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RESEARCH ARTICLE

Reflectance variation in the blue tit crown in relation to feather structure

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ABSTRACT

Structural plumage colour is one of the most enigmatic sexually selected traits. The information content of structural colour variation is debated, and the heterogeneity of the findings is hard to explain because the proximate background of within-species colour differences is very scarcely studied. We combined measurements of feather macrostructure and nanostructure to explain within-population variability in blue tit crown reflectance. We found that sexual dichromatism in aspects of crown reflectance was explained only by feather macrostructure, whereas nanostructural predictors accounted for some of the age-related differences in reflectance. Moreover, we found that both mean reflectance and spectral shape traits reflected a combination of quantity and regularity aspects in macrostructure and nanostructure. This rich proximate background provides ample scope for reflectance to convey various types of information on individual quality.

KEY WORDS: Coherent scattering, *Cyanistes caeruleus*, Macrostructure, Nanostructure, Ultraviolet

INTRODUCTION

In the face of directional selection, sexual signal traits may be constrained by various costs and limitations. Production costs arise in connection with the development of the trait, such as the energy expenditure required for display (Hedenström, 1995) or the necessity of collecting and depositing pigments into some integumental colours at the expense of other physiological functions (Olson and Owens, 1998). In contrast, wearing costs are caused by the trait itself, for example, the additional predation risk imposed by visual conspicuousness (Slagsvold et al., 1995) or the negative effect of dragging a long ornament on flight efficiency (Thomas, 1993). Furthermore, intrinsic individual characteristics may limit the production of certain signal character states. For example, stress and impaired neural development early in life may limit song learning capability in birds (MacDonald et al., 2006), and body size may limit the amplitude and frequency of acoustic signals in amphibians (Hoskin et al., 2009).

Bird plumage colour is a conspicuous example of potential signal traits and has therefore attracted much research (for reviews of non-avian colour, see e.g. Kinoshita and Yoshioka, 2005; Caro et al., 2017). Although there is a classical categorization of plumage colour as pigment-based versus structural, it currently seems that, with very few exceptions, colour variation is generated by the interaction of pigments and structures (Shawkey and D'Alba, 2017). The keratin matrix always contributes to the expression of pigment-based colour (Shawkey et al., 2006a), and pigments play vital roles in the modulation of structural colour (Stavenga et al., 2011; Maia et al., 2012a; Tinbergen et al., 2013). The pigment-based component of colour usually involves carotenoid or melanin deposition, and the honesty of the resulting signal can be derived from the costs of obtaining, synthesizing or metabolically transforming the pigment (Griffith et al., 2006; Guindre-Parker and Love, 2014).

One type of structural colour is achromatic white reflectance produced by incoherent scattering of incident light (e.g. McGlothlin et al., 2007). In this colour type, the quantitative aspects of the feather keratin macrostructure may explain trait expression and honesty (Prum, 2006; Shawkey et al., 2006a). Another type is iridescent structural colour, where the dominant wavelength changes with the relative angles of incident light and observation (Osorio and Ham, 2002), and is typically produced by 2D photonic crystals or multi-layer thin-film interference in feather barbules (Yin et al., 2006; Maia et al., 2009; Eliason and Shawkey, 2012; Lee et al., 2015; Stavenga et al., 2017). Finally, many avian taxa exhibit non-iridescent chromatic structural colour. This is produced by constructive interference of short wavelengths by the spongy keratin layer of feather barbs, which shows short-distance structural regularity (Prum et al., 1999; Saranathan et al., 2012).

The honesty of chromatic structural colour may involve at least two limitations (Fitzpatrick, 1998). The first is the production cost of keratin and melanin required for the nanostructure (Meadows et al., 2012). The second is the accurate self-assembly of the ordered nanostructure necessary for constructive interference (Prum et al., 2009; Maia et al., 2012a). Therefore, predictions concerning the information content (e.g. condition dependence) of structural colouration depend on how feather structural properties translate to aspects of reflectance variation at the within-species level. However, despite extensive data at the species level (e.g. Saranathan et al., 2012), studies of within-species variation of colour and nanostructure are currently restricted to one species each for iridescent (satin bowerbird, *Ptilonorhynchus violaceus*; Doucet et al., 2006) and non-iridescent structural colour (eastern bluebird, *Sialia sialis*; Shawkey et al., 2003, 2005), which allows little generalization.

In addition to nanostructure, feather macrostructure, i.e. the size and density of coloured feather parts, may also contribute to colour expression. Macrostructure is certainly not the mechanistic basis of the overall colour, but it may still grossly modify the visual attributes of the given individual, principally through quantity and density aspects of the reflective surfaces. Macrostructure of contour feathers has been shown to be condition dependent (Vágási et al., 2012) and

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environment dependent (common garden experiment; Broggi et al., 2011). Moreover, in the only experimental study that measured feather nanostructure, manipulation of food access at moulting impaired feather macrostructure but not nanostructure (D'Alba et al., 2014). Still, only a few studies have correlated contour feather macrostructure and reflectance traits, and nearly all of these have focused on dark melanin-based colour produced by pigment-containing barbules in high densities (Galván, 2011; D'Alba et al., 2014; sex and morph differences in Enbody et al., 2017; but see one macrostructural variable in relation to structural colour in Shawkey et al., 2005). No general conclusion can therefore be drawn on the macrostructural mechanisms of colour determination but, according to the studies mentioned above, the surface (size, density, etc.) of the colour-producing feather parts may play an important role.

