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Brief report

Measuring plumage colour using different spectrophotometric techniques: a word of caution

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1. Introduction

The study of animal colouration has developed rapidly with the proliferation of portable reflectance spectrophotometers and light sources in the past few years. These instruments have almost completely substituted reference colour swatches and other methods of measuring colour based on human standards that were used in early studies of colour. As humans can capture biologically relevant variation in colour, these latter methods are completely correct if used with caution. However, the use of spectrophotometers allows more objective analyses of colour and the inclusion of wavelengths in the ultraviolet (UV) spectral range, to which some birds are sensitive but humans are blind (Andersson & Prager 2006). Reflectance spectra can be thus analysed in detail through many colour variables, which potentially facilitates the comparison of results from different studies.

This improvement of spectrophotometric technology has increased our understanding of the function and evolution of animal colour patterns by dividing spectral data in different wavebands that can be analysed separately. For example, this allows the inference of which colour components reflect the content of carotenoids in the plumage coloured by these pigments (Saks *et al.* 2003, Andersson & Prager 2006, Shawkey *et al.* 2006), or which colour components are related to particular signaling roles (e.g. Stein & Uy 2006). This classification of spectral data (Montgomerie 2006) is useful in studying bird colouration because, although originally conceived for the properties of human vision, the most commonly used tristimulus colour psychometrics (brightness, hue and chroma) seem to be strongly correlated with avian colour discrimination (Andersson & Prager 2006, Montgomerie 2006). Thus, some modifications have been made to adapt these variables to the peculiarities of reflectance of bird plumage and bare parts, and today several indices to describe the shape of reflectance curves can be found in the literature (Montgomerie 2006).

The rapid increase in the number of studies on

bird colouration can be explained by the increased availability of instruments and variables to describe the measurements obtained using them. However, to our knowledge no one has attempted to compare the results of bird colour measurements obtained using different spectrophotometric instruments, as has been done with digital cameras (Stevens *et al.* 2007). Spectrophotometers can use directional or diffuse light to analyse surface reflectance, the reflected light can be collected at a different angle from the light source or can coincide, and with diffuse light source the collected light can be that reflected in all directions or at the direction normal to the sample. On the other hand, there are several light source types available, each with different output intensities and noise across the spectral range (Montgomerie 2006). All of these factors may contribute to discrepancies between results obtained with different spectrophotometric instruments, which should not be problematic if the values were correlated. However, correlations between values obtained from different spectrophotometric instruments have not previously been estimated.

Our aim was to determine the degree of relationship between colour measurements obtained from two commonly-used spectrophotometers (Ocean Optics USB2000, Ocean Optics, USA) and Minolta CM-2600d (Minolta Co. Ltd, Japan) on the plumage of birds to evaluate whether results from different studies can be properly compared. It must be noted that we did not attempt to make a direct comparison between the spectrophotometers, as this would require the use of similar characteristics (light sources, probes, white standards, etc), but to compare results obtained using the default settings of these spectrophotometers as used by many researchers (see references in Andersson & Prager [2006] for examples with the Ocean Optics USB2000, and Arriero & Fargallo [2006], Moreno *et al.* [2006], Velando *et al.* [2006] and Penteriani *et al.* [2007] for examples with the Minolta CM-2600d).

Thus, we compared spectrophotometric measurements obtained from two spectrophotometers rather than specific models of spectrophotometers, which implies the comparison of different methods of analysing surface reflectance and different light sources. Indeed, only a few technical specifications can be modified in these spectrophotometers (see Ocean Optics 2001–2005 and Konica Minolta 2002–2005 for lists of technical specifications for both spectrophotometers). Quesada and Senar (2006) recently found that feather colour measurements obtained from both spectrophotometric instruments are highly repeatable, but to date a detailed comparison between them has been lacking.

