Intra-clutch variation in avian eggshell pigmentation covaries with female quality

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Abstract  Among the most eye-catching traits of avian eggs are their background coloration and pigmentation, consisting in many passerine birds of dark protoporphyrin spots. Although variation in protoporphyrin pigmentation among clutches has been shown to reflect female quality, within-clutch variation in egg pigmentation remains less well understood. Here, we hypothesize that female quality may also be reflected in within-clutch variation in egg pigmentation as a result of energetic constraints and/or increased susceptibility to oxidative stress, and test this hypothesis in a free-living population of Great Tits (Parus major). Within clutches, both pigment ‘darkness’ and ‘spread’ (reflecting intensity, distribution and size of pigment) increased with laying order. For pigment ‘darkness’, this was most strongly so in larger females and in females showing lysis (as a measure of constitutive innate immunity), suggesting that intra-clutch variation in pigment ‘darkness’ positively relates to both structural as well as condition-dependent female traits. In contrast, for pigment ‘spread’, no relationships were detected with body size, body condition, age, and two components of constitutive innate immunity. Among clutches, ‘darkness’ and ‘spread’ of pigments also varied. However, this variation was not related to any of the female characteristics we measured. To the best of our knowledge, this study is the first one to relate intra-clutch variation in protoporphyrin egg pigmentation to structural and condition-dependent traits of laying females. Further experimental study is, however, required to better understand the underlying causal mechanisms.

Keywords Eggshell colour · Great Tit · Laying sequence · Maculation · Protoporphyrin

Zusammenfassung  Unterschiede in der Pigmentierung der Eierschalen innerhalb eines Geleges sind ein Hinweis auf die Qualität des Weibchens

am stärksten bei größeren Weibchen und bei Weibchen, die Lyse zeigten (als Maß einer angeborenen Immunität), was darauf hindeutet, dass Unterschiede in der Dunkelheit der Pigmente innerhalb von Gelegen positiv korreliert sowohl mit strukturellen als auch gesundheits-abhängigen Merkmalen der Weibchen. Dagegen konnte für die Pigment- „Ausbreitung” kein Zusammenhang gefunden werden mit Körpergröße, Alter, Gesundheitszustand oder zwei Komponenten einer angeborenen Immunität. „Dunkelheit” und „Ausbreitung” der Pigmente unterschieden sich auch zwischen den Gelegen. Allerdings zeigten diese Unterschiede keinen Zusammenhang mit einer der gemessenen Größen der Weibchen. Soweit wir wissen ist dies die erste Untersuchung, in der Unterschiede in der Protoporphyrin-Pigmentierung der Eier in Zusammenhang gebracht werden mit strukturellen und gesundheits-abhängigen Merkmalen legender Weibchen. Allerdings sind weitere experimentelle Untersuchungen notwendig, um die zugrunde liegenden kausalen Mechanismen besser zu verstehen.

Introduction

Avian eggshells vary widely in background coloration and pigmentation among species. The two main pigments responsible for egg coloration in birds are biliverdin and protoporphyrin (PP). While biliverdin causes blue-greenish eggs, PP is responsible for reddish and brownish tints and for the dark spots that are often encountered on passerine eggs (Kennedy and Vevers 1976; Underwood and Sealy 2002). Despite their wide occurrence, the precise mechanisms underlying the production and deposition of egg pigments are still equivocal, and so is the question whether the production and deposition of pigments are either detrimental or beneficial to females and chicks. To date, most studies on the significance of avian eggshell pigmentation have focused on structural or signaling properties (reviewed in Underwood and Sealy 2002; Kilner 2006; Reynolds et al. 2009), and it is widely assumed that PP pigmentation serves to reduce eggshell permeability and to strengthen eggshell thinning due to calcium deficiency (structural function hypothesis; sensu Gosler et al. 2005; Higham and Gosler 2006). However, recent experimental studies could only partially confirm statistical associations between calcium availability and PP pigmentation (García-Navas et al. 2011; Mági et al. 2012). A second hypothesis; which states that eggshell colour is a sexually-selected signal indicating a female’s quality to her mate (sexually selected eggshell colouration hypothesis; Moreno and Osorno 2003), is more controversial for explaining PP pigmentation (see Reynolds et al. 2009 for an extensive review).