Here, we examine some potential nanostructural and macrostructural predictors of individual reflectance variation in a well-known structural plumage ornament, the crown of the blue tit (*Cyanistes caeruleus*). The UV–blue reflectance of this trait has been suggested to exhibit strong sexual dichromatism (Andersson et al., 1998) and age dependence (Delhey and Kempenaers, 2006), and be subject to intersexual (Hunt et al., 1999) and intrasexual (Rémy et al., 2010; Midamegbe et al., 2011) selection in both sexes. Furthermore, the expression of the trait in males appears to mediate vocal interactions among neighbours (Poesel et al., 2007), and perhaps also sex ratio adjustment by the female partner (Sheldon et al., 1999; Delhey et al., 2007). Finally, both sexes have been found to react to the partner's crown colour at parental care, although in varying directions (Limbourg et al., 2004, 2013; Mahr et al., 2012). Concerning the information content of blue tit crown colour, its age-related increase seems to reflect within-individual change between years (Delhey and Kempenaers, 2006), and crown colour expression has been found to be influenced by moult speed manipulation (Griggio et al., 2009) and brood size manipulation (Doutrelant et al., 2012), although not by mild nutritional manipulation during moulting (Peters et al., 2011). Therefore, given that different measures of blue tit crown colour have been shown to indicate phenotypic quality and to function as signals in both sexes, the species is ideal for examining the proximate background of variation in non-iridescent structural colour expression. In addition to individual differences, we also investigate whether the sex and age dependence of crown reflectance can be traced back to feather nanostructural properties.

MATERIALS AND METHODS

Field methods

Among other hole-breeders, we have been studying a breeding population of blue tits [*Cyanistes caeruleus* (Linnaeus 1758)] in our nestbox plots in the Pilis–Visegrádi Mountains, Hungary. For the present study, we used data from the year with the largest sample size (2009). In this year, all parents were caught at nestling feeding, and approximately 10 crown feathers were collected in envelopes for spectral measurements in the laboratory. This study was conducted under a long-term research agreement with the Pilis Park Forestry (December 1988 and March 2007), and with a research permit from the regional nature conservation authority (KTVF 35464-1/2007). Blind to the reflectance values of their feathers, we chose 32 individuals for feather structure analyses. These included eight juvenile females, eight adult females, eight juvenile males and eight adult males.

Feather macrostructure

The crown feather was placed on a white sheet with a piece of millimetre scale paper attached for calibration, and it was gently pressed down with a microscope slide. Two feathers of each individual (one at a time) were photographed in RAW format under diffuse natural illumination using a Nikon D70 camera and AF Micro Nikkor 60 mm lens. The resulting photographs (Fig. 1A) and additional scanning electron microscope imaging (Fig. 1B) indicated that there are no or very few barbules (i.e. lower order branches) in the apical part of the feather. The UV–blue colour is probably produced principally by the bare distal sections of the apical barbs, which indeed look blue in the photographs. The rest of the feather is grey and densely covered by dark (likely melanin-containing) barbules. After size

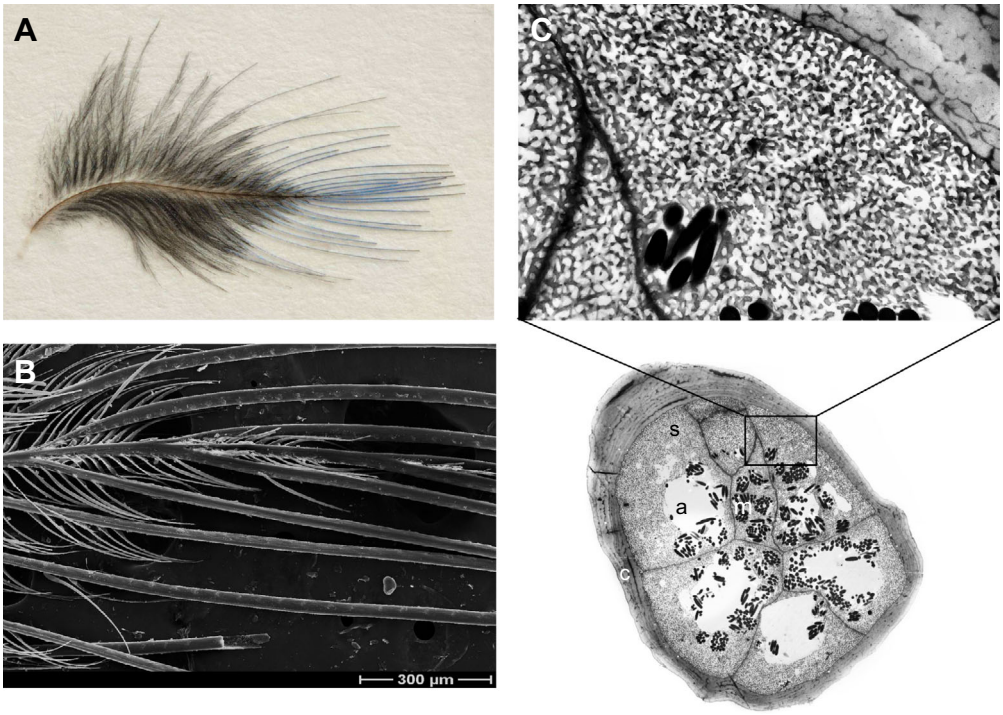

Fig. 1. Structure of blue tit crown feathers. (A) Overall macrostructure, (B) SEM image of the base of the colour-generating bare barb sections (photograph courtesy of Károly Bóka) and (C) TEM image of nanostructure. a, air vacuole; c, barb cortex; m, melanosomes; s, spongy layer.

calibration, two objective measurements were made with a distance tool using SPIP (Image Metrology Inc., Hørsholm, Denmark). We measured the distance from the end of the rachis to the end of the most distal blue barb (hereafter, overhang length), and the distance (parallel to the final section of the rachis) between the most proximal bare point of the most proximal blue barb and the end of the feather (hereafter, blue section length). We also counted the number of blue barbs (with bare sections longer than 1 mm) in the feather (hereafter, number of barbs). All three measurements were repeatable for the two feathers of the same individual (Pearson's r=0.763–0.815, P<0.001).

