ORIGINAL ARTICLE

Shell thickness and pore density in relation to shell colouration, female characteristics, and environmental factors in the Collared Flycatcher *Ficedula albicollis*

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Abstract Avian eggshell structure may have important consequences for embryonic growth and development, but relatively little is known about the factors responsible for variation in eggshell characteristics of wild birds. In this paper, we explored potential causes of variation in eggshell colour and structure (shell thickness and porosity) in the Collared Flycatcher (*Ficedula albicollis*). We analysed if eggshell colour is affected by shell structure or pigment level, and whether female traits, laying date, local breeding density, and clutch size affect shell thickness and pore density. We found that eggshell blue-green and UV colours were unrelated to shell thickness, pore density and egg size. Eggs with higher concentration of biliverdin showed lower UV reflection and higher reflection in the blue-green part of the spectrum. We found that females in better nutritional condition, indicated by their higher mass controlled for tarsus length, laid eggs with thicker shells. It is possible that females in better condition have more time available for searching calcium-rich food, and thus could produce eggs with stronger shells. However, female physical characteristics had no significant relationships with shell porosity. In contrast to our expectation, shell thickness and pore density were unrelated to local breeding density and laying date, though very late and repeat clutches were not sampled in our study. However, we found that eggs in larger clutches had lower pore density than eggs in smaller clutches, which may be expected if the rate of water loss and nest humidity are to remain constant in clutches of different egg numbers.

Zusammenfassung Die Struktur der Eischale von Vögeln kann wichtige Folgen für das Wachstum und die Entwicklung der Embryonen haben, doch über die Faktoren, die für Variation in den Eigenschaften der Eischale bei freilebenden Vögeln verantwortlich sind, ist relativ wenig bekannt. In diesem Artikel haben wir mögliche Gründe für die Variation in Eischalenfarbe und -struktur (Schalendicke und Porosität) beim Halsbandschnäpper (*Ficedula albicollis*) untersucht. Wir haben analysiert, ob die Eischalenfarbe von der Schalenstruktur oder dem Pigmentgehalt beeinflusst wird und ob Weibchenmerkmale, Legedatum, lokale Brutpaardichte und Gelegegröße die Schalendicke und Porendichte beeinflussen. Wir fanden, dass blaugrüne und ultraviolette Farben der Eischale nicht mit der Schalendicke, Porendichte und Eigröße zusammenhingen. Eier mit einer höheren Biliverdinkonzentration zeigten eine geringere UV-Reflexion und stärkere Reflexion im blaugrünen Bereich des Spektrums. Wir fanden, dass Weibchen in besserem Ernährungszustand, angezeigt durch ihre höhere Körpermasse korrigiert für Tarsuslänge, Eier

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mit dickerer Schale legten. Es ist möglich, dass Weibchen in besserer Kondition mehr Zeit haben, kalziumreiche Nahrung zu suchen, und daher Eier mit stärkerer Schale produzieren. Die Körpermerkmale der Weibchen standen jedoch in keiner signifikanten Beziehung zu der Schalenporosität. Entgegen unserer Erwartung standen Schalendicke und Porendichte nicht mit der lokalen Brutpaardichte und dem Legedatum in Zusammenhang, obwohl sehr späte Gelege und Nachgelege in unserer Studie nicht beprobt wurden. Wir fanden jedoch, dass Eier in größeren Gelegen eine geringere Porendichte hatten als Eier in kleineren Gelegen, was zu erwarten wäre, wenn die Wasserverlustrate und die Nestfeuchte in Gelegen mit unterschiedlicher Eizahl konstant blieben.

Introduction

Avian eggs have hard eggshells, which are composed of 95% calcite, one of the crystalline forms of calcium carbonate, and 3.5% proteins, proteoglycans and glycoproteins (Nys et al. 1999). The main function of the shell is to provide mechanical protection for the developing embryo (Ar et al. 1979). In addition, embryos use the shell as a source of calcium for the ossification of the developing skeleton (Packard and Packard 1984). During egg laying, female birds require large amounts of calcium-rich material, such as mollusc shells, woodlice and millipedes (Bureš and Weidinger 2003), as their normal food usually does not contain sufficient calcium for eggshell formation (Graveland and van Gijzen 1994; Graveland and Berends 1997; Bureš and Weidinger 2003). A shortage of calcium during egg formation could result in thinner-shelled eggs, and thus poor hatchability due to excessive water loss or shell breakage (Graveland et al. 1994; Eeva and Lehikoinen 1995).

To provide the necessary communication between the developing embryo and the external environment, the shell is perforated by microscopic pores (Ar and Rahn 1985). Exchange of respiratory gases and water vapour is affected by the porosity of the shell and the partial pressure gradients of the gases across the shell (Ar et al. 1974; Paganelli 1980; Rahn and Paganelli 1982). In addition, the rate of water loss through the eggshell is negatively affected by eggshell thickness, as it lengthens the pathway for water vapour (Ar and Rahn 1985). The oxygen availability and thus the growth rate of the embryo may be limited by the pore number of the shell (Burton and Tullett 1983; Zimmermann and Hipfner 2007).

In many bird species, the cuticle and the calcified layer of the eggshell are pigmented, often with complex patterns. Blue-green eggshell coloration results from the pigment biliverdin, which typically permeates the whole eggshell, while red-brown spots or patches are the result of protoporphyrin (Kennedy and Vevers 1976; Mikšík et al. 1996). Avian eggshell coloration has traditionally been interpreted as a response to selection pressures imposed by nest predators and brood parasites, although other functions, e.g. filtering solar radiation, strengthening the eggshell, or serving as a sexually selected signal of female quality, have also been proposed (Underwood and Sealy 2002; Moreno and Osorno 2003; Gosler et al. 2005; Higham and Gosler 2006; Kilner 2006). In addition to human-visible light, most passerines can also detect near ultraviolet (UV-A) light (Cuthill et al. 2000; Ödeen and Håstad 2003), thus UV reflection may also be an important aspect of egg coloration (Cherry and Bennett 2001; Avilés et al. 2006). Ultraviolet colour is produced by light scattering and it may provide information on the nanostructure of the tissue (Prum et al. 1999; Prum and Torres 2003; Shawkey et al. 2003). The basis of eggshell UV-colour has not yet been determined; it could be affected by the structure of the calcified layer and/or by the pigment content of the shell.