PP is a pro-oxidant whose accumulation in the liver causes oxidative stress (Afonso et al. 1999). Although the origin of eggshell PP is still unclear, the precursor material is thought to be (mainly) derived from the blood, while PP itself is synthesized in the shell gland and transferred to the eggshell during egg formation (Baird et al. 1975; Zhao et al. 2006; Wang et al. 2007, 2009). Based on the haem biosynthesis pathway and the observed relationship between PP pigmentation and a female’s anaemic condition during egg-laying, we suggested that anaemia in females may lead to diminished PP availability from disintegrated red blood cells and hence decreased deposition of PP (De Coster et al. 2012). It has also been shown that increased amounts of circulating PP are followed by increased activity of antioxidant enzymes to avoid or revert oxidative damage (Afonso et al. 1999). Based on these findings, Moreno and Osorno (2003) proposed that high rates of PP deposition may reflect high circulating PP levels in the blood, which may indicate a high antioxidant capacity (or oxidative tolerance) to sustain high blood levels of PP or a female’s ability to efficiently remove damaging PP pigments that accumulate in the blood. Conversely, they may reflect high circulating PP levels in the blood because of an inability of the anti-oxidant system to efficiently remove pigments.

These hypotheses, in combination with the fact that eggshell pigmentation is highly variable within species (Miksik et al. 1994, 1996), have triggered predictions on how PP pigmentation may be related to female quality, either inversely (i.e. ‘poor quality’ hypothesis; low-quality females lay more pigmented eggs; e.g. Martínez-de la Puente et al. 2007) or positively (i.e. ‘good quality’ hypothesis; high-quality females lay more pigmented eggs; e.g. Sanz and García-Navas 2009; López de Hierro and De Neve 2010; Martínez-Padilla et al. 2010). Earlier empirical studies have provided support for both hypotheses. For instance, Martínez-de la Puente et al. (2007) showed that Blue Tits (Cyanistes caeruleus) that laid more strongly pigmented eggs were in poorer body condition, and showed evidence of higher stress levels and lower circulating immunoglobulins levels. However, other studies on Blue Tits (Sanz and García-Navas 2009; Holveck et al. 2012) and studies on Great Tits (Parus major; Stoddard et al. 2012), House Sparrows (Passer domesticus; López de Hierro and De Neve 2010) and Eurasian Kestrels (Falco tinnunculus; Martínez-Padilla et al. 2010) showed positive associations between PP pigmentation and female condition-based traits, such as plumage coloration, body mass, tarsus length, age, clutch size and/or yolk antibody concentrations.

Egg production is a demanding process for many birds, particularly for small passerine birds that lay a clutch of eggs with a total mass that may exceed the female’s own
body mass (Monaghan and Nager 1997). It has also been shown that increased reproductive effort often results in an increased susceptibility to oxidative stress (Dowling and Simmons 2009 and references therein). Energetic constraints and the susceptibility to oxidative stress can thus be assumed to rise through the course of egg-laying, possibly resulting in increased or decreased PP deposition (depending on the pattern in question—see hypotheses below). Furthermore, low-quality females may experience more severe energetic constraints and more oxidative stress, since levels of oxidative stress seem to be tightly linked to ageing and diseases (reviewed in Finkel and Holbrook 2000; Valko et al. 2007). Therefore, it is conceivable that the change in pigment deposition across the laying period is also related to female quality. Under the ‘good quality’ hypothesis, we predict high-quality females to progressively lay more strongly pigmented eggs than low-quality females (Fig. 1). Potentially, the underlying mechanism is that females might become progressively less capable of removing PP by depositing it into the eggs, most strongly so in low-quality females. Under the ‘poor quality’ hypothesis, we predict low-quality females to progressively lay more strongly pigmented eggs compared to their high-quality counterparts (Fig. 1), because the capacity of the antioxidant system decreases more strongly over the laying sequence in the former. As a consequence, PP is accumulating in the blood and, hence, deposited more to the eggs. Such changes in relative difference in egg pigmentation between high- and low-quality females with laying order may be independent of whether the overall level of pigmentation increases or decreases with laying order. Previous studies in several bird species indeed showed both increasing (e.g. Gosler et al. 2005) and decreasing (e.g. López de Hierro and De Neve 2010; Martínez-Padilla et al. 2010) deposition of PP pigments along the laying sequence, alternating patterns (Hanley and Doucet 2009) or the occurrence of a paler last egg (Ruxton et al. 2001 and references therein). So, while we present two competing hypotheses to explain egg pigmentation patterns, we cannot exclude the possibility that both processes alternate during the egg-laying period.

