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**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.

Keywords: biliverdin, eggshell pigmentation, eggshell porosity, eggshell structure, eggshell thickness, *Ficedula albicollis*

Zusammenfassung

Schalendicke und Porendichte in Bezug zu Schalenfärbung, Weibchenmerkmale und Umweltfaktoren beim Halsbandschnäpper *Ficedula albicollis*

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 zusammenhängen. 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 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.

1 **Introduction**

2
3 Avian eggs have hard eggshells, which are composed of 95% calcite, one of the crystalline
4 forms of calcium carbonate, and 3.5% proteins, proteoglycans and glycoproteins (Nys et al.
5 1999). The main function of the shell is to provide mechanical protection for the developing
6 embryo (Ar et al. 1979). In addition, embryos use the shell as a source of calcium for the
7 ossification of the developing skeleton (Packard and Packard 1984). During egg laying, female
8 birds require large amounts of calcium-rich material, such as mollusc shells, woodlice and
9 millipedes (Bureš and Weidinger 2003), as their normal food usually does not contain sufficient
10 calcium for eggshell formation (Graveland and van Gijzen 1994, Graveland and Berends 1997,
11 Bureš and Weidinger 2003). A shortage of calcium during egg formation could result in thinner-
12 shelled eggs, and thus poor hatchability due to excessive water loss or shell breakage
13 (Graveland et al. 1994, Eeva and Lehikoinen 1995).

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

22 In many bird species, the cuticle and the calcified layer of the eggshell are pigmented, often
23 with complex patterns. Blue-green eggshell colouration results from the pigment biliverdin,
24 which typically permeates the whole eggshell, while red-brown spots or patches are the result of

1 protoporphyrin (Kennedy and Vevers 1976, Mikšík et al. 1996). Avian eggshell colouration has
2 traditionally been interpreted as a response to selection pressures imposed by nest predators and
3 brood parasites, although other functions, e.g. filtering solar radiation, strengthening the
4 eggshell, or serving as a sexually selected signal of female quality, have also been proposed
5 (Underwood and Sealy 2002, Moreno and Osorno 2003, Gosler et al. 2005, Higham and Gosler
6 2006, Kilner 2006). In addition to human-visible light, most passerines can also detect near
7 ultraviolet (UV-A) light (Cuthill et al. 2000, Ödeen and Håstad 2003), thus, UV reflection may
8 also be an important aspect of egg colouration (Cherry and Bennett 2001, Avilés et al. 2006).
9 Ultraviolet colour is produced by light scattering and it may provide information on the
10 nanostructure of the tissue (Prum et al. 1999, Prum and Torres 2003, Shawkey et al. 2003). The
11 basis of eggshell UV-colour has not been determined yet; it could be affected by the structure of
12 the calcified layer and/or by the pigment content of the shell.

13 In this study, we explored potential causes of variation in eggshell colour (blue-green and
14 UV chroma) and structure (shell thickness and porosity) in a wild passerine, the collared
15 flycatcher (*Ficedula albicollis*). In a previous study, we have shown that the average blue-green
16 colour of a clutch was unrelated to female morphological and serological characteristics and
17 increased with laying date in the collared flycatcher (Hargitai et al. 2008). Here, one of our aims
18 was to determine if the blue-green and UV reflectances of the eggshell are affected by the
19 pigment concentration and the structure of the shell. We predicted that biliverdin pigment
20 concentration in the eggshell would be positively related to blue-green chroma, as demonstrated
21 in earlier studies (Moreno et al. 2006, López-Rull et al. 2008). In contrast, as biliverdin absorb
22 light in the UV part of the spectrum (Falchuk et al. 2002), we expected that eggs with more
23 pigmentation would show less reflectance in the UV region, which hypothesis has not been
24 tested before to our knowledge. Also, we expected that eggs with thicker shells and less pore

1 channels would contain more pigments and thus be more colourful. Alternatively, biliverdin
2 pigment may strengthen the shell similarly to protoporphyrin (Gosler et al. 2005, Jagannath et
3 al. 2008, Sanz and García-Navas 2009), therefore, thinner-shelled eggs with a larger number of
4 pores may show more intense blue-green colour. Until now, the relationship between blue-green
5 eggshell colour and shell thickness has been tested only in a few species, where no associations
6 were detected (Jagannath et al. 2008, López-Rull et al. 2008, Hargitai et al. 2010).