In this study, we explored potential causes of variation in eggshell colour (blue-green and UV chroma) and structure (shell thickness and porosity) in a wild passerine, the Collared Flycatcher (*Ficedula albicollis*). In a previous study, we have shown that the average blue-green colour of a clutch was unrelated to female morphological and serological characteristics and increased with laying date in the Collared Flycatcher (Hargitai et al. 2008). Here, one of our aims was to determine if the blue-green and UV reflectances of the eggshell are affected by the pigment concentration and the structure of the shell. We predicted that biliverdin pigment concentration in the eggshell would be positively related to blue-green chroma, as demonstrated in earlier studies (Moreno et al. 2006; López-Rull et al. 2008). In contrast, as biliverdin absorb light in the UV part of the spectrum (Falchuk et al. 2002), we expected that eggs with more pigmentation would show less reflectance in the UV region, which hypothesis has not been tested before to our knowledge. Also, we expected that eggs with thicker shells and less pore channels would contain more pigments and thus be more colourful. Alternatively, biliverdin pigment may strengthen the shell similarly to protoporphyrin (Gosler et al. 2005; Jagannath et al. 2008; Sanz and García-Navas 2009), therefore, thinner-shelled eggs with a larger number of pores may show more intense blue-green colour. Until now, the relationship between blue-green eggshell colour and shell thickness has been tested only in a few species, where no associations were detected (Jagannath et al. 2008; López-Rull et al. 2008; Hargitai et al. 2010).

Although eggshell thickness and porosity may have important consequences for embryonic growth and development, relatively little is known about the factors responsible for variation in these eggshell characteristics of

wild birds. We tested if eggshell thickness and shell porosity are influenced by morphological and serological characteristics of the mother, such as body mass, body size, and stress level, and by local breeding density, laying date, and clutch size. As females seek dietary calcium sources during egg formation (Bureš and Weidinger 2003), we expected that females in better nutritional condition, indicated by their higher mass controlled for tarsus length, would lay eggs with stronger shells, as they may also be better foragers of calcium-rich materials. In addition, we hypothesised that females with a higher level of physiological stress would produce eggs with thinner shells. As far as we are aware, these hypotheses have not been tested before for wild passerines.

Breeding density may be related to eggshell thickness through affecting the level of food competition between females (Alatalo and Lundberg 1984; Török and Tóth 1988; Hargitai et al. 2009), and thus having a negative effect on shell thickness; however, this hypothesis has not been tested before for wild songbirds. Alternatively, high density areas may be of higher quality with more resources (Doliguez et al. 2004), where individuals of better quality may be able to settle. In this case, we may expect females to produce eggs with stronger shells in an area of higher density. Nevertheless, we suppose that this hypothesis is less likely in our case, as forest soil, vegetation structure and age of oak trees were homogenous in the study area. We also expected that eggshells of eggs laid later in the breeding season may be thinner as late-breeding females may be lower-quality individuals (Garamszegi et al. 2004a; Verhulst and Nilsson 2008), which may not be able to acquire a sufficient amount of calcium to produce strong-shelled eggs. Eggshell thickness may also vary during the breeding season due to seasonal variation in the availability of calcium-rich resources. Seasonal decline in eggshell thickness has been found in a study of the Blue Tit (*Cyanistes caeruleus*; Dauwe et al. 2004), but other studies found no significant variation in eggshell thickness with laying date (Eeva and Lehikoinen 1995; Mänd 1996; Massaro and Davis 2004).

The seasonal variation in eggshell thickness and pore density could also be explained by some adaptive hypotheses. Later breeding has been shown to result in lower reproductive success in small insectivorous passerines as a result of declining food availability (Norris 1993; Wiggins et al. 1994). In several bird species, there is a seasonal decline in incubation length (Eichholz and Sedinger 1998; Hipfner et al. 2001; Massaro et al. 2004), which could reflect adjustments by late-laying parents to improve the survival prospects of their offspring. Hipfner et al. (2001) hypothesised that later-laid eggs might have higher porosity, which may allow embryos to develop more rapidly as a consequence of a higher gas exchange

rate. In addition, later-breeding females may produce eggs with thinner shells as an adaptation to allow embryos to develop more rapidly and to emerge faster during hatching (Massaro and Davis 2004). So far, these hypotheses have only been tested for non-passerine birds (Mänd 1996; Massaro and Davis 2004; Boersma and Rebstock 2009), although accelerated embryonic development of later-laid clutches would also be beneficial for insectivorous songbirds.

Females of small passerines have to consume enough calcium each day for egg formation during the laying period (Graveland and Berends 1997; Pahl et al. 1997), so we might not expect a decline in shell thickness with increasing clutch size, or there may even be a positive relationship between clutch size and shell thickness as a result of the effect of female quality (Eeva and Lehikoinen 1995). However, females laying more eggs might reduce pore number per egg so that nest humidity remains constant in clutches of different egg numbers. Also, we expected that egg size would be positively related to shell thickness but negatively to pore density in line with previous findings (Tullett and Board 1977; Rahn and Paganelli 1989; Eeva and Lehikoinen 1995). Moreover, eggs with thicker shells may have more pores so as to compensate for the greater length of each pore channel.

Methods

Study species and field procedures

The study was carried out on a population of Collared Flycatchers breeding in a sessile oak (*Quercus petraea*) dominated woodland in the Pilis Mountains (47°43′N, 19°01′E), Hungary, in 2005. The study area was a nestbox plot of approximately 17.2 ha, where altogether 98 Collared Flycatcher pairs bred in the given year. The woodland has clayed brown forest soil on volcanic stones (pH = 5–6). The Collared Flycatcher is a long-distance migratory, insectivorous passerine. This species is predominantly monogamous (Garamszegi et al. 2004b; Herényi et al. 2010), and females usually lay five to seven eggs of one clutch per breeding season (Török et al. 2004). Collared Flycatcher eggs appear light blue-green in the human visible spectrum, and have a peak of reflectance at 490 nm at the visible spectrum, and at 320 nm at UV wavelengths (Hargitai et al. 2008). Females were captured in the nestbox during incubation, weighed with a Pesola spring balance (to the nearest 0.1 g), and the length of the tarsus was measured with a calliper (to the nearest 0.1 mm).

