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RESEARCH ARTICLE

Darker eggshell spotting indicates lower yolk antioxidant level and poorer female quality in the Eurasian Great Tit (*Parus major*)

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ABSTRACT

Protoporphyrin pigment causes the red-brown eggshell colors; however, for many species, the function of this pigment is unknown. It has been proposed that eggshell pigmentation may strengthen the shell or that it may be a sexually selected signal, which advertises the quality of the female and that of her offspring to the male parent. In this study, we aimed to discover whether protoporphyrin-based eggshell pigmentation patterns of Eurasian Great Tits (*Parus major*) were related to female or egg quality. Additionally, we tested whether different methods of eggshell pigmentation estimation could be reliable predictors of eggshell protoporphyrin levels. We found that spot intensity, spot size, spotting coverage, and brown spot chroma indicated the protoporphyrin pigment concentration of the eggshell. Our results revealed that Eurasian Great Tit females that laid eggs with darker pigmentation had more lymphocytes in their circulation and had paler yellow breast and lower UV plumage reflectance, possibly indicating poorer health and individual quality. However, we did not find evidence that eggshell pigmentation patterns indicated the body condition, body size, or plasma oxidative status of females. Furthermore, we found that eggs with darker spots contained lower concentrations of antioxidants in the yolk. High protoporphyrin levels may be detrimental to females as they may cause oxidative damage, and this may be why birds that laid eggs with darker spots deposited lower amounts of antioxidants into the egg yolk. Shell spot darkness may also indicate territory quality, as females that laid smaller clutches also laid eggs with higher eggshell pigmentation levels. Thus, our results suggest that shell spot darkness may indicate the state of health of the female, egg yolk antioxidant level, and possibly also the quality of the territory.

Keywords: eggshell color, female condition, lymphocyte count, plumage coloration, protoporphyrin pigmentation, oxidative status, sexual signalling hypothesis

Manchas más oscuras de la cáscara de huevo indican un nivel más bajo de antioxidantes y una menor calidad de la hembra en *Parus major*

RESUMEN

El pigmento protoporfirina causa el color marrón rojizo de la cáscara de huevo; sin embargo, para muchas especies, la función de este pigmento es desconocida. Se ha propuesto que la pigmentación de la cáscara de huevo fortalece la cáscara o que puede ser una señal seleccionada sexualmente, la cual publicita la calidad de la hembra y la de sus polluelos al macho paterno. En este estudio, nuestro objetivo fue determinar si el patrón de pigmentación de la cáscara de huevo basado en protoporfirina de *Parus major* estuvo relacionado a la calidad de la hembra o del huevo. Además, evaluamos si diferentes métodos de estimación de la pigmentación de la cáscara de huevo podrían ser predictores confiables del nivel de protoporfirina en la cáscara de huevo. Encontramos que la intensidad de las manchas, el tamaño de las manchas, la cobertura de las manchas y el color marrón de las manchas indicaron la concentración del pigmento protoporfirina de la cáscara de huevo. Nuestros resultados revelaron que las hembras de *P. major* que ponen huevos con una pigmentación más oscura tienen más linfocitos en su circulación y tienen un amarillo más pálido pecho y menor plumaje con reflectancia UV, indicando posiblemente un estado de salud y una calidad individual más baja. Sin embargo, no encontramos evidencia de que el patrón de pigmentación de la cáscara de huevo indicara la condición corporal, el tamaño corporal o el estatus oxidativo de la sangre de las hembras. Más aún, encontramos que los huevos con manchas más oscuras contenían concentraciones más bajas de antioxidantes en la yema. El alto nivel de protoporfirina puede ser perjudicial para las hembras ya que podría causar un daño oxidativo, y esta puede ser la razón por la que estas aves depositaron una menor cantidad de antioxidantes en la yema de huevo. La oscuridad de las manchas de la cáscara también puede indicar la calidad del territorio, ya que las hembras con

puestas más chicas pusieron huevos con un nivel más alto de pigmento de la cáscara del huevo. Por ende, nuestros resultados sugieren que la oscuridad de las manchas de la cáscara puede indicar el estado de salud de la hembra, el nivel de antioxidantes de la yema de huevo y posiblemente también la calidad del territorio.

Palabras clave: coloración del plumaje, color de la cáscara de huevo, condición de la hembra, estatus oxidativo, hipótesis de señalización sexual, pigmentación de protoporfirina, recuento de linfocitos

INTRODUCTION

A great number of bird species lay eggs with reddish-brown maculation; however, for many species, the function of this pigmentation is not known. The red, brown, and black eggshell colors are caused by protoporphyrin pigment (Kennedy and Vevers 1976, Mikšík et al. 1996, Gorchein et al. 2009), which is a metabolite intermediate in the biosynthesis of heme (Baird et al. 1975), or may derive from the transformation of heme (Wang et al. 2009). Several hypotheses have been proposed to explain the pigmentation of bird eggshells (Kilner 2006, Reynolds et al. 2009, Maurer et al. 2011a); these hypotheses may not be mutually exclusive and could share similar predictions. Protoporphyrin-based eggshell pigmentation may promote camouflage (Stoddard et al. 2011, Lovell et al. 2013); it may enhance egg mimicry or permit egg recognition in species exposed to brood parasites (Stokke et al. 1999, Soler et al. 2000, Moskát et al. 2008, 2014); it may help resist bacterial penetration into the egg (Ishikawa et al. 2010, Fargallo et al. 2014); or it may structurally strengthen eggshells (Gosler et al. 2005, García-Navas et al. 2010, Bulla et al. 2012, Hargitai et al. 2013), the latter of which is possibly the most plausible explanation for shell spotting patterns in cavity-nesting birds (Solomon 1997, Cherry and Gosler 2010). Furthermore, Moreno and Osorno (2003) proposed that eggshell color could be a sexually selected signal, which may advertise the quality of the female and that of her offspring to the male parent in order that he contribute more to the breeding attempt (sexually selected eggshell coloration [SSEC] hypothesis). The possibility of a signaling function of eggshell protoporphyrin has received much less attention than that of the biliverdin-based blue-green eggshell coloration (e.g., Riehl 2011). However, in recent years, studies have started to test this signaling hypothesis for protoporphyrin-based eggshell coloration too.

Contrary to blue-green biliverdin, which is an antioxidant (McDonagh 2001, Kaur et al. 2003), protoporphyrin possesses pro-oxidant properties (Afonso et al. 1999, Pimentel et al. 2013). The accumulation of protoporphyrin has been reported to induce oxidative stress by generating superoxid, and to lead to an increase in the activity of antioxidant enzymes to avoid oxidative damage (Afonso et al. 1999). During the laying period, the metabolism of the oviduct, including the shell gland, is very active, and thus large amounts of reactive oxygen species are produced (Scanes et al. 1987). The deposition of high amounts of protoporphyrin into the eggshell may indicate a female's oxidative tolerance to a sustained elevated level of this pro-oxidant in the blood and uterus, and thus a high capacity of the antioxidant system of the female (Moreno and Osorno 2003). On the other hand, high eggshell protoporphyrin content may signal poor physiological condition and a high level of oxidative stress in a female, inducing her to remove large amounts of this pro-oxidant from her system (Moreno and Osorno 2003).

Thus, there are 2 opposing hypotheses regarding the signal revealed by eggshell protoporphyrin pigmentation: Darker spotting may indicate either higher quality (greater oxidative tolerance and antioxidant capacity) or lower quality (higher level of oxidative stress) of the female bird (Moreno and Osorno 2003). To date, these hypotheses have been tested with ambiguous results. Sanz and García-Navas (2009) demonstrated that pigmentation darkness was positively related to female tarsus length in Blue Tits (*Cyanistes caeruleus*), and suggested that darker eggshell pattern indicated higher female quality. In contrast, Martínez-de la Puente et al. (2007) found that Blue Tit females that laid less spotted eggs had better body condition and lower concentrations of stress proteins. In a study of British Great Tits (*Parus major newtoni*), it was found that heavier females laid paler eggs (Stoddard et al. 2012). These latter studies support the view that darker pigmentation indicates poorer condition of the female. Additionally, female body condition and body size were not related to eggshell coloration in Eurasian Kestrels (*Falco tinnunculus*; Martínez-Padilla et al. 2010) or in a Belgian population of Eurasian Great Tits (*Parus major*; De Coster et al. 2013). However, to our knowledge, no study has tested the relationship between red-brown eggshell pigmentation and female oxidative status.

