Short communication

The mantle colour of female European Pied Flycatchers *Ficedula hypoleuca* (also) becomes darker with age

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Running Title: Plumage colour of female Pied Flycatchers

1 Although variation in the dorsal plumage colour of male European Pied Flycatchers
2 *Ficedula hypoleuca* has received a great deal of attention, females of the species have
3 been usually considered as nearly uniformly monochromatic brown. By using
4 reflectance spectrophotometry, we describe and explore the age dependent variation of
5 the plumage colour in females. We disentangle the within- and between-individual
6 effects of this pattern and show a within-individual darkening of the mantle colour with
7 age whereas differences between individuals underlie the trend for a more reflective
8 white in the females' breast plumage with advancing age. The darkening of the dorsal
9 plumage as females age reflects the most common pattern of age-related variation in
10 males in most European populations of the species. We hypothesize that a genetic
11 correlation between the sexes involving ontogenetic changes in androgen levels may
12 underlie variation in the female dorsal plumage colour in this species.

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15 **Keywords:** ageing, ornaments, reflectance spectrophotometry, sexual dimorphism
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Secondary sexual traits expressed in females are now receiving increased attention, after an emerging consensus that the long-suspected between-sex (genetic) correlations, leading to their sometimes ‘vestigial’ expression in females (Darwin 1871, Lande 1987), may have important evolutionary bearing hitherto largely neglected (Amundsen & Pärn 2006, Clutton-Brock 2007, Potti & Canal 2011). In short, if sex-related traits are heritable and expressed in both sexes, selection on them and preferences can no longer be considered by default as acting only on one sex, and a broader perspective on the causes and consequences of phenotypic variation in both sexes is therefore needed (Kraaijeveld *et al.* 2007, Laczi *et al.* 2011, Potti *et al.* 2013). Documenting this variation in females, the usually ignored sex when it comes to secondary sexual traits and selection (Amundsen 2000), is much needed, in particular for those taxa widely used as ‘model’ species in sexual selection and the ecology of speciation, as are Palearctic black-and-white flycatchers in the genus *Ficedula* (Newton 2003, Price 2008).

The European Pied Flycatcher *Ficedula hypoleuca* has been extensively investigated in Europe with respect to the large geographic variation among populations in the dorsal plumage colour (Lundberg & Alatalo 1992, Cramp & Perrins 1993, Lehtonen *et al.* 2009). However, that variation has exclusively been addressed in males, with little consideration as to the possibility of within- or between-population plumage differences among females of the species. In fact, handbooks and field guides (e.g. Svensson 1992, Cramp & Perrins 1993) usually describe females of the species as uniformly brownish or with a slight greyish tinge, without further consideration as to the possibility of individual variation in overall plumage colour in some populations. This may partially be due to relatively limited sampling in the most southern parts of its range (i.e. Iberian and North African populations; but see Curio 1960, Potti & Montalvo 1991a) until recently (Sætre *et al.* 2001), in comparison with central and northern

European populations where Pied Flycatchers have been intensively studied since before the mid 20th century (Drost 1936), albeit with little focus on female plumage variation (Lundberg & Alatalo 1992).

During our long-term (1984-2013) work with European Pied Flycatchers in central Spain (e.g. Camacho *et al.* 2013), the first author noted with the bird in the hand clear differences in the female plumage colour, with some looking much darker than others (Fig. 1). In general, some females display a greyer hue in their dorsal body plumage, instead of the more extended (and reported) colour perception of females as ‘brownish’ (Fig. 1). However, to measure objectively such variation we had to wait for the advent of advanced colorimetric approaches (e.g. spectrophotometers; Montgomerie 2006). We present here the first analysis of the ontogeny of female plumage variation in female Iberian Pied Flycatchers. We aim to draw attention to the existence of plumage differences among females that may be of a wider interest in broadening our understanding and implications of the phenotypic variation in this already well-studied, model species in ornithology, ecology and behaviour.