Eggs were marked according to laying order with a waterproof marker, and two eggs (third and fourth) per

clutch were collected on the day of laying from 24 clutches. For ethical reasons and due to legal restrictions, no whole clutches were collected in this study. Egg length and breadth were measured with a calliper (to the nearest 0.1 mm). Egg size was estimated from Hoyt's formula (Hoyt 1979; size = length × breadth^2 × 0.51).

Female Collared Flycatchers defend a small breeding territory around their nestbox against conspecific females, particularly during the nest-building and egg-laying periods (Král et al. 1996) similarly to their sibling species, the Pied Flycatcher (*Ficedula hypoleuca*; Slagsvold et al. 1992; Rätti 2000). However, the foraging areas of flycatchers often overlap with those of neighbouring pairs (von Haartman 1956). Previously, it has been shown that egg yolk mass was negatively related to the number of neighbouring pairs in the Collared Flycatcher (Hargitai et al. 2009), which could indicate that high local breeding density could limit food availability in this species. We calculated a local breeding density index by summing up the number of breeding Collared Flycatcher pairs in neighbouring (i.e. adjacent) nestboxes that started egg laying until the laying of the fifth egg of the clutch in question. As nestboxes were placed in a grid system, each nestbox, except for those located at the edge of the study plot, had eight nestboxes adjacent to it. Distance between nestboxes was approximately 30–50 m.

Serological analysis

A blood sample was taken from the female's brachial vein during incubation. A drop of blood was smeared on a microscope slide, air dried, fixed in absolute methanol, and later stained with Giemsa's solution. The proportion of different types of leucocytes was assessed by examining a total of 50 leucocytes under ×1,000 magnification with oil immersion. An index of the relative abundance of heterophil granulocytes to lymphocytes (H/L ratio) was used to assess the stress level of the females (Gross and Siegel 1983; Maxwell 1993).

Egg colour measurement

Egg colour was measured on the day of laying using a portable battery-driven USB2000 spectrometer with a Mini DT deuterium-halogen light source (Ocean Optics Europe, Duiven, The Netherlands). The reflectance spectrum was determined between 320 and 700 nm at 0.38-nm intervals. Inclusion of ultraviolet (UV) is necessary as most bird species are sensitive to near UV light (Cuthill et al. 2000). Eggs were placed with their side in the hole of a brick-formed aluminium reflection probe holder (RPH-1; Ocean Optics Europe), so that eggs completely filled the hole. A bifurcated 400-μm fibre optic probe was used, and the

sampling optic was held perpendicular and at a distance of 3 mm from the eggshell surface. Three measurements were taken from each egg by slightly changing the position of the egg in the hole. Reference calibration was performed with a WS-1 white standard after every second egg. Mean reflectance was calculated by averaging the three measurements. The spectral curves were generated by using the OOIBase32 software with a 3,500-ms integration time. The reflectance spectrum was double peaked with a prominent peak around 400–580 nm (blue-green), and around 320 nm (UV). Blue-green chroma ($R_{400–580}/R_{320–700}$) and UV chroma ($R_{320–400}/R_{320–700}$) were calculated as the proportion of the reflectance occurring in the given part of the spectrum, corresponding to purity or saturation. Variations in eggshell colour variables were higher among eggs than within eggs (blue-green chroma: $r = 0.90$, $F_{47,96} = 28.10$, $P < 0.001$, UV chroma: $r = 0.85$, $F_{47,96} = 17.69$, $P < 0.001$; Lessells and Boag 1987).

Eggshell thickness and pore density

Pieces of eggshell were taken from the equatorial region of two eggs (third and fourth) per clutch. Shell thickness was measured with a Mitutoyo Digimatic Micrometer (395–271) with a ball-point end at a precision of 0.001 mm at four places of the shell. Variation in eggshell thickness was greater among eggs than within eggs ($r = 0.68$, $F_{46,139} = 9.48$, $P < 0.001$), indicating that these measurements were repeatable (Lessells and Boag 1987). We used the mean of the four measurements as an average eggshell thickness of an egg.

Organic shell membranes were removed by immersing shells in household bleach (5.4% aqueous sodium hypochlorite) for 1–2 min. Eggshells were then rinsed in tap water, drained and allowed to dry. The inner surface of shell fragments was painted with 1% aqueous Evans blue. Capillary forces filled the pore channels rendering them visible as fine blue dots on the external surface of the shell. A paper mat with an exposed area of 25 mm^2 was placed over the shell. Pores were counted in two such areas using a stereomicroscope with a magnification of ×40. Six shell samples were too small, and only one area was used for pore density measurement. Variation in pore density was higher among eggs than within eggs ($r = 0.41$, $F_{40,41} = 2.41$, $P = 0.003$), showing that these measurements were repeatable (Lessells and Boag 1987).

Biliverdin analysis

Biliverdin analysis was based on the method described by Mateo et al. (2004) with some modifications. Biliverdin was extracted from 7- to 19-mg eggshell pieces from the equatorial region of one egg (fourth) per clutch by adding

315 μl acetonitrile and 263 μl HCl 3N. After 15 min of the addition of the reagents, the tubes were capped and subsequently vortexed for 15 s and sonicated for 10 s in an Ultrasons-H. Samples were centrifuged for 10 min at 13,520g and the supernatant, containing the pigments, was transferred to glass vials for later high pressure liquid chromatography (HPLC) analysis. Extraction was repeated twice by adding 400 μl acetonitrile to the samples. The acetonitrile phases were combined and HPLC analysis was conducted with an Agilent 1100 system with a Waters Spherisorb 5-μm ODS2 (4.6 × 100 mm) column at a 1.5-ml/min flow rate. The initial mobile phase composition was ammonium-acetate (1M, pH: 5.16) 75% and methanol 25% for 2 min after sample injection, and then a gradient was applied with a linear change in 8 min to 95% methanol and 5% ammonium acetate, followed by 2 min at these conditions. At this moment, the phase composition returned to the initial conditions in 3 min, and was then stabilised for another 3 min before the next sample injection. The column was maintained at 70°C and the detection was performed at 400-nm wavelength. The quantification of samples was performed using calibration curves of biliverdin standard added to white eggshells of domestic hen (Moreno et al. 2006). The concentration of biliverdin was expressed as nmol/g of dry weight of eggshell.