Eggshell pigmentation may also reflect the quality of the egg and, thus, the reproductive value of the clutch. For example, it has been reported that biliverdin-based eggshell coloration may signal egg yolk antioxidant (Hargitai et al. 2008, 2010, Navarro et al. 2011, Butler and McGraw 2013), yolk immunoglobulin (Morales et al. 2006), and yolk testosterone (López-Rull et al. 2008) levels. For protoporphyrin-based shell coloration, to our knowledge there have been only 2 studies to date that have tested the association between eggshell pigmentation and the concentrations of biomolecules in the egg. Holveck et al. (2012) found no relationship between eggshell spotting

pattern and yolk carotenoid concentration in Blue Tits, but they did find that eggs with larger and more concentrated spotting contained more antibodies. Krištofík et al. (2013) reported that the brightness of Eurasian Reed-Warbler (*Acrocephalus scirpaceus*) eggshells was positively related to yolk testosterone levels and negatively to the level of an egg immune compound (lysozyme), indicating that more pigmented eggs had increased antimicrobial protection.

The possible lack of consistency among studies of protoporphyrin-based eggshell spotting could be due to differences in the methods used for measurement of eggshell spotting patterns. Several studies measured eggshell reflectance using a spectrophotometer and expressed it as brightness and red or brown chroma (Avilés et al. 2007, Martínez-de la Puente et al. 2007, Hanley and Doucet 2009, 2012, Maurer et al. 2011b, Holveck et al. 2012, Duval et al. 2013, Krištofík et al. 2013). Others determined pigment concentration of the eggshell by chemical analysis (Maurer et al. 2011b, Cassey et al. 2012, Duval et al. 2013, Brulez et al. 2014), or analyzed digitized pictures of eggshells to determine the percentage of spotted eggshell surface (Martínez-de la Puente et al. 2007, Sanz and García-Navas 2009, Bulla et al. 2012, Holveck et al. 2012, Brulez et al. 2014), or to determine hue, saturation, and brightness derived from HSB and RGB (Martínez-Padilla et al. 2010, Cassey et al. 2012, Holveck et al. 2012), or to determine other spotting pattern variables, such as pattern contrast and marking size (Stoddard et al. 2012). Finally, many studies used visual scoring of photographs, and spotting pattern was expressed as spot intensity, spot size, and spot distribution (Gosler et al. 2005, Sanz and García-Navas 2009, López de Hierro and De Neve 2010, Bulla et al. 2012, Holveck et al. 2012, De Coster et al. 2013, Hargitai et al. 2013, Brulez et al. 2014).

In this study, we aimed to discover whether protoporphyrin-based eggshell pigmentation patterns of Eurasian Great Tits (hereafter, Great Tits) were related to the quality of the female or the egg. Great Tits lay white eggs speckled with red-brown spots. The species breeds in cavities, therefore it is unlikely that egg maculation serves as camouflage to conceal eggs from nest predators. In additon, the Great Tit has no brood parasites, and birds do not eject distinct-looking eggs (Kempenaers et al. 1995, R. Hargitai personal observation). In a previous study, we showed that the distribution of eggshell spots was related to eggshell thickness in our Great Tit population (Hargitai et al. 2013), suggesting a structural function of shell pigmentation (Solomon 1997, Gosler et al. 2005), but the intensity and size of spots were not related to eggshell thickness (Hargitai et al. 2013). In this study, we first tested whether eggshell spotting pattern was related to female characteristics that may indicate condition and state of health (body condition, body size, plasma oxidative status, plasma leukocyte count, and clutch size). Second, we tested whether eggshell spotting pattern was related to the carotenoid- and melanin-based and nanostructural plumage coloration of females, which may indicate the intrinsic quality of the individual (Hõrak et al. 2001, Galván 2011, Hegyi et al. 2015). Third, we tested whether eggshell spotting pattern was related to the quality of the egg (yolk antioxidant level and yolk mass). According to the "high quality" hypothesis (Moreno and Osorno 2003), more intense eggshell pigmentation may be related to higher female quality and higher maternal investment into her eggs. Alternatively, based on the "low quality" hypothesis, females that lay eggs with more intense eggshell pigmentation may be in poorer condition and a worse state of health as they may be exposed to higher levels of oxidative stress due to higher levels of protoporphyrin; thus, they may deposit lower amounts of antioxidants into the egg yolk. Finally, we also aimed to test whether the qualitative visual scoring of shell spottiness proposed by Gosler et al. (2005) and brown spot chroma measured by a spectrophotometer were reliable estimators of the concentration of protoporphyrin in the eggshells of Great Tits.

METHODS

Field Procedures

Our study was carried out on a nestbox-breeding population of Great Tits in a sessile oak–dominated woodland in the Pilis Mountains (47°43′N, 19°01′E) of Hungary in 2009 ($n = 19$ nests), 2011 ($n = 25$ nests), and 2012 ($n = 28$ nests). Females were captured in the nestbox when nestlings were 6–9 days old, rather than during the laying or incubation periods, in order to prevent nest desertion. Birds were weighed to the nearest 0.1 g with a Pesola spring balance (Pesola, Baar, Switzerland), and the length of the tarsus was measured to the nearest 0.1 mm with a pair of calipers. Female body condition was expressed as the residual of the regression of body mass on body size (tarsus length). Most females in our study were yearlings (2009: yearling $n = 12$, adult $n = 1$; 2011: yearling $n = 3$, adult $n = 7$, age unknown $n = 1$; 2012: yearling $n = 22$, adult $n = 0$, age unknown $n = 3$), so we did not have enough data for adult females to be able to test the effect of female age on eggshell pattern. Feather samples were collected from the black crown and breast stripe and the yellow breast (see details in Hegyi et al. 2015), and stored in a dry, cool, and dark place until later spectrophotometric analysis. Blood samples (30–60 μl) were taken from the brachial veins of females and centrifuged at 10,000 rpm for 10 min on the same day. Plasma was extracted and stored at −80°C until later analysis. A drop of blood from each breeding female was smeared on a microscope slide, air dried, fixed in absolute methanol, and later stained with Giemsa's solution.

Eggs were numbered with a waterproof marker according to laying order, and 4 eggs (1st, 4th, 7th, and 10th, in 2009 and 2011) or 2 eggs (1st and 10th, in 2012) were collected. Based on our 3 study years, modal clutch size was 11 eggs (range: 6–15 eggs) in our Great Tit population. Collected eggs were replaced with dummy eggs. In 3 nests each in 2009 and 2012, the 10th egg could not be collected as females had already started incubation, and we did not want to disturb them to avoid nest desertion. In 2012, instead of the 10th egg, the 7th egg was collected from 2 nests, the 8th from 2 nests, and the 9th from 4 nests, as there was no 10th egg in those clutches. First, 4th, and 7th eggs were collected on the day of laying of the 7th egg, before incubation began. The 10th egg was collected on its laying date. In nests in which the 8th or 9th egg was collected instead of the 10th, these eggs had been incubated for at most 1 day before collection. Each collected egg was opened, the yolk, albumen, and eggshell were separated, and the yolk was weighed and stored at −80°C until later analysis.

Blood Leukocyte Count

In each blood smear, the proportion of different types of leukocytes was assessed by examining a total of 50 leukocytes under 1000× magnification with oil immersion. Total white blood cell count was estimated in relation to 10,000 red blood cells. Heterophil granulocyte and lymphocyte numbers were estimated by multiplying their proportions with total white blood cell count. An elevated lymphocyte count is associated with infection, while an increase in heterophil number is commonly observed in response to inflammation or stress (Ots and Hõrak 1998, Fudge 2000, Campbell 2012). However, it is also possible that a higher lymphocyte count reflects better immunocompetence of an individual for coping with potential infection (Hale and Briskie 2007, Tella et al. 2008). We had data for leukocyte count of females from 2009 and 2012.

