymers that constitute the most ubiquitous and ancient pigments found
106 in nature¹⁵ and are synthesized by animals in specialized cells called melanocytes¹⁶, so
107 we use these molecules as study models. Our aim could be achieved as a consequence
108 of recent advances in the study of melanins. First, the slope of the reflectance spectra of
109 feathers and hairs containing melanins has been shown to be a good predictor of the
110 perceived variation in the colours that they generate¹⁷ (see also Experimental).
111 Additionally, recent analyses of Raman scattering have proved that specific signal from the
112 two main chemical forms of melanins (i.e., eumelanin, polymers composed of indole units,
113 and pheomelanins, composed of sulphur-containing heterocycles¹⁸) can be detected by
114 non-destructive spectroscopic examination of biological tissues¹⁹⁻²³.

115 We therefore obtained feather samples from 47 diverse species across the avian
116 phylogeny (12 orders and 30 families), comprising a comprehensive diversity of colours
117 that natural melanins can generate (Table S1 in Supporting Information). We quantified
118 the expression of colour by analysing the feathers by reflectance spectroscopy and
119 calculating the slope of the reflectance spectra (Fig. 1). Then we determined the
120 vibrational characteristics of melanins contained in the same feathers by non-destructive
121 micro-Raman spectroscopy (Fig. 1, Table S2 in Supporting Information), and finally
122 determined the concentration of melanins in the feathers by high-performance liquid
123 chromatography (HPLC) after a chemical degradation of the pigments. Partial least-
124 squares regression (PLSR) analyses²⁴ allowed us to quantify the relative contribution of
125 melanin vibrational characteristics and of melanin concentration to generate the observed
126 variation in colour expression. To confirm the conclusions obtained from our empirical
127 analyses, we finally conducted simulations of Raman spectra given the chemical structure
128 of melanin monomers and calculated the Huang-Rhys factors for each vibrational mode,
129 which provide an indication of the contribution of these modes to the vibrational
130 broadening of the UV-visible electronic transitions (i.e., resulting colours). Huang-Rhys

factors reflect the structural displacement associated to the electronic transition for each normal mode and, consequently, they are directly related to the extent of the progressions of such modes in the vibronic spectra²⁵. Large Huang-Rhys factors imply large progressions, and thus determine the involvement of the modes tuning the spectral shape.

Experimental

Sampling

For each species, we obtained two feathers from one bird specimen deposited in the bird collection of the National Museum of Natural Sciences (MNCN, Madrid, Spain; mean specimen age $\pm$ se: 56.9 years $\pm$ 4.2) or the Icelandic Institute of Natural History (RM, Reykjavic, Iceland; mean specimen age $\pm$ se: 60.7 years $\pm$ 2.1), excepting feathers from two species (*Sitta europaea* and *Anas strepera*), which were taken from wild birds. Specimens from MNCN and RM did not differ in age (ANOVA: $F_{1,37} = 0.09$, $P = 0.759$). The species were chosen on the basis of homogeneity in the colour patches that were analysed, i.e. avoiding complex plumage patterns consisting in differently perceived colour hues. We avoided iridescent colourations, as these are generated by melanosome morphology and not by melanin chemistry²⁶. The number of species was thus chosen on the basis of these criteria while comprising the full palette of colours produced by melanins¹⁷.

Analysis of colour expression

We measured the colour expression of feathers by reflectance spectrophotometry, using an Ocean Optics (Dunedin, FL, USA) Jaz spectrophotometer (range 220-1000 nm) with UV (deuterium) and visible (tungsten-halogen) lamps and a bifurcated 400 μ m fiber optic probe. The fiber optic probe both provided illumination and obtained light reflected from the sample, with a reading area of c. 1 mm². Feathers were mounted on a light absorbing

157 foil sheet (Metal Velvet coating, Edmund Optics, Barrington, NJ, USA) to avoid any
158 background reflectance. Measurements were taken at a 90° angle to the sample. All
159 measurements were relative to a diffuse reflectance standard tablet (WS-1, Ocean Optics),
160 and reference measurements were frequently made. An average spectrum of five readings
161 on different points of the target colour patches in feathers was obtained for each bird,
162 removing the probe after each measurement. Only one specific colour patch was
163 measured in each species (Table S1). The analyses were made on individual feathers
164 separately, and mean spectra were then calculated. Reflectance curves were obtained by
165 calculating the median of the percent reflectance in 10 nm intervals.

