Methods in Ecology and Evolution

BRITISH ECOLOGICAL SOCIETY

Methods in Ecology and Evolution 2017

doi: 10.1111/2041-210X.12801

Colour patch size and measurement error using reflectance spectrophotometry

Arnaud Badiane*,†,[1,2], Guillem Pérez i de Lanuza†,[3], María del Carmen García-Custodio[2], Pau Carazo[2] and Enrique Font[2]

[1]*Department of Biological Sciences, Macquarie University, Sydney, NSW 2109, Australia;* [2]*Ethology Lab, Cavanilles Institute of Biodiversity and Evolutionary Biology, University of Valencia, Valencia APDO 22085, 46071, Spain; and* [3]*CIBIO, Research Centre in Biodiversity and Genetic Resources, InBIO, University of Porto, Institute of Agrarian Sciences of Vairão, R. Padre Armando Quintas, 4485-661 Vairão, Portugal*

Summary

1. Over the past 20 years, portable and relatively affordable spectrophotometers have greatly advanced the study of animal coloration. However, the small size of many colour patches poses methodological challenges that have not, to date, been assessed in the literature. Here, we tackle this issue for a reflectance spectrophotometry set-up widely used in ecology and evolution (the beam method).

2. We reviewed the literature on animal coloration reporting the use of reflectance spectrophotometry to explore how the minimum measurable size of a colour patch is determined. We then used coloured plastic sheets to create artificial colour patches, and quantify the relationship between colour patch size and distortions induced by resulting chimeric spectra (spectra contaminated by an adjacent colour patch). Finally, we assessed the generality of our findings using natural colour spots in the lizard *Podarcis muralis*, as a biologically realistic model.

3. We found a lack of consensus in the literature, frequently resulting in the rejection of valid data or the potential inclusion of unreliable data. As expected, we show that decreasing colour patch size reduces the reliability of reflectance measurements, but also that spectral distortions resulting from chimeric spectra depend on patch/background colour combinations. We found similar results using natural colour spots in *P. muralis*.

4. We propose a series of steps to avoid the pitfalls described above. First, we provide guidelines on how to identify chimeric spectra and estimate the minimum size of a measurable colour patch in order to avoid them. Second, we show that reducing the probe-to-surface distance allows for more accurate measurements and therefore improves the spectrophotometric assessment of small colour patches. Third, we suggest that, as a general rule of thumb, very small (< 2 mm) colour patches should be avoided when using traditional spectrophotometry methods.

Key-words: coloration, colour, reflectance, spectrometry

Introduction

Since the advent of relatively affordable, portable spectrophotometers, reflectance spectrophotometry has been widely used for the objective measurement of animal coloration in many taxa (Endler & Thery 1996; Hunt *et al.* 1998, 1999; Macedonia 2001; Marshall *et al.* 2003; Siddiqi *et al.* 2004; Lim & Li 2006; Whiting *et al.* 2006; Rutowski *et al.* 2007; Kemp, Reznick & Grether 2008). Although different research groups currently active in animal coloration research use various spectrophotometers and recording set-ups, most use a modification of the beam method described by Endler (1990) and adhere to the recommendations issued by Andersson & Prager (2006; see also Eaton & Lanyon 2003; Eaton 2005).

Briefly, this entails placing the probe of a reflectance spectrophotometer at a fixed distance and angle over the target colour patch. There are two options regarding colour patch illumination. One is to use separate reading and illumination probes, which offers multiple options regarding colour measurement geometry since the angle of both probes can be changed relative to each other and to the target patch (azimuth and elevation; Johnsen 2016). This set up is often used in studies measuring iridescent or glossy colours (Fleishman, Leal & Sheehan 2006; Kemp & Rutowski 2007; Cummings *et al.* 2008; Pérez i de Lanuza & Font 2016). Most modern spectrophotometers (e.g. Ocean Optics, Dunedin, FL, USA), however, offer the possibility of using dual-purpose probes that contain both reading and illumination optical fibres. A widely used dual-purpose reading-illumination probe is Ocean Optics model R200-7-VIS-NIR, a bifurcated (Y-shaped) probe with six illumination optical fibres (200 μm in diameter) surrounding a single reading fibre (200 μm in diameter). With dual-purpose probes, researchers select a constant angle between the measured surface and the probe – the same angle for the reading and illumination fibres. The most common measuring

*Correspondence author. E-mail: arnaud.badiane@gmail.com

†Both authors contributed equally to this work.

geometries use angles of 90° (coincident normal: Andersson & Prager 2006) or 45° (coincident oblique: Andersson & Prager 2006) between the measured surface and the probe. The probe must be positioned so that readings reflect only the radiance from the target colour patch, excluding surrounding coloration. This implies that the effective size of the reading area must be as large as or smaller than the colour patch being measured.

It is often erroneously assumed that the reading area matches the area illuminated by the reflection probe. However, since illumination fibres in dual-purpose probes surround the reading fibre and project a cone of light with a solid angle of 25°, the illuminated area will necessarily be larger than the reading area. The effective size of the reading area hence depends on the acceptance angle of the reading fibre (i.e. sensor field angle; Endler 1990), often supplied by the manufacturer (e.g. 12·7° acceptance angle or 25·4° full acceptance angle for Ocean Optics fibres), the distance between the tip of the probe and the target surface, and the diameter of the reading fibre. While some manufacturers provide guidelines for estimating the approximate size of the reading area (e.g. half the distance between the tip of the probe and the measured surface – Ocean Optics Inc.), the exact diameter D of the reading area can be calculated via the following formula:

$$D = 2 \times d \times \tan\theta + l$$

with d being the distance between the probe and the measured surface, θ being the acceptance angle (i.e. half the full acceptance angle) of the reading fibre, and l being the diameter of the reading fibre. For example, using an Ocean Optics dual-purpose 200-μm-reading/illumination probe with a distance of 5 mm between the probe and the measured surface, the effective size of the reading area is 2·45 mm in diameter, which corresponds to the minimum size of a colour patch that can be reliably measured. Alternatively, to estimate the size of the reading area, in some cases it may be possible to connect the reading fibre to the light source and measure the diameter of the projected light spot, which will, due to the symmetry of optics, correspond to the reading area.

When the target colour patch is smaller than the size of the probe's reading area, the outcome is a chimeric spectrum whose spectral properties are a combination of the reflectance of both the target patch and its surroundings. The spectrum is therefore contaminated. Researchers often eschew measuring very small colour patches because they may yield unreliable data (e.g. Eaton & Lanyon 2003; Prum, Andersson & Torres 2003; Mullen 2006; Burns & Shultz 2012). However, many relatively small colour patches may be biologically relevant. This includes some of the most interesting colour patches displayed by insects, arachnids, fish, amphibians or lizards, such as the conspicuous ultraviolet (UV)-blue patches found on some of the outer-ventral scales (OVS) of many lacertid lizards, which probably function as social signals (Pérez i de Lanuza, Carazo & Font 2014; Martin *et al.* 2015). In these circumstances, researchers using standard equipment must estimate based on intuition the minimum size of a colour patch that can be reliably measured (S_{min}), with two major shortcomings. If S_{min} is overestimated (i.e. too large), potentially reliable data will be lost and final sample size can be affected. Alternatively, if S_{min} is underestimated (i.e. too small), chimeric, potentially unreliable data will be incorporated into the results.