METHODS

A population of Pied Flycatchers breeding in nestboxes in La Hiruela, about 100 km northeast of Madrid, central Spain, was studied in the breeding seasons from 2006 to 2011 (e.g. Camacho *et al.* 2013). Breeding females were captured with a spring trap inside their nestboxes while feeding nestlings aged 8-13 days old and marked with a numbered metal ring if previously unringed. The exact age of many individuals was known due to high natal philopatry (Potti & Montalvo 1991b). Unringed females were aged as first-year (yearlings) or older based on the shape of the primary coverts (Karlsson *et al.* 1986). Based on the patterns of age at first breeding assessed in birds of

exactly known age, unringed females older than 1 year-old at first capture were ascribed an age of 2 years (Potti & Montalvo, 1991b). We also recorded whether the female displayed a quantifiable forehead patch (Potti & Canal 2011).

A portable Minolta CM-2600d (Minolta Co. Ltd, Japan) reflectance spectrophotometer was used to quantify the colour spectrum of the plumage of breeding females in 2006-2008 and 2010-2011. The spectrophotometer reads an area of 8 mm with sensitivity in the 360-740 nm spectral range. Standard calibration was taken with a white plate (CM-A145; Minolta Co. Ltd, Japan) according to the user's manual. The bird was held by one person while another observer applied the lens against the dorsal plumage at an angle of 90° to obtain readings at two fixed points, one in the middle rear of the neck and the other in the middle point of the back between both wings. Another reading was obtained from the female breast plumage. As both measures of the female back ('mantle') did not differ, we averaged the two spectra to give a unique estimate for each parameter. The software extracts readings within the resulting spectra for every 10 nm from 360 to 740 nm which were imported into a spreadsheet. We focused our analyses in the range between 360-700 nm since previous work has shown that visual sensitivity in several bird species reaches up to 700 nm (e.g. Hart *et al.* 2000). Following Delhey and Kempenaers (2006) we computed: (i) brightness, an estimate of the total light reflected by the feathers, calculated as the average reflectance between 360 and 700 nm; (ii) hue, determined as the wavelength of peak reflectance; (iii) chroma or colour saturation, computed as the difference between peak and minimum reflectance divided by total reflectance $((R_{\max} - R_{\min}) / \text{brightness})$. Average reflectance spectra of yearling and older females are represented in Fig. 2).

Statistical analyses

We used generalized linear mixed models (GLMMs) to investigate age-related patterns of variation in the spectral characteristics of the female plumage (dependent variables). GLMMs (normal errors, identity link function) were fitted in R (version 2.14.0; R Core Team 2011) using the function *lmer* in the package 'lme4' (Bates et al. 2011). Female age and its squared term were included as explanatory variables to look for linear and curvilinear relationships with age, respectively, and female identity and year were fitted as random effects to account for repeated measurements of individuals and annual heterogeneity. Forehead patch expression (presence/absence) was included in these models to test if differences in plumage colour could be associated with concurrent age variation in the expression of this ornament (only a few females display the patch in their first breeding season and its expression is also related to increasing age; Potti *et al.* 2013).

To disentangle whether the age-dependent plumage variation (Table 1) was due to within-individual or between-individual effects, or a combination of both (Dreiss & Roulin 2010, Evans *et al.* 2011), we split female age into two new fixed effects: mean age (between-individual term calculated over all records of an individual) and deviation from mean age of each individual's sample ("Delta age", within-individual effect) following the within-subject centring method (Van de Pol & Wright 2009). To obtain *P*-values, we fitted the models using Maximum Likelihood (Bolker *et al.* 2009). *P*-values for the individual effects were based on Markov Chain Monte Carlo (MCMC) sampling (10000 iterations) and derived using the function *pvals.fnc* in the package 'languageR' (Baayen 2011).

RESULTS

We obtained 393 and 379 readings of the mantle and breast plumage, respectively, corresponding to 263 and 254 different females. Details of results of the GLMMs are given in Table 1 and average reflectance spectra of yearlings and 4-year old females in Figure 2. Females displayed significantly less light (i.e. appearing darker) mantles and more reflecting bellies as they aged, as shown by the sign of the estimates for brightness, hue and chroma (Table 1). However, while the breast/belly plumage brightness and hue increased steadily until the older ages, mantle brightness and hue showed a steep increase in females aged five or more years, as shown by the significance of the quadratic term in the GLMMs models (Table 1; Fig. 3).

