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Brief report

Eggshell blue-green colouration fades immediately after oviposition: a cautionary note about measuring natural egg colours

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The colour fading in eggs of the Pied Flycatcher was studied in Spain. Egg colour for >400 eggs was estimated at the moment of oviposition, at the onset of incubation, and at the end of incubation. Results, obtained using a spectrophotometer, showed that egg colours tended to fade significantly during the study period. For unhatched eggs over longer time periods of 6 and 23 months after incubation, the blue-green colouration faded further still. These figures suggest that studies on egg colouration should account for temporal changes in egg colouration.

1. Introduction

The function of eggshell colouration has received recent attention (e.g., Underwood & Sealy 2002, Moreno & Osorno 2003) as museum egg collections are increasingly used to test hypotheses concerning the proposed functions of egg colouration and the possibility that birds detect colour differences. For example, Cassey *et al.* (2009) estimated perceptual discriminability for reflectance measures from different eggs within a clutch and between clutches using museum collections. Such studies may shed light on avian colour perception, but the assumption that eggs from museum collections are accurate representations of eggs in the wild underlies these analyses. Carotenoid pigments in avian integuments, for example, degrade with exposure to light when removed from the protection of uropygial gland oils (Doucet & Hill 2009). Egg colours may also fade after oviposition due to pigment degradation with light exposure. This degradation possibly continues after collection and during storage in egg collections. The degradation of eggshell pigments, mainly protoporphyrins and biliverdin, may affect the shape of the relationship between colour variation in eggs stored for varying time periods in museum collections and natural variation in freshly laid eggs.

Here we show for the first time that egg colour in a cavity-nesting passerine starts to fade immediately after oviposition, and that the process continues at least until hatching, the maximum period for which eggs exist in the wild. Any intraspecific test of hypotheses relating to adaptiveness of egg colouration (see Underwood & Sealy 2002 for a review) should be tested in the wild. Using eggs from collections that have been subjected to uncontrolled fading effects due to random light exposure should be avoided.

The present study was conducted on Pied Flycatchers *Ficedula hypoleuca*, a species ovipositing blue-green eggs (Moreno *et al.* 2005, 2006). Our aim was to estimate colour fading in eggs across the period for which eggs exist in natural nests, from oviposition to hatching.

2. Material and methods

Egg colouration in Pied Flycatcher was studied in 2005 in Valsaín, central Spain, where a population of this species has been studied since 1991 (Sanz *et al.* 2003). Clutch size in the study population ranges from 4 to 7 eggs, with a mode of 6 eggs. Colouration measurements were done three times between oviposition and the end of incubation.

(1) A total of 428 eggs in 92 clutches were marked with a felt pen and weighed using a portable electronic balance to the nearest 0.1 g on the day of oviposition or the day after (measurement at the oviposition). The exact oviposition day could be identified, as recently-laid eggs have clearly smaller air cells than eggs laid on the previous day (Pitts 1995). Their colour was measured using a portable, battery-driven Minolta spectrophotometer CM-2600d (Minolta Co. Ltd., Osaka, Japan), which covered the reflectance spectrum above 360 nm. Measurements of blue-green chroma (BGC) with this apparatus are positively correlated with measurements taken by a spectrophotometer including reflectances below 360 nm, and are significantly associated with eggshell biliverdin content (Moreno *et al.* 2006). Eggs were placed directly with their broad pole on a target mask of the spectrophotometer with a diameter of 1 cm, so that eggs completely filled the space covered by the specimen measuring port. Reflectance spectra for each egg were obtained as the mean of three sequential measurements of each egg by changing the position of the egg with respect to the apparatus. Reference calibrations against a white standard were performed periodically according to apparatus specifications.

(2) Four hundred and thirty-three eggs, from 77 of the 92 original clutches, were again measured in the same way on the day of oviposition of the 6th egg (measurement at the onset of incubation), at which time most females were already incubating. Thus, the interval between first and second measurement declined with oviposition order. Accordingly, oviposition order was included in all analyses. Only eggs 1–5 were considered in the statistical analyses, as there were no independent first measurements for subsequent eggs in the laying sequence.