Feather nanostructure

One undamaged crown feather of each of the individuals was prepared for transmission electron microscopy (TEM). Feather barbs were cut from the upper 5 mm of contour feathers and incubated in 0.25 mol l^{-1} sodium hydroxide and 0.1% Tween-20 for 30 min on a bench-top shaker. These barbs were then changed into 2:3 (v/v) formic acid and ethanol for 2.5 h. Feather barbs were dehydrated by incubation in 100% ethanol for 2×15 min and 100% propylene oxide for 2×15 min, and infiltrated with Spurr Low-Viscosity resin (EM0300, Sigma-Aldrich) in successive concentrations of 25%, 50%, 75%, 90% and 100%. Each of these infiltration steps was performed for 24–48 h except the last one, which was only 6 h. Barbs were placed into moulds with the most distal tip of the barb at the top of the mould, and then the blocks were cured in an oven at 80°C for 48 h. Sections of 80 nm thickness were cut oriented perpendicularly to the barbs of the blue apical portion of the feather using a diamond knife on an Ultracut E ultramicrotome (Reichert-Jung, Leica Microsystems, Cambridge, UK). Sections were placed on a 150-mesh copper grid with Formvar support, post-stained in 2.5% water-based uranyl acetate for 10 min and Reynold's lead citrate for 3 min, and photographed on a Jeol JEM1011 transmission electron microscope (Jeol, Tokyo, Japan).

For one feather, the preparation for TEM was unsuccessful, so this individual was omitted from the dataset. Two undamaged barb cross-sections (4000× or 5000× magnification, see Fig. 1C) were chosen for analysis from each feather. The images were processed in SPIP, and the following nanostructural parameters were measured. Thickness of the homogeneous outer keratin matrix and the distance between the cortex and the melanosomes beneath the spongy layer (keratin–air layer beneath the cortex) were recorded five times per cross-section using a distance tool (yielding cortex thickness and spongy layer thickness, respectively). Spongy layer thickness was not recorded in a subset of the individuals (N=9) where the interior air vacuoles beneath the spongy layer were not filled by the resin, and therefore the melanosomes were displaced and dispersed by the ultramicrotome.

Two magnifications of a fixed scale (25,000×) and a fixed size (corresponding to 1.5 µm picture length and width) were prepared from the spongy layer of each cross-section. In these detail images, after noise reduction, we measured the density of the spongy layer (percentage of the picture area covered by keratin) using a detection tool (by manually adjusting the detection threshold value). We also manually measured the minimum diameter of 20 approximately circular keratin rods and air spaces using a distance tool. Twenty was the approximate number of circular rods in a detail image, and fewer than 20 rods were found in some images. We measured the minimum diameter as the spongy layer is a branching structure in which the exactly perpendicular intersection of rods or cavities is unlikely, and therefore the minimum diameter best approximates the true diameter. We only focused on circular rods and air spaces because branching ones probably correspond to the 'joints' of the air or keratin network, and are therefore not representative of gross nanostructure. From these data, we calculated mean and standard deviation (s.d.) values for keratin rod and air space diameter. Finally, 'distance between scatterers' (i.e. surfaces with a shift in refractive index) was estimated by summing the mean rod and air space diameters. This measure indicates periodicity in the spongy layer. Although it is a derived measure and therefore correlated with its component traits, we included it for comparison with the findings of Shawkey et al. (2005).

The repeatability of these measurements could be tested on different levels. Cortex and spongy layer thicknesses were first subjected to repeatability testing at the cross-section level (five data per cross-section) using the rptR procedure (Nakagawa and Schielzeth, 2011) in R (https://www.r-project.org/). Spongy area parameters were tested for repeatability between the two detail images of each cross-section. Repeatability was significant for all parameters, so the data were averaged and the repeatability testing was repeated at the level of cross-sections (two per individual). All of the measured structural variables were tested at this level and all repeatabilities were again significant, indicating that measurements were sufficiently accurate and that the two cross-sections were representative. All details on measurement repeatabilities are presented in Table S1. We then used overall averages of each parameter at the individual level to characterize feather nanostructure.

Reflectance

Reflectance measurements were made using at least eight collected feathers from each individual, using methods detailed and validated in Hegyi et al. (2015). Briefly, the feathers were put on top of one another in a parallel position on a piece of black velvet. Spectral data were collected using OOIBase software, a USB2000 spectrometer, a bifurcated fibre optic probe fitted with a black plastic tube to standardize measurement distance, and a DH2000 deuterium and tungsten halogen light source (Ocean Optics Europe, Ostfildern, Germany). Percent reflectance was given relative to a WS-1 spectralon white reflectance standard (Ocean Optics Europe). Three reflectance spectra were taken from each sample. We calculated three spectral variables from the obtained spectra: mean reflectance (average reflectance from 320 to 700 nm), UV chroma (UV reflectance from 320 to 400 nm, divided by total reflectance) and dominant wavelength (wavelength of maximum reflectance). All of these variables were repeatable (tested in rptR; r_I=0.787–0.955, P<0.001), and were therefore averaged at the sample level. We illustrate variation in the three spectral variables in Fig. 2.

Five juvenile individuals (one male and four females) had dominant wavelength values greater than 400 nm. All of these samples had been collected late in the season. The reflectance spectra of these five samples showed a characteristic shape difference from the rest of the samples (Fig. S1), suggesting that an environmental impact on the crown feathers had caused a UV cutoff in these birds. We present the analyses without these individuals in the paper, and results including their data are given in Tables S2 and S3.

Statistical analyses

All statistical analyses were conducted in Statistica 5.5 (StatSoft Inc., Dell, Rock Round, TX, USA). To improve normality, we log transformed dominant wavelength, cortex thickness, spongy area thickness and mean keratin rod diameter before analysis. We first tested age and sex effects (and their interaction) on each reflectance