Several recent reviews have provided practical guidelines for obtaining reflectance spectra from biological samples (Andersson & Prager 2006; Fleishman, Leal & Sheehan 2006; Kemp *et al.* 2015; White *et al.* 2015), but none have addressed the issue of colour patch size (but see Galván & Sanz 2010). The overarching aim of this study is to fill this gap by addressing the most common problems arising from the spectrophotometric assessment of small colour patches. First, we review estimates of S_{min} in the literature in order to explore whether there is a consensus regarding the minimum size of a measurable colour patch (and its relationship with the effective measurement size used) across available studies. Second, we empirically quantify the relationship between colour patch size and the distortions introduced by chimeric spectra on the 'true' hue, luminance ('brightness') and chroma of a measured colour patch. Chimeric spectra could affect different colour variables in different ways. Luminance and chroma should be particularly sensitive to spectral contamination because they depend on the shape of the entire spectral curve, whereas the most common estimation of hue depends on primary or secondary peak location (i.e. maximum slopes, Endler 1990). Third, we study how different combinations of patch and background colour may affect spectral distortions caused by chimeric spectra (i.e., are particular combinations of patch vs. background more prone to spectral distortion?). Fourth, we estimate whether reducing the distance between the probe and the measured surface, by decreasing the effective size of the measuring area, allows the measurement of smaller patches without spectral distortion. Fifth, we explore the effect of chimeric spectra using the UV-blue patches of *P. muralis* as a realistic biological model to test our conclusions regarding minimum patch size. Finally, we outline a series of guidelines to avoid the potential caveats identified in this study.

Materials and methods

LITERATURE REVIEW

We reviewed 152 papers published between 1999 and 2016 in peer-reviewed journals, in which the beam method was used to assess the coloration of macroscopic living organisms. We searched for papers with the Google Scholar (Google Inc.) search engine, using the key words 'spectrophotometry', 'colour signals' and 'coloration', and supplemented the results with those of a search through our own private libraries. This searching method allowed us to achieve a sufficiently large sample size for our purposes, but it must be noted that our aim was not to conduct an exhaustive search. We only considered articles in which at least one of the following procedural details was provided: the angle between the reading fibre and the measured surface, the distance between the reading fibre and the measured surface, or the minimum size of colour patches measured. We excluded methodological studies. We also excluded studies using collimating and microscopic lenses (e.g. Fleishman, Leal & Sheehan 2006). The reviewed articles encompass a wide variety of taxa, including mammals, birds, squamates, chelonians,

amphibians, arachnids, insects, crustaceans, ray-finned fishes, and plants. Different types of surfaces were measured in these studies, including scales, eggshells, exoskeletons, feathers, skins, wings, and tongues. We calculated the percentage of studies that provided information about both the S_{min} used and the distance between the tip of the probe and the measured surface (*d*). Within this subset, we calculated the percentage of studies that over-estimated, under-estimated, or estimated the S_{min} correctly according to the manufacturer's specifications (i.e. diameter of the effective reading area equal to half the distance between the probe and the measured surface).

REFLECTANCE MEASUREMENTS

We performed two experiments – with artificial materials and with natural samples – using spectral reflectance measurements. Reflectance spectra were obtained indoors, in a darkened room, using a standard USB 2000 portable diode-array spectrometer with a R200-7-VIS-NIR reading-illumination probe (Ocean Optics Inc.) and a notebook computer running Ocean Optics SpectraSuite software (Ocean Optics Inc.). Full spectrum illumination was provided by a PX-2 xenon strobe light source (Ocean Optics Inc.). Spectra were recorded in 0·37-nm intervals and expressed as the percentage of light reflected relative to a certified Spectralon white diffuse reflectance standard (Labsphere, North Sutton, NH, USA). A dark current reading was also taken and subtracted from the signal just prior to gathering the spectral data. Reflectance measurements were averaged over 5 nm, using a kernel smoothing function. We set integration time according to the distance used (i.e. 50 ms at 3 mm, 34 ms at 5 mm), scans to average to 20, and boxcar width to 10.

For data acquisition, the probe was hand-held over the centre of the colour patch, and approximately perpendicular to the patch surface (hence illumination and recording angles were both 90°). An entomological pin attached to the side of the probe (nylon head down) allowed us to maintain a constant distance between the tip of the probe and the surface of the target colour patch.

EXPERIMENT 1: RELIABILITY OF MEASUREMENT OF SMALL COLOUR PATCHES

To determine the smallest colour patch that can be reliably measured with a dual-purpose beam spectrophotometer, we took reflectance measurements from artificial patches varying in colour (i.e. spectral distribution independent of intensity) and size. To create artificial colour patches, we used six Lambertian matte-coloured sheets (black, white, blue, green, yellow and red) made of rigid opaque plastic (thickness = 0·8 mm). In each sheet, we used a laser-cutting machine (Chanke, CO_2 laser tube, 100 W, beam intensity set at 10%) to create five circular holes of different diameters (1, 1·5, 2, 2·5, 3 mm). We obtained artificial patches by overlaying a sheet with a hole (i.e. background 'surround' colour) over an unperforated sheet (i.e. focal patch 'centre' colour – Appendix S1, Supporting Information). We recorded reflectance spectra of all the artificial patches resulting from the factorial combination of the 30 different artificial surround-centre combinations (excluding same colour combinations) and five different patch sizes, and repeated the measurements with a 5 and 3 mm distance between the probe and the focal patch (i.e. centre). We repeated each measurement five times (i.e. lifting then repositioning the probe) for a total of 1800 measurements.

For statistical analyses, spectral data were treated in R v.2.3.2 (R Core Team, 2014) using the software package PAVO (Maia *et al.* 2013). First, each spectrum was further smoothed using a span of 0·2. Then, five colorimetric variables were extracted, namely luminance (i.e. brightness or spectral intensity, defined as the sum of individual wavelength amplitudes between 400 nm and 700 nm, $R_{400–700}$), blue chroma (C_{BLUE}, $R_{400–510}/R_{400–700}$), green chroma (C_{GREEN}, $R_{510–605}/R_{400–700}$), red chroma (C_{RED}, $R_{605–700}/R_{400–700}$) and hue (defined as the wavelength at the maximum reflectance peak; H). The three chroma variables were defined arbitrarily, as implemented as a default option in PAVO. As none of the colour sheets had any relevant reflection in the UV range, we did not include any UV metric.