7 Although eggshell thickness and porosity may have important consequences for embryonic
8 growth and development, relatively little is known about the factors responsible for variation in
9 these eggshell characteristics of wild birds. We tested if eggshell thickness and shell porosity
10 are influenced by morphological and serological characteristics of the mother, such as body
11 mass, body size, and stress level, and by local breeding density, laying date, and clutch size. As
12 females seek dietary calcium sources during egg formation (Bureš and Weidinger 2003), we
13 expected that females in better nutritional condition, indicated by their higher mass controlled
14 for tarsus length, would lay eggs with stronger shells, as they may be better foragers of calcium-
15 rich materials too. In addition, we hypothesized that females with a higher level of physiological
16 stress would produce eggs with thinner shells. As far as we are aware, these hypotheses have
17 not been tested before for wild passerines.

18 Breeding density may be related to eggshell thickness through affecting the level of food
19 competition between females (Alatalo and Lundberg 1984, Török and Tóth 1988, Hargitai et al.
20 2009), and thus having a negative effect on shell thickness, however, this hypothesis has not
21 been tested before for wild songbirds. Alternatively, high density areas may be of higher quality
22 with more resources (Doliguez et al. 2004), where individuals of better quality may be able to
23 settle. In this case, we may expect females to produce eggs with stronger shells in an area of
24 higher density. Nevertheless, we suppose that this hypothesis is less likely in our case, as forest

1 soil, vegetation structure and age of oak trees were homogenous in the study area. We also
2 expected that eggshells of eggs laid later in the breeding season may be thinner as late-breeding
3 females may be lower-quality individuals (Garamszegi et al. 2004a, Verhulst and Nilsson
4 2008), which may not be able to acquire sufficient amount of calcium to produce strong-shelled
5 eggs. Eggshell thickness may also vary during the breeding season due to seasonal variation in
6 the availability of calcium-rich resources. Seasonal decline in eggshell thickness has been found
7 in a study of the blue tit (*Cyanistes caeruleus*; Dauwe et al. 2004), but other studies found no
8 significant variation in eggshell thickness with laying date (Eeva and Lehikoinen 1995, Mänd
9 1996, Massaro and Davis 2004).

10 The seasonal variation in eggshell thickness and pore density could also be explained by
11 some adaptive hypotheses. Later breeding has been shown to result in lower reproductive
12 success in small insectivorous passerines as a result of declining food availability (Norris 1993,
13 Wiggins et al. 1994). In several bird species, there is a seasonal decline in incubation length
14 (Eichholz and Sedinger 1998, Hipfner et al. 2001, Massaro et al. 2004), which could reflect
15 adjustments by late-laying parents to improve the survival prospects of their offspring. Hipfner
16 et al. (2001) hypothesized that later-laid eggs might have higher porosity, which may allow
17 embryos to develop more rapidly as a consequence of a higher gas exchange rate. In addition,
18 later-breeding females may produce eggs with thinner shells as an adaptation to allow embryos
19 to develop more rapidly and emerge faster during hatching (Massaro and Davis 2004). So far,
20 these hypotheses have only been tested for non-passerine birds (Mänd 1996, Massaro and Davis
21 2004, Boersma and Rebstock 2009), although accelerated embryonic development of later-laid
22 clutches would be beneficial for insectivorous songbirds too.

23 Females of small passerines have to consume enough calcium each day for egg formation
24 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

1 Pesola spring balance (to the nearest 0.1 g), and the length of the tarsus was measured with a
2 calliper (to the nearest 0.1 mm).

