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Eggshell spot scoring methods cannot be used as a reliable proxy to determine pigment quantity

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Eggshell maculation of most passerines is due to the deposition of the pigment protoporphyrin which is produced during biosynthesis of blood haem. Its functional significance has only received empirical attention in recent years. This interest has generated a number of hypotheses of which some remain untested partly because the quantification of protoporphyrin is analytically challenging and can be prohibitively expensive. Many studies have therefore used the extent of eggshell spotting as a proxy for total eggshell protoporphyrin concentration, although this has not been formally tested. Pigment scoring involves recording visible eggshell pigment attributes, such as spot intensity, distribution and size. Since even immaculate eggs can contain some protoporphyrin, there remains doubt over the degree to which visible pigment correlates with total pigment content of the shell. In this study, we test whether visible pigment scoring can be used as a proxy for protoporphyrin concentration of an eggshell. We use pigmented eggshells of two common British passerine species to compare eggshell spot intensity, distribution and spot size (as used by the visual pigment scoring method) with direct measures of eggshell protoporphyrin concentration. In addition, we compared an alternative method of pigment scoring, the pixel pigment scoring method, using a computer programme to quantify the number of pixels exceeding a specified colour threshold. We demonstrate that although results from both scoring methods were positively correlated with eggshell protoporphyrin concentrations, the correlations were not sufficiently strong to be used as surrogates in studies where actual pigment concentrations are required.

The coloration and patterning of avian eggshells is caused by two main types of pigments. These are protoporphyrin IX (brownish hues) and biliverdin (blue and green hues) (Kennedy and Vevers 1973, Gorchein et al. 2009). Protoporphyrin, produced during the biosynthesis of blood haem (Burley and Vadhera 1989), occurs in both the calcite and cuticular layers of the eggshell (Roberts 2004), and is often localized as maculation (i.e. pigment spots) either in distinct layers within or upon the eggshell (Kennedy and Vevers 1976, Kilner 2006). Maculated eggs are represented in all of the 22 passerine families of the Holarctic (Sibley and Monroe 1990).

As the direct measurement of protoporphyrin concentration can be analytically challenging and financially expensive, most studies have used alternative methods to quantify the amount of pigment in eggshells. Visual pigment scoring is such a method and records for example eggshell pigment intensity, distribution and spot size. Scoring can be carried out on eggs in situ (Gosler et al. 2000) or retrospectively from photographs (Mägi et al. 2012). One such method was described by Gosler and colleagues (2000, 2005) for the eggs of great tits *Parus major*, and has been used in studies of eggshell thickness (Gosler et al. 2005, Mägi et al. 2012), the ability of females to counter anaemia during egg-laying (De Coster et al. 2012), and the inheritance of eggshell patterning (Gosler et al. 2000). This method has also been applied to the eggs of other species including blue tits *Cyanistes caeruleus* (Sanz and García-Navas 2009, García-Navas et al. 2011, Holveck et al. 2012), house sparrows *Passer domesticus* (López de Hierro and De Neve 2010), and northern lapwings *Vanellus vanellus* (Bulla et al. 2012). The original method was designed to quantify the appearance of eggshells, and was never intended to replace direct measurement of protoporphyrin concentration. However, it has subsequently been widely inferred to reflect the quantity of pigment in eggshells (Reynolds et al. 2009). It has long been known that even apparently immaculate eggshells contain some protoporphyrin (Kennedy and

Vevers 1973), so that while it seems reasonable to assume that eggshells with larger, darker, and/or more spots contain more protoporphyrin (Cassey et al. 2012a), the strength and linearity of this relationship has never been determined empirically.

Pigment scoring has provided additional useful information but is not necessarily related to the quantity of protoporphyrin in eggshells. In great tits, protoporphyrin-pigmented spots have been found to demarcate thinner areas of the shell, with darker spots covering thinner areas than paler spots, and both spot darkness and spread were negatively correlated with local soil calcium (Gosler et al. 2005). These maculation traits were found to be heritable down the female line (Gosler et al. 2000). In blue tits, females laying more maculated eggs were found to be in lower body condition (Martínez-de la Puente et al. 2007), and eggs with larger and less evenly distributed spots had higher anti-body concentrations (Holveck et al. 2012).

Bird eggs provide an effective bio-monitoring tool (Ormerod and Tyler 1990, Van den Steen et al. 2010) due to their high lipid contents, which concentrate hydrophobic contaminants (Van den Steen et al. 2006). Eggshells, in particular, are sensitive to persistent organic pollutants, either directly, by blocking calcium uptake to the shell gland (Ratcliffe 1970, Lundholm 1997, Jagannath et al. 2008) or indirectly, by affecting the haem biosynthesis pathway and consequently shifting pigment concentrations (Casini et al. 2003). Resident passerine species, such as great tits and blue tits, might be particularly effective for monitoring local environmental contamination because of their small territories and foraging areas (Moore 1966, Dauwe et al. 2006).

Pigment scoring offers the possibility of a non-destructive bio-assay of the health of the egg, the laying female and/or the environment. For instance, the presence of spotting on eggs is negatively related to eggshell thickness which is sensitive to environmental calcium availability (Gosler et al. 2005) and pollutants (Eeva and Lehikoinen 1995), and has been identified as an indicator of egg quality (Sanz and García-Navas 2009). Eggshells of Eurasian sparrowhawks *Accipiter nisus* with protoporphyrin spots as an internalised layer, showed a strong correlation between DDE content of eggshells and their thickness (Jagannath et al. 2008). Eggshell coloration is also a good predictor of environmental contamination in herring gulls *Larus argentatus*, although a positive correlation was found only with blue-green chroma and not with brown chroma (Hanley and Doucet 2012). Porphyrins in particular, can be used as a marker of bio-chemical effects (De Matteis and Lim 1994). They are capable of binding metals and can be detected in a range of biological materials, even at low concentrations (De Matteis and Lim 1994), suggesting their potential as bio-markers of pollutants in birds (Casini et al. 2003). Protoporphyrin has already demonstrated its potential as such a bio-marker (Wayland et al. 1998, Casini et al. 2001). Establishing visual pigment scoring methods as a reliable proxy for eggshell protoporphyrin content will create a powerful tool in field ecology and hence could be applied to conservation practices.