We here test whether inter- and intra-clutch variation in eggshell pigmentation is related to female phenotypic quality in a free-living population of Great Tits. This passerine bird lays one white egg pigmented with PP spots each day upon clutch completion (4–12 eggs; Gosler 1993). The costs of egg production can be considerable as each egg can weigh more than 10 % of the female’s body mass and the total clutch mass can outweigh that of the female (Gosler 1993; Nilsson and Råberg 2001; Visser and Lessells 2001). For each incubating female, we measured both structural (body size) and condition-related traits [body condition and two components of constitutive innate immunity, i.e. natural antibodies (hereafter NAbs) and the activation of the complement cascade]. Both immunity components provide the first defence against a broad spectrum of pathogens and are therefore assumed to be critical to survival (Ochsenbein and Zinkernagel 2000). Since NAbs and complement activation were earlier shown to be positively related to body condition (Pomeroy et al. 1997) and negatively related to nutritional stress (Bourgeon et al. 2010), we consider females with higher agglutination (NAbs) and lysis (complement) scores to be of higher quality. Indices of body condition are assumed to reflect the overall health, nutritional status and fat content of individuals (Brown 1996). Finally, we also determined female age, which is known to affect reproductive performance in Great Tits (Bouwhuis et al. 2009).

Methods

Field procedures

The study was conducted in spring 2008 in a Great Tit population breeding in nest-boxes in a forest near Ghent, Belgium (for details, see De Coster et al. 2010). Prior and during egg-laying (starting during the last week of March), 30 nests were visited daily until clutch completion (range 7–11 eggs; only first clutches considered) to mark the newly-laid egg. After 3 days of incubation, assessed by the presence of the female at the nest or the presence of warm
eggs during three consecutive days, all females were captured while incubating their eggs, ringed, and a blood sample (100–150 μl) was collected in heparinised capillary tubes from the brachial vein and transferred to a microfuge tube. Afterwards, females were aged according to plumage characteristics, weighed, and measured following Svensson (1992). Subsequently, the entire clutch was collected for biochemical analysis (see De Coster et al. 2012 for a more detailed description of the methodology). Tarsus and wing lengths were combined into a composite measure of body size by means of a principal component analysis. As the first principal component (PC1) explained 57% of the total variation in wing and tarsus length, it was used as a measure of body size (Costantini et al. 2010). Afterwards, we calculated body condition as the residual of the linear regression of body mass on body size (PC1). Because female body condition and mass were strongly positively correlated (r = 0.91; P < 0.0001), only body condition was used in statistical modelling. However, results were very similar when body mass instead of body condition was used (analyses not shown). As part of another study (De Coster et al. 2012), half of the nests had been randomly assigned to an experimental parasite treatment. However, as there was no relationship between parasite counts and any of the female traits measured (all P > 0.18), we can safely assume that the treatment was randomised over female quality, and hence did not bias the results of this study.

Quantification of immune function

Blood samples were centrifuged (5 min at 10,000g) and plasma was separated from the cells and frozen at −20 °C on the day of collection. Levels of circulating NAbs and complement were estimated from blood plasma by means of the haemolysis–haemagglutination assay (Matson et al. 2005). This assay is based on red blood cell agglutination and NAb-mediated complement activation (see De Coster et al. 2010 for a detailed description of the protocol). Subsequently, digitised images of the assay plates were scored based on the highest dilution at which agglutination (NAb) or lysis (complement) of red blood cells was observed. Half scores were assigned if the termination of the reaction was intermediate between two wells. As only two different scores were obtained from lysis titres, i.e. ‘0’ (no lysis) or ‘1’ (only lysis in undiluted plasma), lysis score was further treated as a binary variable. One individual that showed an ambiguous lysis reaction was excluded from further analysis.

