



Variation in eggshell traits between geographically distant populations of pied flycatchers *Ficedula hypoleuca*

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The expression and impact of maternal effects may vary greatly between populations and environments. However, little is known about large-scale geographical patterns of variation in maternal deposition to eggs. In birds, as in other oviparous animals, the outermost maternal component of an egg is the shell, which protects the embryo, provides essential mineral resources and allows its interaction with the environment in the form of gas exchange. In this study, we explored variation of eggshell traits (mass, thickness, pore density and pigmentation) across 15 pied flycatcher populations at a large geographic scale. We found significant between-population variation in all eggshell traits, except in pore density, suggesting spatial variation in their adaptive benefits or in the females' physiological limitations during egg laying. Between-population variation in shell structure was not due to geographic location (latitude and longitude) or habitat type. However, eggshells were thicker in populations that experienced higher ambient temperature during egg laying. This could be a result of maternal resource allocation to the shell being constrained under low temperatures or of an adaptation to reduce egg water loss under high temperatures. We also found that eggshell colour intensity was positively associated with biliverdin pigment concentration, shell thickness and pore density. To conclude, our findings reveal large-scale between-population variation of eggshell traits, although we found little environmental dependency in their expression. Our findings call for further studies that explore other environmental factors (e.g. calcium availability and pollution levels) and social factors like sexual selection intensity that may account for differences in shell structure between populations.

A mother can greatly influence her offspring phenotype through non-genetic contributions, the so-called 'maternal effects' (Mousseau and Fox 1998). These are modulated by the mother's condition and genetic quality, but also by the environment she experiences. Environmental variation may constrain maternal resource allocation as well as influence the adaptive benefits of maternal effects and thereby the evolutionary trajectories of populations (Räsänen and Kruuk 2007). Thus, although little explored in empirical work, the expression of maternal effects is expected to vary across populations and environments.

Birds are excellent models to study maternal effects because mothers must allocate all the resources required for

embryonic development in a single package, the egg. While most work has focussed on egg size and yolk components, such as antibodies, carotenoids and hormones, much less is known about variation in eggshell maternal components between populations (but see Anderson and Hickey 1970, Davies and Randall 1989, Ryckman et al. 1998, at a relatively large geographic scale). Importantly, the shell is the outermost component of an egg and its first key function is to protect the embryo and all the resources allocated inside the egg (Ar et al. 1979). Other crucial shell functions are to supply the embryo with a reservoir of calcium and magnesium, and to facilitate gas and water transfer through the shell pores (Rahn et al. 1987, Deeming 2002). Thus, females

must fine-tune resource allocation to the shell according both to their own physiological limitations and to local environmental conditions (Packard et al. 1977, Arad et al. 1988, Gosler et al. 2005, Higham and Gosler 2006).

Maternal investment in shell mass and thickness is limited by calcium availability (Graveland et al. 1994, Tilgar et al. 1999a, b, Mänd et al. 2000, García-Navas et al. 2011) and thus should vary across habitats with different nutrient abundance. For example, pied flycatchers lay thicker shells in calcium-rich deciduous forests than in calcium-poor evergreen ones (Tilgar et al. 1999a). Other environmental factors like low ambient temperatures may impair resource allocation to shell thickness (Ojanen 1983; see also Sahin et al. 2002, 2003, in poultry). On the other hand, high ambient temperatures increase egg water loss (Higham and Gosler 2006). Thus, given that shell thickness determines pore length and is inversely related to shell conductance (Ar et al. 1974), thicker eggs might be adaptive in hot and dry environments to prevent water loss. Likewise, less porous shells should be adaptive in these environments (Davis et al. 1984, Davis and Ackerman 1985). Previous findings in wild birds show either a positive effect of temperature on shell mass (Ojanen 1983) and thickness (Arad et al. 1988) or no effect at all (Stein and Badyaev 2011). Bourgault et al. (2007) found that blue tits *Cyanistes caeruleus* laid eggs with heavier shells in a colder deciduous forest compared with a coniferous forest; yet, the effects of temperature and habitat type could not be disentangled. Studies exploring the effect of ambient temperature on shell structure are required in wild populations.

Mothers also transfer pigments to the eggshell and the function of this allocation is still under debate. Traditionally, predation and brood parasitism have been proposed as major selective pressures on shell pigmentation (Wallace 1889). Other hypotheses invoke a signalling role (Moreno and Osorno 2003, Hanley et al. 2010), reinforcement of shell strength (Gosler et al. 2005) or direct benefits to the embryo (Maurer et al. 2011). The blue-green pigment biliverdin possesses strong in-vitro antioxidant and antiviral properties (Stocker et al. 1987, McDonagh 2001), and in amphibians, it favours embryo development (Falchuk et al. 2002). In birds, females that lay intensively coloured biliverdin-pigmented eggs show higher antioxidant capacity (Hanley et al. 2008). For these reasons, biliverdin pigmentation could be an adaptive maternal effect in birds. Whatever its function, shell biliverdin-based pigmentation seems costly for female birds (Morales et al. 2008) and varies with environmental conditions. For example, females enhance biliverdin-based pigmentation when food abundance and carotenoid availability increase (Moreno et al. 2006, Morales et al. 2011). Additionally, a study performed with eggs from a museum collection suggests that shell biliverdin pigmentation is influenced by rainfall and temperature (Avilés et al. 2007). Thus, we may expect that geographic variation in habitat type and climate drives variation in shell biliverdin pigmentation. However, to our knowledge, this has not been explored to date.