Leukocyte count was not related to body size or body condition of females (lymphocyte: all $P > 0.24$; heterophil: all $P > 0.23$). We found that females with darker melanin-based plumage coloration (see below) had lower lymphocyte counts (PC3: $r = 0.43$, $n = 24$, $P = 0.04$), but that yellow and structural plumage colors were not significantly correlated with leukocyte counts (lymphocyte: all $P > 0.30$; heterophil: all $P > 0.51$).

Spectral Measurements of Plumage

The reflectance of feather samples was measured using a portable Ocean Optics USB2000 spectrophotometer (Ocean Optics Europe, Ostfildern, Germany) with a bifurcated fibre-optic probe (QR400-7-SR-BX; Ocean Optics Europe) and a DH-2000 deuterium-halogen light source (Ocean Optics Europe). The probe was held at a 90° angle to the measured feather surface. To standardize the measurement distance (3 mm) and exclude ambient light, we fixed a black plastic tube onto the probe. Three consecutive scans were taken from each set of feather samples (black crown, black breast stripe, and yellow breast), removing the probe between each scan. Each sample set contained at least 8 feathers, which were placed on top of one another on a black velvet background. The reflectance of the background did not confound the measurements of the feather samples. Reflectance data were computed relative to a white WS-1-SS diffuse reflectance standard (Ocean Optics Europe) and a dark reference (excluding incoming light from the detector). We used OOIBase32 software (Ocean Optics Europe) to record the reflectance spectra. We checked and remeasured the standards at regular time intervals to calibrate the system. All feather spectral measurements were taken by the same person (M. Laczi).

We created spectral variables from the raw reflectance spectra data. For the black crown and black breast stripe, we calculated brightness (average reflectance, $R_{320–700}$) and UV chroma ($R_{320–400}/R_{320–700}$), and for the yellow breast, we calculated brightness and yellow chroma ($(R_{700} - R_{450})/R_{700}$). Because repeatabilities of measurements are high (Hegyi et al. 2015), we used the average from each individuals' 3 scans in the following analyses. All plumage reflectance data from individuals analyzed in the present study were included in a larger (7-yr) dataset of plumage spectral data of our Great Tit population (Hegyi et al. 2015). Correlations among spectral traits have been found to be very consistent across years, seasons, sexes, and ages (Hegyi et al. 2015). Therefore, in order to increase the reliability of our derived plumage color measures (principal components [PC] axes), we decided to use the axes obtained using the 7-yr dataset to describe plumage reflectance of individuals included in the present 3-yr dataset. Spectral variables from the larger dataset were standardized within years, seasons, sexes, and ages before principal components analysis (PCA) to remove any absolute differences among groups (see table 1 in Hegyi et al. [2015] for details). The first 3 principal components (PC1, PC2, and PC3) explained 61% of the variation in plumage coloration (see table 3 in Hegyi et al. [2015] for full details). Higher values of the 3 PCs principally indicated: (1) higher UV chroma of the black feathers of the crown and breast stripe (PC1: loadings $\text{abs}(r) > 0.76$; for all other spectral variables, $\text{abs}(r) < 0.21$); (2) higher yellow chroma and lower brightness of the breast (PC2: $\text{abs}(r) > 0.76$; all other $\text{abs}(r) < 0.10$); and (3) higher brightness of the black feathers of the crown and breast stripe (PC3: $\text{abs}(r) > 0.73$; all other $\text{abs}(r) < 0.11$).

Plasma OXY Test

Total plasma antioxidant capacity was measured by the OXY-Adsorbent test (Diacron, Grosseto, Italy) within 1

mo of blood collection. This test measures the ability of the antioxidant compounds of the plasma (e.g., carotenoids, vitamins A, C, and E, proteins, and thiols) to cope with the oxidizing action of hypochlorous acid (HClO). Unlike other methods, this test does not overemphasize the contribution of uric acid to the total antioxidant capacity of the sample (Costantini 2011). First, 2 μl of plasma was diluted 1:100 with distilled water. Then, 200 μl of HClO solution was pipetted into the wells of a microplate and 2 μl of the diluted sample was added to each well. The microplate was then incubated at 37°C for 10 min with continuous shaking of moderate intensity. After that, 2 μl of a chromogenic mixture was added to the wells. The alkyl-substituted aromatic amine solubilized in the chromogen was oxidized by the residual HClO of the solution and thus transformed into a pink derivative. Accordingly, the intensity of the pink color was inversely related to the total antioxidant capacity of the plasma. After 5 min of continuous shaking at moderate intensity, the absorbance was read by a BioTek ELX808 spectrophotometer with Gen5 software (BioTek, Winooski, Vermont, USA) at 540 nm. Absorbances were compared with those of a dilution series of a calibrator (stabilized protein solution). Samples were tested in duplicates (percent CV $< 5\%$), and results were expressed as mM HClO neutralized.

Plasma ROM Test

We measured the plasma level of reactive oxygen metabolites (ROMs; primarily hydroperoxides [ROOH]) via the d-ROM assay (Diacron International, Grosseto, Italy) following the protocols of previous studies (Costantini et al. 2006, 2011). ROMs are intermediate oxidative damage compounds, which are generated by the peroxidation of biological macromolecules (lipids, proteins, and nucleic acids) by reactive oxygen species (Halliwell and Gutteridge 2007). Plasma samples were analyzed within 6 mo of blood collection. First, we mixed the reagents (chromogenic mixture [aromatic alkyl-amine] and acetate buffer) in a 1:100 ratio and added 200 μl of this solution to Eppendorf tubes. We added 5 μl of plasma to the reagent mix in the Eppendorf tubes. Samples were then incubated in a water bath at 37°C for 75 min with continuous shaking of moderate intensity. Iron and copper ions were released from plasma proteins in the presence of the acidic buffer, and alkoxyl and peroxyl radicals were generated from hydroperoxides. These highly reactive pro-oxidant compounds oxidized the aromatic alkyl-amine and transformed it into a pink derivative. Thus, the intensity of the pink color was directly proportional to the concentration of ROMs in the sample. Precipitates (lipids and proteins) were present in the solution, so all samples were centrifuged for 2 min at 10,000 rpm after incubation, including both the calibrator and the blank. Then, 190 μl of the clear solution was transferred to the wells of a microplate. Absorption was read immediately with a microplate spectrophotometer (BioTek ELX808 with Gen5 software) at 490 nm, and compared with that of a dilution series of a calibrator (lyophilized serum). We did not use duplicates, as the percent CV of the samples was very low (mean percent CV $= 1.7\%$, $n = 6$ duplicates). Plasma hydroperoxide concentration was expressed as mM of hydrogen peroxide (H_2O_2) equivalents.

Plasma Oxidative Status (OS)

Hydroperoxides may also function as pro-oxidants as metal ions may catalyze their cleavage, leading to the production of radicals (Halliwell and Gutteridge 2007). The increase in plasma radical concentrations may mobilize the antioxidant defense system, thus elevating plasma antioxidant capacity (OXY) in order to counteract the oxidant attack and maintain the redox-homeostasis of the organism. We calculated an index of oxidative status (OS) as a ratio between plasma ROM and OXY values, as applied in previous studies (Costantini et al. 2006, Markó et al. 2011). Higher values of OS indicate higher levels of oxidative molecules in relation to the antioxidant capacity of the plasma.

Eggshell Pigmentation Scoring

Photographs of the whole clutch were taken with a digital camera in the field in 2009 and 2012, and of 4 collected eggs in the laboratory in 2011. Eggshells were scored from photographs by 1 observer (R. Hargitai) following the methods of Gosler et al. (2005). Eggshell pigmentation was scored on the basis of 4 categories from the side view of the egg: (1) mean spot intensity (from 1 for the palest to 5 for the darkest); (2) mean spot size (scored in increments of 0.5 from 1.0 for the smallest spots to 3.0 for the largest spots); (3) spot distribution (from 1 for aggregated spotting distribution to 5 for even spotting distribution); and (4) spotting coverage (scored in increments of 0.5 from 1.0 for few spots to 3.0 for many spots). The within-clutch repeatability values of pigmentation scoring variables were significant ($0.41 < r < 0.65$, all $P < 0.001$).