Chroma was lower in female breasts after their first year but increased again in the oldest age class (Table 1) whereas the mantle chroma decreased linearly with age.

Decomposing the above described age variation in plumage colour in within- vs. between-individual components revealed that plumage variation in the mantle colour was due to ontogenetic changes within females (Table 1). Further, these analyses revealed an association in the breast colour with age due to differences among females, with little evidence of within-individual changes except for the breast chroma (Table 1). An analysis using only birds of exactly known age showed the same trends but lower significances in both mantle (within-individuals effects: hue: $P = 0.074$, chroma: $P = 0.024$; brightness: $P = 0.29$; between-individuals effects all $P < 0.95$) and breast reflectance (between-individuals effects: hue: $P < 0.001$, chroma: $P = 0.17$; brightness: $P = 0.002$; within-individuals effects: all $P < 0.46$) because large samples size are needed for the detection of age effects in natural populations with moderate to large mortality (Nussey et al. 2008) as our study population.

The expression (yes/no) of the forehead patch was associated with the spectral characteristics of the breast and mantle since females expressing the forehead patch had

a darker dorsal plumage (except for the brightness component) and a more reflective breast (Table 1). We infer from this that the mechanisms underlying the variable expression across ages of the forehead patch and plumage colour may be correlated within females.

DISCUSSION

Variation in plumage colour of male Pied Flycatchers has received a great deal of attention but females of the species have been traditionally considered as lacking any variation in the (visible) colour of the dorsal plumage. Here we have shown that the mantle colour of female Pied Flycatchers becomes increasingly darker as they age, paralleling findings for males wherein individuals become darker with age in most European populations (Drost 1936, Lundberg & Alatalo 1992; Galván & Moreno 2009, Lehtonen *et al.* 2009; but see Potti & Montalvo 1991a). Significant age-related variation was also revealed for the female breast plumage that, as in males (authors' unpubl. data), grows more reflectively white with age but, unlike the mantle colour, mainly due to between-individual changes. This may be due to selective appearance/disappearance of certain colour phenotypes, which is under investigation. Overall contour plumage variation, besides that shown by the age-related expression of the white forehead patch in females (Potti *et al.* 2013) makes the combination of an 'obscure', greyer dorsal plumage, a whitish ventral hue and the displaying of a forehead patch reliable indicators of middle to very advanced ages (2-10 years in our study population) in female Pied Flycatchers.

The similarity in the back plumage variation with age in males and females, with individuals generally becoming darker as they age, begs the question whether dorsal plumage colouration may be genetically correlated between the sexes. This has been

recently found for another secondary sexual trait, the size of the forehead patch, formerly reported as nearly exclusive to males but now known to be expressed by about one third of the females in Iberian populations (Potti *et al.* 2013). As forehead patch expression is also age-dependent, plumage ‘maturation’ in females overall tends to produce more ‘masculinized’ individuals, i.e. expressing ‘male’ traits (white forehead patch, dark mantle colour) or to recapitulate patterns of age expression similar to those found in males (see also Laczi *et al.* 2011). This is consistent with the hypothesis of an underlying genetic correlation between the sexes (Roulin & Dijkstra 2003, Potti & Canal 2011) although, at another level of explanation, could also be mediated by the action of sex steroid hormones (Kimball & Ligon 1999). In addition, both levels of explanation can be reconciled if female plumage darkening with age is due to increased levels of androgens and the genes responsible for ontogenetic changes in androgens titres are involved in the genetic correlation between the sexes. The fact that females become darker with age also suggests that with increasing age females might produce more eumelanin pigment, responsible for dark colours, than pheomelanin, which is involved in brown colorations. Because a dark or pale plumage coloration is associated with the expression of different genes (Emaresi *et al.* 2013) our results raise the possibility that females change the expression levels of important genes with age (Ducrest *et al.* 2008). Work is ongoing to investigate if the hormonal and/or the between-sex genetic correlation levels of explanation of female plumage maturation may apply in our study population.