166 As the reflectance of melanins steadily increases from 300 to 700 nm and shows no
167 spectral peaks²⁷, variation in the perceived colour generated by melanins may be given to
168 a large extent by variation in the slope of the reflectance curves. We therefore calculated
169 the slope of percent reflectance regressed against wavelength and used it as a descriptive
170 measurement of melanin-based colour expression. To determine if this measurement
171 actually collects the perceived variation in melanin-based colouration, we assigned the
172 studied species to one of six colour categories that increased with decreasing darkness as
173 perceived by humans (i.e. increasing from black to orange). Thus, colour categories and
174 their corresponding values were: black (1), grey (2), dark brown (3), dark orange (4), light
175 brown (5) and light orange (6) (Table S1)¹⁷. We therefore regressed slope values against
176 this scale, and found a significant correlation when slope was calculated considering the
177 visible spectral range (400-700 nm) only ($r = 0.64$, $n = 47$, $P < 0.0001$) as well as when
178 near UV wavelengths (300-400 nm) were also considered ($r = 0.66$, $P < 0.0001$). The
179 categories based on the human perception of color are shown here only to illustrate that
180 the measurement of color used in the analyses (slope of percent reflectance regressed
181 against wavelength) collect color variation that might be biologically relevant, as human
182 perception reliably detects variation in the plumage color of birds²⁸. Indeed, the correlation

183 between slope and human-based color categories indicates that our measurement of
184 slope reliably explains the perceived variation in melanin-based color phenotypes. The
185 most commonly used parameter to quantify colour expression from melanin reflectance
186 spectra, namely the sum of reflectance values (frequently referred to as brightness²⁷) was
187 weakly correlated with the human-based colour categories (300-700 nm: $r = 0.32$, $P =$
188 0.027 ; 400-700 nm: $r = 0.39$, $P = 0.006$), as previously reported¹⁷. This suggests that the
189 slope is a more biologically relevant parameter to quantify variation in reflectance spectra
190 than the sum of reflectance values. Furthermore, PLSR models (see below) did not result in
191 any significant component when brightness instead of slope was considered as the
192 response variable. We therefore calculated the mean slope of the two feathers analysed
193 for each specimen.

194

195 **Analysis of vibrational characteristics**

196 The same feathers analysed for colour expression were then analysed in a Thermo Fisher
197 DXR confocal dispersive Raman microscope (Thermo Fisher Scientific, Madison, WI,
198 USA) with a point-and-shoot Raman capability of 1 μm spatial resolution and using a near-
199 infrared excitation laser of 780 nm. Laser power was set at 6-8 mW when obtaining
200 pheomelanin spectra, and at 2-3 mW when obtaining eumelanin spectra. The integration
201 time was 5 s for pheomelanin spectra and 3 s for eumelanin spectra, with 8 accumulations
202 for both cases. The single spectra were obtained using a 50x confocal objective and a slit
203 aperture of 25 μm . These conditions produced an average spectral resolution of 2.2-4.4
204 cm^{-1} in the wavenumber range of 150-2500 cm^{-1} . The average Raman linewidth (FWHH)
205 obtained from four spectra in two bands of polystyrene centered at 1002.30 cm^{-1} and
206 1603.06 cm^{-1} was 6.2 cm^{-1} and 8.9 cm^{-1} , respectively. The system was operated with
207 Thermo Fisher OMNIC 8.1 software. Calibration and aligning of the spectrograph were
208 checked using pure polystyrene.

209 The laser beam was focused at different barbs and barbules chosen at random until
210 obtaining five single spectra of pheomelanin and five single spectra of eumelanin for each
211 feather. Thus, every single Raman spectra was categorized as either pheomelanin-based
212 or eumelanin-based. We used the diagnostic bands 1, 2 and 3²⁰ to assign the Raman
213 spectra to either pheomelanin or eumelanin. Some eumelanin spectra also showed a band
214 around 2000 cm⁻¹ coinciding with pheomelanin's band 3^{19,20,23}, probably as a result of
215 collecting mixed Raman signal from pheomelanin and eumelanin. However, we treated
216 this band as an additional, fourth band for eumelanin in the statistical analyses.

