Females mate with males with diminished pheomelanin-based coloration in the Eurasian nuthatch *Sitta europaea*

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Running title: Female preference for diminished color expression

Declarations

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Abstract

Sexual selection can drive the evolution of phenotypic traits because of female preferences for exaggerated trait expression in males. Sexual selection can also lead to the evolutionary loss of traits, a process to which female preferences for diminished male trait expression are hypothesized to contribute. However, empirical evidence of female preferences for diminished male traits is virtually lacking. Eurasian nuthatches *Sitta europaea* provide an opportunity to test this possibility, as a chestnut flank patch produced by the pigment pheomelanin is present since the first plumage of these birds and its color is more intense in nestlings in poor condition in our study population. It has been proposed that developing birds in poor condition may increase their production of pheomelanin as a detoxifying strategy. Female nuthatches may thus prefer mating with males showing flank feathers of diminished color, as this could indicate that males experienced good conditions early in development, which can positively affect the fitness of future generations. Here we show results according with this prediction in a wild population of Eurasian nuthatches, as adult males with lighter chestnut feathers paired earlier in the season, while chestnut coloration had no effect on female mating success. Chestnut color expression was not affected by the body condition of birds, suggesting that females obtain information on the body condition in early life of their potential mates and not on their current body condition. This constitutes one of the few examples of females mating with males showing diminished traits and provides the only explanation so far by which this process can occur.

Key-words: early life effects, female preference for diminished traits, nuthatch, plumage coloration, pheomelanin, sexual selection

Introduction

Understanding variation in female preferences for male traits helps explain how phenotypes evolve by sexual selection (Jennions and Petrie 1997). Some models of sexual selection predict that this occurs as an endless process in which periods of female preference for exaggerated phenotypic traits (e.g., large morphological or intense color characters) alternate with periods of preference for diminished traits (i.e., perpetual evolution; Mead and Arnold 2004). Indeed, phylogenetic comparisons between species show frequent losses of sexually selected traits (Wiens et al. 2011), a phenomenon to which female preferences for diminished male traits may contribute (Wiens 2001). However, almost two decades after the proposal of this mechanism, the empirical evidence of female preferences for diminished male traits not associated to heterospecific mating avoidance or predation risk remains limited to one example already echoed by Wiens (2001): a population of the house sparrow *Passer domesticus* in which females, in contrast to females in other populations (Nakagawa et al. 2007), prefer mating with males displaying smaller black bib plumage patches (Griffith et al. 1999). This may suggest that the theoretical possibility of female preference for diminished male traits has not been assumed by researchers, as findings in this direction may be disregarded as incoherent evidence of sexual selection.

Although female preferences can drive the evolution of male traits by an arbitrary attractive-only effect (Fukamachi et al. 2009), empirical studies suggest that in most cases females obtain adaptive benefits from their choices, either directly by getting genetic pools that increase the viability of their offspring or indirectly by getting resources that help raising the offspring (e.g., Jaquiéry et al. 2010). In this regard, understanding the mechanisms that regulate the expression of male traits is essential to get insight into female preferences (Folstad and Karter 1992), as these preferences are mostly understood through the information that male traits honestly signal about their ability to

produce the traits (Zahavi 1975). This information is in turn necessary to evaluate the likelihood that female preferences for diminished male traits actually operates at any time.

Recent findings in the Eurasian nuthatch *Sitta europaea* provide a unique opportunity to test a possibility of female preference for a diminished male traits. These birds are sexually dichromatic regarding a chestnut (dark orange) flank plumage patch produced by the deposition of the pigment pheomelanin in feathers, whose color intensity is higher in males than in females (Fig. 1). This color patch is already present in the first plumage developed by nestlings, which are virtually identical to adults (Fig. 1). However, the color intensity of this trait depends on the physical condition, a predictor of survival prospects (Matthysen 1989), in nestlings but not in adults: nestling nuthatches in poorer body condition develop more intense flank feathers than nestlings in better condition in at least one population (Galván 2017). As the synthesis of pheomelanin and its deposition in inert integumentary structures like feathers can help removing the amino acid cysteine, which is toxic if in excess and this excess is more likely to arise during the nesting stage (i.e., under low relative stress levels), it has been proposed that nestling nuthatches in poor condition increase their production of pheomelanin to pigment their plumage as an adaptive detoxifying strategy (Galván 2017). Thus, plumage coloration in adult nuthatches may be a developmental consequence of selection operating in juveniles.