The aim of this study was to investigate between-population variation of eggshell traits in 15 pied flycatcher populations at a large geographic scale. We studied shell thickness, mass, pore density and pigment concentration

in relation to latitude, longitude, habitat type (deciduous or coniferous) and minimum temperature at laying. The pied flycatcher is a long-distance migrant that breeds in Western Palaearctic woodlands, from temperate to subarctic regions (Cramp and Perrins 1993). In this species, previous studies have reported geographic and habitat-specific variation in various reproductive traits. For instance, timing of breeding is later and clutch size smaller from central Europe towards the north, where breeding seasons are short and weather conditions unpredictable (Järvinen 1989, Sanz 1997, Eeva et al. 2002). Likewise, maternal allocation of carotenoids to the egg yolk is reduced from central Europe outwards (Eeva et al. 2010, Ruuskanen et al. 2011). Also, eggshells are thicker in calcium-rich deciduous forests (Tilgar et al. 1999a, b), and ambient temperature prior to egg laying exerts a positive effect on shell mass in one of the study populations (Ojanen 1983). This species lays blue biliverdin-pigmented eggs and shell colour intensity accurately reflects biliverdin concentration (Moreno et al. 2006). Shell colour intensity is limited by food availability at laying (Moreno et al. 2006) and is subject to environmental variation across years (Morales et al. 2010). Besides, it mirrors the amount of maternal antibodies transferred to the egg (Moreno et al. 2005, Morales et al. 2006). Given that egg colouration results from maternal allocation of biliverdin to the shell and likely from other aspects of shell structure, a secondary goal of this study was to investigate if egg colour intensity was related to eggshell traits in the study populations.

We predicted that maternal resource allocation to costly shell traits, such as thickness, mass and pigment concentration would diminish towards the northern boundary of the species distribution and in populations experiencing low ambient temperatures at laying. Given that food availability is usually higher in deciduous forests than in coniferous ones (Mänd et al. 2005, Veen et al. 2010), we expected that populations breeding in coniferous habitats would reduce resource allocation to shell mass, thickness and biliverdin concentration, as previously found for yolk carotenoids in the study populations (Eeva et al. 2010). Finally, we expected that shell colour intensity was positively associated with pigment concentration (Moreno et al. 2006) and likely with other shell traits.

Methods

Study sites, egg collection and storage

Eggs were collected in 2007 from 15 nest-box study populations (on average, 24 eggs per population; total $n = 358$; Table 1) to analyze eggshell structure and some other maternal components in the yolk and white (Eeva et al. 2010, Ruuskanen et al. 2011). The sampling area ranged in latitude from 41°N to 69°N and in longitude from -4°E to 60°E (Fig. 1), thus covering a large part of the species breeding range. It must be noted that the locations of the study populations and in general the breeding range of pied flycatchers in Europe are not equally distributed with respect to latitude and longitude (Fig. 1). The population locations show a trend from south-west to north-east and,

Table 1. Geographic location, minimum temperature at laying (min. T) and mean \pm SD (n) values of the eggshell traits studied for 15 pied flycatcher populations.