Egg pigmentation

On the day of clutch collection (see above), all 264 eggs were weighed and digital photographs were taken indoors at fixed distance, light conditions, lateral position, and (dark) background. Egg pigmentation was assessed from the digital photographs following the criteria of Gosler et al. (2000, 2005), i.e. scores were assigned for pigment intensity (I; scored in 0.5 increments from 1 for the palest to 5 for the darkest spots), distribution (D; scored in 0.5 increments from 1 for >90% spots concentrated at one end to 5 for evenly distributed spots) and spot-size (S; scored in 0.5 increments from 1 for the smallest to 5 for the largest spots). As pure white eggs were not present in the dataset, zero scores were not assigned. All eggs were scored by two observers (between-observer repeatability: I 92%; D 87%; S 98%) and the mean I, D and S scores were calculated (correlations: I − D = −0.44, P < 0.0001; I − S = 0.32, P < 0.0001; D − S = 0.08, P = 0.18), summarised by means of a principal component analysis, and the first two principal components (PCs) were retained and used as response variables in the statistical analyses (see below). PC1 (PC1 = 0.73I − 0.57D + 0.37S; 50% of total variation explained) reflects the ‘darkness’ of the pigmentation with higher values indicating increasing overall darkness of the eggshell pigment (Gosler et al. 2000, 2005), while PC2 (PC2 = 0.04I + 0.58D + 0.81S; a further 36% of total variation) reflects the pigment ‘spread’ with higher values corresponding with larger and more evenly dispersed spots over the egg (Gosler et al. 2000, 2005). Hence, eggs with larger values for both pigment ‘darkness’ and ‘spread’ can be considered as being more pigmented.

Statistical analysis

We first tested whether variation in egg pigmentation among clutches was related to female quality by fitting linear mixed models (LMM) with pigment ‘darkness’ or ‘spread’ as response variables and agglutination, lysis, body size, body condition and age (1st-year or older) as explanatory variables. Laying sequence was thereby included as covariate to account for the variability within clutches. The quadratic term of laying sequence was also included to account for non-linear effects of laying sequence on eggshell pigmentation. Second, a set of models was built to test whether variation in female quality was related to variation in eggshell pigmentation within clutches by including the interactions of laying sequence with all female traits in the corresponding mixed model. Because of the significant quadratic effect of laying sequence on pigment ‘darkness’ (see “Results”), the quadratic term of laying sequence and the interactions between this quadratic term and all female traits were included when modelling pigment ‘darkness’. Because clutch size differed between females, we additionally ran general linear models with pigment ‘darkness’ or pigment ‘spread’ from the first or last egg. All models were corrected for
laying date and clutch size. Because data from eggs of the same clutch are not statistically independent, we included clutch-specific (i.e., random) intercepts in all mixed models. Clutch-specific linear and quadratic slopes were also included in cases in which laying sequence and its quadratic term were included as fixed effects in the model, respectively (Schielzeth and Forstmeier 2009). The percentage of the total variation present between nests was calculated as intraclass correlations coefficients. Degrees of freedom were estimated following Kenward and Roger (1997; not necessarily integers). We used restricted maximum likelihood (REML) parameter estimation for LMM to obtain unbiased estimates of variance components, and likelihood ratio test statistics to test if variances significantly differed from zero (Verbeke and Molenberghs 2000). The most parsimonious model was obtained by removing non-significant fixed and random effects with a sequential step-down procedure. All statistical analyses were performed in SAS 9.2 (SAS Institute, 2002–2003, Cary, NC, USA).

Results

Female identity explained 71 and 53% of the total variation in pigment ‘darkness’ and ‘spread’ among clutches, respectively (both \( P < 0.0001 \)). However, this variation was not significantly related to any of the female traits (agglutination, lysis, body condition, body size or age; agglutination \( P = 0.08 \); all other \( P > 0.10 \)). Within clutches, a significant quadratic effect of laying sequence on pigment ‘darkness’ was observed (\( F_{1,226} = 53.01; P < 0.0001 \)). After an initial increase, pigment ‘darkness’ attenuated from the fifth egg onwards (Fig. 2a). Furthermore, in the four largest clutches (all 11 eggs), the last egg was paler (Fig. 2a). In contrast, pigment ‘spread’ increased in a linear way with laying sequence (\( F_{1,29.7} = 50.43; P < 0.0001 \); Fig. 2b). Hence, along the laying sequence eggs became pigmented with larger and more evenly dispersed spots.