(3) Ten days after the second measurement (incubation period is normally two weeks), a total of 106 eggs in 18 clutches were measured again in the same manner (measurement at the end of incubation). Not all clutches were used here due to time constraints.

To estimate colour fading for time periods longer than the normal duration of eggs in the wild, we collected 11 unhatched eggs in 2008. As detailed above, the reflectance of these eggs had been measured the day after oviposition. Eggshells were removed and kept in total darkness in individual tubes. Reflectance of these eggs was again measured six months later, i.e., December 2008, and again 23 months after oviposition, i.e., in April 2010.

SPECTRAMAGIC software (Minolta Co. Ltd., Osaka, Japan)was used to obtain reflectance spectra. The spectrophotometer obtains the reflectance spectrum in the range 360–740 nm in intervals of 10 nm. BGC was calculated as the proportion of total reflectance that is in the blue-green region ($R_{400-570}/R_{360-700}$) of the spectrum. BGC was used to describe egg reflectance data because this region corresponds to the region of least absorbance (and therefore greatest reflectance) of biliverdin (Falchuk *et al.* 2002), and because the eggs of Pied Flycatcher reflect light maximally in this region (Moreno *et al.* 2005).

For the data collected in 2005, to ascertain if slopes differed across measurements, the dependence of measurements at oviposition (dependent variable) on measurements at the onset and end of incubation (predictor variable with two levels) were analysed using the Homogeneity of Slopes Regression module in STATISTICA 7.0. There were 424 measurements taken during the incubation period – either onset or end – for which a measurement of the same egg was taken shortly after oviposition. A significant interaction between the measurement time (onset or end of incubation) and the oviposition measurement would imply that the regression slopes are different.

Then, the similarity of colour measures between the time of measurement was analysed using time of measurement (oviposition, incubation onset, incubation end) as a fixed factor in a GLM. Subsequently, either the first two measurements or all measurements (note different sample sizes of eggs with 2 or 3 measurements; see above) were analysed using the Variance Components module of GLM in STATISTICA 7.0, using nest as a random factor. In these analyses, the degrees of freedom were estimated using the Satterthwaite method.

The measurement time (oviposition, onset of incubation, end of incubation) was introduced as a fixed factor, while the oviposition sequence, egg mass and clutch size were introduced as covariates. Non-significant variables were removed sequentially until a final model with only significant associations was obtained. Only eggs for which an independent measurement at oviposition had been obtained and for which the exact laying order was known were included in analyses.

A repeated-measures ANOVA was also conducted to reveal within-egg differences. For the data collected in 2008, correlations between measurements at oviposition and six months afterwards, and at oviposition and 23 months afterwards were conducted and mean values were compared using *t*-tests.

3. Results

The three BGC measurements were highly intercorrelated (Fig. 1). In the homogeneity-of-slopes analysis, both the incubation measurement and the time of incubation measurement (onset or end of incubation) significantly explained measurements at oviposition (incubation measurement: $F_{1,423}$ = 920, P <0.001; type of incubation measurement: $F_{1,423}$ = 27.3, P <0.001). There was also a highly significant interaction between the type of measurement (onset or end of incubation) and the incu-