3 Eggs were marked according to laying order with a waterproof marker, and two eggs (third
4 and fourth) per clutch were collected on the day of laying from 24 clutches. For ethical reasons
5 and due to legal restrictions, no whole clutches were collected in this study. Egg length and
6 breadth were measured with a calliper (to the nearest 0.1 mm). Egg size was estimated from
7 Hoyt's formula (Hoyt 1979; $\text{size} = \text{length} \times \text{breadth}^2 \times 0.51$).

8 Female collared flycatchers defend a small breeding territory around their nestbox against
9 conspecific females, particularly during the nest-building and egg-laying periods (Král et al.
10 1996) similarly to their sibling species, the pied flycatcher (*Ficedula hypoleuca*; Slagsvold et al.
11 1992, Rätti 2000). However, the foraging areas of flycatchers often overlap with those of
12 neighbouring pairs (von Haartman 1956). Previously, it has been shown that egg yolk mass was
13 negatively related to the number of neighbouring pairs in the collared flycatcher (Hargitai et al.
14 2009), which could indicate that high local breeding density could limit food availability in this
15 species. We calculated a local breeding density index by summing up the number of breeding
16 collared flycatcher pairs in neighbouring (i.e. adjacent) nestboxes that started egg laying until
17 the laying of the fifth egg of the clutch in question. As nestboxes were placed in a grid system,
18 each nestbox, except for those located at the edge of the study plot, had eight nestboxes adjacent
19 to it. Distance between nestboxes was approximately 30-50 m.

20 21 Serological analysis

22
23 A blood sample was taken from the female's brachial vein during incubation. A drop of blood
24 was smeared on a microscope slide, air dried, fixed in absolute methanol, and later stained with

1 Giemsa's solution. The proportion of different types of leucocytes was assessed by examining a
2 total of 50 leucocytes under $\times 1000$ magnification with oil immersion. An index of the relative
3 abundance of heterophil granulocytes to lymphocytes (H/L ratio) was used to assess the stress
4 level of the females (Gross and Siegel 1983, Maxwell 1993).

5 6 Egg colour measurement

7
8 Egg colour was measured on the day of laying using a portable battery-driven USB2000
9 spectrometer with a Mini DT deuterium-halogen light source (Ocean Optics Europe, Duiven,
10 The Netherlands). The reflectance spectrum was determined between 320 and 700 nm at 0.38
11 nm intervals. Inclusion of ultraviolet (UV) is necessary as most bird species are sensitive to near
12 UV light (Cuthill et al. 2000). Eggs were placed with their side in the hole of a brick-formed
13 aluminium reflection probe holder (RPH-1, Ocean Optics Europe), so that eggs completely
14 filled the hole. A bifurcated 400- μ m fibre optic probe was used, and the sampling optic was
15 held perpendicular and at a distance of 3 mm from the eggshell surface. Three measurements
16 were taken from each egg by slightly changing the position of the egg in the hole. Reference
17 calibration was performed with a WS-1 white standard after every second egg. Mean
18 reflectance was calculated by averaging the three measurements. The spectral curves were
19 generated by using the OOIBase32 software with a 3,500 ms integration time. The reflectance
20 spectrum was double peaked with a prominent peak around 400-580 nm (blue-green), and
21 around 320 nm (UV). Blue-green chroma ($R_{400-580}/R_{320-700}$) and UV chroma ($R_{320-400}/R_{320-700}$)
22 were calculated as the proportion of the reflectance occurring in the given part of the spectrum,
23 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 mm² was placed over the shell. Pores were counted in two such areas using a stereomicroscope with a magnification of 40x. 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