There was a strong correlation between spot intensity and spot size ($r = 0.53$, $n = 603$, $P < 0.001$), and between spot distribution and spotting coverage ($r = 0.68$, $n = 603$, $P < 0.001$). The other spotting variables were weakly correlated (intensity–coverage: $r = -0.09$, $P = 0.04$; size–coverage: $r = 0.14$, $P < 0.001$; intensity–distribution: $r = -0.15$, $P < 0.001$; all $n = 603$) or showed no significant correlation (size–distribution: $r = -0.01$, $n = 603$, $P = 0.83$). We decided not to apply a principal components analysis (PCA), but instead to use the original scored variables in the statistical analyses. Had we applied PCA, we would have lost some of the variance of eggshell spotting patterns, and would have mixed variables that may be related to different physiological mechanisms, which may have led to committing type II statistical error.

Eggshell Spectrophotometric Analysis

Eggshell spot color was measured with the same apparatus that we used for measuring feather reflectance (see above). Only eggs collected in 2012 (2 eggs per clutch) were measured. Eggshells were stored at −20°C for 1 yr before measurement. The sampling optic was held perpendicular to and at a distance of 3 mm from the eggshell surface. Reference calibration was performed with a white standard after measuring every egg. Mean reflectance was calculated by averaging the measurements of 3 spots. The reflectance spectrum of brown spots had a series of peaks in the long wavelengths (Figure 1), as expected from the absorbance properties of protoporphyrin (Scalise and Durantini 2004). We thus used brown chroma ($R_{600–700}/R_{320–700}$) to describe the reflectance data because this region corresponded to the region of greatest reflectance of protoporphyrin (Scalise and Durantini 2004). This method of color estimation has previously been employed in other studies of species with speckled eggs (Hanley and Doucet 2009, 2012).

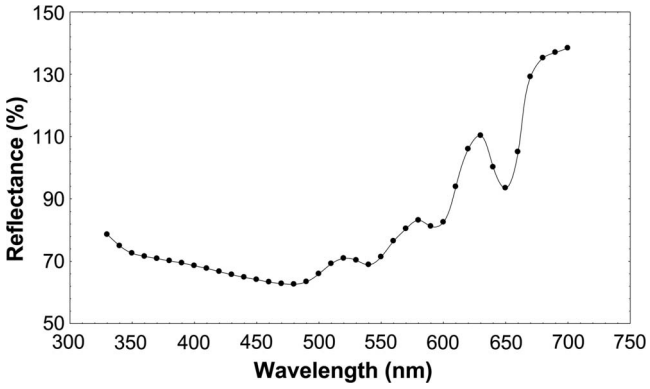

FIGURE 1. Typical reflectance spectrum of a brown spot of a Eurasian Great Tit eggshell. Data were averaged in 10 nm steps.

Eggshell Protoporphyrin Analysis

Eggshells were stored for 18 mo in darkness at −20°C before analysis. Only eggs collected in 2012 (2 eggs per clutch) were analyzed for protoporphyrin content. Eggshell samples were ground in an agate mortar and put into a centrifuge tube. They were solubilized by adding 750 µl of acetic acid and 13.3 µl of dimethyl sulfoxide and undergoing sonication for 10 min. Eggshell proteins were precipitated by adding 1.24 ml of ethyl acetate and vortexing for 30 s. The samples were then centrifuged for 5 min at 5,500 rpm. A 1.5 ml aliquot of the resulting supernatant (ethyl acetate and formic acid) was evaporated under a gentle stream of nitrogen. The residue was redissolved in 190 µl of dimethyl sulfoxide. After vortex shaking for 30 s, the samples were transferred into a 1.5 ml autosampler vial.

Reversed-phase high performance liquid chromatography (HPLC) was performed using an HP 1050 system (Hewlett Packard, Palo Alto, California, USA) with an Agilent Eclipse XDB-C18 liquid chromatography column (100 mm × 4.6 mm, 3.5 µm; Agilent Technologies, Santa Clara, California, USA). The HPLC system consisted of a degasser, a quaternary pump, an autosampler, a column termostat, and a UV-VIS detector, and was controlled using ChemStation A10.02 software (Agilent Technologies). A 5 µl sample was injected onto the column and eluted with a gradient starting at 60% for 0.05% (*V*/*V*) trifluoroacetic acid in water purified with a Direct-Q system (Merck Millipore Corporation, Darmstadt, Germany; eluent A), and starting at 40% for acetonitrile (eluent B). This composition was kept for 0.2 min after which it was gradually changed to 100% eluent B over a period of 4.8 min. This final composition was kept constant for 2.5 min before ending the run. The flow rate was 1.0 ml min^{-1}, and the column was kept at 50°C. The detection wavelength was 384 nm for the first 5 min (for biliverdin) and 400 nm afterwards (for protoporphyrin). Peaks were identified and quantified by comparisons with retention times and peak areas of reference materials (Frontier Scientific, Logan, Utah, USA). Shell protoporphyrin concentration was repeatable within clutches ($r = 0.53$, $F_{18,19} = 3.28$, $P = 0.007$).

Egg Yolk Antioxidant Analysis

Egg yolk antioxidant concentration was analyzed by HPLC (protocol slightly modified from Hargitai et al. [2010]). A reversed phase Develosil C_{30} (150 mm × 4.6 mm, 3 µm) column was used with a Zorbax C_{18} (12.5 mm × 4.6 mm, 5 µm) precolumn. First, an internal standard (astaxanthin) was added to a 100–200 mg yolk sample. The sample was placed in a mortar containing 500 mg of C_{18} sorbent and manually blended. The blended material was then transferred into a column suitable for conducting sequential elution and pressed into a compact column bed between 2 frits. Next, the column was washed with 3 ml of water to elute the polar impurities. Before eluting the analytes, a 2nd column packed with sodium sulfate was placed under the 1st column to remove the water residue from the effluent. The carotenoid fraction was eluted with 8 ml of dichloromethane. After evaporating the solvent to dryness under a nitrogen stream at 40°C, the residue was redissolved in 200 µl of dichloromethane:methanol (1:4) solution. A reversed phase Develosil C30 (150 mm × 4.6 mm, 3 µm; Nomura Chemical Company Ltd., Seto, Japan) column was used with a Zorbax C18 (12.5 mm × 4.6 mm, 5 µm; Agilent Technologies) precolumn in an HP1050 system (see above). A 10 µl sample was injected onto the column and eluted with a gradient starting at 10% water (eluent A) and 90% acetonitrile:ethyl acetate (2:1) with 0.05% (*V*/*V*) triethylamine (eluent B). This composition was kept for 3 min with a flow rate of 0.5 ml min^{-1}, after

which it was gradually changed to 100% eluent B and a flow rate of 1.0 ml min^{-1} over a period of 6 min. This final composition was kept constant for 11 min, during which the flow rate was 1.0 ml min^{-1} for 1 min, after which it was gradually changed to 1.5 ml min^{-1} over 3 min, and this flow rate was kept constant for 7 min before ending the run. The column was kept at 50°C. The UV-VIS detector was operated at 295 nm (tocopherol), 325 nm (retinol), and 450 nm (carotenoids). Peaks were identified and quantified by comparisons with retention times and peak areas of carotenoid, retinol, and tocopherol standards (Sigma-Aldrich, St. Louis, Missouri, USA, and CaroteNature, Ostermundigen, Switzerland).