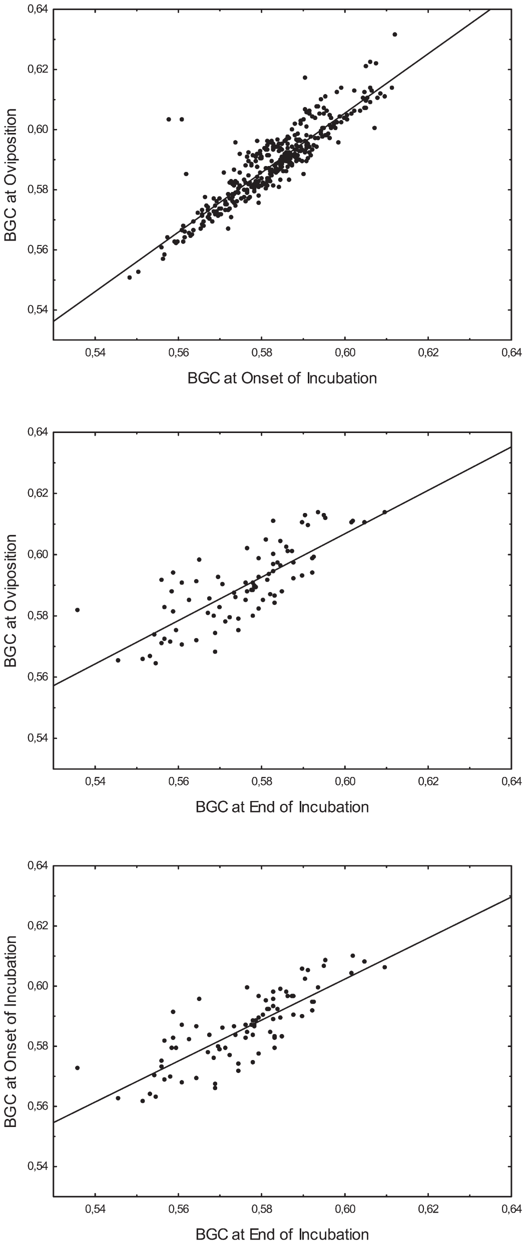

Fig. 1. Correlations between (a) BGC at oviposition and at onset of incubation (r_{338} =0.90, P <0.001), (b) BGC at oviposition and at end of incubation (r_{82} =0.78, P <0.001), and (c) BGC at onset and at end of incubation (r_{82} =0.81, P <0.001). Regression lines are extrapolated to allow easy visualisation of slopes.

Table 1. ANOVA for synthesised errors with df error computed using the Satterthwaite method. Only eggs 1–5 were included. The measurements 1–3 refer to colour measurements (1) at oviposition, (2) at the onset of incubation, and (3) at the end of incubation.

Measurement/Factor	df effect	df error	*F*	*P*
Meas. 1 and 2				
Clutch size	1	84	4.29	0.041
Laying sequence	1	629	198.7	<0.001
Type of measurement	1	148	75.1	<0.001
Nest	84	70	23.2	<0.001
Type of measurement × Nest	75	665	0.96	0.583
Meas. 1, 2 and 3				
Egg mass	1	93	9.7	<0.01
Laying sequence	1	620	202	<0.001
Type of measurement	2	117	15.2	<0.001
Nest	91	95	29.1	<0.001
Type of measurement × Nest	72	585	0.84	0.814

bation measurement, indicating that the two regression slopes of measurements at the moment of oviposition on those at either onset or end of incubation were different ($F_{1,423}$ = 24.9, P <0.001). Thus, the slope of the association declined significantly across measurements from 0.989 ± 0.026 SE for the onset of incubation to 0.709 ± 0.062 SE for the end of incubation (Fig. 1a–b).

Considering only the first two measurements, clutch size showed a weak positive effect, while laying sequence and nest were highly significant (Table 1). BGC decreased with laying order of eggs (Table 1; see also Moreno *et al.* 2005). The type of measurement was also significant and did not significantly interact with nest (Table 1). BGC decreased significantly from the moment of oviposition to the onset of incubation (Fig. 2). A full model including the three measurements (oviposition, and the onset and end of incubation) suggested significant effects of laying sequence and egg mass on egg colouration (Table 1). BGC increased with egg mass, and the main effects of nest and type of measurement were highly significant, but the interaction between nest and measurement was not significant (Table 1). BGC continued to decrease after the onset of incubation (Fig. 2). A repeated-measures ANOVA revealed highly significant within-egg differences among measurements ($F_{2,166}$ = 158, P < 0.001) meaning egg colouration changed with time.

Eggshells from 2008 were significantly less blue-green after 6 months compared with their colour at oviposition (0.553 ± 0.004 SE versus 0.579 ± 0.004 SE; t = 4.57, P < 0.001), and even paler 23 months after oviposition (0.541 ± 0.007 SE; t = 4.92, P < 0.001). Measurements at oviposition and 6 months later were significantly correlated ($F_{1,9}$ = 11.06, r = 0.74, P = 0.009), but there was no significant association between measurements at oviposition and those made 23 months after oviposition ($F_{1,9}$ = 0.67, r = 0.26, P = 0.43).