However, the plumage of nuthatches is sexually dichromatic as mentioned above, indicating that sexual selection operates on this trait (Heinsohn et al. 2005). The absence of condition-dependence in adult nuthatches suggests that preferences of adult females for male flank coloration might be related to information different from the current body condition of males. Instead, adult females may obtain information about the condition experienced in early life by their potential mates by assessing the color intensity of their flank patches, as suggested for some morphological traits in other birds with marked sexual dimorphism (Carranza and Hidalgo de Trucios 1993). This would only be possible if

plumage color expression was correlated between years in individual males, and this is indeed likely, as bird color traits involved in sexual selection uses to be consistent across years (Galván and Møller 2009, Gladbach et al. 2010, Chaine and Lyon 2015). This information may be highly relevant for the fitness of female nuthatches, as conditions experienced in early life can have transgenerational effects (Burton and Metcalfe 2014). In birds specifically, it is known that stress experienced early during development can lead to poor body condition in the affected offspring and negative fitness-related effects in subsequent generations (Naguib et al. 2006, Goerlich et al. 2012). Therefore, female nuthatches would benefit from mating with males with a diminished expression of flank coloration, as this would be indicative of good physical condition during development (Galván 2017). We thus tested this prediction in a wild population of Eurasian nuthatches using laying date as a measure of mating success, as laying date reflects pairing date (Potti and Montalvo 1991, Johnsen et al. 1998).

Material and Methods

Field methods

The study was carried out during three consecutive breeding seasons (April-May 2015-2017) in the Natural Park of Sierra Norte de Sevilla, southern Spain (37°47' N, 06°04' W). Frequent checks of wood nest boxes placed in the study area provided data on dates of clutch initiation (laying date) for all breeding pairs.

12-15 days after hatching, adults were captured and banded with numbered rings. The adults were weighed with a portable electronic balance to the nearest 0.1 g and their tarsus length was measured to the nearest 0.01 mm with a digital calliper. Sex was determined on the basis of the color intensity of the flank plumage patch, which is darker in males (Galván 2017). We plucked 5-6 chestnut flank body feathers and stored them in the dark until reflectance measurements were made (see below). In total, 37 Eurasian

104 nuthatches (17 males and 20 females) belonging to 27 breeding pairs were captured and
105 examined for flank coloration. Flank feathers could not be taken in one female, and body
106 mass could not be measured in other two females.

107

108 **Analysis of plumage color expression**

109 To analyze the color expression of flank feathers, we used an Ocean Optics Jaz
110 spectrophotometer (range 220-1000 nm) with ultraviolet (deuterium) and visible (tungsten-
111 halogen) lamps and a bifurcated 400 micrometer fiber optic probe. The fiber optic probe
112 both provided illumination and obtained light reflected from the sample, with a reading
113 area of ca. 1 mm². Feathers were mounted on a light absorbing foil sheet (Metal Velvet
114 coating, Edmund Optics, Barrington, NJ) to avoid any background reflectance.
115 Measurements were taken at a 90° angle to the sample. All measurements were relative
116 to a diffuse reflectance standard tablet (WS-1, Ocean Optics, Dunedin, FL), and reference
117 measurements were frequently made. An average spectrum of five readings on different
118 points of the feathers was obtained for each bird, removing the probe after each
119 measurement. Reflectance curves were determined by calculating the median of the
120 percent reflectance in 10 nm intervals. Spectral data was summarized as the summed
121 reflectance across the 300-700 nm range. As pheomelanin reflectance spectra are
122 approximately linear and spectral variation largely correspond to variation in the slope of
123 these lines (Galván and Wakamatsu 2016), we also calculated the slope of percentage
124 reflectance regressed against wavelength as an alternative measure of pheomelanin-
125 based color expression. In both parameters, summed reflectance and reflectance slope,
126 lower values denote darker colors and higher color intensity. Then we determined which
127 parameter was a better indicator of the pheomelanin content of feathers (see below). To
128 minimize observer bias, reflectance measurements were made by a technician blindly from
129 the aims of the study.