Statistical modelling was done using the R v.2.3.2 packages LME4 (Bates *et al.* 2015) and Multcomp (Hothorn, Bretz & Westfall 2008). First, in order to run linear models, we grouped our data by focal patch colour. We included as response variables the five colorimetric variables, namely B, C_{BLUE}, C_{GREEN}, C_{RED}, and H. Chroma variables were not considered for black and white focal patch colours. For other colour combinations we used the chroma variables appropriate to each centre patch colour (i.e. C_{GREEN} and C_{RED} for yellow, C_{BLUE} for blue, C_{GREEN} for green, and C_{RED} for red). As predictors, we set the interaction (including main effects) between focal patch diameter and surround colour. We then performed an ANOVA and post hoc Tukey tests on the models. In total, we ran 34 models (17 at a 5-mm probe-to-surface distance and 17 at a 3-mm distance).

In order to explore the differences within each patch diameter (i.e. 1, 1·5, 2, 2·5, 3), we created a subset of measurements for each diameter. Each subset also included a reference, which is a measurement obtained for a centre patch of 3 mm. According to the spectrophotometer manufacturer (Ocean Optics Inc.), at a 5-mm distance, the reading spot diameter should be 2·5 mm, which allows for a non-contaminated measurement for the 3-mm wide reference. We included the colorimetric variables as response variables, and the surround colour as predictor. We then performed ANOVA and post-hoc Tukey tests on the fitted models.

EXPERIMENT 2: EFFECT OF CHIMERIC SPECTRA ON A LIZARD'S COLOUR PATCHES

In order to quantify the impact of chimeric spectra on spectral analyses of small natural patches, we used the common wall lizard, *Podarcis muralis* as a model. *P. muralis* is a small lacertid with a widespread distribution across Europe, and shows a cryptic dorsal coloration and conspicuous ventral and lateral coloration (Pérez i de Lanuza & Font 2015). Ventral coloration is polymorphic, with three pure morphs (i.e. orange, yellow, and white) and two intermediate morphs (i.e. orange-white and orange-yellow), and seems to play a role in mating patterns (Pérez i de Lanuza, Font & Carazo 2013; Pérez i de Lanuza, Font & Carretero 2016). Laterally, these lizards display small UV-blue spots on some of the OVS, interspersed with black spots and the long-wavelength-biased ventral background. The UV-blue spots are believed to act as social signals and are sexually dimorphic (i.e. they differ in colour between males and females and are smaller and less numerous in females – Pérez i de Lanuza, Carazo & Font 2014; Martin *et al.* 2015). The size of these UV-blue spots can vary from a few mm to less than 0·5 mm in diameter (personal observation), and are therefore a challenge for the acquisition of reliable spectrophotometric measurements.

We measured the reflectance of the UV-blue spots in the OVS of 47 adult males of *P. muralis* from the Pyrenees (France) ensuring that the reading area included a single UV-blue spot. Additionally, we forced chimeric spectral measurements by intentionally displacing the illuminated area so that it overlaid the UV-blue spot and part of the adjacent background coloration (black for 34 lizards and white, yellow or orange background for 46 lizards). From the recorded spectra, we

extracted hue (i.e. wavelength of maximum reflectance, H), UV chroma (C_{UV}, $R_{300–400}/R_{300–700}$) and luminance (B). We used correlation (Spearman or Pearson) and paired comparison tests (Wilcoxon or paired *t*-test) to evaluate the impact of using variables obtained from chimeric spectra.

Results

LITERATURE REVIEW

Of the 152 articles reviewed, 59 (39%) provided information on the S_{min} used, with reported values varying between 0·8 and 6 mm in diameter depending on the probe to target distance. One hundred and forty-seven (97%) studies provided information about the angle between the probe and the measured surface (30–90°). However, the great majority of studies used 45° (33%) and 90° (57%) angles. Information about the distance between the probe and the measured surface was provided by 61 (40%) studies, and it varied between 1 and 17 mm with the most commonly used distance being 5 mm (29 studies). Only 32 (21%) studies provided information about both the S_{min} and the distance between the probe and the measured surface. From these 32, 4 (12·5%) studies correctly estimated the effective size of the reading probe according to the manufacturer's specifications, while 6 (19%) overestimated it (i.e. set a S_{min} larger than necessary), and 22 (69%) underestimated it (i.e. set a S_{min} smaller than required, thus potentially leading to some chimeric spectra). Among the 22 studies underestimating S_{min}, 13 (59%) did so by 20% or less, while 9 (41%) did so by more than 20%.

EXPERIMENT 1

Our results show that decreasing colour patch size reduces the reliability of reflectance measurements (Figs 1 and 2). At a 5-mm distance, luminance measurements begin to be unreliable at a patch size of 2·5 mm (e.g. for a red patch against a blue surrounding; Fig. 2). At a 5-mm distance, consistent spectrum distortions (i.e. deformations of the spectral curve compared to the reference spectral curve) occur for colour patches with a diameter of 1·5 mm or less. For a colour patch 1·5 mm in diameter, luminance and chroma are unreliable whereas hue remains relatively unaffected because the general spectral shape is conserved (Fig. S2). However, for a colour patch smaller than 1·5 mm, spectral distortions are generalized and none of the colorimetric variables can be considered reliable. The resulting chimeric spectra are the result of spectral contamination by the background colour. Interestingly, the magnitude of this effect depends on the colorimetric properties of the background colour, as discussed below.

The most striking spectral distortions are obtained at a 5-mm distance for a 1-mm diameter colour patch. Figure 1 illustrates different types of distortions. First, a second reflectance peak appears in the blue range of the spectrum (400–500 nm), as a consequence of contamination by the blue background. This is probably the most critical distortion because it affects directly the hue at shorter wavelengths, causing an increase in luminance and a decrease in chroma (Fig. 2). Second, in the red part of the spectrum (600–700 nm), the reflectance peak is reduced by