Previous studies have highlighted the complications of using visually based measures as a proxy for eggshell pigment concentration. Cassey et al. (2012b) found that pigment concentrations were not consistently associated with blue-green chroma and brightness as measured with spectrophotometry of eggshells of two thrushes *Turdus* spp. A further study using 49 species of British breeding non-passerines showed that increased maculation on the eggshell corresponds to an increase in eggshell protoporphyrin and biliverdin concentrations. However, the variability detected in pigment concentrations was considerable (Cassey et al. 2012a). Our study focuses on a commonly used method to quantify eggshell pigment content, the visual pigment scoring method (as described by Gosler and colleagues 2000, 2005), and investigates whether this is a reliable proxy for the measurement of the quantity of protoporphyrin in eggshells. Using eggshells of two common British species of tit, we compared eggshell spot intensity, distribution and spot size (as used by the visual pigment scoring method) with direct measures of protoporphyrin. Furthermore, we examined an additional method of pigment scoring, the pixel pigment scoring method (PPSM), using a specially designed computer program, which quantified the number of pixels exceeding a specified threshold colour gradient. This method has already been used (e. g. Stoddard and Stevens 2010, Cassey et al. 2012a) to quantify eggshell pigment spotting.

Material and methods

Egg sampling

The study was conducted in the 2010 breeding season at Chaddesley Woods National Nature Reserve (NNR), a 101-ha mixed woodland in Worcestershire, UK (UK Ordnance Survey Grid Reference: SO914736, 52°36′N, 2°14′W). Wooden nestboxes are mounted on tree trunks approximately 2 m off the ground with 32 mm entrance holes facing northeast, away from the prevailing south-western winds (see Harrison et al. 2010 for more details). Nestboxes were checked every 3–5 d for signs of nest building and then checked daily from the half-nest stage onwards (Fig. 1 in Smith et al. 2013). Eggs 1, 2 and 3 were numbered according to laying order using a waterproof marker. Fourth-laid eggs in clutches were removed under licence (Natural England Permit 20100857) on the day of laying.

Eggs were photographed using a digital camera with a 105 mm lens under standardised conditions following Cassey et al. (2010a). The camera was mounted on a camera stand, surrounded by two photographic umbrellas with silver-white and flat white linings. Eggs were lit to the right and front using two 11 W energy saving light bulbs. Photographs were taken at ISO 400 with an aperture of f16 and the exposure was set to automatic. To ensure that the whole eggshell was recorded, four photographs were taken per eggshell, rotating the egg approximately 90° between photographs. Eggs were cut longitudinally into halves using a disposable razor-blade, their contents removed and eggshells were washed in water, and dried to constant mass.

Pigment analysis

The quantity of protoporphyrin IX present in the eggshell was determined by chromatography as described by Mikšík

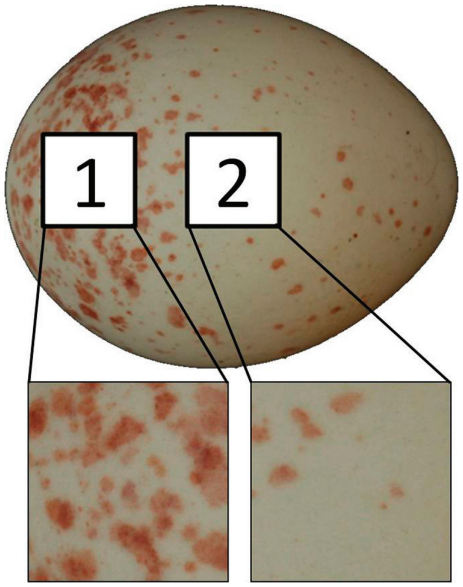

Figure 1. Two squares per egg were used to analyse eggshell pigment spotting of great tits and blue tits. A square centred on (1) the blunt end (crown) and (2) the 'equator' (i.e. the widest point) of the eggshell.

et al. (1996). Briefly, half eggshells were extracted (and esterified) in the dark in 5 ml of absolute methanol containing 5% concentrated sulphuric acid at room temperature under nitrogen for 24 h. Extracts were decanted and 4 ml of chloroform and 4 ml of distilled water were added and then shaken. The lower (chloroform) phase was collected, and the higher (water) phase was again extracted with chloroform (chloroform phases from both extractions were collected). These phases were washed with 2 ml of 10% sodium chloride, followed by distilled water until the solution was neutral. Extracts were evaporated to dryness and reconstituted in 0.5 ml of chloroform with an internal standard (5, 10, 15, 20-tetra (4-pyridyl)-21H,23H-porphine, 0.01 mg ml^{-1}). Standards for quantification were treated with the same procedure.

Pigments were determined and quantified by reversed-phase high-performance liquid chromatography (HPLC) using an LC system and a multi-wavelength detector coupled to an ion-trap mass spectrometer. Chromatographic separation was conducted in a column (250 × 2.0 mm ID). The 10 µl sample was injected into the column and eluted using a linear gradient (X = water with 0.1% formic acid, and Y = acetonitrile with 0.085% formic acid), a flow rate of 0.35 ml min^{-1} and at a temperature of 55°C. The gradient started at X/Y 80:20 reaching 10:90 rations after 15 min and reaching 100% Y after 5 min. For the next 10 min the elution was isocratic. Elution was monitored by absorbance at 410 nm. Atmospheric pressure ionization-electrospray ionization (API-ESI) positive mode ion-trap mass spectrometry in MRM (multiple reaction monitoring) mode was used when precursor ions were 619 m/z (internal standard), and 591 m/z (protoporphyrin IX).