Statistical Analyses

Yolk lutein and retinol concentrations, female body condition, heterophil count, plumage coloration variables (see Hegyi et al. 2015), and clutch size differed among years, so these variables were standardized within years. Standardization within years was carried out by subtracting the mean value of the particular year from the actual value and dividing the result by the standard deviation for that year. Lutein, tocopherol, and protoporphyrin concentrations, female OS, and lymphocyte and heterophil counts were log-transformed for normality. We carried out the analyses with 6 different dependent variables reflecting eggshell spotting: spot intensity, spot size, spot distribution, spotting coverage, brown spot chroma, and eggshell protoporphyrin concentration. For spot intensity, spot size, spot distribution, and spotting coverage, we had data for whole clutches from 2 yr (2009 and 2012), and for 4 eggs per clutch from 1 yr (2011); for brown spot chroma and eggshell protoporphyrin concentration we had data for 2 eggs per clutch from 1 yr (2012). Although some dependent variables were correlated with each other (see "Eggshell Pigmentation Scoring" in Methods and "Relationships among Eggshell Pigmentation Traits" in Results), due to the unclear proximate physiological background and therefore meaning and possible function of the different eggshell pigmentation descriptors, we analyzed all of them to gain a more comprehensive picture.

We ran general linear mixed models with nest identity as a random factor. We applied maximum likelihood estimation, which is considered more suitable for mixed models than restricted maximum likelihood estimation (Singer and Willett 2003). In order to use as much data as possible and to avoid collinearity of variables, we analyzed 6 separate models with the following predictor variables: (1) female body condition, tarsus length, and clutch size (controlled for laying date); (2) female plasma OXY and ROM; (3) female OS; (4) female leukocyte (lymphocyte and heterophil) count; (5) female plumage coloration (PC1, PC2, and PC3); and (6) egg traits (yolk mass and egg yolk antioxidant concentrations [lutein, tocopherol, and retinol]). For the 1st and 5th models, we had data from 3 yr; for the 4th and 6th models, we had data from 2 yr; and for the 2nd and 3rd models we had data from 1 yr. Laying order and clutch size were included in all models as covariates. Year and year × predictor variable interactions were included in all models with multiple years, but they were nonsignificant, so they are not reported. In all models, a stepwise analysis based on a backward deletion procedure was employed, removing nonsignificant ($P > 0.05$) effects from the model 1 by 1 in decreasing order of P-value. To avoid nonsignificance due to overparameterization, we re-entered nonsignificant effects into the final model 1 by 1, and present these F- and P-values. We calculated standardized effect sizes as R = SQRT((F * df1) / ((F * df1) + df2)) following McNeil et al. (1996), and computed the 95% confidence limits (CL) for R-values (Alvir 1993). Analyses were performed in SPSS 19.0 (SPSS, Chicago, Illinois, USA) and STATISTICA 5.5 (StatSoft, Tulsa, Oklahoma, USA).

TABLE 1. Correlation between shell protoporphyrin concentration (μg per gram of eggshell) and scoring variables of eggshell spotting and brown spot chroma of Eurasian Great Tit eggshells.

		r	n	P
Shell protoporphyrin concentration	Spot intensity	0.42	45	0.004
	Spot size	0.54	45	<0.001
	Spot distribution	0.05	45	0.75
	Spotting coverage	0.33	45	0.03
	Brown spot chroma	0.50	44	0.001

RESULTS

Relationships among Eggshell Pigmentation Traits

The concentration of protoporphyrin pigment in Great Tit eggshells was 102.09 ± 67.06 μg per gram (mean ± SD). The amount of protoporphyrin per individual eggshell was 9.35 ± 5.33 μg (mean ± SD). Biliverdin was not detectable in the eggshells. Eggshell protoporphyrin concentration was significantly positively correlated with spot intensity, spot size, spotting coverage, and brown spot chroma, but was not correlated with spot distribution (Table 1). Brown spot chroma was correlated with spot size and tended to be correlated with spot intensity, but was not correlated with spot distribution or spotting coverage (spot intensity: $r = 0.27$, $P = 0.06$; spot size: $r = 0.36$, $P = 0.01$; spot distribution: $r = -0.04$, $P = 0.80$; spotting coverage: $r = 0.11$, $P = 0.43$; $n = 50$ in all cases).

Eggshell Spotting and Female Traits

We found that females that laid larger clutches deposited significantly less protoporphyrin into the eggshell and laid eggs with paler spots (shell protoporphyrin concentration: $F_{1,24.11} = 23.08$, $P < 0.001$, $R = -0.70$, 95% CL: −0.85, −0.44; Figure 2A; brown spot chroma: $F_{1,25.23} = 5.96$, $P = 0.02$, $R = -0.44$, 95% CL: −0.69, −0.09; all other variables, $P > 0.27$).

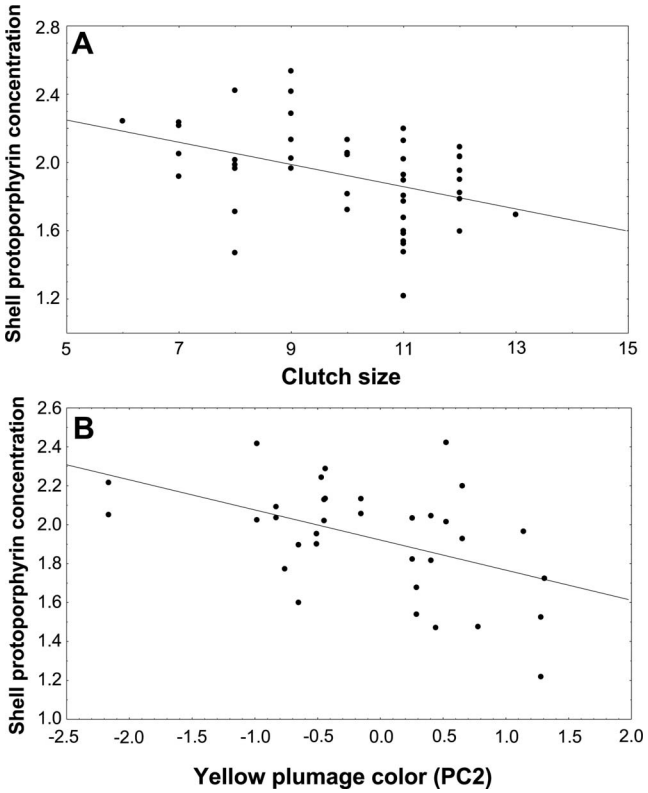

FIGURE 2. Correlation between Eurasian Great Tit eggshell protoporphyrin concentration (μg per gram of eggshell) and (**A**) clutch size of Great Tits, and (**B**) yellow plumage color (PC2) of female Great Tits. Eggshell protoporphyrin concentration was log-transformed.

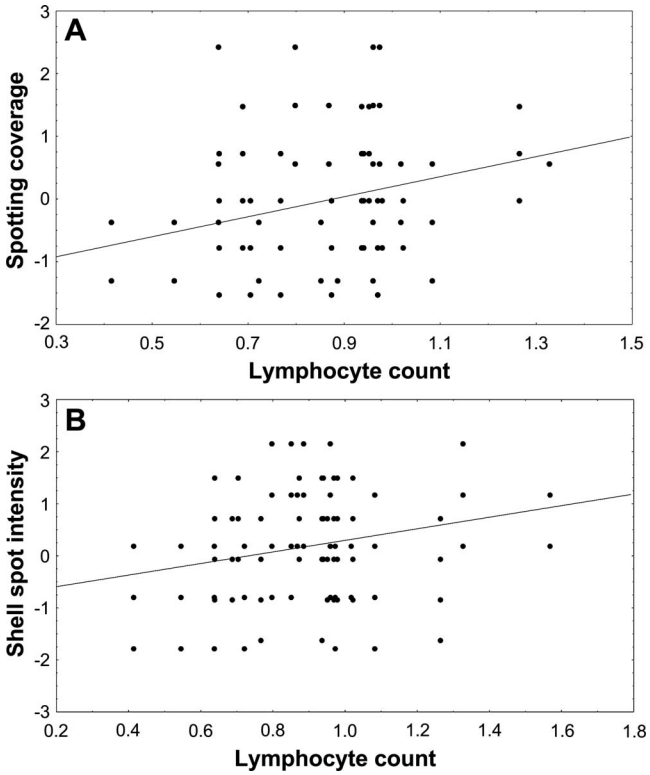

FIGURE 3. Correlation between blood lymphocyte count (in relation to 10,000 red blood cells) of Eurasian Great Tit females and (**A**) eggshell spotting coverage, and (**B**) eggshell spot intensity. Lymphocyte count was log-transformed. Shell spotting coverage and shell spot intensity were year-standardized.