Statistical analyses

Data were analysed with Pearson's correlation and general linear models. Separate analyses were performed with female characteristics (body mass, tarsus length, and H/L ratio; Table 1) and environmental factors (laying date and local breeding density; Table 1) as the independent variables with pore density and shell thickness as the dependent variable. Eggshell thickness, pore density, egg size, and biliverdin concentration were entered together as explanatory variables in separate models with blue-green chroma and UV chroma as the dependent variable. In the models involving biliverdin concentration, data of the fourth egg of each clutch were used; in all other cases, the mean values of the third and fourth eggs per clutch were entered into the models. In all models, a stepwise analysis

based on backward deletion procedure was employed, removing nonsignificant ($P > 0.05$) effects from the model one by one in decreasing order of P. To avoid nonsignificance due to overparameterisation, we re-entered nonsignificant effects to the final model one by one, and present these F and P values. We also report standardised regression coefficients (beta) as effect size estimates and the 95% confidence intervals around those measures to facilitate the interpretation of the results (Nakagawa and Cuthill 2007). Log-transformed biliverdin concentration and H/L ratio were used in order to obtain normal distribution of data. Analyses were performed by STATISTICA, version 5.5 (StatSoft).

Results

Our results revealed that blue-green eggshell colour was unrelated to shell thickness, pore density and egg size (Table 2; for egg size, see also Hargitai et al. 2008), but showed a positive relationship with biliverdin concentration in the shell (Table 2; Fig. 1). We also tested if UV reflection of the shell was influenced by shell structure or biliverdin pigment concentration. Similarly, we found that shell thickness, pore density and egg size had no significant associations with UV chroma (Table 2), but there was a negative correlation between biliverdin concentration and UV chroma (Table 2; Fig. 2). Thus, eggs with higher concentration of biliverdin pigment showed lower UV reflection and higher reflection in the blue-green part of the spectrum. Moreover, we found no significant correlation between the biliverdin concentration of the eggshell and shell thickness ($r = -0.01$, $n = 23$, $P = 0.96$) or pore density ($r = -0.15$, $n = 23$, $P = 0.49$).

Concerning female morphological and serological characteristics, female H/L ratio and tarsus length did not influence shell thickness (Table 3), but we found that females of higher body mass laid eggs with thicker shells (Table 3; Fig. 3). We also tested the effect of female condition by using female mass as an independent variable and tarsus length as a covariate; the effect of female condition was also significant ($F_{1,18} = 4.69$, $P = 0.044$,

Table 1 Sample sizes (n), minimum, maximum, mean and standard deviation (SD) values of the independent variables from Collared Flycatchers (*Ficedula albicollis*) analysed in this paper

	n	Minimum	Maximum	Mean	SD
Clutch size	23	5	7	6.3	0.8
Laying date (1 = 30 April)	24	1	11	4.8	2.3
Local breeding density (number of occupied neighbour nests)	24	1	6	3.4	1.1
Tarsus length (cm)	23	1.68	1.88	1.80	0.06
Body mass (g)	23	13.9	17.7	15.3	0.94
H/L ratio (%)	21	0.06	1.63	0.45	0.37

Table 2 Results of separate GLMs with blue-green chroma and UV chroma as dependent variables, and eggshell thickness, pore density, egg size and biliverdin concentration as independent variables

	Blue-green chroma					UV-chroma				
	F	df	P	Beta	CI (lower, upper)	F	df	P	Beta	CI (lower, upper)
Eggshell thickness	0.23	1, 20	0.64	−0.09	(−0.51, 0.32)	0.23	1, 20	0.64	0.10	(−0.32, 0.52)
Pore density	0.02	1, 20	0.90	0.03	(−0.39, 0.45)	0.01	1, 20	0.98	−0.01	(−0.43, 0.42)
Egg size	1.23	1, 20	0.28	−0.22	(−0.63, 0.19)	0.59	1, 20	0.45	0.16	(−0.27, 0.58)
Biliverdin concentration	5.61	1, 21	**0.027**	0.46	(0.06, 0.86)	4.60	1, 21	**0.044**	−0.42	(−0.83, −0.01)

Final models (from stepwise deletion procedure) with significant effects are marked in bold. We present the effects of nonsignificant variables after re-entering them to the final model. Effect size estimates (*Beta*) and their 95% confidence intervals (*CI*) are also reported
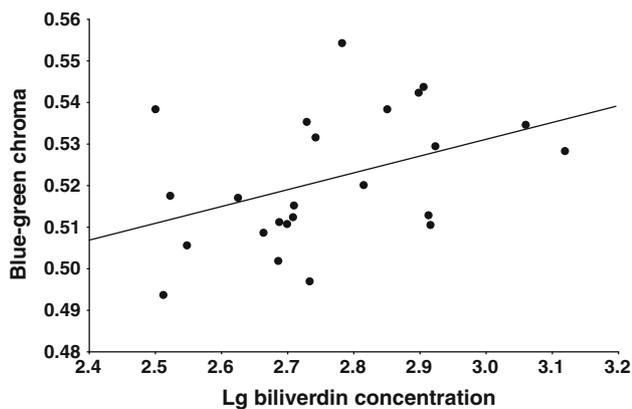
Fig. 1 Relationship between eggshell blue-green chroma ($R_{400–580}/R_{320–700}$) and eggshell biliverdin concentration (lg-transformed) in the Collared Flycatcher (*Ficedula albicollis*). Values correspond to the fourth egg in each clutch
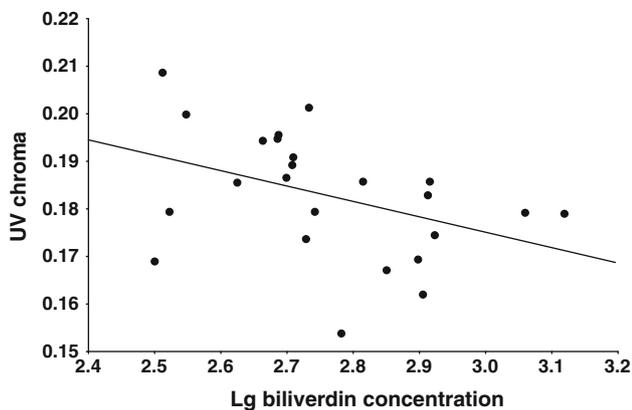
Fig. 2 Relationship between eggshell UV chroma ($R_{320–400}/R_{320–700}$) and eggshell biliverdin concentration (lg-transformed) in the Collared Flycatcher. Values correspond to the fourth egg in each clutch

beta = 0.45, CI 0.01, 0.89). Female body mass, tarsus length and H/L ratio had no significant relationships with shell porosity (Table 3).