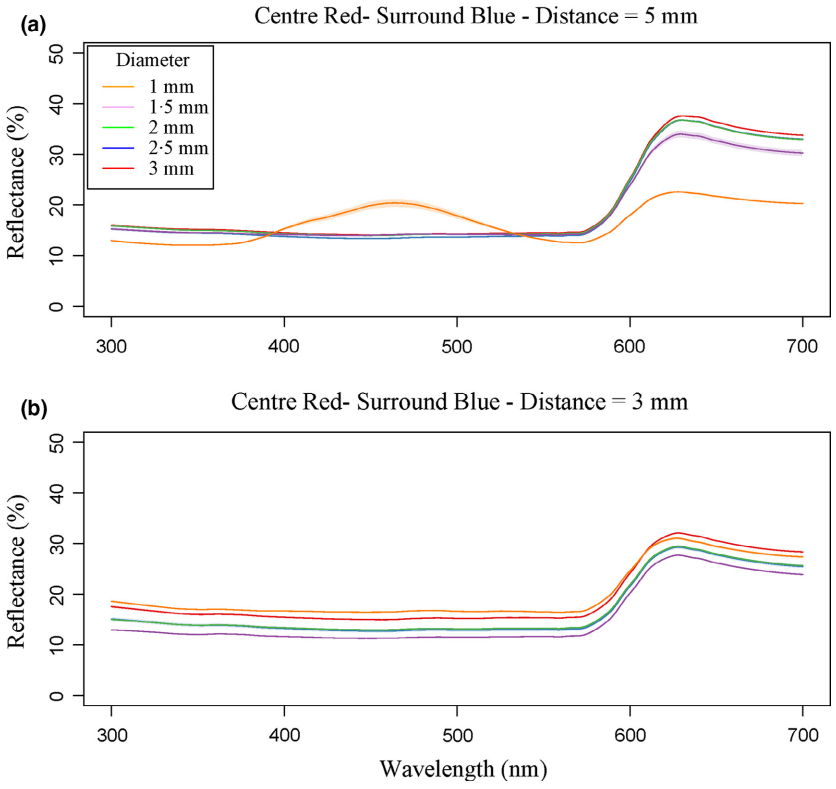

Fig. 1. Spectra obtained for a red colour patch with a surrounding blue colour at all five patch's diameters (i.e. 1, 1·5, 2, 2·5, 3 mm), at a distance of 5 (a) and 3 mm (b). A secondary reflectance peak appears at a distance of 5 mm for a 1-mm patch diameter corresponding to the reflectance peak location of the surrounding blue colour. Also, reflectance values for the primary peak corresponding to the red focal patch are strongly reduced.

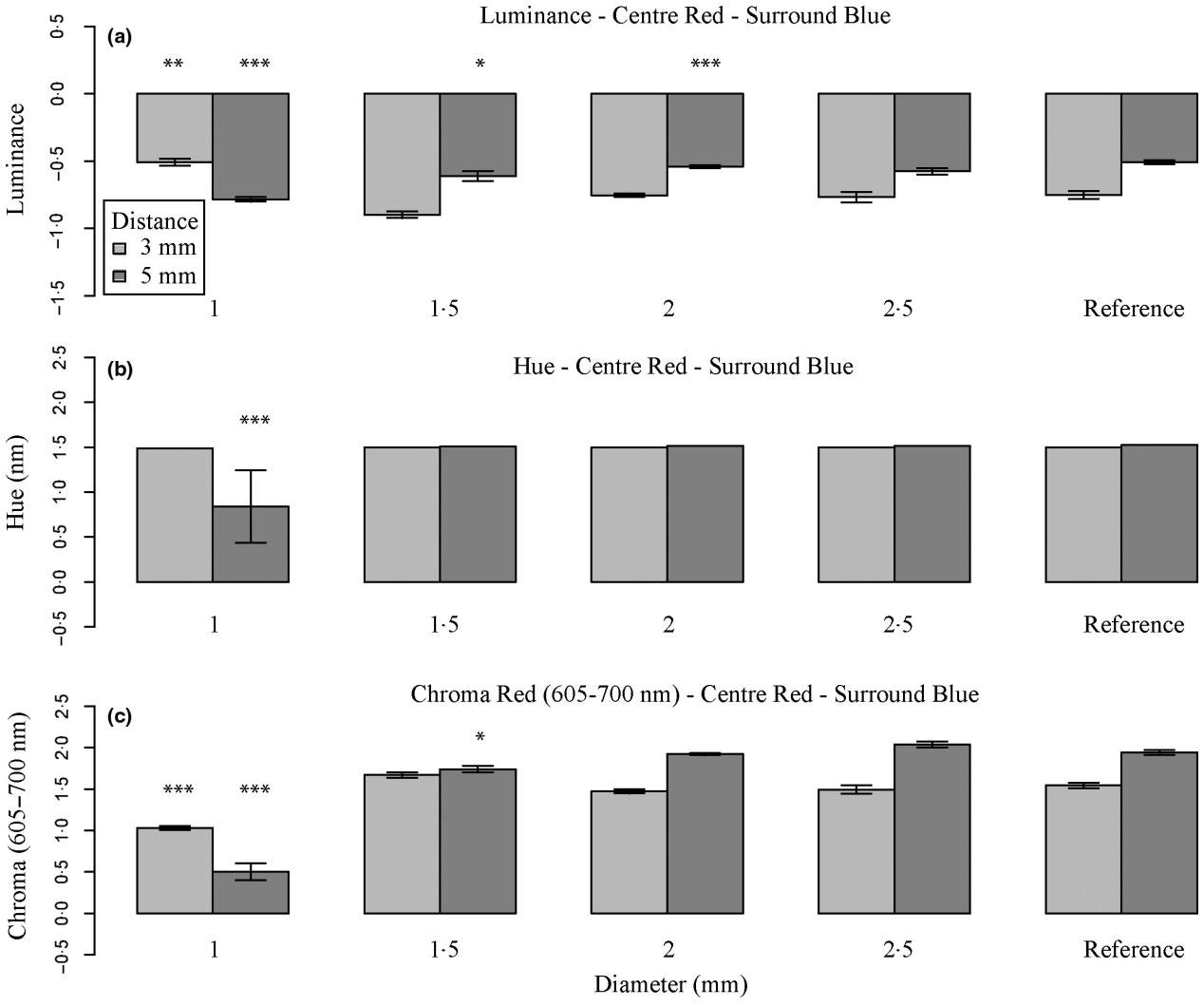

Fig. 2. Plots presenting luminance (a), hue (b), and chroma-red (c) for a red centre patch (focal patch) and a blue surround colour at distances of 3 and 5 mm, and for different colour patch's diameters (i.e. 1, 1·5, 2, 2·5 mm) and a reference obtained at colour patch diameter of 3 mm. Significant differences with the reference are indicated (i.e. * < 0·05; ** < 0·01; *** < 0·001).

approximately half of its non-contaminated values, which inevitably affects luminance and chroma. Nevertheless, if peak shape is conserved and if no secondary peak is present, hue may remain unaffected and can therefore be trusted, at least with these colour combinations. This is illustrated in Fig. 1 with the spectrum obtained at a 5-mm distance and a 1·5-mm diameter. Peak shape remains very similar, only with lower luminance values. The consequence is an increase in chroma, but hue is conserved.

Unsurprisingly, our results also suggest that a smaller distance between the reflection probe and the colour patch (3 mm vs. 5 mm) provides more accurate measurements (Figs 1 and S2). For a colour patch of 1·5 mm in diameter, the distortions obtained at 5 mm disappear at a 3-mm distance. Only minor alterations affect the spectrum and colorimetric variables can be accurately estimated. For a colour patch 1 mm in diameter, luminance and chroma often become unreliable while hue can still be accurately measured.