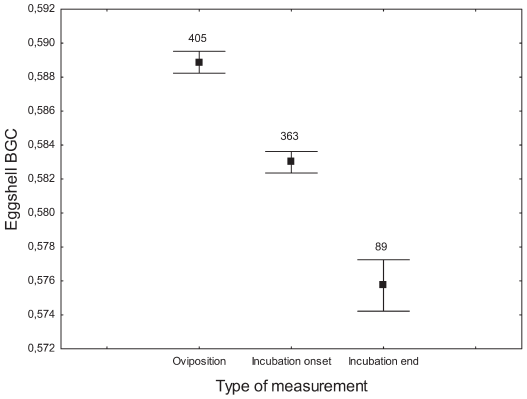

Fig. 2. Eggshell BGC (means ± SE, sample size above symbols) in relation to the type of colour measurement. Means are raw averages of BGC for the three types of measurements. Only eggs 1–5 are represented.

4. Discussion

This study showed that egg BGC may significantly decrease from oviposition to the onset of incubation, and that it may decline further during the incubation period. These declines appeared consistent among clutches as shown by the absence of significant interactions with nest in the analyses. This general decline is probably due to the chemical degradation of biliverdin, the pigment responsible for eggshell colouration in pied flycatchers (Moreno *et al.* 2006). Bilirubin, the precursor of biliverdin, may be degraded by light as in phototherapy of jaundice (Salih & Pillay 2004), and photo-oxidation of bilirubin in a variety of solvents leads to colourless products (Lightner & Cu 1977). Biliverdin on eggshells may likewise degrade chemically through light incidence, explaining the almost immediate colour fading after oviposition reported here. The decline in BGC appears continuous during the first two weeks after exposure to air and light and amounts to 2.5% of initial values. BGC decline is faster during the laying period (1% decline in only a few days) than during incubation (1.25% during 10 days). Moreover, the slope of the association of the measurement at oviposition with subsequent measurements declined significantly with time, indicating that BGC estimates become less predictive of oviposition values with time. A continuous erosion of predictive value would render estimates less accurate with egg storage time. Although based on a relatively small sample of eggshells, it appears as if storage for more than a year removes associations in colours of eggshells between several-months storage period and oviposition.

Egg colours in the wild can only be observed for the duration of oviposition and the incubation period if nests are not deserted or eggs fail to hatch. Accordingly, eggshell colours cannot have been selected to have any fitness-enhancing effect after this time. Any hypothesis about adaptation of eggshell colours (Underwood & Sealy 2002) should therefore be tested during this time interval. Measuring the colour of eggs for months or years after collection is fraught with problems of interpretation even if collection time is well known and can be statistically controlled for. Accumulated light incidence on eggs in collections may be considerable if samples are taken out repeatedly for measurements during many years of storage. Moreover, light incidence on collected eggs cannot be fully controlled for because variable exposition of eggs to light throughout storage will occur. Thus, eggs in collections are presumably not only paler than in natural nests, but paler to an unknown degree. Interspecific comparisons of egg colours suffer less from the complicating effects of fading. Colour differences are presumably much more marked between than within species. Only strong interspecific biases in collection time would constitute a problem for these tests.

To conclude, researchers willing to test hypotheses about the adaptive value of eggshell colours based on intraspecific comparisons should measure egg colours in natural nests, and preferably immediately after oviposition (e.g., Moreno *et al.* 2006, Krist & Grimm 2007). The cautionary note regarding measurements of plumage colours based on samples under long-term storage (Doucet & Hill 2009) can be extended to eggshell colours.

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Munankuoren sinivihreä väri haalistuu välittömästi haudonnan jälkeen: varoituksen sana luontaisen munanvärin mittaamisesta

Munanvärin haalistumista tutkittiin kirjosiepolla (*Ficedula hypoleuca*) Espanjassa. Munanväri arvioitiin >400 munasta spektrofotometriä käyttäen munintahetkellä, haudonnan alkaessa ja haudonnan päättyessä. Munien väri haalistui mittausten välillä merkitsevästi. Pidemmällä aikavälillä (6 ja 23 kuukautta vanhoja, kuoriutumattomia munia) väri haalistui edelleen. Tulosten perusteella munanväritutkimuksissa on syytä huomioida haalistumisen ajallinen eteneminen.

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