217 We fitted Lorentzian deconvolution functions to the mean Raman curves for
218 pheomelanin and eumelanin obtained from the two feathers of each bird specimen,
219 considering the three bands for pheomelanin and eumelanin described above (Fig. 1), and
220 a fourth band for eumelanin when present. From the deconvolution functions, we obtained
221 the spectral position, the intensity, the width at half maximum and the area for each
222 diagnostic Raman band. The analyses of the Raman spectra were made with Thermo
223 Fisher OMNIC 8.1 software. When no Raman signal from pheomelanin or eumelanin was
224 found in any sample, a zero value was assigned to the vibrational characteristics of the
225 corresponding pigment.

226

227 **Analysis of melanin concentration**

228 The same feathers analysed for colour expression and vibrational characteristics were
229 finally analysed by HPLC, which allows the detection of specific degradation products of
230 melanins that are specific to the different structural units (monomers) of eumelanins and
231 pheomelanins. In particular, pyrrole-2,3,5-tricarboxylic acid (PTCA) and pyrrole-2,3-
232 dicarboxylic acid (PDCA), which are specific markers of the indole units of eumelanins
233 (5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) moieties),
234 and 4-amino-3-hydroxyphenylalanine (4-AHP) and thiazole-2,4,5-tricarboxylic acid (TTCA),

235 which are specific of benzothiazine and benzothiazole pheomelanin moieties,
236 respectively^{29,30}. The feathers were first homogenized with Ten-Broeck glass homogenizer
237 at a concentration of 10 mg/ml water (removing barbs and rachis parts not corresponding
238 to the target colour patch), and then using alkaline H₂O₂ oxidation of eumelanin and
239 pheomelanin to measure PTCA, PDCA and TTCA levels³⁰ and reductive hydrolysis of
240 pheomelanin with hydriodic acid (HI) to measure 4-AHP levels²⁹.

241 HI reductive hydrolysis products were analysed with an HPLC system consisting of
242 a JASCO 880-PU liquid chromatography, a JASCO C18 column (JASCO Catechopak; 4.6
243 x 150 mm; 7 µm particle size) and an EICOM ECD-300 electrochemical detector (Eicom,
244 Kyoto, Japan). The mobile phase was 0.1 M sodium citrate buffer, pH 3.0, containing 1
245 mM sodium octanesulphonate and 0.1 mM Na₂EDTA: methanol, 98:2 (v/v). The
246 electrochemical detector was set at +500 mV vs. an Ag/AgCl reference electrode.

247 H₂O₂ oxidation products were analysed using a Shiseido C18 column (Shiseido
248 Capcell Pak MG; 4.6 x 250 mm; 5 µm particle size; Shiseido Co., Ltd., Tokyo, Japan) and
249 a JASCO UV detector. The mobile phase was 0.1 M potassium phosphate buffer, pH 2.1:
250 methanol, 99:1 (v/v). The absorbance of the eluent was monitored at 269 nm.

251

252 **Statistical analyses**

253 We calculated the differential contribution of vibrational characteristics (spectral position,
254 intensity, width at half maximum and area of each Raman band) and melanin
255 concentration (PTCA, PDCA, 4-AHP and TTCA levels) (predictor variables) to explain
256 variability in colour expression (slope; response variable) using PLSR models. This is an
257 appropriate statistical technique to analyse the predictive capacity of highly intercorrelated
258 predictor variables²⁴, as in the case of vibrational characteristics and markers of melanin
259 concentration^{20,31}. PLSR is an extension of multiple regression analysis in which
260 associations are established with components extracted from predictor variables that

261 maximize the explained variance in the response variable. These components are defined
262 as a linear combination of predictor variables, so the original multidimensionality is
263 reduced to a small number of orthogonal components to detect structure in the
264 relationships between predictor variables and between these factors and the response
265 variable. All predictor variables were $\log_{10}$ -transformed prior to analyses to achieve
266 normality assumptions. The significance of the extracted PLSR components was
267 determined with two criteria. First, a cross-validation test of the parameter Q_2 was carried
268 out to determine if a component was significant. Then, we tested the significance of the
269 correlation coefficient of the relationship between PLSR scores for the response variable
270 and PLSR component scores, thus determining if the amount of variance explained in the
271 response variable was significant. We also tested the statistical significance of the
272 regression coefficients of the predictors in the PLSR models to determine the degree of
273 correlation between the response variable and these predictors, which was made by
274 bootstrapping using 1000 replications³¹.