Moreover, we analysed if environmental factors influenced the thickness and pore density of the eggshell, but neither laying date nor local breeding density was related to

eggshell characteristics (Table 3). We found that eggshell thickness was unrelated to clutch size ($r = -0.07$, $n = 23$, $P = 0.74$). However, pore density showed a negative relationship with the number of eggs within a clutch ($r = -0.57$, $n = 22$, $P = 0.005$); that is, eggs in clutches of 7 eggs had less pores than eggs in clutches of 5 eggs. As clutch size declined with laying date ($F_{1,21} = 6.18$, $P = 0.021$, beta = −0.48, CI −0.88, −0.08), we examined whether the negative relationship between pore density and clutch size could be accounted for the confounding effect of laying date. However, we found that the effect of laying date on pore density was nonsignificant, while the effect of clutch size remained significant (laying date: $F_{1,19} = 0.41$, $P = 0.53$, beta = −0.14, CI −0.58, 0.31; clutch size: $F_{1,19} = 9.10$, $P = 0.007$, beta = −0.64, CI −1.09, −0.20).

Our results showed that eggshell thickness was unrelated to shell porosity ($r = -0.05$, $n = 23$, $P = 0.83$), but there was a trend for larger eggs to have thicker shells, although it was non-significant ($r = 0.36$, $n = 24$, $P = 0.081$). However, we detected no significant association between egg size and the porosity of the eggshell ($r = 0.15$, $n = 23$, $P = 0.51$).

Discussion

Our study showed that eggshell biliverdin concentration was positively related to blue-green chroma, in line with the findings of earlier studies (Moreno et al. 2006; López-Rull et al. 2008). In addition, eggshells containing a higher concentration of biliverdin pigment showed less intense UV reflection. However, neither shell thickness nor shell porosity was related to eggshell coloration or the biliverdin pigment concentration of the shell. In agreement with our results, blue-green eggshell coloration was unrelated to shell thickness in a study of the Sparrowhawk (*Accipiter nisus*; Jagannath et al. 2008), the Spotless Starling (*Sturnus unicolor*; López-Rull et al. 2008), the Cuckoo (*Cuculus canorus*; Hargitai et al. 2010) and the Great Reed Warbler

Table 3 Results of separate GLMs with eggshell thickness and pore density as dependent variables, and environmental factors (laying date and local breeding density) and female traits (tarsus length, body mass and H/L ratio) as independent variables

	Eggshell thickness					Pore density				
	F	df	P	Beta	CI (lower, upper)	F	df	P	Beta	CI (lower, upper)
Laying date	0.07	1, 22	0.80	0.05	(−0.39, 0.50)	0.56	1, 21	0.46	0.16	(−0.29, 0.61)
Local breeding density	1.19	1, 22	0.29	0.23	(−0.20, 0.66)	0.84	1, 21	0.37	−0.20	(−0.64, 0.25)
Tarsus length	0.50	1, 18	0.49	0.15	(−0.29, 0.59)	2.53	1, 18	0.13	−0.35	(−0.81, 0.11)
Body mass	5.85	1, 19	**0.026**	0.49	(0.07, 0.91)	0.65	1, 18	0.43	−0.19	(−0.67, 0.30)
H/L ratio	0.61	1, 18	0.45	−0.16	(−0.59, 0.27)	0.71	1, 18	0.41	−0.19	(−0.68, 0.29)

Final models (from stepwise deletion procedure) with significant effects are marked in bold. We present the effects of nonsignificant variables after re-entering them to the final model. Effect size estimates (Beta) and their 95% confidence intervals (CI) are also reported
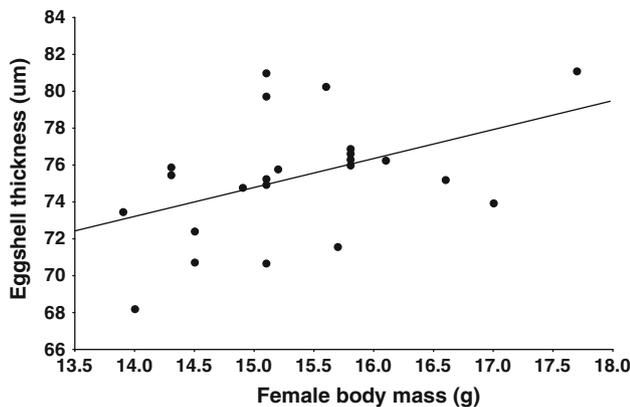

Fig. 3 Relationship between female body mass and eggshell thickness (mean value of the third and fourth eggs) in the Collared Flycatcher

(Acrocephalus arundinaceus; Hargitai et al. 2010). In several studies, the brown-red eggshell pigment protoporphyrin was shown to be associated to shell thickness (Gosler et al. 2005; Jagannath et al. 2008; Sanz and García-Navas 2009), suggesting that protoporphyrin might have a structural function in strengthening the eggshell (Solomon 1997; Gosler et al. 2005). However, despite the similar molecular structure of the two pigment types (Lang and Wells 1987), it appears that biliverdin pigmentation of the eggshell does not have such a function.

Our results suggest that UV reflectance of the shell is generated by the structure of the calcified layer, as biliverdin pigmentation decreased UV chroma. This hypothesis is also supported by Siefferman et al. (2006), who found that white eggs also reflected light in the UV range. Egg pigments appear to decrease the intensity of UV reflectance by absorbing light in these wavelengths. However, we could not detect any associations between UV chroma and shell thickness or pore density, suggesting that nanostructural characteristics of the shell and shell pigmentation may be responsible for the UV reflectance, and shell thickness and porosity do not influence it.