Country	Area	Lat. (°N)	Long. (°E)	min. T (°C)	Thickness (mm)	Mass (mg)	Biliverdin conc. (nmol g ⁻¹)	Pore density (no. pores mm ⁻²)	Colour intensity (blue-green chroma)
Spain	Lozoya (ESP)	40.8	-4.0	8.10	0.066 \pm 0.003 (27)	94.39 \pm 9.47 (27)	384.18 \pm 72.99 (9)	0.81 \pm 0.53 (8)	0.578 \pm 0.008 (27)
Germany	Harz (GER2)	51.9	10.6	3.55	0.065 \pm 0.004 (18)	85.88 \pm 14.53 (18)	180.62 \pm 84.03 (10)	1.03 \pm 0.50 (11)	0.576 \pm 0.006 (18)
Netherlands	Buunderkamp (NL)	52.0	5.8	8.12	0.064 \pm 0.005 (15)	89.30 \pm 8.71 (15)	205.49 \pm 101.0 (13)	0.97 \pm 0.54 (11)	0.578 \pm 0.006 (15)
UK	Powys (UK)	52.2	-3.5	8.11	0.067 \pm 0.004 (20)	97.42 \pm 6.60 (20)	278.28 \pm 88.95 (20)	1.36 \pm 0.22 (10)	0.579 \pm 0.007 (20)
Germany	Lingen (GER1)	52.5	7.3	7.52	0.064 \pm 0.003 (22)	94.95 \pm 8.52 (22)	362.24 \pm 67.11 (10)	0.85 \pm 0.31 (11)	0.579 \pm 0.005 (22)
Russia	Moscow (RU/S2)	55.7	36.9	15.47	0.070 \pm 0.003 (22)	91.65 \pm 10.22 (22)	271.28 \pm 86.19 (9)	0.99 \pm 0.68 (9)	0.570 \pm 0.009 (22)
Latvia	Krāslava (LV)	55.9	27.2	12.66	0.068 \pm 0.005 (18)	91.31 \pm 9.26 (19)	348.51 \pm 44.02 (10)	0.86 \pm 0.54 (7)	0.579 \pm 0.008 (19)
Russia	Revda (RU/S1)	56.8	59.5	8.30	0.067 \pm 0.004 (22)	85.61 \pm 10.45 (22)	310.27 \pm 77.55 (10)	0.79 \pm 0.47 (14)	0.572 \pm 0.006 (22)
Sweden	Öland (SWE)	57.2	17.0	8.78	0.069 \pm 0.004 (9)	104.61 \pm 11.47 (7)	345.79 \pm 67.56 (9)	0.47 \pm 0.48 (8)	0.580 \pm 0.008 (10)
Estonia	Kilingi-Nõmme (EST)	58.1	25.1	9.0	0.069 \pm 0.004 (42)	95.74 \pm 11.13 (42)	221.65 \pm 62.89 (10)	1.03 \pm 0.41 (13)	0.578 \pm 0.007 (42)
Finland	Turku (FIN1)	60.4	22.2	10.15	0.066 \pm 0.002 (20)	95.35 \pm 10.77 (20)	383.51 \pm 97.28 (10)	0.78 \pm 0.58 (12)	0.580 \pm 0.008 (20)
Finland	Harjavalta (FIN2)	61.3	22.2	9.90	0.062 \pm 0.004 (32)	86.63 \pm 7.16 (32)	248.30 \pm 103.0 (20)	1.21 \pm 0.50 (11)	0.578 \pm 0.007 (32)
Finland	Kauhava (FIN3)	63.0	23.0	9.25	0.067 \pm 0.004 (47)	87.33 \pm 7.09 (46)	327.23 \pm 59.16 (8)	0.87 \pm 0.32 (12)	0.579 \pm 0.007 (47)
Finland	Oulu (FIN4)	65.0	25.5	5.21	0.065 \pm 0.004 (20)	98.70 \pm 10.11 (21)	342.87 \pm 86.39 (10)	0.76 \pm 0.33 (12)	0.581 \pm 0.007 (21)
Norway	Skibotn (NOR)	69.3	20.7	5.73	0.065 \pm 0.003 (21)	92.09 \pm 8.17 (21)	239.80 \pm 99.47 (10)	0.94 \pm 0.41 (13)	0.579 \pm 0.006 (21)

thus, latitude and longitude are not totally independent variables (although they are not significantly correlated; $r = 0.48$, $p = 0.07$, $n = 15$). Thus a trend in either one is to some extent affected by the other as well.

We classified study sites as coniferous or deciduous to differentiate purely coniferous habitats from deciduous or mixed forests (Table 1). One of the Russian populations (Revda) was divided in two plots, corresponding to a coniferous and a deciduous forest. The Estonian population also shows two habitats that differed in some breeding parameters (Tilgar et al. 1999a, Mänd et al. 2005). Temperature data for the laying period of each population was acquired from European Climate Assessment and Dataset Project (Klein Tank et al. 2002), using the nearest possible meteorological station. We used the average of daily minimum temperatures one week before and one week after the population average laying date in 2007.

We checked nest-boxes regularly in all populations to monitor the progress of nesting. When eggs were found in the nest they were marked and the nest was visited daily to collect the freshly laid third or fourth egg of each clutch, according to national and international guidelines and under the licenses from environmental authorities and ethical committees in each country (Appendix 1). In each population, egg sampling was conducted evenly throughout the breeding season if possible (leaving out the very first and last nests), to avoid bias in egg composition due to potential seasonal variation in egg components. We recorded the laying date of the egg collected and the clutch size of its nest to include them in statistical analyses. On the day of collection, whole eggs were deposited inside tubes with cotton or paper and stored at -20°C until the separation of the eggshell from the egg content (yolk and white). On the whole, all eggshells were taken out of the tubes twice (and therefore twice exposed to light) from collection to thickness and colour quantification and three times from collection to pigment analyses. These exposures were similar in length and performed under the same conditions for all samples, thus minimizing potential differences in pigment degradation between samples (Cassey et al. 2010, Moreno et al. 2011).

Shell mass, thickness and pore density

Eggshells were carefully halved and separated from the yolk and white at the Dept of Biological and Environmental Science at Jyväskylä Univ. (Finland). The eggshell halves were then washed, air-dried, weighed in a Mettler Toledo XS204 Delta Range scale (accuracy 0.1 mg) and stored in darkness inside tubes. The shell membranes were apparently not affected by the freezing, removal of egg contents and washing, and remained attached to the shell as confirmed by cross-sectional electron microscopy images of 10 shell samples per population. Shell thickness was measured at the Dept of Evolutionary Ecology at Museo Nacional de Ciencias Naturales-CSIC (Madrid, Spain). It was measured including shell membranes in three places on the eggshell with a Mitutoyo digital tube micrometer (model 395-271) with ball-point ends and precision of 0.001 mm. Shell thickness was repeatable within samples ($r = 0.65$; $F_{357, 709} = 6.56$, $p < 0.001$;



Figure 1. Locations of the pied flycatcher populations sampled. Grey area illustrates the breeding range of pied flycatchers in Europe (map modified from *Birds of the Western Palearctic*, ver. 2.0.1., Oxford Univ. Press, 2003). Names refer to populations listed in Table 1.