275

276 **Computational methods**

277 To investigate the role of active Raman modes on the UV-visible spectra of melanins we
278 computed the Huang-Rhys factors associated to the lowest energy bright electronic
279 transitions in the most relevant monomers of eumelanin and pheomelanin. In the case of
280 eumelanin, these monomers are DHI and DHICA and their corresponding orthoquinones:
281 5,6-indolequinone (IQ) and indole-2-carboxylic acid-5,6-quinone (IQCA)³² (Fig. 3). In the
282 case of pheomelanin, the monomers are a benzothiazine and a benzothiazole¹⁸ (Fig. 3).
283 Simulations were performed evaluating the potential energy surfaces of the monomers at
284 the ground and excited electronic states with Density functional theory methods (DFT) and
285 their TD extension (TDDFT) for the excited states, adopting the Becke, three-parameter,
286 Lee-Yang-Parr (B3LYP) hybrid functional³³ along with the 6-31G(d) basis set. The

287 Gaussian 09 program package³⁴ was used to carry out the calculations. Raman spectra
 288 were obtained at the ground state minimum energy structure. The Huang-Rhys factors
 289 account for the geometrical relaxation of the excited states upon excitation, thus requiring
 290 the optimization of each electronic state under consideration. The actual factors are then
 291 computed using the following equation:

292

$$S_i = \left(\frac{2\pi v_i}{2\hbar} \right) (\Delta Q_i)^2$$

293

294 where v_i are the frequencies associated to the normal modes, $\hbar$ is the Planck constant and
 295 ΔQ_i are the normal mode displacements between the equilibrium positions of the ground
 296 and excited states, which are related to the geometrical differences through:

297

$$\Delta Q_i = \sum_{k=1}^{Nat} m_k^{1/2} (L_{xk,i} \Delta x_k + L_{yk,i} \Delta y_k + L_{zk,i} \Delta z_k)$$

298

299 where m_k are the atomic masses, $L_{\alpha k,i}$ are the elements of the normal mode matrix, and
 300 $\Delta \alpha (\alpha = x, y, z)$ are the geometrical differences in Cartesian coordinates.

301

302 Results

303 All feathers analysed contained both eumelanin and pheomelanin at different proportions,
 304 showing different degrees of variability in the vibrational characteristics (Table S2). The
 305 PLSR model resulted in a significant component that accounted for 48.6 % of variance in
 306 the slope of reflectance spectra considering the visible range (400-700 nm), which was
 307 significantly correlated with this component ($r = 0.70$, $n = 47$, $P < 0.0001$; Fig. 2). Among
 308 the 32 potential predictors of slope, only the effect of eight of them was statistically
 309 significant, seven of these being vibrational characteristics of melanins and only one being

310 an index of melanin concentration (Table 1). The seven significant molecular vibration
311 predictors of colour expression together explained 26.1 % of the total variance accounted
312 for by the PLSR model, while the single significant melanin concentration predictor
313 explained 2.8 % of that variance, i.e. 9.3 times less than molecular vibration predictors.
314 Indeed, the individual effect of the melanin concentration predictor was weaker than the
315 individual effect of any single molecular vibration predictor, as indicated by the absolute
316 value of predictor weights (Table 1). In particular, the vibrational characteristics that
317 predicted colour expression were the spectral position of the three diagnostic Raman
318 bands for pheomelanin, which were positively related to the slope, and the area and
319 Raman intensity of diagnostic bands 2 and 3 for eumelanin, which were negatively related
320 to the slope (Table 1). The spectral position of the three diagnostic Raman bands for
321 pheomelanin were also the vibrational characteristics of pheomelanin with lowest
322 coefficients of variation (Table S2). The only melanin concentration index that predicted
323 colour expression was the concentration of the benzothiazole moiety of pheomelanin
324 (TTCA), which was positively related to the slope (Table 1).

325 We obtained similar results when the slope of reflectance spectra was calculated
326 including near ultraviolet (UV) wavelengths (300-400 nm), which some birds though not
327 humans can perceive³⁵, with the exception of the vibrational characteristics of
328 pheomelanin which did not remain as significant predictors (Table 1). The four significant
329 eumelanin vibration predictors of colour expression explained together 18.1 % of the total
330 variance accounted for by the PLSR model, while the pheomelanin concentration predictor
331 explained 1.9 % of that variance, again representing > 9 times less variance than
332 molecular vibration predictors (Table 1).

333 Lastly, our simulations showed high Huang-Rhys factors frequently coinciding with
334 the vibrational modes of melanin monomers (Fig. 3). This was particularly evident in one of
335 the two monomers of pheomelanin (i.e., the benzothiazole) (Fig. 3). The presence of these

336 high Huang-Rhys factors indicates that the molecular vibration of melanins can actually
337 affect their UV-visible spectra.