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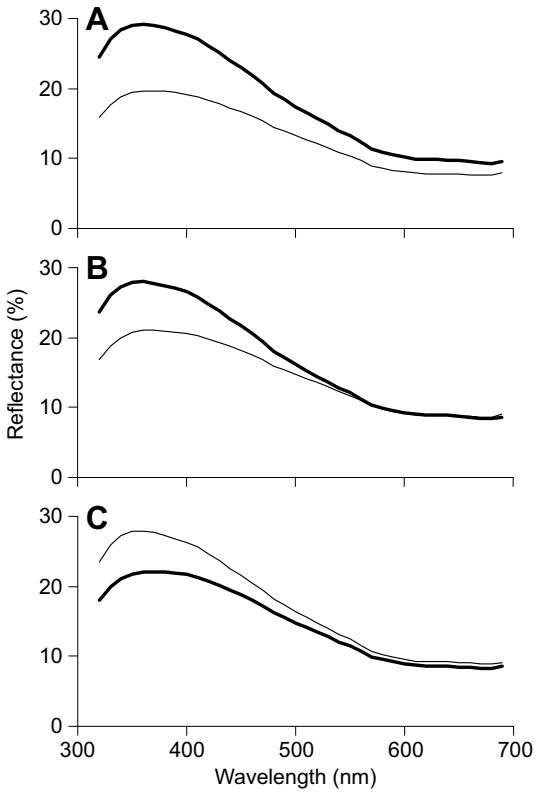

Fig. 2. Averaged reflectance curves of the lower and upper halves of the feather sample after sorting the data by a given spectral variable.
(A) Mean reflectance, (B) UV chroma and (C) dominant wavelength. Thin and thick lines refer to low and high values, respectively.

and structural trait in general linear models with backward stepwise selection and reintroduction. In the second step, we correlated the nanostructural and macrostructural descriptors with one another and with the reflectance traits. Where we found significant age or sex effects on both structure and colour, we repeated the colour–structure correlations using the residuals of the affected variables.

RESULTS

Effects of age and sex

All three spectral traits differed between the sexes (see statistical details in Table 1). We found higher mean reflectance and UV chroma and lower dominant wavelength in males than in females. UV chroma and dominant wavelength (P=0.068) also varied with age, with higher UV chroma and lower dominant wavelength in adults than in juveniles.

Among the nanostructural traits, spongy area thickness and mean air space diameter (Fig. 3A) were greater in adults than in juveniles and there was a similar tendency (P=0.061) for distance between scatterers. No other nanostructural trait varied with age, and no nanostructural trait differed between sexes. By contrast, all three macrostructural traits differed between sexes, with males having a greater number of barbs (Fig. 3B) and greater overhang length (Fig. 3C) and blue tract length than females; however, no macrostructural trait differed between age classes.

Interrelations among structural variables

There were some strong correlations within groups of variables (details in Table 2). Distance between scatterers was positively correlated with mean rod and air space diameters, and all three macrostructural variables were strongly positively correlated. In contrast, relationships among the groups of structural variables were

Table 1. Age- and sex-related variation in aspects of reflectance and feather structure in blue tits: general linear models with backward stepwise selection

	Age		Sex		Age×sex	
	F	d.f.	*F*	d.f.	*F*	d.f.
Mean reflectance	1.955	1, 23	9.106**	1, 24	0.620	1, 22
UV chroma	6.827*	1, 23	38.093***	1, 23	0.642	1, 22
Dominant wavelength	3.665‡	1, 23	6.167*	1, 24	0.798	1, 22
Cortex thickness	0.022	1, 29	1.195	1, 29	0.015	1, 27
Spongy area thickness	9.565**	1, 29	0.761	1, 28	0.223	1, 27
Spongy area density	0.042	1, 29	0.133	1, 29	0.303	1, 27
Mean rod diameter	0.286	1, 29	0.007	1, 29	0.082	1, 27
s.d. of rod diameter	0.214	1, 29	0.046	1, 29	0.000	1, 27
Mean air space diameter	5.645*	1, 29	1.999	1, 28	1.061	1, 27
s.d. of air space diameter	0.080	1, 29	0.648	1, 29	0.643	1, 27
Distance between scatterers	3.452‡	1, 29	1.243	1, 29	0.619	1, 27
Number of barbs	0.251	1, 28	16.845***	1, 29	0.012	1, 27
Overhang length	0.002	1, 28	38.147***	1, 29	0.019	1, 27
Blue section length	0.000	1, 28	29.129***	1, 29	1.620	1, 27

‡P<0.07; *P<0.05; **P<0.01; ***P<0.001.

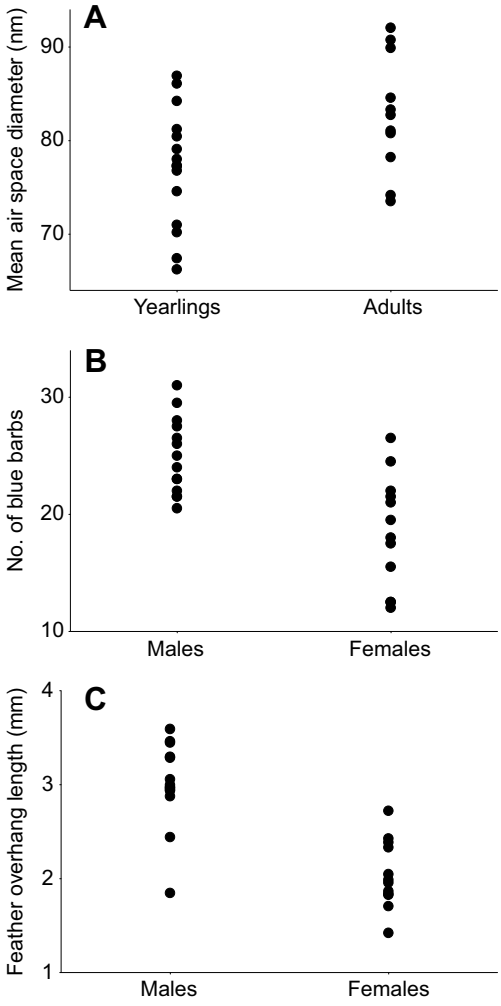

Fig. 3. Age and sex dependence of some structural parameters of blue tit crown feathers. (A) Age dependence of mean air space diameter, and sex dependence of (B) the number of blue barbs and (C) feather overhang length.