The amount of error in pigment quantification was estimated in two ways. First, in instances of high concentrations of protoporphyrin (e.g. 15 000–15 ng ml^{-1}), absorbance at 410 nm was used when calibration curves were linear with regression coefficients in the range of $R^2 = 0.9979$ and 0.9947. Error of quantification (relative standard deviations – RSD), of the whole sample preparation procedure (i.e. methylesterification, extraction, analysis) was calculated based on standards using six independent measures and did not exceed 11%. Samples were re-analysed a month after the first analysis, and compared to each other for repeatability. The RSD values were lower than 5% for all samples, indicating the good repeatability of results from the HPLC methodology. Pigment content of eggshells is expressed as both total content (µg) and as concentration as mass per g of eggshell (µg g^{-1}).

Visual pigment scoring

Eggshell pigmentation was recorded from photographic images. Only one image per egg chosen at random was used. Following Gosler et al. (2000, 2005), we scored the eggshell pigmentation pattern on the basis of three categories: pigment intensity (I, scored in 0.5 increments, from 1 [palest] to 5 [darkest]), distribution (D, scored in 0.5 increments from 1 [> 90% of spots concentrated at a single end] to 5 [spots evenly distributed]), and spot size (S, scored in 0.5 increments from 1 [small spots] to 3 [large spots]). All eggs were scored blind by a single observer (AGG).

Pixel pigment scoring

Eggshell images were used to quantify eggshell coverage by, and intensity of, pigment spots. Spot coverage was defined as the amount of spotting in the foreground compared to the background (based on number of pixels). Spot pigment intensity was defined as the darkness of the spotting based on grayscale intensity (on a scale of 0 [black] to 1 [white]).

Analysis of the eggshell images was conducted in MATLAB (The MathWorks Natick, MA, USA). Each image was loaded and processed individually. Processing comprised two main phases: selection of the regions of the image to analyse, and calculation of the image statistics. In order to select the regions of the image, the image was first partitioned into egg and background regions using a simple binary threshold on a greyscale version of the image. The threshold level was determined using the method of Otsu (1979), to locate where the intra-class variance is minimized, and the inter-class variance is maximized. All images were checked manually to ascertain that the eggs were separated from their background correctly, and if incorrect, were removed from the analysis when detected.

Having identified the egg region of the image, two square sections (square 1 at the blunt end [crown] and square 2 at the equator [shoulder] – Fig. 1) were taken along the long axis of the egg. The squares were equal in size which was determined so that each side was 20% of the total egg length. The squares were placed such that each square fell entirely within the perimeter of the eggshell in the image, and were separated from each other by a distance of 10% of the total egg length. This had the effect of excluding pixels found near the edge of the egg, thereby avoiding parts of the image where the pigment spots may have been distorted due to eggshell curvature.

Table 1. Intra-class correlation (calculated following Lessells and Boag 1987) of eggshell maculation scores across multiple images of the same egg for great tits (n = 45) and blue tits (n = 27) at Chaddesley Woods National Nature Reserve.

	Great tit	Blue tit
Spot coverage (blunt)	0.52	0.76
Spot coverage (equator)	0.51	0.35
Intensity (blunt)	0.60	0.79
Intensity (equator)	0.44	0.40

Finally, pixels in the image were categorised as either maculated or non-maculated using the same greyscale threshold and the method of Otsu (1979). The percentage of squares 1 and 2 which were maculated was then calculated. In addition, the mean greyscale intensity was calculated for maculated and non-maculated eggshell in each square. Eggshell maculation measurements across multiple photographs of individual eggs were repeatable (Table 1) allowing the use of mean pigment scores calculated per square from the four images per eggshell. Photographs containing incorrectly identified eggs were removed from the analysis and were therefore not included in the calculation of an overall mean. Roughly 50% of eggs had at least one photograph which had to be removed from the analysis. As spotting was shown to be repeatable across photographs, we decided that maculation means of eggs calculated from less than four photographs remained reliable. Therefore, these eggs remained in the analysis.

The PPSM approach makes two assumptions: firstly, that pixels with an equivalent grey scale value represented eggshells with equivalent pigment concentration; and secondly, that the distribution and intensity of the spots in the analysed squares represented spotting over the entire eggshell. By using discrete segments centrally located rather than the entire eggshell to quantify spotting, we overcome errors caused by peripheral spotting, such as spots blending in with the background, and repeated quantification of spotting of certain areas of the egg. Squares were located in areas of the eggshell (i.e. the blunt end and the equator) where spotting tends to be concentrated in the two focal species (Gosler et al. 2005). As mean pigment scores were calculated from multiple images per eggshell (i.e. with the eggshell being rotated approximately 90° between images) which covered a large proportion of the eggshell surface area, scores are assumed to be representative of overall eggshell spotting.

Statistical analysis

Fourth-laid eggs were removed from a total of 72 clutches of the two species, 45 from great tits and 27 from blue tits. Pixel pigment scoring data were collected from all 72 eggs. Visual pigment scoring data were collected from a subset of these, totalling 55 eggs, 28 from great tits and 27 from blue tits. Previous studies such as Gosler et al.(2005) and Higham and Gosler (2006) found that the three components I, D and S were less informative than their first and second principal components, which combine information from all three variables. The principal components PC1 (darkness) and PC2 (spread) were determined from the correlation matrix of I, D and S.

Table 2. Mean (+ 1 SE) eggshell protoporphyrin concentrations and eggshell maculation variables for great tits (n = 45) and blue tits (n = 27) at Chaddesley Woods National Nature Reserve.