2. Material and methods

We used the above-described Ocean Optics and Minolta spectrophotometric instruments to measure the plumage colour of museum specimens from the collection of the National Museum of Natural Sciences (CSIC, Madrid, Spain). To detect effects of plumage type on possible differences in colour measurements, we chose skins from 10 species of birds (order Passeriformes) representing two main types of plumage colouration: pigmentary (carotenoid-based and melanin-based) and structural. As examples of yellow colour produced by carotenoids, we used skins of Cirl Bunting (*Emberiza cirlus*), European Serin (*Serinus serinus*), Golden Oriole (*Oriolus oriolus*), Blue Tit (*Parus caeruleus*) and Great Tit (*Parus major*), and skins of Common Bullfinch (*Pyrrhula pyrrhula*) served as examples of red carotenoid-based colour. As examples of reddish colour produced by melanins, we used skins of Robin (*Erithacus rubecula*) and Common Stonechat (*Saxicola torquata*). Skins of Azure-winged Magpie (*Cyanopica cyanus*) and Blue Rock Thrush (*Monticola solitarius*) were used as examples of non-iridescent blue structural colour. Golden Oriole, Common Bullfinch and Blue Rock Thrush are sexually dichromatic, so we only measured the colour of males except for the Golden Oriole in which we also measured some females with uniform patches of yellow feathers similar to males. The nature of colouration of these species was deduced from published and unpublished material and from the shape of the spectral curves (Table 1). Apart from the representativity of different types of plumage colouration, the choice of these species responded to the availability of a sufficient number of skins in well-conserved condition, and deteriorated specimens were avoided.

The two spectrophotometers, considered here,

use different methods to analyse surface reflectance. The Ocean Optics spectrophotometer (hereafter referred to as OOS) uses directional reflectance, while the Minolta spectrophotometer (hereafter referred to as MS) uses diffuse light through a 52-mm integrating sphere. Differences in spectral sensitivity allowed consideration of a spectral range from 300 nm to 700 nm with the OOS and from 360 nm to 700 nm with the MS. The light sources were also different: the MS has three pulsed xenon lamps (flashes), whereas deuterium (UV) and halogen-tungsten (visible) lamps were used as a light source (Ocean Optics DT-MINI) for the OOS.

The spectrophotometers both provided illumination and obtained light reflected from the sample. The OOS had a considerably smaller diameter of the reading area (ca. 1 mm) than the MS (8 mm). A bifurcated 400 micrometer fiber-optic Dunedin probe was used for the OOS. Standard black and white references were made according to recommendations from each apparatus' user manual. Thus, the white reference for the OOS was a white "Spectralon" tablet (WS-1-SS, Ocean Optics, USA), while the white reference for the MS was a white calibration plate 'CM-A145' (Konica Minolta, Japan). Reference measurements were frequently made in both cases.

The measurements were always taken at the same angle (90º) to the sample to minimize differences generated by this factor. The distance between the probe and the sample was held constant by using a probe pointer. An average spectrum of three readings on different points of the breast (right wing in the case of the Azure-winged Magpie) was obtained for each specimen, moving the probes by at least 5 mm before taking each new reading, but always following the same order. Thus, each specimen was measured with both spectrophotometers and at the same points, making our results comparable. The softwares OOIBase and Spectramagic were used to obtain spectral data from the OOS and MS, respectively.

The above methods of measuring colour and apparatus characteristics have been used by other authors in previous studies with the spectrophotometers considered here (e.g., Andersson & Prager 2006, Moreno *et al.* 2006, Velando *et al.* 2006, Penteriani *et al.* 2007; see Hofmann *et al.* 2006, Pryke & Griffith 2006 and Stein & Uy 2006 for examples using the OOS with a different light source).

Spectral data were summarized as measures of brightness, chroma and hue by using the most common procedures in the literature (Montgomerie 2006). Total brightness ($R_{360–700}$) was defined as the summed reflectance across the entire spectral range (360–700 nm). To search for differences in the correlation values between spectral zones, other brightness measures were calculated for the UV-blue range only (short-wavelength brightness; $R_{360–470}$) as the summed reflectance between 360 and 470 nm, and for the rest of the spectral range (long-wavelength brightness; $R_{480–700}$). Similarly, a measure of spectral chroma was calculated for this latter range (long-wavelength chroma; $R_{480–700}/R_{360–700}$), showing the contribution of reflectance at long wavelengths to the total brightness. Since carotenoid chroma, defined as $(R_{700} – R_{450})/R_{700}$, is an index often used to determine the relative reflectance around peak absorbance of these pigments (e.g., Jacot & Kempenaers 2007), we also calculated this variable for skins belonging to species with carotenoid-based plumage.