Besides proteins and lipids, laying birds need to consume large quantities of calcium-rich material in order to possess sufficient calcium for egg formation (Simkiss 1967). Acquiring a sufficient amount of calcium is time-consuming even in a calcium-rich environment; thus, there may exist a trade-off between feeding time and time spent searching for calcium (Graveland and Berends 1997). We found that females in better nutritional condition produced eggs with thicker shells, although body size and stress level showed no significant relationships with shell thickness. It is possible that females in better condition have more time available for searching calcium-rich food items and thus could produce eggs with stronger shells. Moreover, these birds may be better foragers of both nutrients and calcium, as egg size, which is also related to female condition (Hargitai et al. 2005), and shell thickness showed a weak, almost significant positive relationship, which might be significant with a larger sample size. The positive relationship between shell thickness and egg size has also been shown in two other songbirds, the Great Tit (Parus major) and the Pied Flycatcher (Eeva and Lehikoinen 1995). In the Peregrine Falcon (Falco peregrinus), it has also been reported that heavier females laid clutches of larger eggs with thicker shells, although in other falcon species this relationship was not significant (Castilla et al. 2009). Experimental studies have also found that calcium-supplemented birds laid thicker-shelled and also larger eggs, probably as the decrease in searching time for calcium might incur a higher intake of nutrients or a lower energy expenditure (Tilgar et al. 1999; Mänd and Tilgar 2003).

Contrary to our expectation, we found no evidence in Collared Flycatchers that eggshell thickness was related to laying date or local breeding density. This suggests that the availability of calcium-rich materials does not vary significantly during the laying period, and it is not affected by local competition for calcium-rich materials. However, the laying date interval of the analysed clutches (Table 1) might have been too short to detect such differences (see

also later), therefore we must treat our results with caution. Nevertheless, our results are in agreement with previous findings. Eggshell thickness also did not vary with laying date in Great Tits and Pied Flycatchers (Eeva and Lehikoinen 1995), Black-headed Gulls (*Larus ridibundus*; Mänd 1996) and Yellow-eyed Penguins (*Megadyptes antipodes*; Massaro and Davis 2004).

Apart from the effect of physiological constraints, eggshell structure may also vary seasonally due to the strategic adjustment of the female. Since later laying has often been associated with lower reproductive success as a result of declining food availability during the period of parental care (Norris 1993; Wiggins et al. 1994), there may be a reproductive advantage for females breeding later in the season to reduce incubation time. It has been shown in numerous studies that total pore area of the eggshell affects water vapour conductance, and both of them are positively related to the growth rate of embryos and hatchability of eggs (Burton and Tullett 1983; Ar and Rahn 1985; Soliman et al. 1994; Massaro and Davis 2005; Zimmermann and Hipfner 2007). In the Yellow-eyed Penguin, Massaro and Davis (2004) detected an increase in pore density with laying date which may allow embryos to develop more rapidly than earlier laid eggs. Until now, this hypothesis has not been tested for small passerines, although accelerated embryonic development of later-laid clutches would also be beneficial for insectivorous songbirds. However, we could not detect a seasonal variation in eggshell pore density in the Collared Flycatcher. It is possible that late-breeding Collared Flycatcher females reduce incubation length by starting incubation before all eggs within a clutch are laid, thereby producing hatching asynchrony, which may benefit the first hatched nestlings (Slagsvold 1986; Szegedi et al. 2009). Alternatively, the laying date interval of the analysed clutches (11 days; Table 1) might have been too short to detect such differences. The total laying date interval of Collared Flycatchers breeding in our study plot in 2005 was 19 days, and we do not have data of clutches at the end of the breeding season or of clutches of repeat laying, where we may expect the largest increase in pore density. This could limit the interpretation of our result. However, in accordance with our result, eggshell pore density also did not differ between eggs of early and late clutches in the Black-headed Gull (Mänd 1996) and the Magellanic penguin (*Spheniscus magellanicus*; Boersma and Rebstock 2009).

Songbirds do not store calcium for egg formation in the skeleton, they must collect calcium on a daily basis during the laying period (Graveland and van Gijzen 1994; Graveland and Berends 1997; Pahl et al. 1997). Therefore, we might not expect a decline in shell thickness with increasing clutch size as calcium is not utilised from a limited store, and indeed we found no relationship between

number of eggs within a clutch and shell thickness. However, we found that eggs in clutches of more eggs had lower pore density than eggs in clutches of fewer eggs. Porosity of the shell and the gas pressure gradient between the internal environment and the microenvironment of the nest affect gas conductance (Ar et al. 1974). If the rate of water loss and nest humidity are to remain constant in clutches of different egg numbers, then pore density should be reduced in proportion to the increase in egg number.

To sum up, our data showed that the blue-green and UV chroma of the eggshell were related to shell biliverdin concentration, as predicted. We found that females in better nutritional condition produced thicker-shelled eggs, although shell thickness was not related to the body size and stress level of the female. In contrast to our expectation, the structure of Collared Flycatcher eggs was not associated with laying date and local breeding density. However, we found that eggs in larger clutches had lower pore density than eggs in smaller clutches. Further research on eggshell thickness and porosity may provide a better understanding of the factors that influence variation in eggshell characteristics, and their effects on incubation period and hatching success of wild birds.

References

Alatalo RV, Lundberg A (1984) Density-dependence in breeding success of the pied flycatcher (Ficedula hypoleuca). J Anim Ecol 53:969–977

Ar A, Rahn H (1985) Pores in avian eggshells: gas conductance, gas exchange and embryonic growth rate. Resp Physiol 61:1–20

Ar A, Paganelli CV, Reeves RB, Greene DG, Rahn H (1974) The avian egg: water vapor conductance, shell thickness, and functional pore area. Condor 76:153–158

Ar A, Rahn H, Paganelli CV (1979) The avian egg: mass and strength. Condor 81:331–337

Avilés JM, Soler JJ, Pérez-Contreras T (2006) Dark nests and egg colour in birds: a possible functional role of ultraviolet reflectance in egg detectability. Proc R Soc Lond B 273:2821–2829

Boersma PD, Rebstock GA (2009) Magellanic Penguin eggshell pores: does number matter? Ibis 151:535–540