338

339 **Discussion**

340 These results show that the expression of plumage colouration is mainly explained by
341 vibrational characteristics of melanins contained in feathers, and to a lesser extent by the
342 concentration of these pigments. Vibrational and electronic interactions in molecules are
343 known to influence each other (vibronic coupling), and thus molecular vibration
344 characteristics can affect electric properties and hence visible absorption spectra^{12,13}. In
345 fact, vibronic coupling has theoretically been demonstrated to predict the colours produced
346 by the melanin-related pigments termed anthraquinones¹⁴. This may explain the colour
347 predictive capacity of the vibrational characteristics of melanins.

348 In order to obtain a preliminar validation of a possible causal link between the
349 vibrational features of Raman spectra and the resulting colours, we have used
350 computational simulations of the Raman spectra and the Huang-Rhys factors for each
351 vibrational mode associated to the first bright electronic transition of the main monomers of
352 eumelanin and pheomelanin. Huang-Rhys factors indicate which modes will be most
353 displaced between ground and excited state minimum structures and thus contribute to the
354 vibrational broadening of the UV-visible electronic transitions²⁵. Interestingly, our results
355 show that, in some monomers, the active Raman modes related to the diagnostic bands 1
356 and 2 used in our statistical analyses are also characterised by large Huang-Rhys factors.
357 This preliminar investigation opens a route to explain the relationship between the
358 vibrational (Raman) spectra of the pigments and their electronic spectra (i.e., their colour).

359 The out-of-plane deformation and stretching of hexagonal aromatic rings in the
360 pheomelanin polymer gives rise to the diagnostic Raman bands 1 and 2 of this pigment,
361 respectively, while the vibrational characteristics that lead to pheomelanin's band 3 are still

362 unknown^{19,23}. Our findings indicate that the spectral shift of these three diagnostic bands
363 makes feathers produce lighter colour hues, i.e. reflectance spectra with higher slopes. On
364 the other hand, the stretching of hexagonal rings and three of the six C-C bonds within the
365 rings gives rise to eumelanin's Raman bands 2 and 3, respectively^{19,36}, which produce
366 darker hues as their area and intensity increase.

367 Therefore, these findings represent a new view of the visual phenotype of
368 organisms. This study shows that the vibrational characteristics of biomolecules are not
369 only powerful diagnostic features that allow their identification^{37,38}, but they also affect the
370 appearance of organisms by affecting visible optical properties. Interestingly, the
371 vibrational characteristics of pheomelanin that predicted plumage colour expression (i.e.,
372 the frequency of the stretching vibration of hexagonal aromatic rings) were also those with
373 the lowest coefficients of variation for this pigment. This stresses the magnitude of the
374 effect of molecular vibration on colour expression, as small changes in pheomelanin
375 vibration lead to perceptible changes in plumage colouration.

376 This is the first evidence that the same pigment concentration can be associated
377 with different vibrational characteristics and produce different colour phenotypes in
378 animals. At this stage, we can only envision the evolutionary implications that this should
379 have, but these may be relevant from the perspective of both proximate and ultimate
380 mechanisms. From the perspective of proximal mechanisms, the influence of vibrational
381 characteristics on the electronic properties of molecules includes changes in electron
382 delocalization³⁹⁻⁴¹, which in turn affects the capacity of biomolecules to stabilize (i.e.
383 scavenge) free radicals⁴². This is particularly relevant for colour phenotypes produced by
384 melanins, as significant amounts of cytotoxic species, including reactive oxygen species
385 (ROS) such as superoxide and hydrogen peroxide, are formed in melanocytes during the
386 final stages of eumelanin synthesis^{43,44} and also in already formed pheomelanins exposed
387 to energetic radiation⁴⁵. Thus, certain colour phenotypes may be associated to certain

388 vibrational characteristics of melanins that would make these molecules especially prone
389 to free radical-mediated cytotoxicity and cellular damage. These possibilities have recently
390 been tested in the chicken, resulting in certain vibrational characteristics of pheomelanin
391 being associated with mitochondrial ROS production in melanocytes and systemic
392 oxidative stress and damage³¹.