Female body condition and body size (tarsus length) showed no relationships with eggshell spotting pattern (condition: all $P > 0.16$; tarsus length: all $P > 0.16$).

Females that laid eggs with many spots had significantly higher lymphocyte counts (Table 1, Figure 3A), and lymphocyte count also showed a marginally significant positive correlation with eggshell spot intensity ($P = 0.051$; Figure 3B) and spot size ($P = 0.051$; Table 2). Heterophil count was not related to eggshell spotting pattern (Table 2). The plasma level of oxidative stress, plasma antioxidant capacity, and oxidative status of the female showed no significant association with eggshell spotting pattern (ROM: all $P > 0.08$; OXY: all $P > 0.28$; OS: all $P > 0.19$).

We found that females with less intense UV reflection of the melanized plumage (lower PC1) and paler yellow breast plumage (lower PC2) produced eggs with a higher protoporphyrin concentration in the eggshell (PC1: $F_{1,21.00} = 6.16$, $P = 0.02$, $R = –0.48$, 95% CL: –0.73, –0.10; PC2: $F_{1,19.42} = 4.76$, $P = 0.04$, $R = –0.44$, 95% CL: –0.72, –0.04; Figure 2B). Other eggshell spotting variables showed no significant correlations with female UV and yellow plumage coloration (PC1: all $P > 0.29$; PC2: all $P > 0.21$). The brightness of the black plumage regions (PC3) of females was not related to eggshell spotting patterns (all $P > 0.15$).

Eggshell Spotting and Egg Investment

Eggs with darker spots, as indicated by higher spot intensity and greater brown spot chroma, contained significantly lower concentration of lutein in the yolk (Table 3, Figure 4A), although the effect size for spot intensity was small (Table 3). The concentration of protoporphyrin pigment in the shell was negatively related to the tocopherol concentration in the yolk (Table 3, Figure 4B). There were no significant relationships between shell spotting pattern and yolk retinol concentration (Table 3) or yolk mass (Table 3).

DISCUSSION

Relationships among Eggshell Pigmentation Traits

Our results showed that spot intensity, spot size, and spotting coverage were significantly positively correlated with shell protoporphyrin pigment concentration, although the correlations were not strong. Brown spot chroma also was correlated with spot intensity, spot size,

TABLE 2. Relationships between eggshell coloration variables and leukocyte counts of Eurasian Great Tit females, including clutch size, egg laying order, and year (2009, 2011) as covariates in the mixed effects models. We calculated standardized effect size as $R =$ SQRT((*F* * df1) / ((*F* * df1) + df2)) following McNeil et al. (1996), and computed 95% confidence limits (CL) for *R*-values (Alvir 1993).

Dependent variable	Predictor variable	*F* (df)	*P*	*R*	95% CL (lower; upper)
Spot intensity	Lymphocyte count	4.19 (1, 26.24)	0.05	0.37	0.01; 0.64
	Heterophil count	0.08 (1, 25.75)	0.78	0.06	–0.31; 0.40
	Clutch size	0.57 (1, 26.61)	0.46	–0.14	–0.47; 0.22
	Egg laying order	8.73 (1, 252.72)	0.003	0.18	0.06; 0.30
	Year	4.41 (1, 25.83)	0.05	0.38	0.03; 0.65
Spot size	Lymphocyte count	4.21 (1, 25.61)	0.05	0.38	0.01; 0.65
	Heterophil count	2.01 (1, 25.56)	0.17	–0.27	–0.58; 0.10
	Clutch size	0.04 (1, 25.86)	0.84	0.04	–0.33; 0.40
	Egg laying order	27.83 (1, 251.71)	<0.001	0.32	0.20; 0.42
	Year	3.86 (1, 24.86)	0.06	0.37	–0.00; 0.65
Spot distribution	Lymphocyte count	0.96 (1, 25.57)	0.34	0.19	–0.20; 0.53
	Heterophil count	1.33 (1, 25.01)	0.26	–0.22	–0.56; 0.17
	Clutch size	0.06 (1, 25.49)	0.81	–0.05	–0.42; 0.33
	Egg laying order	3.39 (1, 254.50)	0.07	–0.11	–0.23; 0.01
	Year	1.81 (1, 24.73)	0.19	0.26	–0.13; 0.58
Spotting coverage	Lymphocyte count	5.87 (1, 25.83)	0.02	0.43	0.07; 0.69
	Heterophil count	1.12 (1, 25.68)	0.30	–0.20	–0.53; 0.17
	Clutch size	0.75 (1, 26.03)	0.75	0.17	–0.21; 0.50
	Egg laying order	17.08 (1, 250.93)	<0.001	–0.25	–0.36; −0.13
	Year	2.42 (1, 25.65)	0.13	0.29	–0.08; 0.59
Brown spot chroma	Lymphocyte count	1.32 (1, 12.94)	0.27	0.30	–0.21; 0.69
	Heterophil count	1.98 (1, 14.00)	0.18	0.35	–0.14; 0.70
	Clutch size	8.04 (1, 15.10)	0.01	–0.59	–0.83; −0.17
	Egg laying order	32.49 (1, 10.18)	<0.001	0.87	0.62; 0.96
Shell protoporphyrin concentration	Lymphocyte count	1.51 (1, 13.28)	0.24	0.32	–0.20; 0.70
	Heterophil count	0.51 (1, 12.61)	0.49	–0.20	–0.64; 0.34
	Clutch size	3.48 (1, 13.44)	0.08	–0.45	–0.77; 0.05
	Egg laying order	7.16 (1, 9.30)	0.03	0.66	0.11; 0.90

and shell protoporphyrin concentration. These results indicate that spot intensity, spot size, spotting coverage, and brown spot chroma could be used as estimates of the level of eggshell protoporphyrin in studies of maculated eggshells. Similar to our results, Holveck et al. (2012) found that the spectral chroma of brown spots was significantly correlated with spot intensity and spot size, but not with spot distribution, in a French population of Great Tits. Brulez et al. (2014) reported that eggshell protoporphyrin concentration was correlated with spot intensity and spot size, but not with spot distribution, in British Great Tits. However, the authors pointed out that correlations with pigment concentration were not sufficiently strong to be used as reliable surrogates, and in our study we found similarly moderate correlation coefficient values. The relative weaknesses of the correlations could possibly be the result of pigment spots deeper in the eggshell (Jagannath et al. 2008, Bulla et al. 2012) that are less visible on photographs, but can be detected by chemical analysis. Nevertheless, we suggest that inner spots may be similarly less detectable by birds and, thus, for testing the SSEC hypothesis, these variables may be applicable.

Eggshell Spotting and Female Traits

Female Great Tits that laid eggs with many spots had significantly higher lymphocyte counts, and lymphocyte count also showed a marginally significant positive correlation with eggshell spot intensity and spot size. Eggshell spot intensity and spot size were interrelated; moreover, eggshell spot intensity, spot size, and spotting coverage were all related to eggshell protoporphyrin concentration, suggesting that females with higher lymphocyte counts deposited more protoporphyrin pigment into the eggshell. However, we did not find a significant correlation between shell protoporphyrin concentration and lymphocyte count, possibly due to a lower sample size, as the relationship had a medium effect size. We surmise that a higher lymphocyte count signals parasitic infection (Ots and Hõrak 1998, Soler et al. 2003), but it is also possible that it reflects better immunocompetence of an individual for coping with potential infection (Hale and Briskie 2007, Tella et al. 2008). We note that, without knowing the parasitization levels of females, the interpretability of a single measure of leukocyte numbers for an assessment of the state of health has limitations (Ots et al. 1998, Demas et al. 2011). It is possible that females that

TABLE 3. Relationships between eggshell coloration variables and egg yolk antioxidant (lutein, tocopherol, retinol) concentrations and egg yolk mass of Eurasian Great Tits, including clutch size, egg laying order, and year (2011, 2012) as covariates in the mixed effects models. We calculated standardized effect size as $R = \text{SQRT}((F * df1) / ((F * df1) + df2))$ following McNeil et al. (1996), and computed 95% confidence limits (CL) for *R*-values (Alvir 1993).