Finally, hue was defined as the wavelength corresponding to the reflectance midpoint between maximum and minimum reflectance values ($\lambda_{R\text{mid}}$), so that it was not calculated for the species with melanin-based colouration because their reflectance spectra do not present defined peaks. Carotenoid-based plumage spectra present two reflectance peaks (one in the UV region and another in the visible range; Andersson & Prager 2006, Shawkey *et al.* 2006), but hue was calculated here for the visible human region only because the MS only covers the UV range from 360 nm. Hue calculated from measurements taken with the MS was only variable in the case of the Common Bullfinch, but this species presents red plumage and thus maximum reflectance values are always (and as expected for this colour) located at 700 nm, so an alternative measure of hue was used (wavelength at the reflectance midpoint between maximum and minimum reflectance values ($\lambda_{R\text{mid}}$; Montgomerie 2006). $\lambda_{R\text{mid}}$ was not used to calculate hue in the rest of the species because the wavelength corresponding to minimum reflectance did not vary among the specimens.

Relationships between variables of brightness

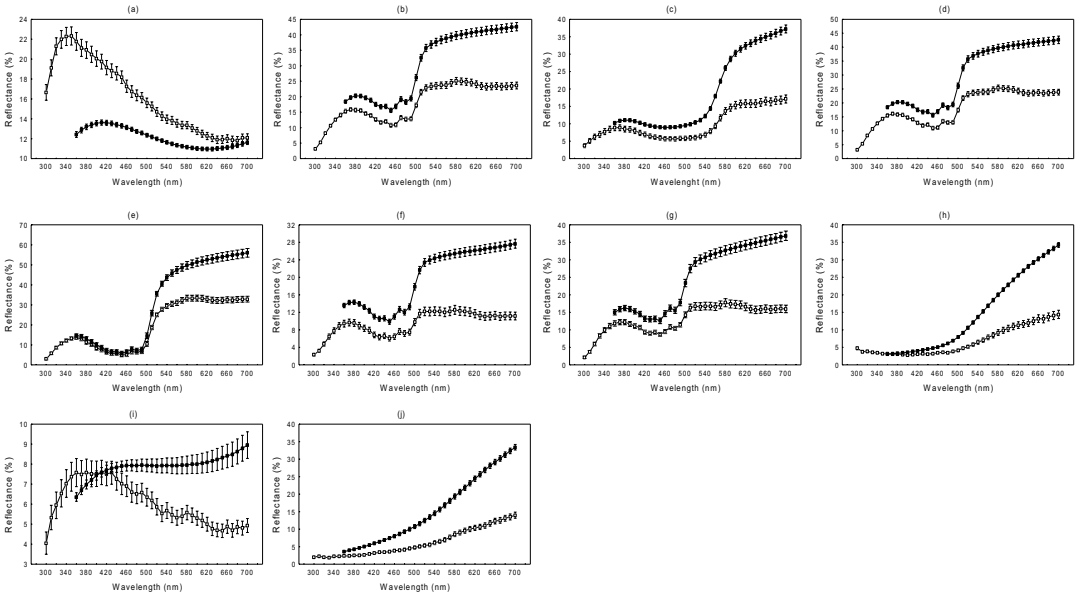

Fig. 1. Spectral reflectance (± SE) of the breast plumage of seven species of passerines obtained with two different spectrophotometers (white squares: Ocean Optics USB2000; black squares: Minolta CM-2600d). (a) *Cyanopica cyanus*; (b) *Emberiza cirlus*; (c) *Pyrrhula pyrrhula*; (d) *Serinus serinus*; (e) *Oriolus oriolus*; (f) *Parus caeruleus*; (g) *Parus major*; (h) *Erithacus rubecula*; (i) *Monticola solitarius*; (j) *Saxicola torquata*. Data are provided in 10 nm intervals.

and chroma obtained from both spectrophotometers were explored through Pearson's correlations tests. Inspections of residuals revealed that normality assumptions were fulfilled. In the case of hue, Spearman rank correlation tests were used.