Bureš S, Weidinger K (2003) Sources and timing of calcium intake during reproduction in flycatchers. Oecologia 137:634–647

Burton FG, Tullett SG (1983) A comparison of the effects of eggshell porosity on the respiration and growth of domestic fowl, duck and turkey embryos. Comp Biochem Physiol A 75:167–174

Castilla AM, Herrel A, Van Dongen S, Furio N, Negro JJ (2009) Determinants of eggshell strength in endangered raptors. J Exp Zool 311A:303–311

Cherry MI, Bennett ATD (2001) Egg colour matching in an African cucko, as revealed by ultraviolet-visible reflectance spectrophotometry. Proc R Soc Lond B 268:565–571

Cuthill IC, Partridge JC, Bennett ATD, Church SC, Hart NS, Hunt S (2000) Ultraviolet vision in birds. Adv Stud Behav 29:159–214

Dauwe T, Janssens E, Kempenaers B, Eens M (2004) The effect of heavy metal exposure on egg size, eggshell thickness and the number of spermatozoa in blue tit Parus caeruleus eggs. Environ Pollut 129:125–129

Doliguez B, Paert T, Danchin E, Clobert J, Gustafsson L (2004) Availability and use of public information and conspecific density for settlement decisions in the collared flycatcher. J Anim Ecol 73:75–87

Eeva T, Lehikoinen E (1995) Egg shell quality, clutch size and hatching success of the great tit (Parus major) and the pied flycatcher (Ficedula hyoleuca) in an air pollution gradient. Oecologia 102:312–323

Eichholz MW, Sedinger JS (1998) Factors affecting duration of incubation in black brant. Condor 100:164–168

Falchuk KH, Contin JM, Dziedzic TS, Feng ZL, French TC, Heffron GJ, Montorzi M (2002) A role for biliverdin IX alpha in dorsal axis development of Xenopus laevis embryos. Proc Natl Acad Sci USA 99:251–256

Garamszegi LZ, Török J, Michl G, Møller AP (2004a) Female survival, lifetime reproductive success and mating status in a passerine bird. Oecologia 138:48–56

Garamszegi LZ, Török J, Tóth L, Michl G (2004b) The effect of timing and female quality on clutch size in the collared flycatcher Ficedula albicollis. Bird Study 51:270–277

Gosler AG, Higham JP, Reynolds SJ (2005) Why are birds' eggs speckled? Ecol Lett 8:1105–1113

Graveland J, Berends JE (1997) Timing of the calcium intake and effect of calcium deficiency on behaviour and egg laying in captive great tits Parus major. Physiol Zool 70:74–84

Graveland J, van Gijzen T (1994) Arthropods and seeds are not sufficient as calcium sources for shell formation and skeletal growth in passerines. Ardea 82:299–314

Graveland J, van der Wal R, van Balen JH, van Noordwijk AJ (1994) Poor reproduction in forest passerines from decline of snail abundance in acidified soils. Nature 368:446–448

Gross WB, Siegel HS (1983) Evaluation of the heterophil/lymphocyte ratio as a measure of stress in chickens. Avian Dis 27:972–979

Hargitai R, Török J, Tóth L, Hegyi G, Rosivall B, Szigeti B, Szöllősi E (2005) Effects of environmental conditions and parental quality on the inter- and intraclutch egg size variation in the collared flycatcher (Ficedula albicollis). Auk 122:509–522

Hargitai R, Herényi M, Török J (2008) Eggshell colouration in relation to female condition, male attractiveness and egg quality in the collared flycatcher (Ficedula albicollis). J Avian Biol 39:413–422

Hargitai R, Arnold KE, Herényi M, Prechl J, Török J (2009) Egg composition in relation to social environment and maternal physiological condition in the collared flycatcher. Behav Ecol Sociobiol 63:869–882

Hargitai R, Moskát C, Bán M, Gil D, López-Rull I, Solymos E (2010) Eggshell characteristics and yolk composition in the common cuckoo Cuculus canorus: are they adapted to brood parasitism? J Avian Biol 41:177–185

Herényi M, Hegyi G, Garamszegi LZ, Hargitai R, Michl G, Rosivall B, Török J (2010) Lifetime offspring production in relation to attractiveness, breeding lifespan and mating status in male collared flycatchers. Oecologia (in press)

Higham JP, Gosler AG (2006) Speckled eggs: water-loss and incubation behavior in the great tit Parus major. Oecologia 149:561–570

Hipfner JM, Gaston AJ, Mardin DL, Jones IL (2001) Seasonal declines in incubation periods of Brünnich's guillemots Uria lomvia: testing proximate causes. Ibis 143:92–98

Hoyt DF (1979) Practical methods of estimating volume and fresh weight of bird eggs. Auk 96:73–77

Jagannath A, Shore RF, Walker LA, Ferns PN, Gosler A (2008) Eggshell pigmentation indicates pesticide contamination. J Appl Ecol 45:133–140

Kennedy GY, Vevers HG (1976) A survey of avian eggshell pigments. Comp Biochem Physiol 55B:117–123

Kilner RM (2006) The evolution of egg colour and patterning in birds. Biol Rev 81:383–406

Král M, Sætre G-P, Bicík V (1996) Intrasexual aggression of female collared flycatchers (Ficedula albicollis): competition for male parental care? Folia Zool 45:153–159

Lang MR, Wells JW (1987) A review of eggshell pigmentation. World Poult Sci J 43:238–246

Lessells CM, Boag PT (1987) Unrepeatable repeatabilities: a common mistake. Auk 104:116–121

López-Rull I, Mikšík I, Gil D (2008) Egg pigmentation reflects female and egg quality in the spotless starling Sturnus unicolor. Behav Ecol Sociobiol 62:1877–1884

Mänd R (1996) Increased eggshell porosity in replacement clutches of the black-headed gull Larus ridibundus. Ornis Fenn 73:131–136

Mänd R, Tilgar V (2003) Does supplementary calcium reduce the cost of reproduction in the pied flycatcher Ficedula hypoleuca? Ibis 145:67–71

Massaro M, Davis LS (2004) The influence of laying date and maternal age on eggshell thickness and pore density in yellow-eyed penguins. Condor 106:496–505