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Table 2. Correlations among descriptors of blue tit crown feather structure

	Spongy area thickness	Spongy area density	Mean rod diameter	s.d. of rod diameter	Mean air space diameter	s.d. of air space diameter	Distance between scatterers	Number of barbs	Overhang length	Blue section length
Cortex thickness	0.311	0.093	0.002	0.221	0.110	−0.257	0.101	−0.367*	−0.065	−0.078
Spongy area thickness		−0.437*	0.045	0.220	0.513*	−0.070	0.506*	0.024	0.338	0.220
Spongy area density			−0.132	−0.240	−0.708***	−0.078	−0.723***	0.078	−0.062	−0.010
Mean rod diameter				0.353	−0.142	0.283	0.395*	−0.216	0.010	−0.099
s.d. of rod diameter					0.070	0.330	0.246	−0.091	0.146	0.063
Mean air space diameter						−0.042	0.853***	−0.210	−0.197	−0.176
s.d. of air space diameter							0.103	−0.102	−0.038	−0.045
Distance between scatterers								−0.303	−0.169	−0.209
Number of barbs									0.483**	0.705***
Overhang length										0.818***

*P<0.05; **P<0.01; ***P<0.001.

less prominent, with only a few exceptions. Spongy area thickness was positively correlated with mean air space diameter and distance between scatterers. Spongy area density was strongly negatively correlated with mean air space diameter and distance between scatterers. There was only one significant relationship between nanostructural and macrostructural traits: the number of barbs was negatively correlated with cortex thickness.

Reflectance traits in relation to nanostructure and macrostructure

Among the nanostructural parameters, mean air space diameter (Fig. 4A) and distance between scatterers were negatively correlated with mean reflectance (see detailed results in Table 3). Cortex thickness was negatively correlated with UV chroma (Fig. 4B), and the pattern remained when using the residual reflectance traits. Spongy area thickness was positively correlated with UV chroma, but this pattern vanished when using standardized reflectance traits. Spongy area density and mean keratin rod diameter were not significantly correlated with reflectance parameters. Finally, the s.d. of rod diameter was negatively correlated with dominant wavelength (Fig. 4C). Among the macrostructural parameters, all three measures were positively correlated with mean reflectance and UV chroma, and negatively correlated with dominant wavelength (marginal between the number of barbs and dominant wavelength). When we corrected both macrostructure and reflectance traits for sex, overhang length continued to show a positive correlation with mean reflectance (raw relationship in Fig. 5A), and the number of barbs remained significantly correlated with UV chroma (raw relationship in Fig. 5B). No other correlation remained significant when corrected for sex.

DISCUSSION

Reflectance of the crown of blue tits is a sexually selected ornamental trait (Hunt et al., 1999) that has been found to show sexual dichromatism (Andersson et al., 1998) and an increased expression with age (Delhey and Kempenaers, 2006). Indeed, all reflectance traits we calculated here differed between the sexes, and spectral shape measures also differed between age classes, all in the expected direction (more exaggerated expression in males and in adults). Here, we aimed to find aspects of feather structure that may explain variation in the reflectance properties of the blue tit crown in three contexts: between sexes, between age groups and among individuals. In addition to explaining variation, the investigation of the pathways of reflectance modulation may also help to identify mechanisms that may limit the production of exaggerated character states (e.g. high mean reflectance, high UV chroma), and may thereby determine the information conveyed by crown reflectance as a sexual signal. We measured feather barb nanostructure that probably generates the UV–blue colour of the area. We also measured aspects of feather macrostructure (size and density traits) that may contribute to reflectance differences among individuals.

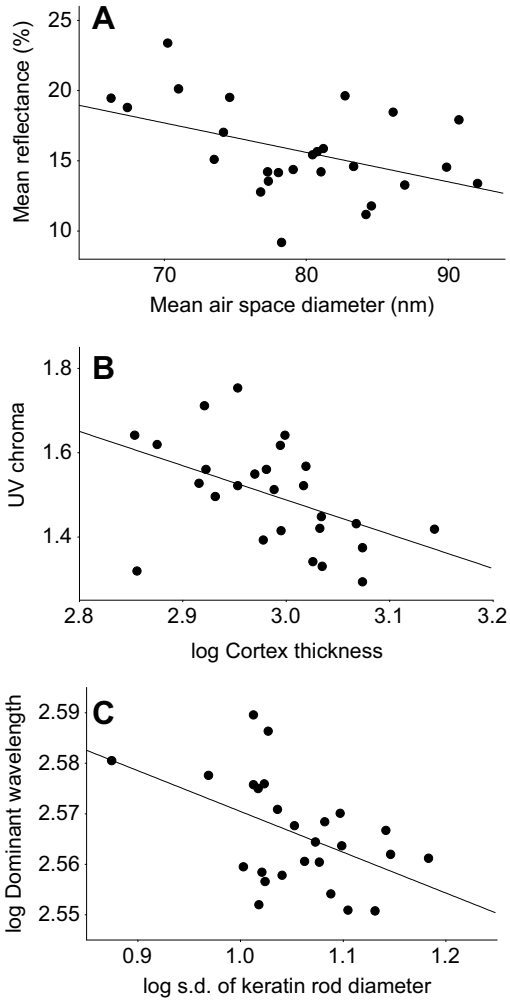

Fig. 4. Feather nanostructural predictors of blue tit crown reflectance. (A) Mean air space diameter and mean reflectance, (B) barb cortex thickness and UV chroma and (C) the s.d. of keratin rod diameter in the barb spongy layer and dominant wavelength.

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Table 3. Correlations (Pearson's *r*) between aspects of feather structure and blue tit crown reflectance

	Mean reflectance	UV chroma	Dominant wavelength
Cortex thickness	−0.125	−0.465*	0.212
Spongy area thickness	0.047	0.527*	−0.414
Spongy area thickness (RES)	n.a.	−0.184	0.066
Spongy area density	0.018	−0.177	0.206
Mean rod diameter	−0.035	−0.193	−0.042
s.d. of rod diameter	0.053	−0.103	−0.471*
Mean air space diameter	−0.442*	0.131	−0.045
Mean air space diameter (RES)	n.a.	0.318	−0.010
s.d. of air space diameter	−0.081	−0.119	−0.246
Distance between scatterers	−0.409*	0.026	−0.059
Distance between scatterers (RES)	n.a.	−0.102	0.072
Number of barbs	0.574**	0.698***	−0.356
Number of barbs (RES)	0.368	0.436*	−0.102
Overhang length	0.644***	0.615***	−0.475*
Overhang length (RES)	0.441*	0.162	−0.221
Blue tract length	0.577**	0.611***	−0.395*
Blue tract length (RES)	0.350	0.241	−0.129

n.a., not applicable; RES, residual variables; *P<0.05; **P<0.01; ***P<0.001.