Eggshell parameter	Great tit	Blue tit
Protoporphyrin (μg g^{-1})	20.42 (1.87)	8.25 (0.78)
Spot coverage (blunt)	28.02 (1.18)	21.69 (1.34)
Spot coverage (equator)	15.65 (1.03)	7.82 (0.80)
Intensity (blunt)	0.46 (0.01)	0.48 (0.01)
Intensity (equator)	0.47 (0.01)	0.49 (0.01)

Statistical analyses were performed in R ver. 2.14.0 (R Development Core Team) using Pearson's product-moment correlation. Scores produced by both methods were correlated against eggshell pigment content, both as total protoporphyrin content (μg) of the half eggshell and as concentration of eggshell mass (μg g^{-1}). Both produced similar results and therefore total protoporphyrin content is not discussed hereafter. Species were analysed separately. Distributions of the protoporphyrin content and concentration data were normalised by square-root transformation.

Results

Mean eggshell protoporphyrin concentrations and eggshell maculation variables for great and blue tits are provided in Table 2.

Scoring principal components of visual pigment

In great tits, increasing PC1 (67.4%, Table 3) represented eggshells with declining spot intensity ($r = -0.93$, $DF = 26$, $p < 0.0001$) but with more highly concentrated spotting at one end of the eggshell ($r = 0.71$, $n = 28$, $p < 0.0001$). Increasing PC2 (24.6%, Table 3) represented eggshells with increasing even-ness of spot distribution across the eggshell ($r = 0.70$, $n = 28$, $p < 0.0001$) and with a tendency for increasing intensity ($r = 0.35$, $n = 28$, $p = 0.065$).

In blue tits, increasing PC1 (51.1%, Table 3) represented eggshells with increasing even-ness of spot distribution across the shell ($r = 0.99$, $n = 27$, $p < 0.0001$) but with decreasing size ($r = -0.47$, $n = 27$, $p = 0.014$). Increasing PC2 (38.6%, Table 3) represented eggshells with decreasing spot intensity ($r = -0.97$, $n = 27$, $p < 0.0001$).

Table 3. Eigenvector loadings on (and % of variance explained by) the first (PC1) and second (PC2) principal component axes from a principal components analysis of the three components of eggshell maculation (spot intensity [I], distribution [D] and size [S]) of great tits (n = 28) and blue tits (n = 27) at Chaddesley Woods National Nature Reserve.

	Loadings	
Eggshell maculation trait	Great tit	Blue tit
PC1	(67.4%)	(51.1%)
I	−0.84	−0.13
D	0.52	0.96
S	−0.17	−0.24
PC2	(24.6%)	(38.6%)
I	0.52	−0.98
D	0.85	−0.16
S	n/a	−0.12

Table 4. Pearson's product-moment correlations (*r*- and associated p-values) between two methods of pigment scoring and eggshell protoporphyrin concentration (total [per half] and per gram of eggshell), of great tits and blue tits at Chaddesley Woods National Nature Reserve. Visible spot scoring methods are given in bold in the column on the left with the explanatory variable tested given below. Highlighted rows indicate a term that is significant ($\alpha = 0.05$).

	Great tit					Blue tit				
		μg (total)		μg g^{-1}			μg (total)		μg g^{-1}	
	n	r	p	r	p	n	r	p	r	p
Visual pigment scoring										
I	28	**0.60**	**0.002**	**0.58**	**0.001**	27	−0.15	0.47	−0.11	0.57
D	28	−0.20	0.32	−0.19	0.33	27	0.08	0.69	0.05	0.80
S	28	**0.45**	**0.02**	**0.47**	**0.01**	27	0.02	0.92	−0.04	0.86
PC1	28	**−0.53**	**0.004**	**−0.54**	**0.003**	27	0.09	0.66	0.056	0.78
PC2	28	0.25	0.20	0.26	0.18	27	0.13	0.53	0.10	0.62
Pixel pigment scoring										
Intensity (blunt)	45	**−0.62**	**<0.001**	**−0.61**	**<0.001**	27	−0.27	0.18	−0.30	0.13
Intensity (equator)	45	**−0.48**	**0.001**	**0.47**	**0.001**	27	0.07	0.72	0.04	0.84
Spot coverage (blunt)	45	0.29	0.05	**0.35**	**0.02**	27	0.31	0.12	**0.41**	**0.04**
Spot coverage (equator)	45	0.06	0.71	0.15	0.32	27	0.14	0.47	0.14	0.50

Visual pigment scoring

Pigment spots had a higher intensity (i.e. were darker) on eggshells that contained more protoporphyrin in eggs laid by great tits (Table 4, Fig. 2a), but no such correlation was found in eggs laid by blue tits (Table 4, Fig. 2a). Pigment spot size was larger on eggshells that contained more protoporphyrin in eggs laid by great tits (Table 4, Fig. 2b), but no such correlation was found in eggs laid by blue tits (Table 4, Fig. 2b). Spot distribution was not correlated with protoporphyrin concentration in either species (Table 4, Fig. 2c).

In eggs laid by great tits, pigment spots were darker (PC1) on eggshells containing higher levels of protoporphyrin (Table 4, Fig. 3a), but not those laid by blue tits. Pigment spot spread (PC2) was not correlated with eggshell protoporphyrin concentration in either great tits (Table 4, Fig. 3b) or blue tits.