1 Biliverdin analysis was based on the method described by Mateo et al. (2004) with some
2 modifications. Biliverdin was extracted from 7-19 mg eggshell pieces from the equatorial
3 region of one egg (fourth) per clutch by adding 315 µl acetonitrile and 263 µl HCl 3N. After 15
4 min of the addition of the reagents, the tubes were capped and subsequently vortexed for 15 s
5 and sonicated for 10 s in an Ultrasons-H. Samples were centrifuged for 10 min at 12000 rpm
6 and the supernatant, containing the pigments, was transferred to glass vials for later high
7 pressure liquid chromatography (HPLC) analysis. Extraction was repeated twice by adding 400
8 µl acetonitrile to the samples. The acetonitrile phases were combined and HPLC analysis was
9 conducted with an Agilent 1100 system with a Waters Spherisorb 5 µm ODS2 (4.6 mm × 100
10 mm) column at a 1.5 ml/min flow rate. The initial mobile phase composition was ammonium-
11 acetate (1 M, pH: 5.16) 75% and methanol 25% for 2 min after sample injection, and then a
12 gradient was applied with a linear change in 8 min to 95% methanol and 5% ammonium
13 acetate, followed by 2 min at these conditions. At this moment, the phase composition returned
14 to the initial conditions in 3 min, and was then stabilized for another 3 min before the next
15 sample injection. The column was maintained at 70° C and the detection was performed at 400
16 nm wavelength. The quantification of samples was performed using calibration curves of
17 biliverdin standard added to white eggshells of domestic hen (Moreno et al. 2006).
18 Concentration of biliverdin was expressed as nmol/g of dry weight of eggshell.

19 20 Statistical analyses

21
22 Data were analysed with Pearson's correlation and general linear models. Separate analyses
23 were performed with female characteristics (body mass, tarsus length, and H/L ratio; Table 1)
24 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 overparameterization, we re-entered nonsignificant effects to the final model one by one, and present these F - and P -values. We also report standardized 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 Inc.).

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 ($F_{1,18} = 4.69$, $P = 0.044$, $\beta = 0.45$, Cnf. Lmt. = 0.01 and 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 ($F_{1,21} = 6.18$, $P = 0.021$, $\beta = -0.48$, Cnf. Lmt. = -0.88 and -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: $F_{1,19} = 0.41$, $P = 0.53$, $\beta = -0.14$, Cnf. Lmt. = -0.58 and 0.31 ; clutch size: $F_{1,19} = 9.10$, $P = 0.007$, $\beta = -0.64$, Cnf. Lmt. = -1.09 and -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 higher concentration of biliverdin pigment showed less intense UV reflection. However, neither shell thickness nor shell porosity was related to eggshell colouration or the biliverdin pigment concentration of the shell. In concert with our results, blue-green eggshell colouration 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 (*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

1 supported by Sieffermann et al. (2006), who found that white eggs also reflected light in the UV
2 range. Egg pigments appear to decrease the intensity of UV reflectance by absorbing light in
3 these wavelengths. However, we could not detect any associations between UV chroma and
4 shell thickness or pore density, suggesting that nanostructural characteristics of the shell and
5 shell pigmentation may be responsible for the UV reflectance, and shell thickness and porosity
6 do not influence it.

7 Besides proteins and lipids, laying birds need to consume large quantities of calcium-rich
8 material in order to possess sufficient calcium for egg formation (Simkiss 1967). Acquiring a
9 sufficient amount of calcium is time-consuming even in a calcium-rich environment, thus, there
10 may exist a trade-off between feeding time and time spent searching for calcium (Graveland
11 and Berends 1997). We found that females in better nutritional condition produced eggs with
12 thicker shells, although body size and stress level showed no significant relationships with shell
13 thickness. It is possible that females in better condition have more time available for searching
14 calcium-rich food items and thus could produce eggs with stronger shells. Moreover, these birds
15 may be better foragers of both nutrients and calcium, as egg size, which is also related to female
16 condition (Hargitai et al. 2005), and shell thickness showed a weak, almost significant positive
17 relationship, which might be significant with a larger sample size. The positive relationship
18 between shell thickness and egg size has also been shown in two other songbirds, the great tit
19 (*Parus major*) and the pied flycatcher (Eeva and Lehikoinen 1995). In the peregrine falcon
20 (*Falco peregrinus peregrinus*), it was also reported that heavier females laid clutches of larger
21 eggs with thicker shells, although in other falcon species this relationship was not significant
22 (Castilla et al. 2009). Experimental studies also found that calcium supplemented birds laid
23 thicker-shelled and also larger eggs, probably as the decrease in searching time for calcium