Lessells and Boag 1987) and thus we calculated the mean of the three measurements of each egg. In most samples (80%), thickness was measured on the equatorial region of the eggshell, although in some cases one or two of the measurements were taken on the shell poles (if only measurements taken on the shell equatorial region are considered, similar results are obtained; data not shown). When the shell samples were accidentally broken into small pieces, it was easy to identify where the shell pieces came from (blunt end, sharp end or equator) due to their curvature.

To estimate pore density, we used on average 11 eggshells from each population (mean \pm SD: 11 ± 2.0). Eggshell porosity was quantified at the Museo Nacional de Ciencias Naturales-CSIC (Madrid, Spain). Shell pores were counted on three different pieces of the equatorial region of each eggshell under $200\times$ magnification with a Scanning Electron Microscope FEI INSPECT. A previous study performed in Powys location suggests that the number of pores is evenly distributed among different parts of the pied flycatcher eggshell (Kern et al. 1992). Under the electron microscope, pores are clearly identified without the need of staining techniques. Despite a few pores can be filled with material, they are still clearly visible under $200\times$ magnification, since the occluding material is usually deep within the pore channel, not like a ‘cap’ (Kern et al. 1992). The samples were observed in the low

vacuum mode with a back scattering electron detector at a voltage of 30 kV and an electron beam spot diameter of 6 μm . The samples totally filled the viewing screen and were always observed at a distance of 12 mm to the electron beam, thus always scanning the same area (2 mm^2). Repeatability of pore density was low but significant ($r = 0.19$; $F_{161, 324} = 1.68$, $p < 0.001$). We thus averaged the pore density (no. of pores mm^{-2}) recorded in the three shell pieces of each egg.

Shell biliverdin concentration

We used on average 11 eggshells from each population (mean \pm SD: 11 ± 3.7) for pigment quantification. Biliverdin concentration was measured at the laboratory of Inst. de Investigación en Recursos Cinegéticos-CSIC (Spain). Each eggshell was first weighed (accuracy 0.1 mg) and between 60 and 80 mg of each sample were introduced inside an Eppendorf tube. Biliverdin pigment was extracted by adding to each sample 250 μl of HCl 3N and 500 μl of acetonitrile to the tube. After five minutes of the addition of reagents, the tubes were capped and subsequently vortexed for one minute and sonicated for another minute in an Ultrasons-H (Selecta, Barcelona, Spain). Samples were then centrifuged for ten minutes at 12000 r.p.m. in a Biofuge Pico Heraeus (Kendro Laboratory Products,

Osterode, Germany), after which 400 μl of organic supernatant was transferred to 1.5 ml glass vials (Fisherbrand). This process was repeated twice more to maximize pigment extraction, but this time adding 400 μl of acetonitrile (the same amount of supernatant collected) instead of 500 μl . The final volume of extract was 1200 μl . High-performance liquid chromatography (HPLC) was performed following the protocol described by Mateo et al. (2004), with few modifications (Moreno et al. 2006, in pied flycatcher eggs). The column was maintained at 63°C, and the UV detection was done at 377 nm wavelength (the peak of absorbance of biliverdin). Calibration curves were constructed by adding 0, 10, 20, 40 and 80 nmol of biliverdin standard (Frontier Scientific Europe, Carnforth, UK) to 80 mg of white eggshell of domestic hens and processed as samples. The recovery of biliverdin with the described method based on three consecutive extractions was 97.3%. Calibrations were injected at the beginning of the analytical sequence and every 12 injected samples. Concentration of biliverdin was expressed as nmol g^{-1} of dry weight of eggshell.

Shell colour intensity

Eggshell colour was measured in all collected eggs with a MINOLTA CM-2600d portable spectrophotometer (Minolta, Osaka, Japan). In most cases, three different pieces of each eggshell were sequentially measured once. In a few cases, just one or two measures could be taken, as the eggshell was broken into small pieces. Eggshell pieces were placed directly on a target mask with a diameter of 3 mm, the mask hole being completely filled. Reference calibrations against zero and a white standard tablet (Minolta) were performed periodically according to the apparatus instructions. The SPECTRAMAGIC software (Minolta) was used to obtain the reflectance spectra from 360 nm in intervals of 10 nm. From the reflectance spectra, we calculated blue-green chroma as the proportion of total reflectance that is in the blue-green region of the spectrum ($R_{400-570}/R_{360-700}$), following Siefferman et al. (2006). This corresponds to the region with least absorbance (and therefore greatest reflectance) of biliverdin (Falchuk et al. 2002), coincident with the region of maximum reflectance of pied flycatcher eggs (Moreno et al. 2005). Blue-green chroma of pied flycatcher eggs measured in this way correlates with reflectance measures using a spectrophotometer that includes the UV range (300–360 nm) (Moreno et al. 2006). Blue-green chroma was repeatable within samples and thus we averaged the three colour measurements taken for different eggshell pieces ($r = 0.85$, $F_{351, 699} = 17.28$, $p < 0.001$).