130

131 Analysis of feather pheomelanin content

132 The flank feathers of 16 nuthatch nestlings from 2017 were analyzed by micro-Raman
133 spectroscopy to determine their relative content of melanins, as both pheomelanin and the
134 non-sulphurated form of melanin (eumelanin) exhibit distinctive Raman signal that can be
135 used to non-invasively identify and quantify them (Galván et al. 2013, Galván and Jorge
136 2015). We used a Thermo Fisher DXR confocal dispersive Raman microscope (Thermo
137 Fisher Scientific, Madison, WI, USA) with a point-and-shoot Raman capability of 1 μm
138 spatial resolution and using a NIR excitation laser of 780 nm. Laser power was set at 5-7
139 mW, integration time at 1 s, and number of accumulations at 8. The spectra were obtained
140 using a 50x confocal objective and a slit aperture of 50 μm . The system was operated with
141 Thermo Fisher OMNIC 8.1 software. Calibration and alignment of the spectrograph were
142 checked using pure polystyrene.

143 A total of five flank feathers were analyzed per nestling. In each feather, we
144 obtained five Raman spectra from different barbs and barbules. Pheomelanin shows a
145 distinctive Raman spectrum comprising three diagnostic bands located at 500, 1500 and
146 2000 cm^{-1} , and eumelanin shows different spectra with other diagnostic bands (Galván et
147 al. 2013, Galván and Jorge 2015, Wang et al. 2016, Polidori et al., 2017). In the nuthatch
148 feathers, we only detected Raman signal from pheomelanin, together with bands that can
149 be assigned to keratin (Hsu et al. 1976) (Fig. 2). Therefore, we used the three diagnostic
150 bands of pheomelanin and four diagnostic bands of keratin (Fig. 2a) to fit Voigt
151 deconvolution functions to the Raman curves to obtain spectral parameters derived from
152 each spectrum. An average Raman spectrum was calculated for the spectra obtained from
153 the five feathers of each nestling.

154 In the analyses, we used the intensity (maximum value at the vertical axis) of the
155 band at 2000 cm^{-1} calculated from the deconvolution functions as an index of pheomelanin

content (Figure 2a), as this is a good predictor of pheomelanin concentration in feathers (Galván et al. 2013) and the band is located within the so-called Raman "silent region" of biomolecules (i.e., 1800-2700 cm^{-1} ; Wang et al. 2016). The intensity of this band increases with increasing the concentration of pheomelanin in feathers (Galván et al. 2013).

We thus regressed the slope and the summed reflectance of feathers against the intensity of the 2000 cm^{-1} Raman band to determine which color trait better reflects the pheomelanin content of feathers. The slope of reflectance spectra was significantly correlated with the intensity of the 2000 cm^{-1} Raman band ($r = -0.51$, $n = 16$, $P = 0.042$; Fig. 2b), while the summed reflectance was not ($r = -0.35$, $n = 16$, $P = 0.177$; Fig. 2c). This indicates that the slope of the regression of reflectance values against wavelength decreases as the pheomelanin content of feathers increases (Fig. 2b). Although the effect size is not large, its statistical significance with a sample size as low as 16 birds suggests that the association might be biologically relevant. We therefore used reflectance slope as an index of pheomelanin-based color expression in the analyses.

Statistical analyses

We tested for a potential dependence of laying date on the flank color expression of males and females separately by using general linear models (GLM). We standardized laying date (mean = 0, s.d. = 1) to compare values between the three years of study. Standardized laying date values thus represent deviations from the yearly means. We also tested for an association between flank color expression and body condition, the latter being calculated as the residuals of body mass regressed against tarsus length (Galván 2017). Males and females were pooled in these models for body condition, and the sex of birds was added as a covariate to account for the effect of sexual dichromatism. Inspections of residuals from the models confirmed that normality assumptions were fulfilled. Lastly, we tested for a

correlation between the color expression of males and females in 10 pairs in which we could capture both sexes.

Results

Flank color expression explained 32.8 % of variance in the laying date of male nuthatches, exerting a significant and negative effect ($b = -68.88$, $F_{1,15} = 7.31$, $P = 0.016$). This means that males displaying lighter chestnut plumage patches paired earlier in the season (Fig. 3). In contrast, flank color expression in females only explained 21.8 % of variance in their laying date, and the effect was not significant ($b = 15.98$, $F_{1,17} = 1.64$, $P = 0.218$; Fig. 3).