EXPERIMENT 2

With our natural sample (UV-blue ventrolateral spots of *P. muralis*), chimeric spectra result in distorted measurements of hue, chroma and luminance. The impact of using chimeric spectra differs depending on the variables and the colours involved in the chimeric measurement (Fig. 3). When a UV-blue and a black patch are involved in a chimeric spectrum, the shape of the spectrum (e.g. peak location) is generally conserved but luminance values drop dramatically ($-40 \pm 3\%$). Remarkably, when UV-blue and orange patches are involved in a chimeric spectrum, a secondary reflectance peak appears, which corresponds to the peak location of the orange belly. Reflectance values obtained from correctly measured (i.e. non-chimeric) UV-blue patches and chimeric spectra are always positively correlated, but strong correlations are restricted to hue for chimeric spectra from black patches and chimeric spectra from belly, and UV chroma for belly chimeric spectra (Table 1). However, values of UV chroma from non-chimeric

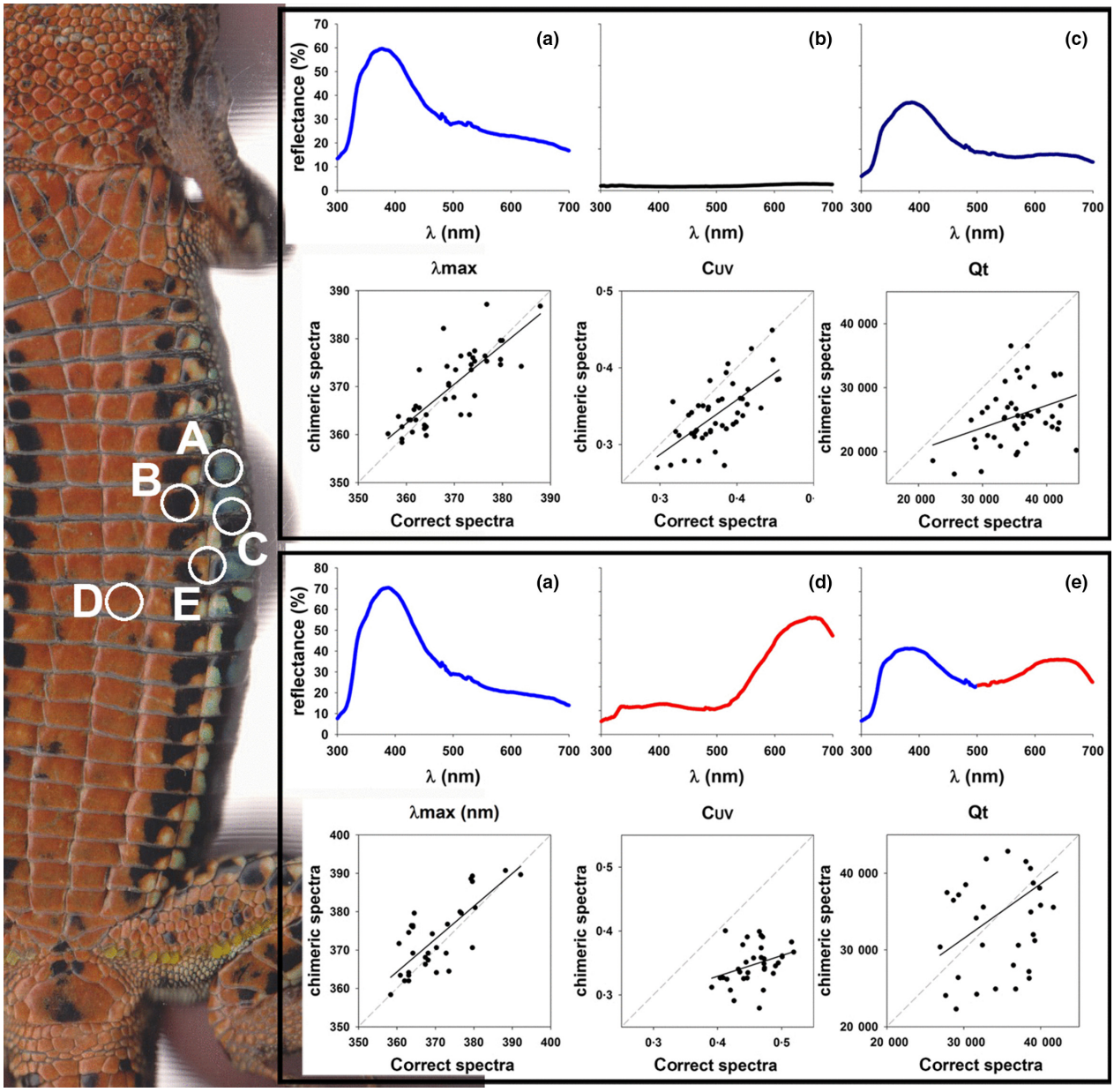

Fig. 3. On the left: a photograph of the outer-ventral scales of *Podarcis muralis* indicating (white empty circles) the colour patches targeted by the reflectance probe, namely a UV-blue patch (a), a black patch (b), a UV-blue and black patch (c), an orange patch (d), and a UV-blue and orange patch (e). On the right: graphs present the spectrum of each colour patch (a, b, c, d, and e), as well as correlation tests between chimeric and nonchimeric spectra for hue, chroma-UV, and luminance. Graph (c) illustrates the typical case of a chimeric spectrum, involving two colours contrasting in brightness, resulting in a drop of reflectance values while conserving the spectral shape. Graph (e) exemplifies the case of a chimeric spectrum of two colours contrasting in terms of peak location (short- vs. long-wavelength reflectance peak), resulting in a secondary peak.

spectra and values from chimeric spectra involving the belly are significantly different, indicating that chroma is strongly affected by chimeric measurements (Table 1).

Discussion

To the best of our knowledge, no study to date has addressed the difficulties arising from the spectrophotometric assessment of small colour patches. Our review of the literature reveals a lack of consensus about the minimum size of a colour patch that can be reliably measured (S_{min}), even among researchers using the same equipment. Our empirical tests also provide a quantification of the relationship between a colour patch size and the probability of obtaining a chimeric spectrum, showing how spectral distortions affect colour patches of decreasing size. Besides, we present evidence that these spectral distortions vary for different colour combinations (i.e. patch centre vs. background colour) and can be reduced by decreasing the distance between the probe and the measured surface. Finally, we show that these experimental results apply to live samples through spectral reflectance measurements of UV-blue spots in the lizard *P. muralis*.