3. Results

The shapes of the reflectance-spectra curves obtained for the 10 bird species were quite similar using both instruments, but the MS provided higher values of reflectance across the entire reflectance range in all cases, except the Azure-winged Magpie (Fig. 1). The curves only overlapped in the case of the Golden Oriole, and only in the range 360–500 nm (Fig. 1e). Differences were found in the shape of the curves of the species with carotenoid-based plumage between 500 nm and 700 nm, as the spectra obtained from the MS provided a uniformly increasing curve in that range, contrasting with the relatively flat curves obtained from the OOS (see Fig. 1b–g). Indeed, this fact prevented us from comparing the hue obtained from both spectrophotometers, as the value of this variable calculated from data obtained from the MS was always 700 nm (mean hue ± SD obtained from the OOS for the Cirl Bunting: 581 ± 4.47 nm; European Serin: 581 ± 4.47 nm; Golden Oriole: 597.5 ± 23.37 nm; Blue Tit: 570 ± 21.02 nm; Great Tit: 577 ± 13.41 nm). Thus, the hue of spectral curves changed dramatically from one apparatus to another, being located at the spectral range perceived as yellow by humans when data were from the OOS and at the red range (without variance) when they were from the MS. Hue values for the Common Bullfinch calculated with data from both spectrophotometers were not correlated (OOS: 588 ± 5.08 nm, MS: 551 ± 2.49 nm; $r_s = -0.16$, $N = 14$, $P = 0.584$).

As expected from the differences in the spectral curves (Fig. 1), not all colour variables were correlated. In particular, for the total and long-wavelength brightness, no significant correlations were found for any of the species with carotenoid-based plumage, except for Great Tit (total brightness) and Golden Oriole and Blue Tit (long-wavelength brightness). In contrast, the measures of short-wavelength brightness were highly correlated, except for Blue Tit (Table 1). None of the

Table 1. Pearson's correlation tests of colour measurements between two spectrophotometers (Ocean Optics USB2000 and Minolta CM-2600d) on the plumage of museum specimens of passerine birds. Significant correlations are marked in bold. C = carotenoid-based; M = melanin-based; S = structural. Superscript numbers indicate source of information on the colour-producing mechanism (see footnote).

Family	Species	*N*	Colour type	Total brightness	Short-wavelength brightness	Long-wavelength brightness	Long-wavelength chroma	Carotenoid chroma
Corvidae	*Cyanopica cyanus*	20	$S^{1,2}$	$r = 0.41$, $P = 0.070$	**$r = 0.45$, $P = 0.047$**	$r = 0.39$, $P = 0.085$	**$r = 0.50$, $P = 0.026$**	–
Emberizidae	*Emberiza cirlus*	20	$C^{1,3}$	$r = 0.44$, $P = 0.053$	**$r = 0.58$, $P = 0.007$**	$r = 0.40$, $P = 0.077$	**$r = 0.61$, $P = 0.005$**	**$r = 0.73$, $P < 0.001$**
Fringillidae	*Pyrrhula pyrrhula*	14	C^{4}	$r = -0.18$, $P = 0.528$	$r = -0.00$, $P = 0.996$	$r = -0.16$, $P = 0.574$	$r = 0.38$, $P = 0.180$	$r = 0.49$, $P = 0.073$
	Serinus serinus	20	C^{4}	$r = 0.40$, $P = 0.080$	**$r = 0.54$, $P = 0.014$**	$r = 0.37$, $P = 0.111$	**$r = 0.61$, $P = 0.005$**	**$r = 0.73$, $P < 0.001$**
Oriolidae	*Oriolus oriolus*	20	C^{4}	$r = 0.37$, $P = 0.106$	**$r = 0.71$, $P < 0.001$**	**$r = 0.52$, $P = 0.018$**	**$r = 0.83$, $P < 0.001$**	**$r = 0.79$, $P < 0.001$**
Paridae	*Parus caeruleus*	20	C^{4}	$r = 0.44$, $P = 0.055$	$r = 0.27$, $P = 0.248$	**$r = 0.47$, $P = 0.036$**	$r = 0.22$, $P = 0.358$	$r = 0.31$, $P = 0.183$
	Parus major	20	C^{4}	**$r = 0.46$, $P = 0.039$**	**$r = 0.56$, $P = 0.009$**	$r = 0.43$, $P = 0.059$	**$r = 0.48$, $P = 0.033$**	**$r = 0.55$, $P = 0.012$**
Turdidae	*Erithacus rubecula*	20	$M^{1,5,6}$	$r = 0.14$, $P = 0.541$	$r = 0.13$, $P = 0.595$	$r = 0.13$, $P = 0.586$	$r = -0.14$, $P = 0.550$	–
	Monticola solitarius	14	$S^{1,2}$	$r = -0.42$, $P = 0.130$	$r = 0.03$, $P = 0.905$	$r = -0.38$, $P = 0.174$	**$r = 0.88$, $P < 0.001$**	–
	Saxicola torquata	20	$M^{1,5,6}$	**$r = 0.74$, $P < 0.001$**	**$r = 0.73$, $P < 0.001$**	**$r = 0.75$, $P < 0.001$**	**$r = 0.69$, $P < 0.001$**	–