Strikingly, nanostructural traits showed some age differences, but no significant sex differences, while the reverse was true for macrostructural traits, all of which were strongly sex dependent but age independent. Based on the interrelations of age-corrected data, the s.d. of keratin rod diameter is age dependent and correlated with the dominant wavelength both among and within groups. Rod diameter may therefore explain some of the age difference in dominant wavelength. Following a similar logic, cortex thickness may explain some age difference in UV chroma. Analogously, relationships with macrostructure remaining after sex correction indicate that feather overhang length and number of barbs may explain some of the sexual dichromatism in mean reflectance and UV chroma, respectively. In summary, sexual dichromatism in the mean reflectance and chromaticity of blue tit crown colour seems to originate at least partly from feather macrostructure, and we found no sign that it originated from nanostructure. Sexual dichromatism represents a substantial proportion of variance in reflectance in the blue tit (Andersson et al., 1998; present study), so our findings highlight a striking discrepancy between the mechanisms generating species-specific overall colour (barb nanostructure) and those leading to variation in colour among individuals (feather macrostructure). As macrostructure should be prone to mechanical deterioration, it will also be very important to examine sex differences in crown macrostructure and reflectance immediately after the summer moult, and their changes until and during the breeding season. We need similar analyses as those done previously on seasonal patterns of reflectance (e.g. Delhey et al., 2010).

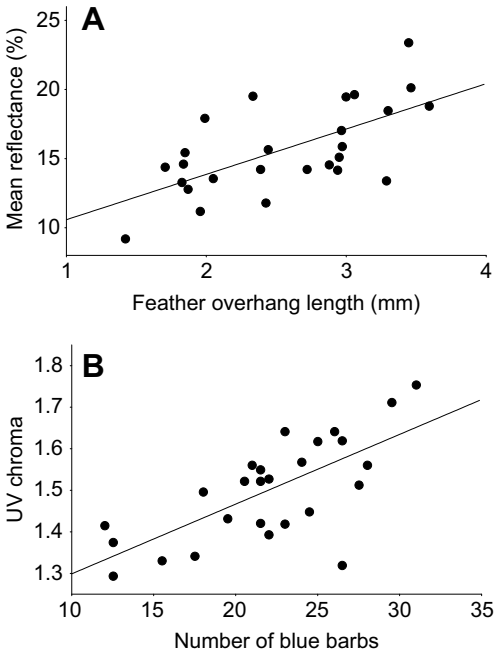

Fig. 5. Feather macrostructural predictors of blue tit crown reflectance. (A) Feather overhang length and mean reflectance and (B) the number of blue barbs and UV chroma.

In contrast to sexual dichromatism, age differences in reflectance (see also Delhey and Kempenaers, 2006) were partly explained by barb nanostructure but were not significantly affected by feather macrostructure. Therefore, it is unlikely that age-related patterns in crown colour are influenced by external feather abrasion. Again, it will be very important to examine the within-individual changes of barb nanostructure and reflectance with subsequent moults (nanostructure is not very likely to substantially change with abrasion but this needs to be confirmed as well). Because of the well-known improvement of individual condition and intrinsic attributes with age (e.g. Forslund and Pärt, 1995), age-dependent nanostructural characters that predict reflectance (here particularly, barb cortex thickness) may be especially likely candidates for indicators of condition or stable aspects of individual quality.

Two previous studies have examined the structural basis of plumage sexual dichromatism. In eastern bluebirds, robust sex differences were found in nanostructural traits, while the only macrostructural measure (number of melanized barbules) was independent of sex (Shawkey et al., 2005). In two species of fairy-wren, sex- and morph-specific differences were suggested in both macrostructure and nanostructure (Enbody et al., 2017). We found no previous studies of age dependence in colour-producing feather nanostructures or macrostructures, so no comparison can be made at this point. It is nevertheless interesting that age and sex differences were produced at different levels of structural organization in our study species.

The most crucial characteristic of a sexually selected signal trait is the information it conveys to the receivers of the signal, which in turn depends on what creates signal variation. Unfortunately, the mechanism that generates within-species variation in 'structural' plumage colour signals is essentially unknown, with only one published study on iridescent colour (Doucet et al., 2006) and two on non-iridescent colour (Shawkey et al., 2003, 2005; but these two concerned the same species and population). Here, we examined a non-iridescent ornament and paid attention to both macrostructure and nanostructure of feathers.

Our macrostructural variables described the amount and density of colour-producing material in the feather. The positive relationship between overhang length and mean reflectance may reflect the increase of the total coloured surface in the crown plumage. The positive correlation of UV chroma with the number of blue barbs in the feather is also logical as there should be a positive link between the density of coloured tissue and the spectral purity of the reflected light. Similar relationships with macrostructure have previously been found for melanin-coloured plumage traits (Galván, 2011; D'Alba et al., 2014).

Concerning the nanostructural predictors of within-species variation in non-iridescent chromatic structural colour, most of

our results were consistent with those of the only published research project, that of Shawkey et al. (2003, 2005) on the eastern bluebird. Mean reflectance was negatively correlated with two variables that were positively correlated with each other: mean air space diameter and the distance between scatterers. These patterns are straightforward as both variables probably correlate negatively with the reflective surface density of the spongy area (given the same thickness of the spongy layer), and a reduced surface density is expected to generate lower overall reflectance from the spongy layer (see e.g. simulations in Yu et al., 2017; see similar empirical results in Shawkey et al., 2005).

UV chroma was negatively correlated with cortex thickness (see also Shawkey et al., 2005). This is presumably due to the selective filtering of UV wavelengths by the keratin matrix of the cortex (Brink and van der Berg, 2004). Concerning dominant wavelength, its only nanostructural predictor was the s.d. of keratin rod diameter, and birds with more variable rod diameter showed more UV-shifted dominant wavelengths. Strikingly, mean values of keratin rod diameter, air space diameter and inter-scatterer distance did not predict dominant wavelength.