393 From the perspective of the ultimate causes, our study points to an unsuspected
394 potential source of evolutionary change. If the same pigment concentration can give rise to
395 different colour phenotypes through variation in the pigment vibrational characteristics
396 (regardless this effect derives or not from changes in the assembly of pigment
397 components), then this may facilitate creating novel colour phenotypes. As animal colour
398 traits often evolve under sexual selection and influence the degree of isolation of incipient
399 species⁴⁶, it is likely that the vibrational characteristics of pigments affect the process of
400 speciation. This new perspective should also be explored by future studies. In particular, it
401 should be investigated if the relative contribution of vibrational characteristics and pigment
402 concentration to colour expression varies among taxa. If the contribution of vibrational
403 characteristics was higher in some groups than in others, that would lead to an increased
404 phenotypic plasticity in the former. This may favour higher speciation rates or a greater
405 capacity to explode a higher diversity of ecological niches in those groups⁴⁷.

406

407 **Acknowledgements**

408 We declare no conflicts of interest. Two anonymous reviewers commented on the
409 manuscript. We thank Josefina Barreiro and Ólafur K. Nielsen their kind collaboration with
410 sampling at the bird collections of the National Museum of Natural Sciences (MNCN,
411 Madrid) and the Icelandic Institute of Natural History (Reykjavic, Iceland), respectively.
412 Rafael Márquez helped with the spectrophotometric analyses of feathers. We
413 acknowledge funding from Fundación BBVA ('Convocatoria 2015 de ayudas Fundación

414 BBVA a investigadores y creadores culturales', reference IN[15]CMA_CMA_1422) to IG.
 415 IG is also supported by a Ramón y Cajal Fellowship (RYC-2012-10237) and the project
 416 CGL2015-67796-P from the Spanish Ministry of Economy, Industry and Competitiveness
 417 (MINECO). JC acknowledges a fellowship provided by "Fundación Séneca - Agencia de
 418 Ciencia y Tecnología de la Región de Murcia" through the "Saavedra-Fajardo" program
 419 (20028/SF/16).

420

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569 **Table caption:**

570

571 **Table 1.** Results of partial least squares regression (PLSR) models explaining the
 572 contribution of vibrational characteristics and concentration of melanins (predictor
 573 variables) to generate diversity in plumage colour expression (response variable) in 47
 574 species of birds. Plumage expression is measured as the slope of percent plumage
 575 reflectance values regressed against wavelength, excluding (400-700 nm) and including
 576 (300-700 nm) the near UV spectral range. Vibrational characteristics refer to the spectral
 577 position (X), intensity (Y), width at half maximum (W) and area (A) of diagnostic Raman
 578 bands 1, 2 and 3 for pheomelanin and Raman bands 1, 2, 3 and 4 for eumelanin. Melanin
 579 concentration indexes refer to the concentration (ng/mg feather) of degradation products
 580 that are specific of eumelanin (PTCA and PDCA) and pheomelanin (4-AHP and TTCA).
 581 The square of predictor weights indicates the proportion of the total variance accounted for
 582 by the PLSR model (indicated at the bottom of the table) that is explained by each
 583 predictor variable (i.e., the sum of squared predictor weights equals 1). Asterisks indicate
 584 predictors whose regression coefficients are significant at *P*-values of 0.05 (*), 0.01 (**) or
 585 0.001 (***).

<i>Predictor</i>	Slope (400-700 nm)		Slope (300-700 nm)	
	Regression coefficient	Predictor weight	Regression coefficient	Predictor weight
Pheomelanin X1	0.0038	0.169*	0.0028	0.167
Pheomelanin Y1	-0.0034	0.120	-0.0016	0.120
Pheomelanin W1	-0.0073	0.150	-0.0047	0.149
Pheomelanin A1	-0.0006	0.147	0.0006	0.150
Pheomelanin X2	0.0031	0.169*	0.0022	0.167
Pheomelanin Y2	-0.0027	0.145	-0.0036	0.140
Pheomelanin W2	0.0030	0.160	0.0043	0.162
Pheomelanin A2	0.0005	0.163	0.0003	0.163
Pheomelanin X3	0.0030	0.169*	0.0021	0.167
Pheomelanin Y3	-0.0106	0.100	-0.0084	0.089
Pheomelanin W3	-0.0044	0.136	-0.0043	0.133
Pheomelanin A3	-0.0025	0.122	-0.0026	0.112
Eumelanin X1	-0.0014	-0.210	-0.0008	-0.213
Eumelanin Y1	0.0022	-0.164	0.0014	-0.163
Eumelanin W1	-0.0009	-0.199	-0.0028	-0.202