1 = deduced from the shape of the reflectance spectrum; 2 = Prum (2006); 3 = Figuerola *et al.* (1999), 4 = McGraw (2006a); 5 = McGraw (2006b); 6 = J. J. Negro, pers. comm.

brightness variables were correlated with structural colouration, except for Blue Rock Thrush, but measures of short-wavelength brightness were consistent in Azure-winged Magpie. Strong correlations were found for all variables in Common Stonechat, with melanin-based colouration, but none of them were correlated for Robin (Table 1). We also found correlations between the measures of chroma obtained from both spectrophotometers, with the exceptions of Blue Tit and Robin (Table 1).

4. Discussion

We showed that some plumage colour measurements obtained from two commonly-used spectrophotometers with the settings most often employed were not correlated, which is a sufficient argument to call for caution. Our results thus indicate that, while any study that compares the colour of different individuals measured with the same spectrophotometer should be valid, conclusions obtained from studies conducted with different spectrophotometric instruments (spectrophotometers, light sources, etc.) may not be comparable.

A look at the reflectance spectra of the species considered here suggests that the main difference between the two spectrophotometric instruments is due to the shape of the curves at long wavelengths in the species with carotenoid-based plumage (Fig. 1b–g). The reflectance curves of yellow plumage are characterized by a UV peak, minimum reflectance values at short wavelengths (coinciding with the maximum in carotenoid absorbance), followed by a rapid increase in reflectance ending in a plateau at longer wavelengths (e.g., Andersson & Prager 2006, Shawkey *et al.* 2006). This characteristic curve is generated by the absorbance effect of carotenoids on a white underlying light created by the structure of feathers (Shawkey & Hill 2005, Shawkey *et al.* 2006). Because the white light produces a constant reflectance across the spectral range, the plateau arises simply because of the absence of effects of carotenoids on long wavelengths. However, the reflectance spectra that we obtained from the MS showed a steady increase in that region, contrasting with the flat curves that are obtained from the OOS; see Mays *et al.* (2004) and Galván and Sanz (2006) for examples obtaining a similar curve with a technically similar spectrophotometer model and light source used here. Interestingly, the same type of curve has also been obtained by Bleiweiss (2004, 2005) with an integrating sphere (i.e., diffuse light). Because we also found that some colour variables were not correlated (see below), the conclusions drawn in spectrophotometric studies may depend on the type of instrument used.