In the model testing for an effect of body condition on flank color expression, neither the interaction between body condition and sex ($F_{1,30} = 0.16$, $P = 0.688$) nor body condition ($F_{1,31} = 0.88$, $P = 0.356$) had a significant effect. Only the sex of birds had a significant effect ($F_{1,34} = 10.7$, $P = 0.002$), indicating darker flank plumage patches in males (mean $\pm$ s.e.: 0.024 ± 0.003) than in females (0.038 ± 0.003).

The correlation between the flank color expression of paired males and females was not significant ($r = -0.40$, $n = 10$, $P = 0.250$). This suggests that the flank color of males was unrelated to that of their mates.

Discussion

Our results suggest that female Eurasian nuthatches tend to mate with males with a diminished expression of chestnut flank coloration, as predicted on the basis that the intensity of chestnut coloration is higher in nestling nuthatches in poor body condition (Galván 2017). In contrast, the flank coloration of female nuthatches does not affect their mating behavior, suggesting the existence of preferences of females to males but not the opposite. Accordingly, there was no assortative mating regarding flank color, as the color expression values of paired males and females were not correlated. In male birds, color

207 traits that are involved in sexual selection tend to be consistent across years (Galván and
208 Møller 2009, Chaine and Lyon 2015). Thus, and although we could not test this in
209 nuthatches, it is likely that the intensity of flank color expression in male nuthatches is
210 correlated across their multiple plumage molts throughout their lifespans, and therefore
211 females could obtain information on the conditions experienced during early life by
212 assessing the flank color expression of their potential mates. As birds reared under
213 stressful conditions that lead to poor body condition (Krause et al. 2017) can exert a
214 negative effect on the fitness of birds of subsequent generations (Naguib et al. 2006), the
215 tendency of female nuthatches to mate with males with lighter flank coloration may be
216 adaptive, as they may potentially avoid mating with males that provide a negative effect on
217 their offspring. These effects can be exerted through the heritability of epigenetic
218 mechanisms that influence the expression of genes that regulate stress responses
219 (Goerlich et al. 2012).

220 As already noted by Galván (2017), the expression of the chestnut flank coloration
221 does not depend on the current body condition of adult nuthatches, and thus, females
222 cannot obtain information on the current body condition of their potential mates by
223 assessing their flank coloration. This may thus be an exception to the observation that
224 sexually selected traits often, but not always, show heightened condition-dependence and
225 thus signal the capacity to afford the costs of producing the traits (Cotton et al. 2004). In
226 this case, the reason for the absence of condition-dependence in adult nuthatches may be
227 the proposed detoxifying function of pheomelanin, the pigment that produces the flank
228 coloration and whose synthesis increases in developing nuthatches in poor body condition
229 (Galván 2017). Therefore, in our study population the chestnut flank coloration is not a trait
230 costly to produce whose expression is limited to individuals of high genotypic quality as
231 predicted by the handicap principle (Zahavi 1975), but is more intensely produced by
232 individuals in poor condition probably as an adaptive detoxifying strategy. This is in

233 accordance with recent proposals of alternatives to the handicap principle that are based
234 on the knowledge of the physiological mechanisms regulating trait expression (Számadó
235 and Penn 2015, Galván and Alonso-Alvarez 2017). This is also in accordance with the
236 suggestion that the chestnut flank coloration of adult nuthatches is a developmental
237 consequence of natural selection operating in nestlings, while sexual dichromatism in the
238 chestnut flank coloration of nestling nuthatches is a developmental consequence of sexual
239 selection operating in adults (Galván 2017).

240 It must be noted that, in other passerine bird (the barn swallow *Hirundo rustica*), the
241 expression of pheomelanin-based plumage coloration is positively related to body
242 condition in nestlings (Arai et al. 2017) and to annual survival rate in adults (Galván and
243 Møller 2013), and, accordingly, females show a preference for males with intense plumage
244 coloration in at least some populations (Scordato and Safran 2014). Between-species
245 comparisons indicate that the production of large amounts of pheomelanin to pigment
246 plumage constrains physiological processes that require antioxidant protection by
247 glutathione (GSH), the most important intracellular antioxidant, such as the development
248 of large brains (Galván and Møller 2011) or performance under exposure to ionizing
249 radiation (Galván et al. 2014). This is because the sulfhydryl group of cysteine, a
250 constituent amino acid of GSH, enters the pheomelanin synthesis pathway, thus
251 pheomelanin production reduces the availability of cysteine for GSH synthesis and this can
252 cause chronic oxidative stress (Napolitano et al. 2014). However, only an adaptive benefit
253 of pheomelanin can explain its evolution, and such benefit may be the detoxifying capacity
254 of pheomelanin by helping to avoid excess cysteine, which is toxic (Galván et al. 2012).
255 Therefore, it is environmental conditions what determine whether pheomelanin production
256 is physiologically advantageous or detrimental (Galván et al. 2012), which may explain
257 differences in the condition-dependence and signaling content of pheomelanin-based
258 coloration between species. In fact, we have evidence that the expression of color of flank