Statistical analyses

All statistical analyses were performed with SAS software (SAS Inst. ver. 9.1). First we analysed if there was between-population variation in the eggshell traits studied. For normally distributed variables (shell mass, thickness and biliverdin concentration), this was done using general linear models (GLM procedure) with the shell trait as the response variable and population as the explanatory variable. Pore density was not normally distributed and was

analyzed with a generalized linear model with Poisson error (Genmod procedure).

For shell mass, thickness and pigment concentration, we performed three linear mixed models (Mixed procedure) to test their associations with the following independent variables: latitude, longitude, habitat type (coniferous or deciduous), temperature, sample egg laying date and its nest clutch size. Since the effect of habitat type on maternal allocation to eggs may depend on laying onset (Blondel et al. 1993), we also searched for the interaction effect between habitat type and laying date. We standardized individual laying date and clutch size within populations to zero mean and unit standard deviation. Given that the total amount of eggshell produced by females may determine pigment allocation to it, shell mass was included as a covariate in the model of pigment concentration. Also, shell thickness was included as a covariate in the model of shell mass. For pore density, we performed a generalized linear mixed model with Poisson error (Glimmix procedure), including the same covariates that we explored for the other shell traits. To investigate the relationship between egg colour intensity and shell structure we performed another linear mixed model with normal error and the following independent variables: pigment concentration, shell thickness and pore density. In all mixed models, we included population as a random effect and calculated the degrees of freedom with the Satterthwaite method, as recommended by Littell et al. (2006). We obtained the final minimal models by a backward deletion procedure: first, the interaction, and then the main effects were removed from the full model when non-significant ($\alpha = 0.05$). We show F and p values and degrees of freedom of non-significant terms before dropping them from the full models.

Finally, we used the Variogram procedure to check whether there was spatial autocorrelation in the residuals of the final models (i.e. those that retained at least one significant term). Moran's I coefficients ranged from -0.0758 to -0.0161 , indicating weak negative autocorrelation in the data. Thus, we run again the final mixed models with a geospatial analysis that allows controlling for geographic coordinates and testing whether spatial covariance structure gives better model fit than the default structure (variance components). This analysis was implemented in the final mixed models by specifying in the random statement the geographic coordinates of data and an exponential covariance structure (Littell et al. 2006, chapter 11). The gaussian structure led to similar results, but was not considered appropriate for these data (Littell et al. 2006).

Results

Population average and trait variance in eggshell characteristics are shown in Table 1. All eggshell traits studied except pore density showed significant variation between populations (thickness: $F_{14, 340} = 8.00$, $p < 0.001$, $r^2 = 0.25$; shell mass: $F_{14, 339} = 5.86$, $p < 0.001$, $r^2 = 0.20$; biliverdin: $F_{14, 153} = 6.52$, $p < 0.001$, $r^2 = 0.37$; pore density: $\chi^2_{14, 147} = 6.36$, $p = 0.96$). Within-population variation was particularly high in pore density and in biliverdin concentration at various locations (Table 1).

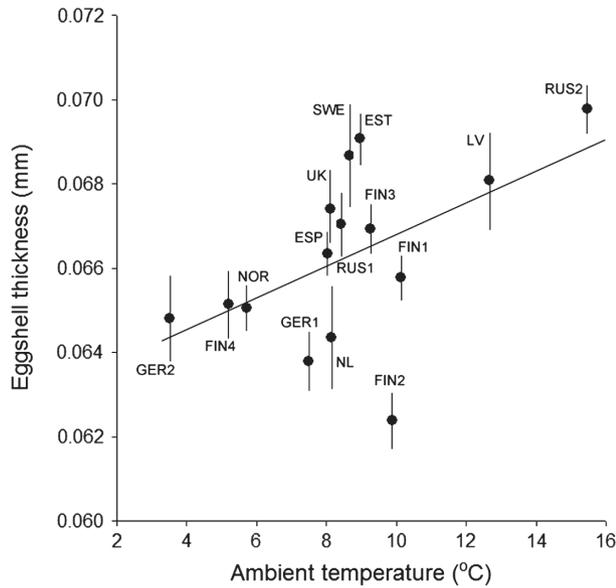


Figure 2. Association between eggshell thickness (mm) and minimum ambient temperature (°C) registered at each population during the laying period. Values are observed population means \pm SE.

Eggshell thickness was the only aspect of shell structure modified by an environmental factor, since it was positively related to minimum temperature at laying (standardized coefficient = 0.24, $F_{1, 14.4} = 5.11$, $p = 0.040$; Fig. 2). Also, shell thickness increased with later laying date (std. coef. = 0.090, $F_{1, 339} = 3.88$, $p = 0.049$). The effects of the other covariates on shell thickness were not significant (laying date \times habitat: std. coef. [coniferous] = 0.045, $F_{1, 328} = 0.21$, $p = 0.64$; clutch size: std. coef. = 0.030, $F_{1, 330} = 0.32$, $p = 0.57$; habitat: std. coef. [coniferous] = -0.12, $F_{1, 98.2} = 0.43$, $p = 0.52$; latitude: std. coef. = -0.10, $F_{1, 11.4} = 0.52$, $p = 0.49$; longitude: std. coef. = 0.068, $F_{1, 12.8} = 0.33$, $p = 0.58$). The geospatial model had worse fit than the mixed model above (as compared with AIC values), but it provided similar results: thickness was positively related to minimum temperature (std. coef. = 0.25, $F_{1, 14.3} = 5.46$, $p = 0.035$) and to laying date (std. coef. = 0.093, $F_{1, 339} = 3.89$, $p = 0.049$).