Table 1. Correlation analyses and paired comparisons between non-chimeric and chimeric spectra obtained combining UV-blue and black patches ($N = 46$) and combining UV-blue and belly background colour ($N = 34$). The variable 'Difference' indicates the magnitude (mean ± 1 SEM) of the distortion resulting from using chimeric spectra, obtained subtracting chimeric spectra values from values of non-chimeric spectra (range in parenthesis). 'Correlation' indicates results from correlation analyses for considering values from non-chimeric and chimeric spectra (r_s corresponding to Spearman's test and r to Pearson's test). 'Comparison' indicates results from paired comparisons between values from non-chimeric and chimeric spectra (Z corresponding to Wilcoxon test and t to paired t-test). In bold, results remaining significant after applying the Holm–Bonferroni correction for multiple comparisons

	Difference	Correlation	Comparison
UV-blue + black patch			
Hue (nm)	0·7 ± 0·7	$\mathbf{r_s = 0{\cdot}74}$	$Z = -2{\cdot}61$
	(−9·7 to 14·4)	$\mathbf{P < 0{\cdot}00001}$	$P = 0{\cdot}009$
UV chroma	−0·03 ± 0·01	$r = 0{\cdot}34$	$\mathbf{t = 17{\cdot}76}$
	(−0·11 to 0·04)	$P = 0{\cdot}046$	$\mathbf{P < 0{\cdot}00001}$
Luminance	−9802 ± 799	$r_s = 0{\cdot}39$	$t = -0{\cdot}06$
	(−24 417 to 2108)	$P = 0{\cdot}022$	$P = 0{\cdot}95$
UV-blue + belly			
Hue (nm)	2·9 ± 1·0	$\mathbf{r_s = 0{\cdot}82}$	$Z = -1{\cdot}18$
	(−9·0 to 15·0)	$\mathbf{P < 0{\cdot}00001}$	$P = 0{\cdot}24$
UV chroma	−0·11 ± 0·01	$\mathbf{r = 0{\cdot}67}$	$\mathbf{t = 12{\cdot}27}$
	(−0·01 to 0·19)	$\mathbf{P < 0{\cdot}00001}$	$\mathbf{P < 0{\cdot}00001}$
Luminance	−19 ± 1296	$r_s = 0{\cdot}35$	$\mathbf{t = 7{\cdot}41}$
	(−12203 to 12958)	$P = 0{\cdot}016$	$\mathbf{P < 0{\cdot}00001}$

The methods reported in published studies using reflectance spectrophotometry are not consistent (White *et al.* 2015). Relatively few studies provide the necessary information to assess the way the authors tackled the problem of S_{min} estimation. Also, very few studies follow guidelines given by the manufacturer to estimate the effective size of the reading area, which in turn can be used to estimate S_{min}. Most studies, in fact, underestimate S_{min}, therefore including in the sample potentially chimeric spectra. Interestingly, our experimental results show that the actual S_{min} is approximately 20% smaller than the effective size of the reading area calculated according to the manufacturer's specifications (i.e. no distortions for a 2-mm patch at a 5-mm distance while the effective reading area is, according to the manufacturer, 2·5 mm in diameter – see Figs 1 and S2). We found that 13 (59%) studies (from a total of 22) underestimating the S_{min} do so by less than 20%, which means that their spectral data should not be subject to the spectral distortions described here. However, the remaining 9 (41%) may have inadvertently included some chimeric spectra, unless an unspecified method was used to improve measurement accuracy. As to the studies overestimating S_{min}, it is possible that the authors overestimated the S_{min} to avoid these issues, but there is no information on how this affected their sample size (i.e. how many candidate measurements were discarded). This lack of consensus in the literature underlines the need to standardize the use of reflectance spectrophotometry.

We found that the combination of patch centre and background colours determines the direction of the spectral distortions. Distortions are more likely to occur in the case of contrasting patch centre and background colours. If the two colours strongly differ in hue (short- vs. long-wavelengths colours; e.g. blue vs. red), a second reflectance peak is likely to arise in the location corresponding to the background colour spectral location (e.g. Fig. 1, see spectrum at a 5-mm distance). If they differ widely in terms of luminance (e.g. black and white), two situations typically occur. First, when the background colour is brighter than the centre colour, the spectrum is prone to severe spectral distortion. Conversely, if the patch centre colour is brighter than the background colour, all reflectance values drop equally, which makes luminance and chroma unreliable, but the spectrum shape – and peak location – is conserved, so hue measurements are reliable (Fig. S2). In the latter case, it may be challenging to identify whether the reflectance spectrum corresponds to a chimeric spectrum or to a colour patch with low brightness. Other cases may pose difficulties in the identification of chimeric spectra such as, for example, when a colour patch naturally shows a secondary reflectance peak.

Our findings indicate that reflectance measurements are generally more accurate when the distance between the reflection probe and the colour patch is reduced because the reading area under the reflection probe is reduced, and thus less prone to contamination. Furthermore, each of the six illumination fibres in a standard 7 × 200 dual-purpose probe projects a 25·4° solid angle cone of light. As the reading fibre is located in the centre of the probe, the distance between the probe and the reading surface must be large enough to allow for the six illumination cones to overlap in the centre of the reading area. Otherwise, the reading spot will not be evenly illuminated and the measurement will not be optimal. Therefore, the minimum distance between the probe and the measured surface will depend on the acceptance angle of the illumination and reading fibres, and the distance separating the illumination and reading fibres. For a dual-purpose reading-illumination probe such as Ocean Optics' model R200-7-VIS-NIR, the six illumination cones will not overlap in the centre of the reading area if the probe-to-surface distance is less than 0·9 mm. Moreover, if the probe-to-surface distance is too low, the reduced measured area will be less representative of the overall colour patch (e.g. less homogeneous due to surface irregularities, dead skin or scales) as perceived by receiver animals.

Choosing the appropriate angle between the probe and the measured surface is not straightforward. In theory, in studies of visual communication, the measurement angle must correspond to the angle at which a receiver most often sees a colour patch (Endler 1990). In practice, this angle is difficult to determine and subject to speculative assumptions. Consequently, different angles are used in coloration studies (mostly 45° and 90° – Whiting *et al.* 2006; Font, Pérez i de Lanuza & Sampedro 2009; Martin *et al.* 2013; Pérez i de Lanuza & Font 2014). Here, we used a 90° angle (coincident normal measuring geometry according to Andersson & Prager 2006) for various reasons. First, as suggested by Pohland (2006), an angle of 90° provides both the brightest reflections and the least variability in the resulting spectra, thus improving the data vs. background noise ratio. Second, as the reading area of the reflection

probe is circular, not elliptic, a 90° angle avoids the potential distorting effects of illumination-reading asymmetries and improves precision (Appendix S3). Third, it is generally easier to maintain a constant angle of 90° than any other angle. Some probe holders allow fixed probe to reading surface angles, but they are not suitable for measuring small colour patches because the patch is hidden when the holder is placed flush with the measured surface. A further complication is the angle-dependence of the spectral reflectance of many colour patches, especially for structural colours (Osorio & Ham 2002). A case in point is iridescent coloration. Iridescence (i.e. a change in hue or intensity with viewing angle) has been reported in many taxa (Doucet & Meadows 2009; Meadows *et al.* 2009), including lacertid lizards (Pérez i de Lanuza & Font 2014, 2016). A single measurement is insufficient to characterize the full angle-dependence of these colours (Kemp, Reznick & Grether 2008). For non-iridescent colours, changes in measuring geometry mostly affect luminance (Pohland 2006). Unless the measured surface is particularly glossy and susceptible to specular reflection (e.g. scales of fish and some squamates), we recommend using a 90° probe to reading surface angle.