Dependent variable	Predictor variable	*F* (df)	*P*	*R*	95% CL (lower; upper)
Spot intensity	Yolk lutein concentration	8.19 (1, 93.16)	0.005	–0.28	–0.46; −0.09
	Yolk tocopherol concentration	0.04 (1, 94.67)	0.84	0.02	–0.18; 0.22
	Yolk retinol concentration	0.24 (1, 85.23)	0.63	–0.05	–0.26; 0.16
	Yolk mass	0.21 (1, 94.09)	0.65	–0.05	–0.24; 0.15
	Clutch size	0.11 (1, 55.03)	0.74	–0.04	–0.30; 0.22
	Egg laying order	2.10 (1, 56.80)	0.15	0.19	–0.07; 0.42
	Year	2.52 (1, 49.92)	0.12	0.22	–0.05; 0.46
Spot size	Yolk lutein concentration	0.23 (1, 93.46)	0.64	–0.05	–0.25; 0.15
	Yolk tocopherol concentration	0.13 (1, 94.98)	0.72	–0.04	–0.23; 0.16
	Yolk retinol concentration	0.65 (1, 90.76)	0.42	–0.08	–0.28; 0.12
	Yolk mass	0.00 (1, 94.89)	0.95	0.01	–0.19; 0.20
	Clutch size	2.23 (1, 52.87)	0.14	–0.20	–0.44; 0.06
	Egg laying order	25.01 (1, 47.02)	<0.001	0.59	0.37; 0.75
	Year	1.52 (1, 48.66)	0.22	0.17	–0.10; 0.43
Spot distribution	Yolk lutein concentration	0.06 (1, 94.65)	0.81	0.03	–0.17; 0.22
	Yolk tocopherol concentration	0.29 (1, 94.07)	0.59	–0.06	–0.25; 0.14
	Yolk retinol concentration	0.09 (1, 86.19)	0.76	–0.03	–0.24; 0.18
	Yolk mass	0.00 (1, 94.81)	0.99	–0.00	–0.20; 0.20
	Clutch size	1.46 (1, 48.14)	0.23	–0.17	–0.42; 0.11
	Egg laying order	4.60 (1, 42.03)	0.04	–0.31	–0.56; −0.02
	Year	6.54 (1, 45.90)	0.01	0.35	0.08; 0.58
Spotting coverage	Yolk lutein concentration	0.19 (1, 92.95)	0.67	0.05	–0.16; 0.24
	Yolk tocopherol concentration	0.00 (1, 87.54)	0.97	–0.01	–0.21; 0.20
	Yolk retinol concentration	1.81 (1, 75.08)	0.18	0.15	–0.07; 0.36
	Yolk mass	0.31 (1, 90.09)	0.58	–0.06	–0.26; 0.15
	Clutch size	2.41 (1, 51.45)	0.13	–0.21	–0.45; 0.06
	Egg laying order	5.03 (1, 45.43)	0.03	–0.32	–0.55; −0.04
	Year	5.03 (1, 49.89)	0.03	0.30	0.03; 0.53
Brown spot chroma	Yolk lutein concentration	5.53 (1, 43.71)	0.02	–0.34	–0.57; −0.05
	Yolk tocopherol concentration	0.27 (1, 43.20)	0.61	–0.08	–0.36; 0.21
	Yolk retinol concentration	0.05 (1, 39.76)	0.83	0.04	–0.26; 0.33
	Yolk mass	0.09 (1, 43.75)	0.77	–0.05	–0.33; 0.24
	Clutch size	1.09 (1, 27.83)	0.31	–0.19	–0.51; 0.17
	Egg laying order	10.71 (1, 21.84)	0.004	0.57	0.23; 0.79
Shell protoporphyrin concentration	Yolk lutein concentration	0.03 (1, 39.62)	0.86	–0.10	–0.39; 0.20
	Yolk tocopherol concentration	15.42 (1, 31.70)	<0.001	–0.57	–0.75; −0.30
	Yolk retinol concentration	0.01 (1, 22.56)	0.93	–0.13	–0.47; 0.24
	Yolk mass	1.60 (1, 38.76)	0.21	–0.15	–0.43; 0.16
	Clutch size	11.25 (1, 26.74)	0.002	–0.54	–0.76; −0.23
	Egg laying order	0.14 (1, 23.36)	0.71	0.08	–0.31; 0.44

bred in poorer-quality territories or that had lower intrinsic quality experienced higher levels of infection and deposited more protoporphyrin into eggshells to remove the potentially harmful pro-oxidants from their bodies. Alternatively, a high level of the pro-oxidant protoporphyrin may use up components of the antioxidant defense system, including carotenoids and tocopherols, which are also effective immunostimulants (Haq et al. 1996, Blount et al. 2003, McGraw and Ardia 2005), thus leading to a weakened state of health of the female.

We found that females with paler yellow breast plumage produced eggs with higher protoporphyrin concentration in the eggshell. Yellow plumage chroma reflects the carotenoid content of the feathers (Partali et al. 1987, Isaksson et al. 2008). Less intense yellow breast plumage could indicate a weaker foraging ability to find carotenoid-rich food in the environment (Slagsvold and Lifjeld 1985), poorer nutritional condition during molt (Senar et al. 2003; in our population specifically, Hegyi et al. [2015]), higher hemoparasitic infection (Hõrak et al. 2001), or lower antioxidant capacity, as females with paler yellow coloration may not have been able to allocate sufficient amounts of carotenoids to the growing feathers during molt. Additionally, we found that females with lower UV

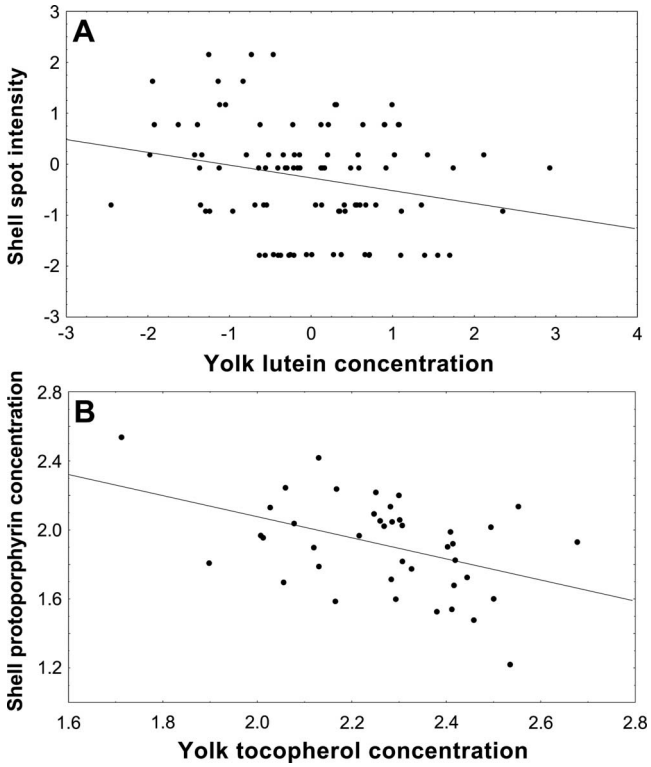

FIGURE 4. Correlation between (**A**) eggshell spot intensity and egg yolk lutein concentration (μg per gram of yolk), and (**B**) eggshell protoporphyrin concentration (μg per gram of eggshell) and yolk tocopherol concentration (μg per gram of yolk) of Eurasian Great Tit eggs. Eggshell protoporphyrin concentration, yolk lutein and tocopherol concentrations were log-transformed. Yolk lutein concentration and shell spot intensity were year-standardized.

chroma of the melanized plumage regions (crown and breast stripe) deposited more protoporphyrin pigment into the eggshell. The relative UV intensity of black feathers is mostly caused by feather nanostructure (Shawkey et al. 2003, Prum et al. 2009), and it may be related to nutritional condition during molt (Johnsen et al. 2003, Shawkey et al. 2003, Siefferman and Hill 2005), but this does not apply to our population (Hegyi et al. 2015). In our population, crown UV reflectance shows a pattern of assortative mating (Hegyi et al. 2007, 2015), suggesting that this trait may be related to individual quality. Structural plumage coloration may also signal developmental stability during feather growth and the quality and abrasion-resistance of feathers (Fitzpatrick 1998, Galván 2011). Accordingly, these associations with plumage ornamentation suggest that females that deposited more protoporphyrin pigment into the eggshell probably had poorer individual quality and health.