Pixel pigment scoring

Pigment spot characteristics on the blunt end (i.e. square 1) and the equator (i.e. square 2) of the eggshells were strongly correlated for both species, both for spot intensity (great tits: $r = 0.72$, $n = 45$, $p < 0.0001$; blue tits: $r = 0.52$, $n = 27$, $p = 0.006$), and coverage (great tits: $r = 0.39$, $n = 45$, $p = 0.007$; blue tits: $r = 0.39$, $n = 27$, $p = 0.04$). Eggshells of both species containing more protoporphyrin had pigment spots covering a greater percentage of the eggshell at the blunt end of the egg (Table 4, Fig. 4a), but

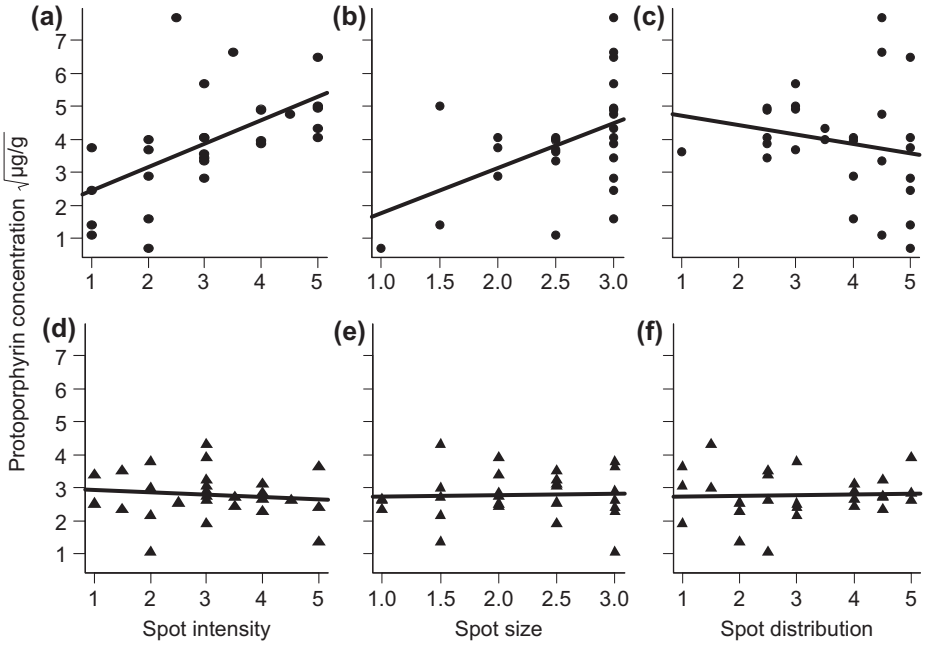

Figure 2. The relationship between eggshell protoporphyrin concentration and pigment spot intensity (a, d), size (b, e), and distribution (c, f), as determined from visual pigment scoring of eggshells produced by great tits (circles) and blue tits (triangles) breeding in Chaddesley Woods National Nature Reserve in Worcs., UK in 2010.

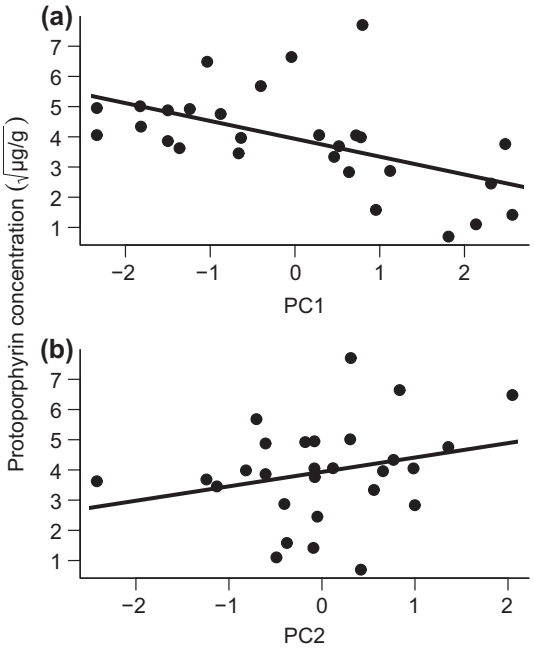

Figure 3. The relationship between eggshell protoporphyrin concentration and pigment (a) darkness (PC1) and (b) spread (PC2), as determined from visual pigment scoring of eggshells produced by great tits breeding in Chaddesley Woods National Nature Reserve in Worcs., UK in 2010.

not at the equator (Table 4, Fig. 4c). In great tits, these spots were darker at both the blunt end of the eggshell (Table 4, Fig. 4b) and at the equator (Table 4, Fig. 4d). In eggs laid by blue tits, eggshell protoporphyrin concentration and pigment spot intensity were not correlated at either the blunt end (Table 4, Fig. 4b) or the equator (Table 4, Fig. 4d).

Discussion

We investigated whether visible pigment scoring can be used as a proxy for measuring the absolute quantity of protoporphyrin within an eggshell. We compared the results of two pigment scoring methods, visual pigment scoring and pixel pigment scoring, with the quantity of protoporphyrin in the eggshell from established techniques in analytical chemistry. Results from both scoring methods were correlated with eggshell protoporphyrin concentration. However, the PPSM produced slightly stronger correlations with protoporphyrin concentration than the visual pigment scoring method did. Furthermore, while results from both methods were correlated with eggshell protoporphyrin concentration of great tit eggs, in blue tits visual pigment scoring of eggshells detected no strong correlations between any eggshell spotting parameter and protoporphyrin concentration.

Although results from both scoring methods were statistically significantly correlated with eggshell protoporphyrin concentration, we observe that these correlations are not sufficiently strong to be used as reliable surrogates. Correlation coefficients measure the strength of a single linear relationship between two variables and are calculated by comparing how closely the data points are located to the line of best fit (Hazewinkel 2001). This means that even moderately strong correlations, such as those found in this study, have a large dispersion of scores, and therefore for each fixed protoporphyrin concentration there will be a variety of corresponding spot scores. The coefficient of determination (R^2) for the strongest correlation ($r = -0.62$) with eggshell protoporphyrin (i.e. PPSM on square 1 for spot intensity of great tit eggs) is 0.38. This means that 62% of the variance in spot intensity was not explained by protoporphyrin concentration. This is likely due, at least in part, to the fact that protoporphyrin is not just present on