Massaro M, Davis LS (2005) Differences in egg size, shell thickness, pore density, pore diameter and water vapour conductance between first and second eggs of Snares penguins Eudyptes robustus and their influence on hatching asynchrony. Ibis 147:251–258

Massaro M, Davis L, Darby JT, Robertson GJ, Setiawan AN (2004) Intraspecific variation in incubation periods in yellow-eyed penguins (Megadyptes antiopes): testing the influence of age, laying date and egg size. Ibis 146:526–530

Mateo R, Castells G, Green AJ, Godoy C, Cristòfol C (2004) Determination of porphyrins and biliverdin in bile and excreta of birds by a single liquid chromatography-ultraviolet detection analysis. J Chromatogr B 810:305–311

Maxwell MH (1993) Avian blood leucocyte responses to stress. World Poult Sci J 49:34–43

Mikšík I, Holáň V, Deyl Z (1996) Avian eggshell pigments and their variability. Comp Biochem Physiol 113B:607–612

Moreno J, Osorno JL (2003) Avian egg colour and sexual selection: does eggshell pigmentation reflect female condition and genetic quality? Ecol Lett 6:803–806

Moreno J, Lobato E, Morales J, Merino S, Tomás G, Martínez-de la Puente J, Sanz JJ, Mateo R, Soler JJ (2006) Experimental evidence that egg color indicates female condition at egg laying in a songbird. Behav Ecol 17:651–655

Nakagawa S, Cuthill IC (2007) Effect size, confidence interval and statistical significance: a practical guide for biologists. Biol Res 82:591–605

Norris K (1993) Seasonal variation in the reproductive success of blue tits: an experimental study. J Anim Ecol 62:287–294

Nys Y, Hincke MT, Arias JL, Garcia-Ruiz JM, Solomon SE (1999) Avian eggshell mineralization. Poult Avian Biol Rev 10:143–166

Ödeen A, Håstad O (2003) Complex distribution of avian color vision systems revealed by sequencing the SWS1 opsin from total DNA. Mol Biol Evol 20:855–861

Packard MJ, Packard GC (1984) Comparative aspects of calcium metabolism in embryonic reptiles and birds. In: Seymour RS (ed) Respiration and metabolism of embryonic vertebrates. Junk, Dordrecht, pp 155–180

Paganelli CV (1980) The physics of gas exchange across the avian eggshell. Am Zool 20:329–338

Pahl R, Winkler DW, Graveland J, Batterman BW (1997) Songbirds do not create long-term stores of calcium in their legs prior to laying: results from the high-resolution radiography. Proc R Soc Lond B 264:239–244

Prum RO, Torres R (2003) Structural colouration of avian skin: convergent evolution of coherently scattering dermal collagen arrays. J Exp Biol 206:2409–2429

Prum RO, Torres RH, Kovach C, Williamson S, Goodman SM (1999) Two-dimensional Fourier analysis of the spongy medullary keratin of structurally coloured feather barbs. Proc R Soc Lond B 266:13–22

Rahn H, Paganelli CV (1982) Role of diffusion in gas exchange of the avian egg. Fed Proc 41:2134–2136

Rahn H, Paganelli CV (1989) Shell mass, thickness and density of avian eggs derived from the tables of Schonwetter. J Ornithol 130:59–68

Rätti O (2000) Characteristics and level of aggression by female pied flycatchers at different distances from the nest hole. Ornis Fenn 77:11–16

Sanz JJ, García-Navas V (2009) Eggshell pigmentatioin pattern in relation to breeding performance of blue tits Cyanistes caeruleus. J Anim Ecol 78:31–41

Shawkey MD, Estes AM, Siefferman LM, Hill GE (2003) Nanostructure predicts intraspecific variation in ultraviolet-blue plumage colour. Proc R Soc Lond B 270:1455–1460

Siefferman L, Navara KJ, Hill GE (2006) Egg coloration is correlated with female condition in eastern bluebirds (Sialia sialis). Behav Ecol Sociobiol 59:651–656

Simkiss K (1967) Calcium in reproductive physiology. Chapman and Hall, London

Slagsvold T (1986) Asynchronous versus synchronous hatching in birds: experiments with the pied flycatcher. J Anim Ecol 55:1115–1134

Slagsvold T, Amundsen T, Dale S, Lampe H (1992) Female-female aggression explains polyterritoriality in male pied flycatchers. Anim Behav 43:397–407

Soliman FN, Rizk RE, Brake J (1994) Relationship between shell porosity, shell thickness, egg weight loss, and embryonic development in Japanese quail eggs. Poult Sci 73:1607–1611

Solomon SE (1997) Egg and eggshell quality. Iowa State University Press, Ames

Szegedi A, Rosivall B, Szöllősi E, Hegyi G, Török J (2009) The effect of environmental conditions on hatching asynchrony in the collared flycatcher. In: 2nd European congress of conservation biology, Prague

Tilgar V, Mänd R, Leivits A (1999) Effect of calcium availability and habitat quality on reproduction in pied flycatcher Ficedula hypoleuca and great tit Parus major. J Avian Biol 30:383–391

Török J, Tóth L (1988) Density dependence in reproduction of the collared flycatcher (Ficedula albicollis) at high population levels. J Anim Ecol 57:251–258

Török J, Hegyi G, Tóth L, Könczey R (2004) Unpredictable food supply modifies costs of reproduction and hampers individual optimization. Oecologia 141:432–443

Tullett SG, Board RG (1977) Determinants of avian eggshell porosity. J Zool 183:203–211

Underwood TJ, Sealy SG (2002) Adaptive significance of egg coloration. In: Deeming DC (ed) Avian incubation, behaviour, environment and evolution. Oxford University Press, Oxford, pp 280–298

Verhulst S, Nilsson J-Å (2008) The timing of birds' breeding season: a review of experiments that manipulated timing of breeding. Philos Trans R Soc Lond B 363:399–410

von Haartman L (1956) Territory in the pied flycatcher Muscicapa hypoleuca. Ibis 98:460–475

Wiggins DA, Pärt T, Gustafsson L (1994) Seasonal decline in collared flycatcher Ficedula albicollis reproductive success: an experimental approach. Oikos 70:359–364

Zimmermann K, Hipfner JM (2007) Egg size, eggshell porosity, and incubation period in the marine bird family Alcidae. Auk 124:307–315