We found no support for the hypothesis that protoporphyrin-based eggshell spotting indicated the body condition or the body size (tarsus length) of female Great Tits. Similar to our results, egg color was not related to female body condition or body size in a Belgian population of Great Tits (De Coster et al. 2013), in the Eurasian Kestrel (Martínez-Padilla et al. 2010), the Ring-billed Gull (*Larus delawarensis*; Hanley and Doucet 2009), or the Eurasian Reed-Warbler (Krištofík et al. 2013). By contrast, in British Great Tits (Stoddard et al. 2012) and Blue Tits (Martínez-de la Puente et al. 2007), heavier females laid less spotted eggs with paler spots. Although, in contrast to our timing of female measurement, which was similar to that of Martínez de la Puente et al. (2007), Stoddard et al. (2012) used body mass of females caught before breeding, their results were qualitatively similar if they used female mass during the nestling rearing period. If we applied these authors' measures of female body condition (body mass was used by Stoddard et al. [2012]; body mass divided by the cube of tarsus length was used by Martínez-de la Puente et al. [2007]), we obtained similar, nonsignificant results (not shown). Thus, it seems that there are differences in the information content of eggshell spotting patterns regarding female condition, even between closely related species or different populations of the same species.

Furthermore, we found that neither the plasma level of oxidative stress nor plasma antioxidant capacity or the oxidative status of the female showed significant associations with eggshell spotting pattern, although we note that oxidative status was estimated 18–21 days after the end of egg laying (during nestling feeding) to avoid nest desertion. Plasma oxidative status may have changed during this time (Sepp et al. 2012), although some studies have demonstrated significant repeatability of the values of OXY and ROM within the same breeding season (Costantini et al. 2007, Saino et al. 2011).

We found that females that laid smaller clutches produced eggs with higher brown spot chroma and eggshell protoporphyrin concentrations, which were interrelated variables. Females that lay smaller clutches may have lower intrinsic quality or they may breed in poorer-quality territories and thus they may be exposed to lower food availability, insufficient for laying more eggs in a clutch (Minot 1981, Perrins and McCleery 1989). Female Great Tits consume high numbers of caterpillars during the breeding period (Török 1986), which are rich in dietary antioxidants (Arnold et al. 2010, Eeva et al. 2010). We may thus assume that females with a lower availability of antioxidant-rich food laid smaller clutches and possibly also possessed a less efficient antioxidant capacity during the laying period. Therefore, these females may have removed more of the pro-oxidant protoporphyrin pigment from their circulation by depositing it into the eggshell. This result also corresponds with our finding that eggs which contained

lower concentrations of yolk antioxidants had more protoporphyrin pigment in the eggshell, although the oxidative status of females during the nestling feeding period showed no relationship with eggshell spotting pattern. Consistent with our results, Duval et al. (2013) found that food-restricted Japanese Quail (*Coturnix japonica*) females deposited more protoporphyrin into their eggshells, suggesting that a higher pigment content in the eggshell may reflect poorer nutritional condition of the female.

In contrast to our results, López de Hierro and De Neve (2010) found that, in the House Sparrow (*Passer domesticus*), eggs in larger clutches had darker pigment spots on the eggshell. Moreover, Blue Tit and House Sparrow females that laid larger clutches laid eggs with a more even distribution of spots (Sanz and García-Navas 2009, López de Hierro and De Neve 2010). In studies of Eurasian Kestrels (Martínez-Padilla et al. 2010), British Great Tits (Stoddard et al. 2012) and Eurasian Reed-Warblers (Krištofík et al. 2013), no significant relationships between eggshell spot color and clutch size were found. Therefore, it appears that there is no clear pattern between clutch size and eggshell spotting pattern among bird species, and the relationship may depend on average clutch size, annual number of breeding attempts, and various physiological mechanisms and limitations during laying, all of which may differ among species and populations.

Eggshell Spotting and Egg Investment

Our results showed that eggs with darker eggshell spots (indicated by higher spot intensity and greater brown spot chroma) and higher shell protoporphyrin pigment concentrations contained lower concentrations of dietary antioxidants (lutein and tocopherol) in the yolk. Eggshell spot intensity and brown spot chroma were correlated and each may signal the protoporphyrin concentration of the eggshell. These results may indicate that eggs with darker spots are lower-quality eggs. Egg yolk carotenoids and tocopherols are important for the rapidly developing bird embryo, as these antioxidants help to protect its vulnerable, lipid-rich tissues from oxidative damage (Surai and Speake 1998, Saino et al. 2003, McGraw et al. 2005). Dietary antioxidants are hypothesized to be a limiting resource (Møller et al. 2000), so the ability of the female to invest these compounds into her eggs may be affected by the antioxidant requirements of the female and the antioxidant availability in the environment.

Females with higher levels of blood protoporphyrin during egg laying may use more antioxidants to compensate for the higher level of this pro-oxidant, and thus could deposit lower amounts of antioxidants into the egg yolk. Alternatively, females that lay eggs with lower amounts of antioxidants may have restricted access to dietary antioxidants in their territory. Therefore, their antioxidant capacity may be lower, and thus they may remove a higher amount of the produced pro-oxidant protoporphyrin from their system to avoid oxidative damage. According to this hypothesis, females use eggshells as an additional way (apart from feces; Scholnick et al. 1971) to remove the potentially harmful protoporphyrin, and the amount of pigment deposited depends on the actual antioxidant capacity of the female. However, we caution that we found no direct evidence for a relationship between shell protoporphyrin concentration and the oxidative status of the female in our study, although oxidative status was estimated during the nestling feeding period, and the level of OXY may have changed between the egg laying and nestling feeding periods (Sepp et al. 2012).

In several previous studies on blue-green eggshell coloration, a positive association between shell biliverdin concentration and yolk antioxidant level was detected (Hargitai et al. 2008, 2010, Navarro et al. 2011, Butler and McGraw 2013). So far, to our knowledge, only one study has tested the association between protoporphyrin-based eggshell spotting and yolk antioxidant level. In Blue Tits, Holveck et al. (2012) found no relationship between eggshell spotting pattern and yolk carotenoid concentration. It is possible that differences in the availability of dietary antioxidants in the breeding environment and variation in species-specific capacity to absorb and metabolize carotenoids caused the difference between these results and those of our study. Further studies are needed to examine whether eggshell spotting patterns reflect antioxidant concentrations of the egg yolk in other species and populations.

It remains unclear whether eggshell spot intensity plays a signaling role to the male parent concerning egg quality. Pigment intensity may be detected by males, and they may adjust their feeding behavior in relation to the expected reproductive quality of the brood (Moreno and Osorno 2003). However, a correlative study on Blue Tits did not find a significant relationship between eggshell pigment darkness and male provisioning effort, although eggshell spot distribution was positively correlated with male parental effort (Sanz and García-Navas 2009). Furthermore, Stoddard et al. (2012) found that neither the feeding behavior of the male parent nor nestling growth were related to eggshell spotting patterns in British Great Tits. Therefore, it is possible that the correlation between shell pigment intensity and yolk antioxidant level is due purely to some physiological constraint (see above), and birds do not use it as a signal of embryonic quality, e.g., because of limitations in the detectability of the intensity of pigmentation within dimly lit cavities (Cassey 2009, Cherry and Gosler 2010, Holveck et al. 2010, Avilés et al. 2011).

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