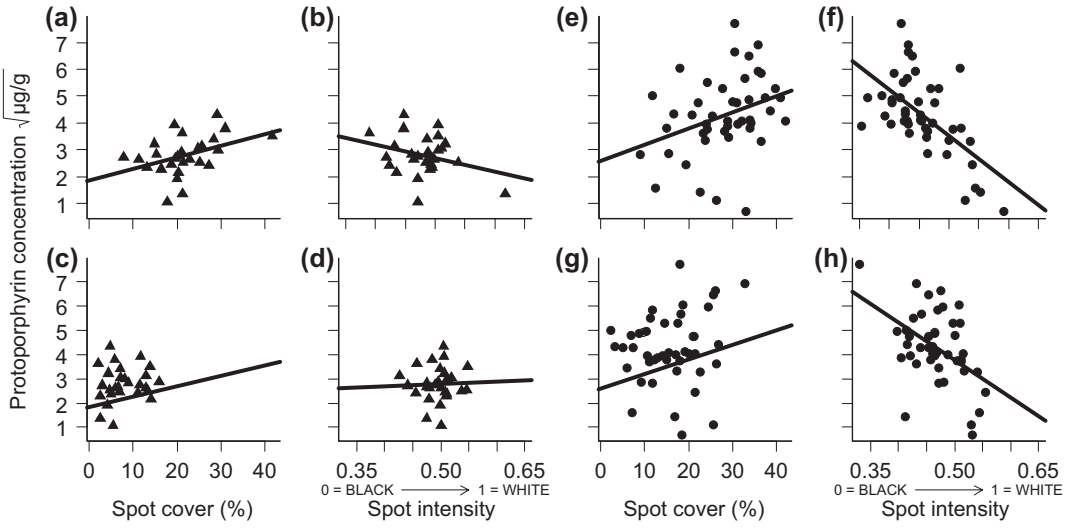

Figure 4. The relationship between protoporphyrin concentration and pigment spotting determined from pixel pigment scoring of eggshells for pigment spot coverage (a, e) and intensity (b, f) for square 1 (top row) and for pigment spot coverage (c, g) and intensity (d, h) for square 2 (bottom row). Eggshells were laid by great tits (circles) and blue tits (triangles) breeding in Chaddesley Woods National Nature Reserve in Worcs., UK in 2010.

the outer (visible) layer of the eggshell but is also present throughout the shell matrix (Kennedy and Vevers 1976, Roberts 2004, Gosler et al. 2011).

Studies of the relationship between pigment content and coloration of bird feathers found that melanin concentration explained significant variation (i.e. 25–78%) in feather brightness, hue and saturation of male and female breast plumage in barn swallows *Hirundo rustica* (McGraw et al. 2005), and carotenoid concentration explained 32–51% of variation in chroma and hue, but not brightness of tail-feathers of wild-caught and captive male greenfinches *Carduelis chloris* (Saks et al. 2003). Here, we were investigating whether visible eggshell spotting could be used as a proxy for pigment concentration and we conclude that the strengths of the correlations produced, despite being statistically significant, are unacceptable for this purpose in both species.

The lack of acceptable correlation strengths between eggshell pigment concentration and visible coloration could be due to structural components rather than the location of deposited pigment or measurement error. Colour is not simply determined by how much pigment is present but it also depends on the structure of the object, its reflective properties (Vevers 1982), and how light is transmitted through it: components of the light can be transmitted forward, absorbed by the molecules or scattered in all directions within the medium (Prum and Torres 2003). In many passerines, plumage is reflected as white due to the keratin-based structure of feathers, this can then change the visible coloration caused by pigment concentration in the feather (Jacot et al. 2010). Likewise, the structure of the eggshell may affect its colour, which therefore may affect the intensity of the protoporphyrin spots present on the eggshell.

Finally, we cannot exclude the possibility of measurement error in the pigment analysis process. However, we are confident that these errors are minimal (see pigment analysis in the materials and methods section) and therefore unlikely to cause the lack of strong correlation between visible spotting and eggshell protoporphyrin.

We must stress that we are not criticising previous studies that have used visible pigment scoring of eggs in species whose eggshell spottiness and protoporphyrin concentration have not been corroborated. Significant findings relating eggshell pigmentation patterns to other variables such as, female body condition (Martínez-de la Puente et al. 2007) and parental investment (Walters and Getty 2010) are important in their own right. Pigment concentration is not necessarily a more instructive measure of adaptive function than visible maculation because pigment concentration is not simply a function of how much is present (Higham and Gosler 2006), but can be dependent on where and how it is deposited on the shell. However, as eggshells often contain protoporphyrin within the eggshell matrix (Roberts 2004), which is not accounted for by visible pigment scoring methods, we argue that caution must be exercised when relating pigment scores directly to eggshell protoporphyrin concentration.

The visual pigment scoring method was originally described for the eggs of great tits (Gosler et al. 2000, 2005), but has subsequently been applied to other species. Therefore, the present study compared methods using the eggs of great tits as well as those of blue tits. Eggshell colour has been found to vary greatly, even between closely related species (Cassey et al. 2010a). As the two focal species exhibit different foraging niches preceding and during egg-laying (Gibb 1954) and blue tits lay smaller, but more, eggs, it is possible that blue tits deposit most of their protoporphyrin within the shell matrix rather than on the eggshell's surface explaining why no strong correlations were found between eggshell pigment spot intensity and its corresponding protoporphyrin concentration. Most of the hypotheses proposed for the evolution of eggshell colour in species, such as brood parasitism, nest site selection and predation pressure, can themselves exhibit considerable intra-specific contemporary variation in eggshell colour (Kilner 2006), suggesting that differences in eggshell colour between populations may exist and must be considered when making interspecific comparisons. Even the relationship we found between spot characteristics as shown by the PCA differed slightly from those found on eggshells laid by great tits at Wytham Woods (Gosler et al. 2005).