1 might incur a higher intake of nutrients or a lower energy expenditure (Tilgar et al. 1999, Mänd
2 and Tilgar 2003).

3 Contrary to our expectation, we found no evidence in collared flycatchers that eggshell
4 thickness was related to laying date or local breeding density. This suggests that the availability
5 of calcium-rich materials does not vary significantly during the laying period, and it is not
6 affected by local competition for calcium-rich materials. However, the laying date interval of
7 the analysed clutches (Table 1) might have been too short to detect such differences (see also
8 later), therefore, we must treat our results with caution. Nevertheless, our results are in
9 concordance with previous findings. Eggshell thickness did not vary with laying date in great
10 tits (Eeva and Lehikoinen 1995), pied flycatchers (Eeva and Lehikoinen 1995), black-headed
11 gulls (*Larus ridibundus*; Mänd 1996) and yellow-eyed penguins either (*Megadyptes antipodes*;
12 Massaro and Davis 2004).

13 Apart from the effect of physiological constraints, eggshell structure may also vary
14 seasonally due to the strategic adjustment of the female. Since later laying has often been
15 associated with lower reproductive success as a result of declining food availability during the
16 period of parental care (Norris 1993, Wiggins et al. 1994), there may be a reproductive
17 advantage for females breeding later in the season to reduce incubation time. It has been shown
18 in numerous studies that total pore area of the eggshell affects water vapour conductance, and
19 both of them are positively related to the growth rate of embryos and hatchability of eggs
20 (Burton and Tullett 1983, Ar and Rahn 1985, Soliman et al. 1994, Massaro and Davis 2005,
21 Zimmermann and Hipfner 2007). In the yellow-eyed penguin, Massaro and Davis (2004)
22 detected an increase in pore density with laying date which may allow embryos to develop more
23 rapidly than earlier laid eggs. Until now, this hypothesis has not been tested for small
24 passerines, although accelerated embryonic development of later-laid clutches would be

1 beneficial for insectivorous songbirds too. However, we could not detect a seasonal variation in
2 eggshell pore density in the collared flycatcher. It is possible that late-breeding collared
3 flycatcher females reduce incubation length by starting incubation before all eggs within a
4 clutch are laid, thereby producing hatching asynchrony, which may benefit the first hatched
5 nestlings (Slagsvold 1986, Szegedi et al. 2009). Alternatively, the laying date interval of the
6 analysed clutches (11 days; Table 1) might have been too short to detect such differences. The
7 total laying date interval of collared flycatchers breeding in our study plot in 2005 was 19 days,
8 and we do not have data of clutches at the end of the breeding season and of clutches of repeat
9 laying, where we may expect the largest increase in pore density. This could limit the
10 interpretation of our result. However, in accordance with our result, eggshell pore density did
11 not differ between eggs of early and late clutches in the black-headed gull (Mänd 1996) and the
12 Magellanic penguin either (*Spheniscus magellanicus*; Boersma and Rebstock 2009).

13 Songbirds do not store calcium for egg formation in the skeleton, they must collect calcium
14 on a daily basis during the laying period (Graveland and van Gijzen 1994, Graveland and
15 Berends 1997, Pahl et al. 1997). Therefore, we might not expect a decline in shell thickness
16 with increasing clutch size as calcium is not utilized from a limited store, and indeed we found
17 no relationship between number of eggs within a clutch and shell thickness. However, we found
18 that eggs in clutches of more eggs had lower pore density than eggs in clutches of fewer eggs.
19 Porosity of the shell and the gas pressure gradient between the internal environment and the
20 microenvironment of the nest affect gas conductance (Ar et al. 1974). If the rate of water loss
21 and nest humidity are to remain constant in clutches of different egg numbers, then pore density
22 should be reduced in proportion to the increase in egg number.