Andersson and Prager (2006) suggested that integrating spheres rather than colour signals should be used in measuring ambient irradiance. Also Bleiweiss (2005) used an integrating sphere to measure colour properties of carotenoid-based plumage and concluded that the observed lack of association between UV and visible hue was due to a restricted variation in the location of the UV peak. However, long wavelength hue may not be a useful measurement in carotenoid-based colour due to the flat shape of this region (Montgomerie 2006). Thus, the use of an integrating sphere by the MS may have caused the differences between the two spectrophotometers compared here. Alternatively, the use of different white standards with both spectrophotometers may also have caused these differences.

A similar explanation can be made for the differences in the spectral curves from skins of the Common Bullfinch. The main carotenoid pigments that this species presents make its plumage appear red (McGraw 2006a). Therefore, the plateau at long-wavelengths, found in the spectra of yellow carotenoid-based colours, does not exist because reflectance steadily increases with wavelength. However, at long wavelengths the curve obtained from the MS had a higher reflectance than that from the OOS. These differences may result from the lack of significant correlations in total and long-wavelength brightness, calculated from data of both spectrophotometers for the majority of species with carotenoid-based plumage and the species with structural colouration.

We found contradictory results in the species with melanin-based colouration. All the colour variables were strongly correlated in the Common Stonechat, while none of the correlations for the Robin were significant. This lack of correlation was not expected, as the absorbance spectrum of melanin is linear and does not present peaks as in the case of carotenoids (McGraw 2006b). In any

case, differences in the output of light sources used in both spectrophotometers may also cause some discrepancies. The problem is especially important for brightness, which is the colour-related variable that better reflects the melanin content of feathers (McGraw 2006b).

The figure for the Blue Rock Thrush, with structural colouration, had clear differences between curves generated by the two spectrophotometers. OOS produced a peak in the UV, followed by a steady decrease in reflectance, but no clues suggest a UV peak in the curve obtained from the MS, not even with an increase in reflectance at long wavelengths that should result in a melanin-based plumage curve. An additional source of error then arises: although the plumage of male Blue Rock Thrush is almost uniform blue, some individuals present a few brown (melanin-based) feathers alternating with blue ones, such that the large diameter of the reading area of the MS (8 mm), compared to that of the OOS (1 mm), could lead us to include these brown feathers in the measurement even after placing the center of the reading area in a blue area. This could explain the discrepancy in curve shapes between the spectrophotometers. However, this is not a caveat of the apparatus, and could be avoided by carefully choosing particularly uniform plumage areas. Indeed, for the Azure-winged Magpie, whose wings have completely uniform blue areas, produced colour measurements that correlated well between the two spectrophotometers. However, discrepancies at long wavelengths again occurred, indicating that measurements of total brightness are not significantly correlated.

In conclusion, generalizations on colour brightness arising from studies carried out with different spectrophotometric instruments should be made with caution. In particular, we do not recommend the use of the MS for the study of brightness and hues of colouration that present unique UV peaks even between 360 nm and 400 nm, which includes both structural and carotenoid-based colours. Chroma variables taken by both spectrophotometers may be compared in any colour type without considering reflectance in the UV range. Finally, any colour variables taken by both spectrophotometers in species presenting melanin-based colouration can be compared. In those cases in which we do not recommend the use of the MS, any comparison of results obtained with the OOS or similar spectrophotometers should only be made after ensuring that all technical parameters (i.e., light sources, probes, white standards, etc.) are similarly set in both instruments.

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Höyhenpuvun värin mittaus eri spektrofotometreillä

Työssä tarkasteltiin kahden spektrofotometrin (Ocean Optics USB2000 ja Minolta CM-2600d) samankaltaisuuksia lintujen höyhenpukujen vertailuissa tavoitteena selvittää, ovatko eri laitteilla tehdyt tutkimukset vertailukelpoisia. Spektrofotometrien tuottamasta aineistosta tutkittiin erityisesti värien kirkkautta, erottelukykyä ja vivahteikkuutta käyttämällä kymmenen varpuslintulajin nahkoja. Joidenkin värimittausten tulokset laitteiden välillä eivät korreloineet kovin voimakkaasti, minkä vuoksi eri tutkimuksien tuloksia tulisi vertailla varovaisesti.

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