We conclude that while visible pigment scoring may give an indication of variability in protoporphyrin concentration, it cannot be used as a reliable proxy for it, at least not in these two species of tit. We encourage comparative methodological studies such as ours to validate eggshell pigment data across different focal species. The techniques examined here are still effective for studies quantifying the appearance of maculated eggshells and may even be valuable for studies on species which are 'endangered' or 'at risk', whose eggs cannot be removed from the wild for destructive use. For these species, we advocate using museum eggshells (Cassey et al. 2010a, b) as an alternative to obtain initial information on eggshell protoporphyrin concentration.

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References

Bulla, M., Šálek, M. and Gosler, A. G. 2012. Eggshell spotting does not predict male incubation but marks thinner areas of a shorebird's shells. – Auk 129: 26–35.

Burley, R. W. and Vadhera, D. V. 1989. The avian egg. – Wiley.

Casini, S., Fossi, M., Gavilan, J., Barra, R., Parra, O., Leonzio, C. and Focardi, S. 2001. Porphyrin levels in excreta of sea birds of the Chilean coasts as nondestructive biomarker of exposure to environmental pollutants. – Arch. Environ. Contam. Toxicol. 41: 65–72.

Casini, S., Cristina Fossi, M., Leonzio, C. and Renzoni, A. 2003. Review: porphyrins as biomarkers for hazard assessment of bird populations: destructive and non-destructive use. – Ecotoxicology 12: 297–305.

Cassey, P., Portugal, S. J., Maurer, G., Ewen, J. G., Boulton, R. L., Hauber, M. E. and Blackburn, T. M. 2010a. Variability in