Eggshell mass was positively related to thickness, as could be expected (std. coef. = 0.41, $F_{1, 350} = 64.91$, $p < 0.001$). Shell mass did not vary with geographic location, although it showed a non-significant tendency to decrease with longitude (std. coef. = -0.22, $F_{1, 11.2} = 4.36$, $p = 0.060$; latitude: std. coef. = 0.14, $F_{1, 10.3} = 1.28$, $p = 0.29$). The effects of the other covariates were not significant (laying date \times habitat: std. coef. [coniferous] = 0.017, $F_{1, 323} = 0.03$, $p = 0.86$; minimum temperature: std. coef. = 0.018, $F_{1, 10.4} = 0.02$, $p = 0.88$; clutch size: std. coef. = 0.029, $F_{1, 325} = 0.34$, $p = 0.56$; habitat: std. coef. [coniferous] = -0.12, $F_{1, 56.8} = 0.57$, $p = 0.45$; laying date: std. coef. = 0.065, $F_{1, 334} = 2.09$, $p = 0.15$). The geospatial analysis did not increase the model fit, and the relationship between shell mass and thickness remained exactly the same as in the final mixed model above (std. coef. = 0.41, $F_{1, 350} = 64.91$, $p < 0.001$).

Eggshell pore density was not related to any covariate (laying date \times habitat: std. coef. [coniferous] = -0.067, $F_{1, 139} = 0.69$, $p = 0.41$; habitat: std. coef. [coniferous] = 0.0036, $F_{1, 21.3} = 0.00$, $p = 0.98$; minimum temperature: std. coef. = 0.020, $F_{1, 10.7} = 0.12$, $p = 0.73$; latitude: std. coef. = 0.025, $F_{1, 10.4} = 0.16$, $p = 0.69$; clutch size: std. coef. = -0.025, $F_{1, 144} = 0.32$, $p = 0.57$; laying date: std. coef. = -0.046, $F_{1, 148} = 1.34$, $p = 0.25$; longitude: std. coef. = -0.060, $F_{1, 11.1} = 1.34$, $p = 0.27$).

Eggshell biliverdin concentration was also not related to any covariate (laying date \times habitat: std. coef. [coniferous] = -0.093, $F_{1, 142} = 0.42$, $p = 0.52$; latitude: std. coef. = -0.011, $F_{1, 11.9} = 0.00$, $p = 0.96$; longitude: std. coef. = -0.030, $F_{1, 11.3} = 0.02$, $p = 0.88$; habitat: std. coef. [coniferous] = -0.12, $F_{1, 49.7} = 0.15$, $p = 0.70$; minimum temperature: std. coef. = 0.099, $F_{1, 12.9} = 0.34$, $p = 0.57$; clutch size: std. coef. = -0.037, $F_{1, 144} = 0.23$, $p = 0.64$; laying date: std. coef. = -0.050, $F_{1, 150} = 0.55$, $p = 0.46$; shell mass: std. coef. = -0.082, $F_{1, 161} = 1.25$, $p = 0.27$).

As expected, shell colour intensity was positively related to pigment concentration (std. coef. = 0.46, $F_{1, 45.8} = 38.38$, $p < 0.001$; Fig. 3a). Also, it was positively associated with shell thickness (std. coef. = 0.18, $F_{1, 101} = 5.16$, $p = 0.025$; Fig. 3b) and pore density (std. coef. = 0.15, $F_{1, 122} = 4.16$, $p = 0.044$; Fig. 3c). These results remained exactly the same in the geospatial analysis, which did not increase the model fit.

Discussion

We found significant between-population variation in all the eggshell traits studied, except in pore density. Population explained higher amount of variance in eggshell biliverdin concentration than other shell traits. Also, population explained more variance in eggshell traits than other maternal effects that have been previously explored in the same study populations (i.e. yolk mass, albumen lysozyme activity, yolk immunoglobulin concentration and yolk androgens; Ruuskanen et al. 2011). This may suggest that there is a relatively high spatial variation in the environmental factors that constrain shell structure and particularly biliverdin pigmentation. We expected that resource allocation to shell structure would be constrained towards the northern boundary of the species distribution range, as found for yolk carotenoids (Eeva et al. 2010, Ruuskanen et al. 2011). However, between-population variation in shell structure was not explained by latitude or longitude, in contrast with previous findings in components of the yolk and albumen (Ruuskanen et al. 2011).