Eumelanin A1	0.0021	-0.167	0.0015	-0.165
Eumelanin X2	-0.0011	-0.210	-0.0006	-0.213
Eumelanin Y2	-0.0039	-0.205**	-0.0034	-0.209*
Eumelanin W2	0.0008	-0.211	0.0005	-0.211
Eumelanin A2	-0.0009	-0.213**	-0.0007	-0.215*
Eumelanin X3	-0.0010	-0.210	-0.0006	-0.213
Eumelanin Y3	-0.0038	-0.208**	-0.0033	-0.211*
Eumelanin W3	0.0011	-0.209	0.0009	-0.210
Eumelanin A3	-0.0010	-0.213***	-0.0008	-0.216**
Eumelanin X4	-0.0010	-0.210	-0.0006	-0.213
Eumelanin Y4	-0.0021	-0.169	0.0004	-0.167
Eumelanin W4	-0.0012	-0.197	-0.0031	-0.200
Eumelanin A4	-0.0005	-0.191	-0.0003	-0.190
PTCA	-0.0131	-0.204	-0.0147	-0.219
PDCA	-0.0008	-0.115	0.0023	-0.128
4-AHP	-0.0066	0.133	-0.0075	0.116
TTCA	0.0434	0.168**	0.0293	0.139*
% variance explained	48.60		45.25	

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603 **Legends to figures:**

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605 **Figure 1.** Upper figure: Mean reflectance spectra of melanin-containing feathers from 47
 606 species of birds, categorized by colour hues as perceived by humans (black, grey, dark
 607 brown, dark orange, light brown and light orange). The colour of symbols only serves as
 608 an orientation to the resulting hues. Error bars are omitted for clarity. The mean slope $\pm$ se
 609 of percent reflectance regressed against wavelength is depicted in the right side of the
 610 figure. The photograph shows a purple sandpiper *Calidris maritima*, species not included
 611 in the study, displaying plumage colouration produced by melanins (credit: Ismael Galván).
 612 Only specific colour patches, not combinations of colours, were analyzed in each species.
 613 Bottom figure: Raman spectra of pheomelanin and eumelanin from feathers (taken from
 614 ref. 20) showing the three diagnostic bands of each pigment and the results of fitting
 615 deconvolution functions to the curves. Red arrows depict a causal link between spectral
 616 characteristics and reflectance spectra.

617

618 **Figure 2.** Relationship between colour expression (measured as the slope of reflectance
 619 values regressed against wavelength in the 400-700 nm spectral range) and the scores of
 620 a significant component from a partial least-squares regression (PLSR) model testing for
 621 the association between colour expression (response variable) and vibrational
 622 characteristics and concentration of melanins in feathers (predictor variables). This PLSR
 623 component is obtained from a linear combination of the predictor variables (the names of
 624 significant predictors below the component indicate which side of the axis increased with
 625 increasing values of these predictors) and maximizes the variance that is explained in the
 626 response variable. The line is the best-fit line.

627

628 **Figure 3.** Computed Raman spectra and Huang-Rhys factors for different monomers
629 constituent of pheomelanin [a benzothiazine (M1) and a benzothiazole (M2)] and
630 eumelanin [5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA)
631 and their corresponding orthoquinones: 5,6-indolequinone (IQ) and indole-2-carboxylic
632 acid-5,6-quinone (IQCA)]. Insert are the chemical structures of the monomers. Huang-
633 Rhys factors correspond to the first bright electronic state (S1) of molecules except in DHI,
634 for which the Huang-Rhys factors for the two most intense states (S1 and S4) are shown.

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641 **Legends to Supplementary Information:**

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643 **Table S1.** Bird species included in the study, colour categories and accession numbers.

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645 **Table S2.** Range and coefficient of variation (CV) for the different vibrational
646 characteristics measured in the feathers of 47 species of birds. Vibrational characteristics
647 refer to the spectral position (X), intensity (Y), width at half maximum (W) and area (A) of
648 diagnostic Raman bands 1, 2 and 3 for pheomelanin and Raman bands 1, 2, 3 and 4 for
649 eumelanin. X and W values are in cm^{-1} and Y and A values are arbitrary units obtained
650 from the Lorentzian deconvolution of Raman spectra.

Figure 1

Figure 2

Pheomelanin

Eumelanin

Figure 3