The strength of the relationship between laying sequence and pigment ‘darkness’ varied with body size (body size \( \times \) LS, \( P = 0.015 \); Table 1) and lysis (lysis \( \times \) LS, \( P = 0.020 \); Table 1), i.e. larger females and those with lysis showed a steeper increase in pigment ‘darkness’ along the laying sequence (Table 1; Figs. 3, 4). In both females with and without lysis, the relationship between pigment ‘darkness’ and laying order showed a significant quadratic term (Fig. 4; lysis: \( P = 0.003 \); no lysis: \( P < 0.0001 \)). However, this quadratic relationship did not differ between both groups of females (lysis \( \times \) LS\(^2\), \( P = 0.37 \); Table 1). In contrast, variation in pigment ‘darkness’ along the laying order was not significantly related to agglutination, body condition or age (all \( P > 0.26 \)), nor was variation in pigment ‘spread’ along the laying sequence related to any attribute of female quality (all \( P > 0.11 \); Table 1). Random intercepts and linear slopes were significant in all models (all \( P < 0.03 \)), while random quadratic slopes were not significant in any of the models (all \( P > 0.12 \)).

Eggs that were laid last in the sequence were significantly darker in females showing lysis compared to those not showing lysis (difference \( \pm \) SE = 1.16 \pm 0.41; \( F_{1,27} = 8.12; P = 0.008 \)). Furthermore, pigment ‘spread’ of the first egg (estimate \( \pm \) SE = 0.32 \pm 0.16; \( F_{1,28} = 4.03; P = 0.055 \)) and last egg (estimate \( \pm \) SE = 0.26 \pm 0.13; \( F_{1,28} = 4.27; P = 0.048 \)) decreased marginally with agglutination. Other female traits were not significantly related to pigment ‘darkness’ or ‘spread’ of the first or last egg (all \( P > 0.09 \)), neither did laying date and clutch size explain significant variation in any of the models (all \( P > 0.49 \)).

Discussion

We tested whether intra-clutch variation in eggshell pigmentation was related to female phenotypic quality in a free-
living population of Great Tits. We found a significant increase in pigment ‘darkness’ and pigment ‘spread’ with the laying order of the eggs, whereby the increase in ‘darkness’ leveled off from the fifth egg onwards. The strength of the increase in pigment ‘darkness’ varied with two indices of female quality, i.e. it was steeper in clutches from larger females and from females that showed lysis. As a consequence, last-laid eggs were significantly darker in clutches from the latter. Average pigment ‘darkness’ and pigment ‘spread’ also varied significantly among clutches; however, there was no relationship with female quality indices measured at that level. Although variation in eggshell pigmentation along the laying order has been reported in a number of passerines before, including the Great Tit (e.g. Gosler et al.)

Table 1 Results of LMM models for eggs of Great Tits (Parus major) with pigment ‘darkness’ (PC1) or pigment ‘spread’ (PC2) as dependent variable, based on a sequential step-down simplification procedure

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pigment ‘darkness’ (PC1)</th>
<th>Pigment ‘spread’ (PC2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>df</td>
</tr>
<tr>
<td>Age</td>
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<td>Agglutination</td>
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<td>BCI</td>
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<tr>
<td>Clutch Size</td>
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</tr>
<tr>
<td>Date</td>
<td>0.23</td>
<td>20.8</td>
</tr>
<tr>
<td>Lysis</td>
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<td>25.7</td>
</tr>
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<td><strong>LS</strong></td>
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</tr>
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<td>BCI × LS²</td>
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<td>Lysis × LS²</td>
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</table>

Fig. 3 Interaction between laying sequence and female body size on pigment ‘darkness’ (PC1). The surface depicts fitted values

Fig. 4 Variation in pigment ‘darkness’ (PC1) with laying sequence and lysis. Each dot represents the average value (±SE) for a particular combination of laying sequence and lysis (lysis filled dots; no lysis open dots)
2005; López de Hierro and De Neve 2010; Martínez-Padilla et al. 2010), it has, to the best of our knowledge, never been directly associated with female quality.