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Table 1. Results from different GLMMs examining (a) cross-sectional effects of age on spectral characteristics of the mantle and breast plumage of female European Pied Flycatchers; and (b) whether the age dependency of those characteristics is due to within-individual or between-individual effects (see 'Statistical analyses'). In all models, female identity and year were entered as random factors. Sample sizes are 393 and 379 readings of the mantle and breast plumage, respectively, corresponding to 263 and 254 different females. Estimates and *P*-values of non-significant and hence removed variables are from when they were added alone to the final model. Significant *P*-values are shown in bold.

Dependent variable	Explanatory variable	Estimate	SE	<i>t</i>	<i>P</i> _{MCMC}
Mantle brightness	a) Intercept	8.143	0.207	39.420	0.0001
	Female age	-0.351	0.114	-3.080	0.0034
	Female age ²	0.044	0.018	2.480	0.0172
	Forehead patch (1/0)	-0.074	0.086	-0.860	0.3432
	b) Between-individual age	0.006	0.061	0.090	0.9014
Mantle hue	Within-individual age	-0.141	0.070	-2.020	0.1000
	a) Intercept	11.948	0.340	35.110	0.0001
	Female age	-0.665	0.156	-4.260	0.0001
	Female age ²	0.078	0.024	3.250	0.0016
	Forehead patch (1/0)	-0.240	0.184	-1.310	0.0270
Mantle chroma	b) Between-individual age	-0.054	0.087	-0.623	0.5584
	Within-individual age	-0.275	0.096	-2.878	0.0140
	a) Intercept	0.914	0.030	30.346	0.0001
	Female age	-0.019	0.007	-2.835	0.0032
	Female age ²	0.004	0.003	1.144	0.2016
Breast brightness	Forehead patch (1/0)	-0.042	0.016	-2.598	0.0172
	b) Between-individual age	-0.013	0.012	-1.135	0.2384
	Within-individual age	-0.031	0.013	-2.276	0.0470
	a) Intercept	33.205	0.717	46.310	0.0001
	Female age	0.937	0.248	3.780	0.0001
Breast hue	Female age ²	-0.105	0.131	-0.800	0.5134
	Forehead patch (1/0)	2.606	0.622	4.190	0.0001
	b) Between-individual age	1.334	0.446	2.991	0.0006
	Within-individual age	0.687	0.401	1.713	0.1760
	a) Intercept	38.723	0.785	49.340	0.0001
	Female age	0.864	0.292	2.960	0.0030
	Female age ²	0.0039	0.157	0.025	0.8524

Breast chroma		Forehead patch (1/0)	2.407	0.742	3.240	0.0012
	b)	Between-individual age	1.106	0.523	2.116	0.0102
		Within-individual age	0.669	0.464	1.442	0.4098
	a)	Intercept	0.621	0.039	16.091	0.0001
		Female age	-0.075	0.021	-3.550	0.0004
		Female age ²	0.009	0.003	2.900	0.0038
		Forehead patch (1/0)	-0.033	0.015	-2.129	0.0130
	b)	Between-individual age	-0.016	0.011	-1.503	0.0924
		Within-individual age	-0.028	0.012	-2.318	0.0240

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Legends to the figures

Figure 1. Two female European pied flycatchers with contrasting mantle colour, brownish (a) and greyish (b); (c) one of the more melanic breeding females recorded in the area, also displaying a white forehead patch

Figure 2. Average reflectance spectra of back (a) and breast (b) plumage of one-year and four-year old female European Pied Flycatchers. Both age classes were chosen to maximize visual differences in plumage colour between females (see Fig. 3).

Figure 3. Cross-sectional patterns of dorsal plumage hue and breast plumage hue in female European Pied Flycatchers of known age. Shown are observed, unadjusted means ± 1 SE; figures beside bars are numbers of females.

312 **Fig. 1**

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315 **Fig. 2.**

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319 **Fig. 3**

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