avian eggshell colour: a comparative study of museum eggshells. – PLoS One 5: e12054.
Cassey, P., Maurer, G., Duval, C., Ewen, J. and Hauber, M. 2010b. Impact of time since collection on avian eggshell color: a comparison of museum and fresh egg specimens. – Behav. Ecol. Sociobiol. 64: 1711–1720.
Cassey, P., Thomas, G. H., Portugal, S. J., Maurer, G., Hauber, M. E., Grim, T., Lovell, P. G. and Mikšík, I. 2012a. Why are birds' eggs colourful? Eggshell pigments co-vary with life-history and nesting ecology among British breeding non-passerine birds. – Biol. J. Linn. Soc. 106: 657–672.
Cassey, P., Mikšík, I., Portugal, S. J., Maurer, G., Ewen, J. G., Zarate, E., Sewell, M. A., Karadas, F., Grim, T. and Hauber, M. E. 2012b. Avian eggshell pigments are not consistently correlated with colour measurements or egg constituents in two *Turdus* thrushes. – J. Avian Biol. 43: 503–512.
Dauwe, T., Japers, V. L. B., Covaci, A. and Eens, M. 2006. Accumulation of organochlorines and brominated flame retardants in the eggs and nestlings of great tits, *Parus major*. – Environ. Sci. Technol. 40: 5297−5303.
De Coster, G., De Neve, L. and Lens, L. 2012. Intraclutch variation in avian eggshell pigmentation: the anaemia hypothesis. – Oecologia 170: 297 − 304.
De Matteis, F. and Lim, C. 1994. Porphyrins as non-destructive indicators of exposure to environmental pollutants. – In: Fossi, M. C. and Leonzio, C. (eds), Non-destructive biomarkers in vertebrates. Lewis Publishers, pp. 93–128.
Eeva, T. and Lehikoinen, E. 1995. Egg shell quality, clutch size and hatching success of the great tit (*Parus major*) and the pied flycatcher (*Ficedula hypoleuca*) in an air pollution gradient. – Oecologia 102: 312–323.
García-Navas, V., Sanz, J., Merino, S., Martínez–De La Puente, J., Lobato, E., Del Cerro, S., Rivero, J., Ruiz De Castañeda, R. and Moreno, J. 2011. Experimental evidence for the role of calcium in eggshell pigmentation pattern and breeding performance in blue tits *Cyanistes caeruleus*. – J. Ornithol. 152: 71–82.
Gibb, J. 1954. Feeding ecology of tits, with notes on treecreepers and goldcrests. – Ibis 96: 513–543.
Gorchein, A., Lim, C. K. and Cassey, P. 2009. Extraction and analysis of colourful eggshell pigments using HPLC and HPLC/electrospray ionization tandem mass spectrometry. – Biomed. Chromatogr. 23: 602–606.
Gosler, A. G., Barnett, P. R. and Reynolds, S. J. 2000. Inheritance and variation in eggshell patterning in the great tit *Parus major*. – Proc. R. Soc. B 267: 2469–2473.
Gosler, A. G., Higham, J. P. and Reynolds, S. J. 2005. Why are birds' eggs speckled? – Ecol. Lett. 8: 1105–1113.
Gosler, A. G., Connor, O. R. and Bonser, R. H. C. 2011. Protoporphyrin and eggshell strength: preliminary findings from a passerine bird. – Avian Biol. Res. 4: 214–223.
Hanley, D. and Doucet, S. M. 2012. Does environmental contamination influence egg coloration? A long-term study in herring gulls. – J. Appl. Ecol. 49: 1055–1063.
Harrison, T. J. E., Smith, J. A., Martin, G. R., Chamberlain, D. E., Bearhop, S., Robb, G. N. and Reynolds, S. J. 2010. Does food supplementation really enhance productivity of breeding birds? – Oecologia 164: 311–320.
Hazewinkel, M. 2001. Correlation in statistics. Encyclopedia of mathematics. – Kluwer.
Higham, J. P. and Gosler, A. G. 2006. Speckled eggs: water-loss and incubation behaviour in the great tit *Parus major*. – Oecologia 149: 561–570.
Holveck, M.-J., Grégoire, A., Stasewski, V., Guerriro, R., Perret, P., Boulinier, T. and Doutrelant, C. 2012. Eggshell spottiness reflects maternally transferred antibodies in blue tits. – PLoS One 7: e50389.
Jacot, A., Romero-Diaz, C., Tschirren, B., Richner, H. and Fitze, P. S. 2010. Dissecting carotenoid from structural components of carotenoid-based coloration: a field experiment with great tits (*Parus major*). – Am. Nat. 176: 55–62.
Jagannath, A., Shore, R. F., Walker, L. A., Ferns, P. N. and Gosler, A. G. 2008. Eggshell pigmentation indicates pesticide contamination. – J. Appl. Ecol. 45: 133–140.
Kennedy, G. Y. and Vevers, H. G. 1973. Eggshell pigments of the araucano fowl. – Compar. Biochem. Physiol. B 44: 11–25.
Kennedy, G. Y. and Vevers, H. G. 1976. Survey of avian eggshell pigments. – Compar. Biochem. Physiol. B 55: 117–123.
Kilner, R. M. 2006. The evolution of egg colour and patterning in birds. – Biol. Rev. 81: 383–406.
Lessells, C. M. and Boag, P. T. 1987. Unrepeatable repeatabilities: a common mistake. – Auk 104: 116–121.
López De Hierro, M. and De Neve, L. 2010. Pigment limitation and female reproductive characteristics influence egg shell spottiness and ground colour variation in the house sparrow (*Passer domesticus*). – J. Ornithol. 151: 833–840.
Lundholm, C. E. 1997. DDE-induced eggshell thinning in birds: effects of p,p'-DDE on the calcium and prostaglandin metabolism of the eggshell gland. – Compar. Biochem. Physiol. C 118: 113–128.
Mägi, M., Mänd, R., Konovalov, A., Tilgar, V. and Reynolds, S. 2012. Testing the structural-function hypothesis of eggshell maculation in the great tit: an experimental approach. – J.Ornithol. 153: 645–652.
Martínez-De La Puente, J., Merino, S., Moreno, J., Tomás, G., Morales, J., Lobato, E., García-Fraile, S. and Martínez, J. 2007. Are eggshell spottiness and colour indicators of health and condition in blue tits *Cyanistes caeruleus*? – J. Avian Biol. 38: 377–384.
McGraw, K., Safran, R. and Wakamatsu, K. 2005. How feather colour reflects its melanin content. – Funct. Ecol. 19: 816–821.
Mikšík, I., Holáň, V. and Deyl, Z. 1996. Avian eggshell pigments and their variability. – Compar. Biochem. Physiol. B 113: 607–612.
Moore, N. 1966. A pesticide monitoring system with special reference to the selection of indicator species. – J. Appl. Ecol. 3: 261–269.
Ormerod, S. J. and Tyler, S. J. 1990. Environmental pollutants in the eggs of Welsh dipper *Cinclus cinclus*: a potential monitor of organochlorine and mercury contamination in upland rivers. – Bird Study 37: 171−176.
Otsu, N. 1979. A threshold selection method from gray-level histograms. – IEEE Trans. Systems Man Cybernetics 9: 62–66.
Prum, R. O. and Torres, R. H. 2003. A Fourier tool for the analysis of coherent light scattering by bio-optical nanostructures. – Integr. Comp. Biol. 43: 591–602.
Ratcliffe, D. A. 1970. Changes attributed to pesticides in egg breakage frequency and eggshell thickness in some British birds. – J. Appl. Ecol. 7: 67−115.
Reynolds, S. J., Martin, G. R. and Cassey, P. 2009. Is sexual selection blurring the functional significance of eggshell coloration hypotheses? – Anim. Behav. 78: 209–215.
Roberts, J. R. 2004. Factors affecting egg internal quality and egg shell quality in laying hens. – J. Poult. Sci. 41: 161–177.
Saks, L., McGraw, K. and Hõrak, P. 2003. How feather colour reflects its carotenoid content. – Funct. Ecol. 17: 555–561.
Sanz, J. J. and Garcá-Navas, V. 2009. Eggshell pigmentation pattern in relation to breeding performance of blue tits *Cyanistes caeruleus*. – J. Anim. Ecol. 78: 31–41.
Sibley, C. G. and Monroe, B. L. J. 1990. Distribution and taxonomy of the birds of the world. – Yale Univ. Press.
Smith, J. A., Harrison, T. J. E., Martin, G. R. and Reynolds, S. J. 2013. Feathering the nest: food supplementation influences

nest construction by blue (*Cyanistes caeruleus*) and great tits (*Parus major*). – Avian Biol. Res. 6: 18–25.
Stoddard, M. C. and Stevens, M. 2010. Pattern mimicry of host eggs by the common cuckoo, as seen through a bird's eye. – Proc. R. Soc. B 277: 1387–1393.
Van Den Steen, E., Dauwe, T., Covaci, A., Jaspers, V. L., Pinxten, R. and Eens, M. 2006. Within- and among-clutch variation of organohalogenated contaminants in eggs of great tits (*Parus major*). – Environ. Pollut. 144: 355–359.
Van Den Steen, E., Pinxten, R., Covaci, A., Carere, C., Eeva, T., Heeb, P., Kempenares, B., Lifjeld, J. T., Massa, B., Norte, A. C., Orell, M., Sanz, J. J., Senar, J. C., Sorace, A. and Eens, M. 2010. The use of blue tit eggs as a biomonitoring tool for organohalogenated pollutants in the European environment. – Sci. Total Environ. 408: 1451–1457.
Vevers, H. G. 1982. The colours of animals. – Edward Arnolds.
Walters, L. A. and Getty, T. 2010. Are brighter eggs better? Egg color and parental investment by house wrens. – J. Field Ornithol. 81: 155–166.
Wayland, M., Trudeau, S., Marchant, T., Parker, D. and Hobson, K. A. 1998. The effect of pulp and paper mill effluent on an insectivorous bird, the tree swallow. – Ecotoxicology 7: 237–251.