The results of this study hence revealed a stronger increase in pigment ‘darkness’ with laying order in clutches from high-quality females. Given that PP pigments are pro-oxidants (Afonso et al. 1999), progressively more pigmentation on eggs from high-quality birds (‘good quality’ hypothesis; Fig. 1) might reflect oxidative tolerance or a female’s ability to get rid of damaging PP pigments (Moreno and Osorno 2003). Alternatively, the variation in female traits observed in this study may be related to calcium availability, which is known to be essential for many physiological functions (Sigel and Sigel 1984) and to trigger relationships between pigment ‘darkness’ and water loss during incubation, which may vary within clutches (Higham and Gosler 2006).

While pigment ‘darkness’ increased in a linear way with laying order in the first eggs, attenuation was observed from the fifth egg onwards. This pattern was unexpected as it deviated from the monotonous linear increase observed by Gosler et al. (2005) in the same species. It is currently unclear whether this discrepancy implies a persistent difference between populations or rather reflects variability between years. In any case, this difference supports the notion that the ‘good quality’ and ‘poor quality’ patterns may both occur within the same species, or possibly even within the same population or individual. Consistent with other species, the shells of the ultimate egg in the four largest clutches were paler (Ruxton et al. 2001 and references therein). Egg production entails high costs for female Great Tits (Nilsson and Råberg 2001; Visser and Lessells 2001), and stronger constraints towards the end of egg-laying might result in a depletion of PP (Nice 1937), which is in agreement with the ‘good quality’ hypothesis. Potentially, the shell gland can only produce intensely-pigmented eggs during a limited period, after which it should be ‘recharged’ to proper function. While we cannot exclude that physiological changes in the female at the end of egg-laying (which often coincide with the beginning of incubation in Great Tits; Gosler 1993), may affect the functioning of pigment glands resulting in a paler last egg (Lowther 1988), this would not explain why the decline in pigment ‘darkness’ was only apparent in the four largest clutches.

Pigment ‘spread’ also increased, but, as opposed to pigment ‘darkness’, this increase was not related to any of the female quality indices measured. Likewise, many other studies have shown that pigment ‘darkness’ and ‘spread’ represent two independent components of eggshell pigmentation that are often related to different variables. For example, while pigment ‘darkness’ has been shown to vary with female age and tarsus length (Sanz and García-Navas 2009; López de Hierro and De Neve 2010), and to be inherited via the maternal line (Gosler et al. 2000; but see Mahler et al. 2008), pigment ‘spread’ has been related to hatching probability and individual characteristics of nestlings and males (Sanz and García-Navas 2009). Possibly, pigment ‘spread’ is associated with the orientation of the egg in the uterus (Kennedy and Vevers 1973 and references therein), whereas pigment ‘darkness’ reflects the accumulation of PP pigment in the shell gland during egg formation (Baird et al. 1975; Wang et al. 2007). If pigment ‘darkness’ and ‘spread’ are triggered by mechanisms operating at different stages during ontogeny, it can indeed be expected that neither components of egg pigmentation are equally related to female quality.

In this study, intra-clutch variation in pigment ‘darkness’ was positively related to structural (i.e. body size) as well as condition-dependent (i.e. lysis score) traits of breeding females, while inter-clutch variation was not related to any female trait. This contrasts with results from various other studies that showed positive relationships between inter-clutch variation in pigment ‘darkness’ and structural traits which change over long timespans (e.g. tarsus length; Sanz and García-Navas 2009; plumage characteristics; Martínez-Padilla et al. 2010), and inverse relationships with condition-dependent ones (e.g. body condition, stress protein and immunoglobulin levels; Martínez-de la Puente et al. 2007). Such a pattern suggests that physiological mechanisms underlying inter- and intra-clutch variation in eggshell pigmentation may differ. Remarkably, while effects of female quality on inter-clutch variation in eggshell pigmentation have been recorded in several other bird species, the absence of such effect in our study is in agreement with results from another study on the same species (Gosler et al. 2000; but see Gosler et al. 2005). Thus, while the broad physiological mechanisms underlying egg pigmentation might be comparable among bird species, their relative relationships with female quality seem to be species-specific.

To summarise, we showed for the first time that variation in PP egg pigmentation within clutches reflects female quality in Great Tits, thereby supplementing similar findings on variation in eggshell pigmentation among clutches. Because of the correlative nature of our study, we cannot make firm conclusions on the mechanisms underlying the observed pattern. Therefore, experimental studies are required to better understand the association between female quality and variation in egg pigmentation along the laying sequence.

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