The small UV-blue reflecting spots in the OVS of *P. muralis* provide a good illustration of the issues arising from chimeric spectra. Indeed, chimeric spectra resulting from the combination of contrasting colours (i.e. short- vs. long-wavelength peak for UV-blue and orange belly, and high vs. low luminance for UV-blue and black spots) match what we would predict based on results of *Experiment 1* – namely, the appearance of a secondary peak for the short wavelength-based UV-blue and the long wavelength-based orange belly, and a decrease in reflectance values in the case of UV-blue and low-luminance black spot. As spectral variables of these UV-blue spots are related to quality indicators of males *P. muralis* (Pérez i de Lanuza, Carazo & Font 2014), chimeric spectra will have harmful consequences on the results and biological interpretations. Furthermore, the use of spectrophotometry to assess small colour patches of live specimens must take into account other aspects. If a study organism presents colour patches varying only in size, we encourage researchers to rely preferably on the larger ones if they are using a standard setup as described here or to use alternative methods that allow measuring small patches (e.g. Marshall *et al.* 2003). If researchers have to measure very small colour patches (< 1 mm), we encourage the use of alternative techniques, for example, techniques involving microscopic or collimating lenses (Fleishman, Leal & Sheehan 2006). In this case, a more complex experimental protocol is often required (e.g. animals may need to be anesthetized) because animals must stand still to obtain reliable measurements. It must also be noted, however, that such methods frequently make it impossible to acquire spectral data in the field. Besides, these methods can raise issues regarding animal ethics, thus the use of less invasive techniques should be preferred when possible.

In summary, to measure small colour patches using standard reflectance spectrophotometry techniques, we recommend that researchers estimate S_{min} in order to avoid potential chimeric spectra, and, when possible, avoid measuring colour patches less than 2 mm in diameter when the probe distance is set at 5 mm. It is also worth stressing that the S_{min} is smaller than the illuminated area under the probe. Finally, we agree with White *et al.* (2015) that researchers need to be more transparent about the settings and methods they use in studies of animal coloration, using spectrophotometry. These simple steps will help ensure reproducibility in biological coloration research.

Authors' contributions

E.F. and G.P.L. conceived and designed the experiments; A.B. reviewed the literature; A.B., G.P.L., and M.G.C. collected the data; A.B., E.F., G.P.L., and P.C. analysed the data. A.B., E.F., G.P.L., and P.C. interpreted the results, drafted and revised the manuscript. All authors gave final approval for publication.

Acknowledgements

We thank Martin Whiting for his invaluable comments on the experimental design and the manuscript. We also thank Ramón Martínez for kindly perforating the holes on the plastic sheets used for Experiment 1. A.B. is funded by an iMQRES doctoral scholarship from Macquarie University. G.P.L. holds a post-doctoral grant (SFRH/BPD/94582/2013) from FCT (Fundação para a Ciência e a Tecnologia, Portugal) under the Programa Operacional Potencial Humano – Quadro de Referência Estratégico Nacional funds from the European Social Fund and Portuguese Ministério da Educação e Ciência. P.C. is funded by a 'Ramon y Cajal' fellowship from the Spanish Government (RYC-2013-12998). Permit to capture and measure the lizards was provided by the Préfecture des Pyrénées Orientales and the Préfecture de l'Ariège (Arrêté n° 2016-2-09).

Data accessibility

Data publicly available in the Dryad Repository https://doi.org/10.5061/dryad.vb549 (Badiane *et al.* 2017).

References

Andersson, S. & Prager, M. (2006) Quantifying colors. *Bird Coloration*, **1**, 41–89.

Badiane, A., Pérez i de Lanuza, G., Garcia-Custodio, M., Carazo, P. & Font, E. (2017) Data from: Colour patch size and measurement error using reflectance spectrophotometry. *Dryad Digital Repository*, https://doi.org/10.5061/dryad.vb549

Bates, D., Maechler, M., Bolker, B. & Walker, S. (2015) Fitting linear mixed-effects models using LME4. *Journal of Statistical Software*, **67**, 1–48.

Burns, K.J. & Shultz, A.J. (2012) Widespread cryptic dichromatism and ultraviolet reflectance in the largest radiation of Neotropical songbirds: implications of accounting for avian vision in the study of plumage evolution. *The Auk*, **129**, 211–221.

Cummings, M.E., Jordão, J.M., Cronin, T.W. & Oliveira, R.F. (2008) Visual ecology of the fiddler crab, *Uca tangeri*: effects of sex, viewer and background on conspicuousness. *Animal Behaviour*, **75**, 175–188.

Doucet, S.M. & Meadows, M.G. (2009) Iridescence: a functional perspective. *Journal of The Royal Society Interface*, **6**, 115–132.

Eaton, M.D. (2005) Human vision fails to distinguish widespread sexual dichromatism among sexually 'monochromatic' birds. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 10942–10946.

Eaton, M.D. & Lanyon, S.M. (2003) The ubiquity of avian ultraviolet plumage reflectance. *Proceedings of the Royal Society of London B: Biological Sciences*, **270**, 1721–1726.

Endler, J.A. (1990) On the measurement and classification of colour in studies of animal colour patterns. *Biological Journal of the Linnean Society*, **41**, 315–352.

Endler, J.A. & Thery, M. (1996) Interacting effects of lek placement, display behavior, ambient light, and color patterns in three neotropical forest-dwelling birds. *American Naturalist*, **148**, 421–452.

Fleishman, L.J., Leal, M. & Sheehan, J. (2006) Illumination geometry, detector position and the objective determination of animal signal colours in natural light. *Animal Behaviour*, **71**, 463–474.