23 To sum up, our data showed that the blue-green and UV chroma of the eggshell were related
24 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.

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1

2 Table 1

	<i>n</i>	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

3

4

5 Table 2

	Blue-green chroma					UV-chroma				
	Cnf. Lmt.					Cnf. Lmt.				
	<i>F</i>	<i>df</i>	<i>P</i>	Beta	(lower,upper)	<i>F</i>	<i>df</i>	<i>P</i>	Beta	(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)

6

Table 3

	Eggshell thickness					Pore density				
	Cnf. Lmt.					Cnf. Lmt.				
	<i>F</i>	<i>df</i>	<i>P</i>	Beta	(lower,upper)	<i>F</i>	<i>df</i>	<i>P</i>	Beta	(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)

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

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. 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 (Conf. Lmt.) are also reported.

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. 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 (Conf. Lmt.) are also reported.

Figure legends

Fig. 1 Relationship between eggshell blue-green chroma ($R_{400-580}/R_{320-700}$) and eggshell biliverdin concentration (lg-transformed) in the collared flycatcher. Presented 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. Presented values correspond to the fourth egg in each clutch

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

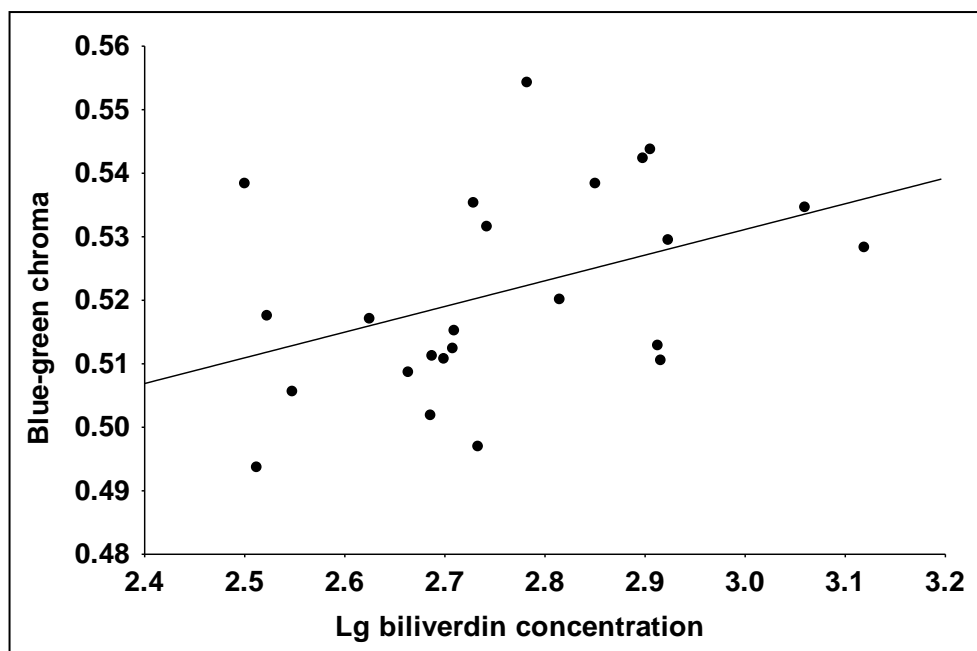


Fig. 1

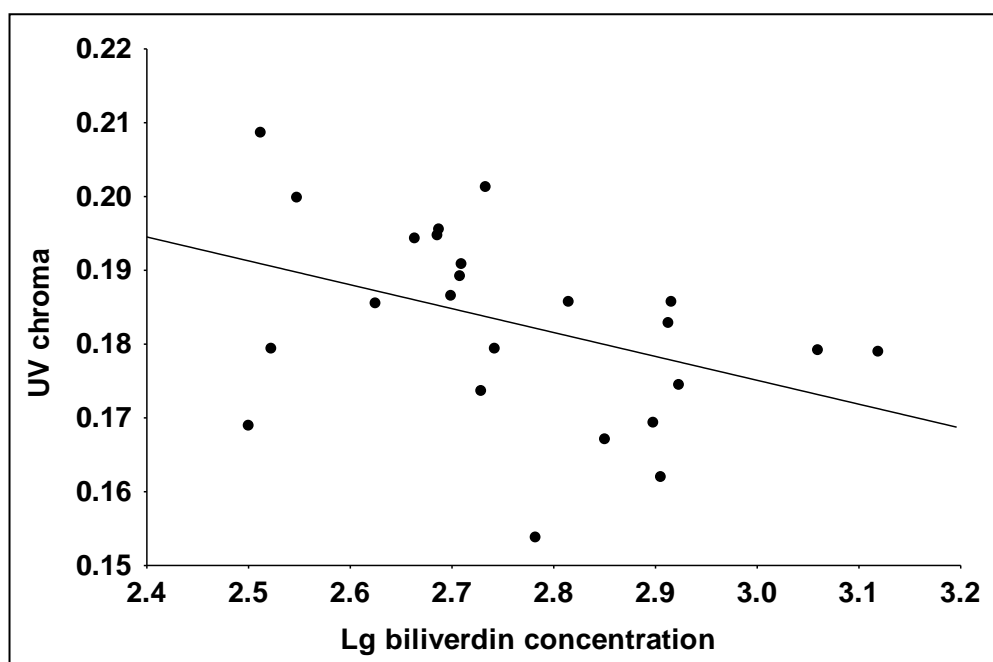


Fig. 2

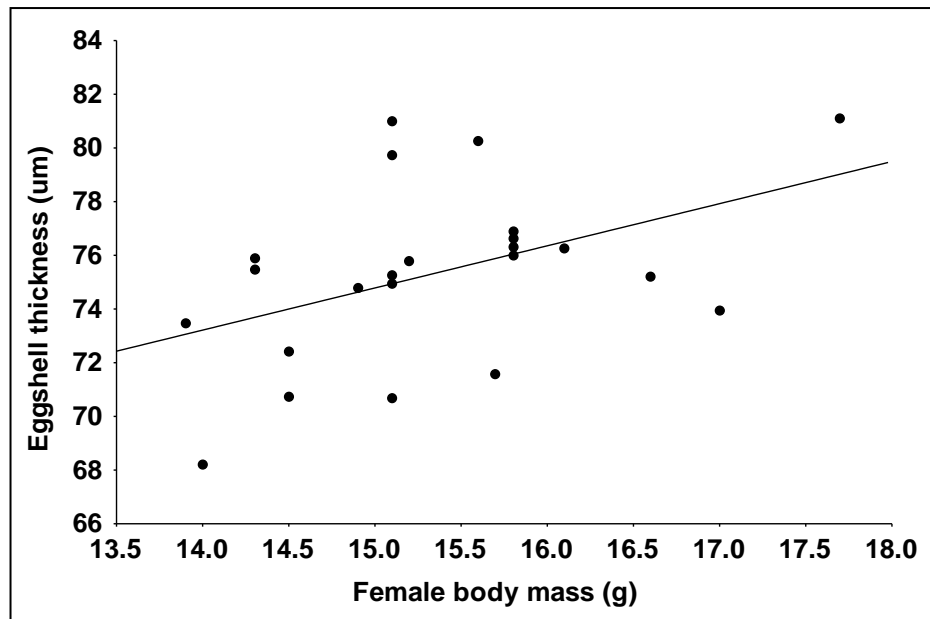


Fig. 3