259 feathers in Eurasian nuthatch nestlings reared in a population different from the population
260 of the present study, with different levels of environmental oxidative stress, shows the
261 reversed pattern to that found here (i.e., color intensity is positively associated to body
262 condition; Galván and Sanz, unpublished results). Future studies should thus consider
263 environmental gradients of oxidative stress when explaining the signaling content of
264 pheomelanin-based coloration.

265 To our knowledge, our study is the only finding suggesting that females may show
266 preferences for a diminished male trait not associated to heterospecific mating avoidance
267 or predation risk since the results provided by Griffith et al. (1999) in a population of house
268 sparrows. This possibility, however, theoretically exists in certain models of sexual
269 selection (Mead and Arnold 2004) and may actually be responsible for the evolutionary
270 loss of sexually selected traits observed in some clades (Wiens 2001). The potential
271 detoxifying function of pheomelanin-based pigmentation provides a mechanism by which
272 females tend to mate with males with a diminished expression of pheomelanin-based
273 coloration as we found here, thus representing the first mechanistic justification for such
274 female mating behavior. Indeed, the evolution of signals can be understood only by
275 considering the physiological mechanisms that regulate their expression (Galván and
276 Alonso-Alvarez 2017).

277 The question now arises why the flank plumage patch of nuthatches does not
278 become unpigmented, if females tend to mate with males with lighter flanks and this
279 potentially provide them with higher fitness benefits. The answer might be in the stressful
280 factors that create differences in the body condition of nuthatches during development.
281 Nuthatch populations may differ in the expression of flank coloration, which would suggest
282 that populations may differ in the intensity of stressful factors and maybe also in the
283 intensity of sexual selection (Galván and Sanz, unpublished results). Thus, similar to the
284 black bib of male house sparrows (Griffith et al. 1999, Nakagawa et al. 2007), preferences

of female nuthatches for the flank coloration of males may differ between populations with different intensity of stressful factors affecting body condition in early life. This should be investigated by future studies, as it may help understanding seemingly paradoxical findings in sexual selection and obtaining a more complete view of the influence of this process in phenotypic evolution.

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Legends to figures:

Fig. 1. Male (a) and female (b) Eurasian nuthatches from the study area showing chestnut flank feathers (credits: Ismael Galván).

Fig. 2. a: Raman spectra of flank covert feathers (shown in the insert photograph) of 16 Eurasian nuthatch nestlings. Each color corresponds to a different bird. The spectra show the three diagnostic Raman bands of pheomelanin at about 500, 1500 and 2000 cm^{-1} . The intensity of the band at 2000 cm^{-1} (marked with a red arrow) was used as an index of pheomelanin concentration in feathers. Other bands at about 1350, 1430, 1600 and 1700 cm^{-1} , corresponding to keratin, were also used to deconvolute Raman spectra. b: relationship between pheomelanin concentration and the summed percentage of reflectance across the 300-700 nm in the same feathers. The line is the best-fit line. c: relationship between pheomelanin concentration and the slope of reflectance spectra of the same feathers. The line is the best-fit line.

Fig. 3. Relationship between laying date (standardized to reflect deviations from yearly means) and color expression level of chestnut flank feathers (measured as summed reflectance in the 300-700 nm spectral range) in male (red symbols and line) and female (black symbols and line) Eurasian nuthatches. Lower reflectance levels indicate darker (i.e., more intense) coloration. Lines are best-fit lines.

a

b

Figure 1

a

Standardized laying date

2
1
0
-1

0.015 0.020 0.025 0.030 0.035 0.040

Reflectance slope of chestnut flank feathers

b

Standardized laying date

1.5
1.0
0.5
0.0
-0.5
-1.0

0.02 0.03 0.04 0.05 0.06 0.07

Reflectance slope of chestnut flank feathers

Figure 3