Font, E., Pérez i de Lanuza, G. & Sampedro, C. (2009) Ultraviolet reflectance and cryptic sexual dichromatism in the ocellated lizard, *Lacerta* (*Timon*) *lepida* (Squamata: Lacertidae). *Biological Journal of the Linnean Society*, **97**, 766–780.
Galván, I. & Sanz, J.J. (2010) Measuring plumage colour using different spectrophotometric techniques: a word of caution. *Ornis Fennica*, **87**, 69–76.
Hothorn, T., Bretz, F. & Westfall, P. (2008) Simultaneous inference in general parametric models. *Biometrical Journal*, **50**, 346–363.
Hunt, S., Bennett, A.T., Cuthill, I.C. & Griffiths, R. (1998) Blue tits are ultraviolet tits. *Proceedings of the Royal Society of London B: Biological Sciences*, **265**, 451–455.
Hunt, S., Cuthill, I.C., Bennett, A.T. & Griffiths, R. (1999) Preferences for ultraviolet partners in the blue tit. *Animal Behaviour*, **58**, 809–815.
Johnsen, S. (2016) How to measure color using spectrometers and calibrated photographs. *Journal of Experimental Biology*, **219**, 772–778.
Kemp, D.J., Herberstein, M.E., Fleishman, L.J., Endler, J.A., Bennett, A.T.D., Dyer, A.G., Hart, N.S., Marshall, J. & Whiting, M.J. (2015) An integrative framework for the appraisal of coloration in nature. *The American Naturalist*, **185**, 705–724.
Kemp, D.J., Reznick, D.N. & Grether, G.F. (2008) Ornamental evolution in Trinidadian guppies (*Poecilia reticulata*): insights from sensory processing-based analyses of entire colour patterns. *Biological Journal of the Linnean Society*, **95**, 734–747.
Kemp, D.J. & Rutowski, R.L. (2007) Condition dependence, quantitative genetics, and the potential signal content of iridescent ultraviolet butterfly coloration. *Evolution*, **61**, 168–183.
Lim, M.L. & Li, D. (2006) Extreme ultraviolet sexual dimorphism in jumping spiders (Araneae: Salticidae). *Biological Journal of the Linnean Society*, **89**, 397–406.
Macedonia, J.M. (2001) Habitat light, colour variation, and ultraviolet reflectance in the Grand Cayman anole, *Anolis conspersus*. *Biological Journal of the Linnean Society*, **73**, 299–320.
Maia, R., Eliason, C.M., Bitton, P.P., Doucet, S.M. & Shawkey, M.D. (2013) pavo: an R package for the analysis, visualization and organization of spectral data. *Methods in Ecology and Evolution*, **4**, 906–913.
Marshall, N.J., Jennings, K., McFarland, W.N., Loew, E.R. & Losey, G.S. (2003) Visual biology of Hawaiian coral reef fishes. II. Colors of Hawaiian coral reef fish. *Copeia*, **2003**, 455–466.
Martin, M., Meylan, S., Gomez, D. & Le Galliard, J.F. (2013) Ultraviolet and carotenoid-based coloration in the viviparous lizard *Zootoca vivipara* (Squamata: Lacertidae) in relation to age, sex, and morphology. *Biological Journal of the Linnean Society*, **110**, 128–141.
Martin, M., Meylan, S., Perret, S. & Le Galliard, J.F. (2015) UV coloration influences spatial dominance but not agonistic behaviors in male wall lizards. *Behavioral Ecology and Sociobiology*, **69**, 1483–1491.
Meadows, M.G., Butler, M.W., Morehouse, N.I., Taylor, L.A., Toomey, M.B., McGraw, K.J. & Rutowski, R.L. (2009) Iridescence: views from many angles. *Journal of The Royal Society Interface*, **6**, 107–113.
Mullen, P. (2006) Spectrophotometric studies of avian plumage coloration with special emphasis on the ultraviolet range. *zur Erlangung des Doktorgrades (Dr. rer. nat.) der Mathematisch Naturwissenschaftlichen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn*, pp. 1–116.
Osorio, D. & Ham, A.D. (2002) Spectral reflectance and directional properties of structural coloration in bird plumage. *Journal of Experimental Biology*, **205**, 2017–2027.
Pérez i de Lanuza, G.P., Carazo, P. & Font, E. (2014) Colours of quality: structural (but not pigment) coloration informs about male quality in a polychromatic lizard. *Animal Behaviour*, **90**, 73–81.
Pérez i de Lanuza, G. & Font, E. (2014) Now you see me, now you don't: iridescence increases the efficacy of lizard chromatic signals. *Naturwissenschaften*, **101**, 831–837.
Pérez i de Lanuza, G. & Font, E. (2015) Differences in conspicuousness between alternative color morphs in a polychromatic lizard. *Behavioral Ecology*, **26**, 1432–1446.
Pérez i de Lanuza, G.P. & Font, E. (2016) Iridescent (angle-dependent reflectance) properties of dorsal coloration in *Podarcis muralis* (Laurenti, 1768). *Amphibia-Reptilia*, **37**, 441–445.
Pérez i de Lanuza, G., Font, E. & Carazo, P. (2013) Colour-assortative mating in a colour-polymorphic lacertid lizard. *Behavioural Ecology*, **24**, 273–279.
Pérez i de Lanuza, G., Font, E. & Carretero, M.Á. (2016) Colour assortative pairing in a colour polymorphic lizard is independent of population morph diversity. *The Science of Nature*, **103**, 82–88.
Pohland, G. (2006) Spectral data of Avian plumage. PhD dissertation, Rheinische riedrich-Wilhelms-Universität Bonn.
Prum, R.O., Andersson, S. & Torres, R.H. (2003) Coherent scattering of ultraviolet light by avian feather barbs. *The Auk*, **120**, 163–170.
R Core Team (2014) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. Available at: https://www.R-project.org (accessed 2 January 2017).
Rutowski, R.L., Macedonia, J.M., Merry, J.W., Morehouse, N.I., Yturralde, K., Taylor-Traft, L., Gaalema, D., Kemp, D.J. & Papke, R.S. (2007) Iridescent ultraviolet signal in the orange sulphur butterfly (*Colias eurytheme*): spatial, temporal and spectral properties. *Biological Journal of the Linnean Society*, **90**, 349–364.
Siddiqi, A., Cronin, T.W., Loew, E.R., Vorobyev, M. & Summers, K. (2004) Interspecific and intraspecific views of color signals in the strawberry poison frog *Dendrobates pumilio*. *Journal of Experimental Biology*, **207**, 2471–2485.
White, T.E., Dalrymple, R.L., Noble, D.W., O'Hanlon, J.C., Zurek, D.B. & Umbers, K.D. (2015) Reproducible research in the study of biological coloration. *Animal Behaviour*, **106**, 51–57.
Whiting, M.J., Stuart-Fox, D.M., O'Connor, D., Firth, D., Bennett, N.C. & Blomberg, S.P. (2006) Ultraviolet signals ultra-aggression in a lizard. *Animal Behaviour*, **72**, 353–363.

Received 12 January 2017; accepted 21 April 2017
Handling Editor: Robert Freckleton

Supporting Information

Details of electronic Supporting Information are provided below.

Appendix S1. Assessment of the maximum potential effect of the sheets thickness (0·8 mm) on the resulting colorimetric variables (i.e. hue, chroma, and luminance).

Appendix S2. Graphs showing the spectra of the artificial coloured sheets (red, yellow, green, blue, black, and white) for each centre vs. surround colour combination, at a probe-to-surface distance of 5 and 3 mm, and for five colour patch diameters (i.e. 1, 1·5, 2, 2·5, and 3 mm).

Appendix S3. Assessment of the asymmetry in spectral contaminations of chimeric spectra due to an elliptical sampling area when a 45° angle between the probe and the measured surface is used.