According to the accepted theory on the generation of non-iridescent structural plumage colour, the reflectance curve directly mirrors the distribution of light path lengths (i.e. inter-scatterer distances) in the spongy layer (Prum et al., 1998, 1999). Looking at the reflectance curves of the blue tit crown (see Fig. 2 for characteristic spectra), we see that these must originate from light path distributions of varying shapes. Assuming a direct correspondence with reflectance curve shape, light path distributions probably become more and more right-tailed as dominant wavelength decreases. This increasing tail may have caused the negative correlation of keratin rod s.d. with dominant wavelength in our dataset. There are multiple complementary explanations for the lack of correlation of dominant wavelength with mean particle diameters, pore diameters and estimated path lengths. For example, means may poorly reflect the true peaks of the non-normal distribution. The assumed short-range but not long-range order of the spongy layer (Noh et al., 2010) may also make it difficult to reliably derive the path length distribution of light from the observed distributions of particle and pore diameters in 2D planes (Shawkey et al., 2009). Even more sophisticated approaches than ours (2D Fourier analysis of TEM images) to directly estimate the path length distribution have previously failed to accurately estimate the dominant periodicity of the 3D spongy layer, with the result that calculated mean dominant wavelength was (roughly) accurate at the species level (e.g. Shawkey et al., 2006b), but individual differences could not be reliably discerned (Shawkey et al., 2003; see also Shawkey et al., 2009). To our knowledge, the only known method to reliably estimate the distributions of path lengths is the high-energy, high-resolution small-angle X-ray scattering (SAXS) screening of single, intact feather barbs (Saranathan et al., 2012). This method may give sufficient precision to capture individual differences in dominant wavelength, but it currently requires a synchrotron, limiting its application for evolutionary ecologists. Further technical developments are clearly needed in this area.

The information content of structural plumage reflectance, including its condition dependence, is unclear. If energetic constraints dominate, condition dependence may be most likely in reflectance traits that depend on the quantity of keratin invested (Meadows et al., 2012). It could be assumed that mean reflectance is a direct function of the quantity and density of scattering or absorbing elements at the macrostructural and nanostructural level (Prum, 2006), whereas the chromaticity of structural colour should instead more strongly depend on the regularity of nanostructure (Prum et al., 1999). Finally, the wavelength of maximum reflectance (dominant wavelength) is assumed to originate from the geometry of the spongy layer, i.e. the distances between scatterers that reinforce certain wavelengths by constructive interference (Prum et al., 1999). With these reflectance modulation pathways in mind, we would expect the greatest condition dependence for mean reflectance and the lowest for dominant wavelength, whereas regularity-dependent measures of spectral purity should be sensitive to stress during moulting.

However, the few existing studies of within-species variation show more complex interrelationships between nanostructure and reflectance. Mean reflectance indeed correlates negatively with quantity and/or density traits such as cortex thickness, keratin rod size and distance between scatterers, whereas UV chroma correlates with aspects of the regularity of the spongy layer (Shawkey et al., 2005; this study). However, UV chroma is also affected by quantity aspects of the nanostructure (cortex thickness: Shawkey et al., 2005; this study; number of circular keratin rods: Shawkey et al., 2003; possibly also spongy layer density: Yin et al., 2012). Finally, the main predictor of dominant wavelength in our study is apparently not one dominant structural parameter of the spongy layer (inter-scatterer distance; Shawkey et al., 2005) but rather its composition (s.d. of keratin rod diameter). Dominant wavelength has also been shown to correlate with quantity/density aspects of the nanostructure (spongy layer thickness: Shawkey et al., 2005; cortex thickness: Doucet et al., 2006). Concerning macrostructural traits, most previous quantitative studies of such traits examined melanin-based colour and are therefore not relevant (Galván, 2011; D'Alba et al., 2014). The only macrostructural trait measured by Shawkey et al. (2005), the number of melanin-containing barbules, reduced both mean reflectance and spectral purity of structural colour. Our present data also indicate that quantitative aspects of feather macrostructure may influence both mean reflectance (overhang length) and spectral shape (number of barbs).

Table 4. Some studies that provided correlative or experimental support for the phenotypic plasticity of structural plumage colour

Experimental?	Independent variable	Dependent	Effect	Subjects	Species	Reference
No	Stress hormone level	Mean reflectance	Negative	Nestlings	Eastern bluebird	Siefferman et al. (2013)
No	Stress hormone level	UV chroma	Negative	Adults	Blue tit	Henderson et al. (2013)
Yes	Brood removal	Dominant wavelength	Positive	Adults	Blue tit	Doutrelant et al. (2012)
Yes	Brood size manipulation	Mean reflectance	Negative	Adults	Eastern bluebird	Siefferman and Hill (2007)
Yes	Brood size manipulation	UV chroma	Negative	Nestlings	Blue tit	Jacot and Kempenaers (2007)
Yes	Food deprivation	Structural colour PC	Negative	Adults	Eastern bluebird	Siefferman and Hill (2005)
Yes	Food supplementation	Mean reflectance	Positive	Nestlings	Eastern bluebird	Doyle and Siefferman (2014)
Yes	Moult acceleration	UV chroma	Negative	Adults	Blue tit	Griggio et al. (2009)
Yes	Social stress at moult	Blue chroma	Positive	Adults	Blue-black grassquit	Maia et al. (2012b)
Yes	Testosterone implant	Mean reflectance	Negative	Nestlings	Eastern bluebird	Siefferman et al. (2013)

It therefore currently seems that all descriptors of structural colour may depend on keratin quantity and density, and thus all of them may show condition dependence during moulting via this pathway. Experimental studies on condition dependence are few, and the results obtained were mixed. It has been suggested that structural plumage colour is dependent on developmental stress rather than body condition at moulting (Peters et al., 2011). Correlations with stress hormone levels and significant effects of various experimental manipulations (see examples in Table 4) indicate that structural colour may depend on both stress and true body condition. Mean reflectance was somewhat more frequently influenced by manipulations than dominant wavelength or chromaticity (Table 4). This suggests that condition or stress during moulting may indeed be more likely to affect the quantity than the regularity aspects of feather structure.