Yet, pied flycatcher populations could differ in other environmental variables not necessarily related to geographic location. Interestingly, minimum local air temperature (which was not related to latitude or longitude; both correlations: $p > 0.2$, $n = 15$) had a positive effect on shell thickness. This result agrees with experimental studies in poultry performed under 6°C (Sahin et al. 2002, 2003) and may indicate that maternal resource allocation to the shell is limited due to the high energy demands imposed by cold weather (Järvinen and Väisänen 1984, Järvinen 1996). Note that a female laying under low temperatures must use

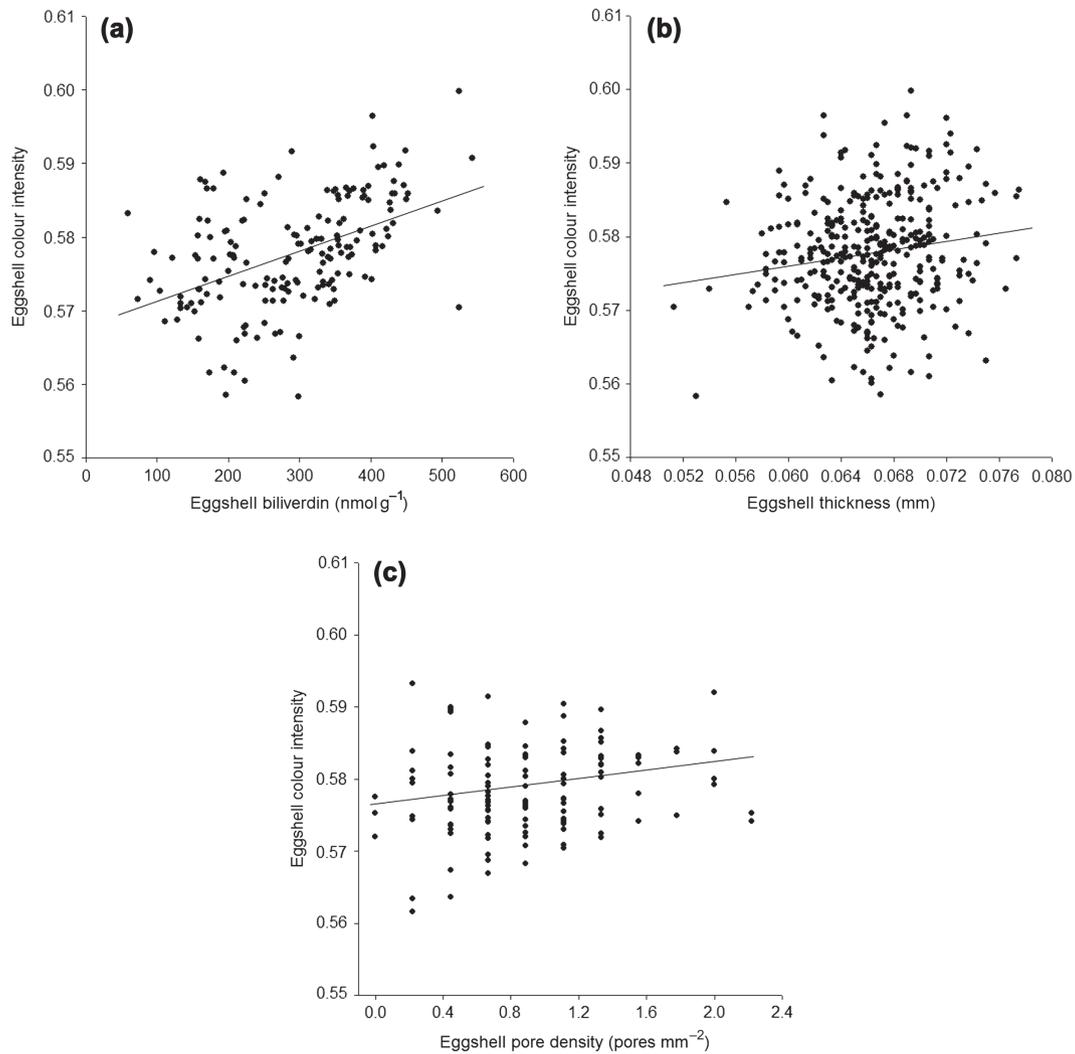


Figure 3. Association between eggshell colour intensity (blue-green chroma) and (a) eggshell biliverdin concentration (nmol g⁻¹), (b) eggshell thickness (mm), and (c) pore density (no. pores mm⁻²). Values of blue-green chroma for (c) are residuals from a model with blue-green chroma as dependent variable and biliverdin concentration and thickness as covariates.

about twice the energy needed for egg formation simply for temperature regulation (Ojanen 1983). Additionally, shell thinning under low temperature could result from reduced availability of invertebrates (e.g. snails) that serve as a calcium source for laying females. Alternatively, females produced thick shells under high temperature to avoid excessive water loss (Davis and Ackerman 1985, Arad et al. 1988). However, the mean ambient temperatures registered in the study areas during egg laying were usually below 20°C, while the temperature of a female's brood patch in contact with an egg is much higher, probably around 40°C (Deeming 2002). Hence, the rate of egg water loss during laying and incubation might not be determined by ambient temperature in these populations, but most likely by the female's behaviour (Higham and Gosler 2006). Further studies are needed to determine whether the relationship between shell thickness and temperature is due to immediate environmental constraints (as found in previous poultry studies; Hempleman et al. 1993) or to adaptive evolution of shell structure (as suggested by Stein and Badyaev 2011).

We found no other environmental effect on shell traits. Shell mass was not related to air temperature, despite being strongly and positively associated with shell thickness. It might be that there was some temperature-related variation in egg shape or volume rather than in total shell mass. Pore density was not associated with ambient temperature either. Actually, pore density did not vary between breeding sites and its within-population variation was relatively high. These results should be taken with caution, though, because repeatability of pore density within sample eggs was low. Anyway, the mechanisms that constrain shell porosity are little understood and it is even uncertain whether producing high or low pore numbers is energetically costly for females. Nevertheless, studies performed in other bird species in the wild suggest that factors such as humidity (Stein and Badyaev 2011), female age (Massaro and Davis 2004), the probability to lay a replacement clutch (Mänd 1996) or laying order (Hargitai et al. 2011) may account for within-population variation in shell porosity. We found no evidence that habitat type affected resource allocation to shell structure, despite insect prey at laying is usually much higher

in deciduous forests compared with coniferous ones (Mänd et al. 2005, Veen et al. 2010). Also, our findings suggest that individual phenology and clutch size exerted little effect on shell structure. We only found a positive association between shell thickness and individual laying date, which may indicate that early females suffered higher physiological constraints (e.g. cold spells, scarce calcium resources) than late females in their population.

We may speculate that between-population variation in shell traits, especially in shell mass and thickness, could be modulated by differences in natural or human-induced soil acidification and thus in calcium availability (as previously found with the study species; Eeva and Lehikoinen 1995, Tilgar et al. 1999a, b). Environmental pollution may also produce direct toxic effects in birds (Eeva and Lehikoinen 2004) and constrain maternal resource allocation to shell structure. In accordance with this we recorded low shell mass and thickness in Harz and Buunderkamp locations, which are highly polluted areas with scarce calcium resources (Graveland et al. 1994, Zang 1998). We also found that biliverdin concentration was low and extremely variable in these locations. Interestingly, two previous studies have reported negative relationships between blue-green eggshell pigmentation and the levels of environmental contaminants found in the eggs of Eurasian sparrowhawks *Accipiter nisus* (Jagannath et al. 2008) and herring gulls *Larus argentatus* (Hanley and Doucet 2012).

As expected, shell colour mirrored the amount of shell biliverdin concentration. Also, it reflected shell thickness and pore density. Despite being more intensively coloured, thicker shells did not have higher pigment concentration. Thick shells probably absorb more light than thin ones, since they have a thicker layer of pigmented material, and this may result in lower total reflectance and higher blue-green chroma. Additionally, the relationship between colour intensity and shell thickness and porosity could be due to other shell characteristics that influence reflectance, like shell smoothness. Previous studies exploring the relationship between biliverdin-based colour intensity and the amount of yolk and albumen maternal components have found significant relationships for antibodies in pied flycatchers (Morales et al. 2006), carotenoids in collared flycatchers (Hargitai et al. 2008) and hormones and antioxidants in other species (López-Rull et al. 2008, López-Rull and Gil 2009, Hargitai et al. 2010). Our findings suggest that egg colour intensity mainly reflects maternal pigment allocation to the eggshell, but also resource allocation to shell thickness and presumably to shell strength (Ar et al. 1979). Our findings together with those of previous studies suggest that shell colour provides valuable information about maternal resource allocation to eggs and seems a good candidate to evaluate egg quality in the field.

To conclude, we found that the eggshell traits studied, with the exception of pore density, varied significantly across the study populations. The most interesting finding was that shell thickness was related to minimum local air temperature, eggshells being thinner in populations that experienced low temperatures at laying. On the other hand, variation in eggshell structure was not due to habitat type or geographic location. However, we cannot discard year-specific geographical trends in eggshell traits in years when

there is for instance a clear temperature gradient between low and high latitudes. Future studies could consider other environmental factors (e.g. pollution levels, calcium availability and humidity) or social factors like sexual selection intensity that may account for between-population variation in eggshell structure.

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Appendix 1

Licenses from environmental authorities and ethical committees in each country. UK: Countryside Council for Wales, license number OTH:SB:06:200; Sweden: Swedish National Board for Laboratory Animals and the Bird Ringing Centre of the Swedish Museum of Natural History, license number Dnr 33-07; Spain: Consejería de Medio Ambiente y Ordenación del Territorio, Comunidad de Madrid, license number 10/289334.9/07; Russia: Dept of Rosprirodnadzor in Sverdlovsk oblast and Council of Zvenigorod Biological Station of Moscow State Univ. and bio-ethic codex of Biological Faculty of MSU, license numbers N 212/647-249, protocol no. 04 16 November, 2005; NL: Animal Experimental Committee of the KNAW,

license number DEC protocol no CTE 07-04; Latvia: Ethical Committee for Research in Ecology and Evolution, Daugavpils Univ., license number 4/ZOO-2009; Germany: Landkreis Goslar, Niedersachsen, Bundesrepublik Deutschland, license number Az. 66 24 03-2.3.1.1; Finland and northern Norway (Lapland): West Finland Regional Environment Centre, Environmental Center of Southwest Finland, Environmental Center of North Ostrobothia and Environmental Center of Lapland, license numbers LSU-2006-L-509 (254), LOS-2007-L-264-254, PPO-2007-L-400-254 and LAP-2007-L-261-254; Estonia: Estonian Environmental Board, license number 1-4.1/11/100.