In summary, our results surprisingly indicate that feather macrostructure may explain a similar amount of phenotypic variation in 'structural' plumage colour traits as nanostructure. Moreover, in our study population, macrostructure seemed to be an important determinant of sexual dichromatism in mean reflectance and spectral purity. Studies of feather macrostructure and structural colour are effectively lacking, although macrostructure is technically much more accessible than nanostructure. More research is urgently needed on this topic as the expected information content, vulnerability and seasonal expression profile of structural colour signals strongly depend on whether their main origin is macrostructural or nanostructural variation. In the blue tit, further observational and experimental studies are needed to elucidate the phenotypic plasticity and quality indicator value of the structural determinants of crown colour we identified here, and the consequences for signal information content.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: G.H., M.L., D.K., T.C., P.L., B.R., E.S., J.T.; Investigation: G.H., M.L., D.K., T.C., P.L., B.R., E.S., J.T.; Writing - original draft: G.H., M.L., D.K., T.C., P.L., B.R., E.S., J.T.; Writing - review & editing: G.H.; Supervision: G.H.; Funding acquisition: G.H., B.R.

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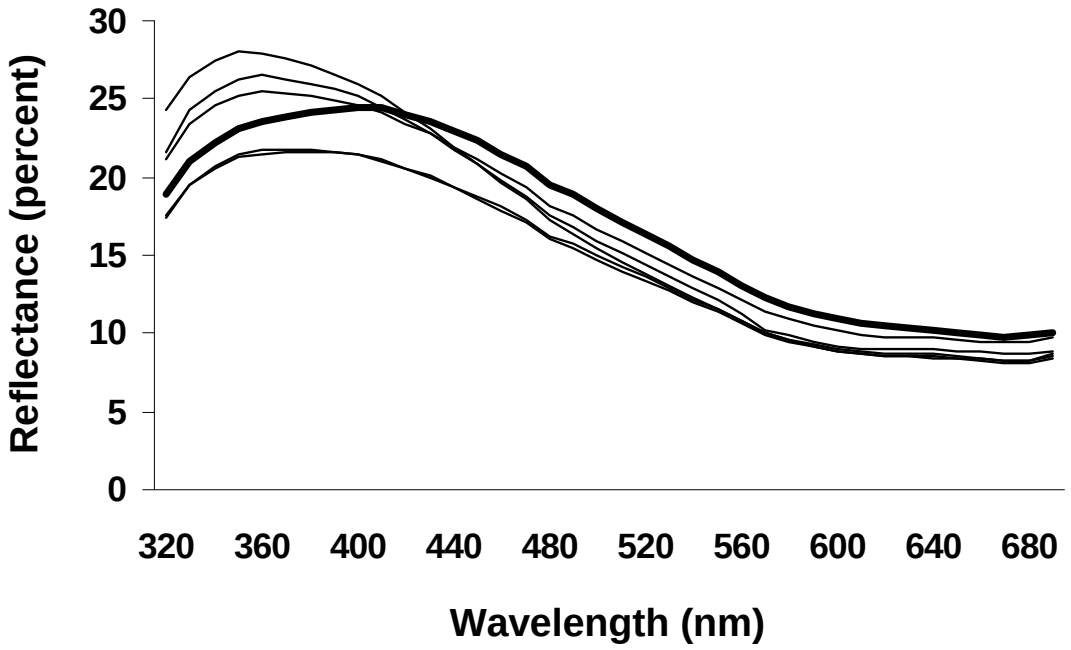

Fig. S1. Mean reflectance spectra of groups of five or six individuals each, averaged after ranking the individuals by dominant wavelength. The curve marked with bold is the mean of the five outlying individuals with dominant wavelength values greater than 400nm. Every single individual in this group has this characteristic curve shape

Table S1. Repeatabilities (r_I) of nanostructural measurements at two different levels

	Within cross-sections	Between cross-sections
Cortex thickness	0.233***	0.601**
Spongy area thickness	0.356***	0.697***
Spongy area density	0.791***	0.681***
Mean rod diameter	0.579***	0.656**
SD of rod diameter	0.255*	0.342*
Mean air space diameter	0.721***	0.526***
SD of air space diameter	0.240*	0.354*

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

Table S2. Effects of age and sex on spectral traits when including five individuals with dominant wavelength > 400 nm

	Age		Sex		Age x sex	
	F	df	*F*	df	*F*	df
Mean reflectance	0.909	28	9.284**	29	0.101	27
UV chroma	3.557†	28	29.624***	29	1.059	27
Dominant wavelength	0.910	29	3.774†	29	0.466	27

†, $P < 0.07$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

Table S3. Correlations (Pearson *r*) between aspects of feather structure and reflectance when including five individuals with hue > 400 nm

	Mean reflectance	UV chroma	Dominant wavelength
Cortex thickness	-0.025	-0.387*	0.130
Spongy area thickness	0.201	-0.011	0.327
Spongy area thickness (RES)	NR	-0.399	0.442*
Spongy area density	-0.059	0.043	-0.235
Mean rod diameter	-0.124	-0.083	-0.059
SD of rod diameter	0.010	-0.065	-0.230
Mean air space diameter	-0.307	-0.065	0.233
Mean air space diameter (RES)	NR	-0.009	0.168
SD of air space diameter	-0.109	-0.108	-0.020
Distance between scatterers	-0.345	-0.097	0.184
Distance between scatterers (RES)	NR	-0.048	0.122
Number of barbs	0.473**	0.706***	-0.487**
Number of barbs (RES)	0.252	0.488**	-0.376*
Overhang length	0.653***	0.486**	-0.120
Overhang length (RES)	0.493*	-0.117	0.220
Blue tract length	0.555***	0.564***	-0.238
Blue tract length (RES)	0.336	0.129	0.004

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$