

ORIGINAL ARTICLE

Maternal influence on eggshell maculation: implications for cryptic camouflaged eggs

Camille Duval^{1,6} · Phillip Cassey² · P. George Lovell³ · Ivan Mikšík⁴ · S. James Reynolds⁵ · Karen A. Spencer¹

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Abstract Egg camouflage may explain the adaptive significance of avian eggshell pigmentation in ground-nesting species. Eggshell maculation (spots) is predominantly due to protoporphyrin, but both biliverdin (antioxidant) and protoporphyrin (pro-oxidant) may be present in spotted eggshells. Because of their role in oxidative stress, the deposition of eggshell pigments might be condition-dependent. However, because of the fitness benefits of eggshell coloration, cryptic eggshell appearance should be strongly conserved in ground-nesting species regardless of female condition and eggshell pigment concentrations. We investigated whether Japanese quails (*Coturnix coturnix japonica*) maintained eggshell maculation under food

restriction. We quantified eggshell maculation (i.e., percentage of spot coverage) using digital photography, and both protoporphyrin and biliverdin concentrations of eggs laid by females either on a food-restricted or an ad libitum diet. Females on a high quality diet, which are known to decrease the deposition of eggshell protoporphyrin, decreased eggshell maculation compared with food-restricted females that maintained it. For the first time, we propose an experimental study which suggests that eggshell maculation depends on female body condition and that manipulating eggshell maculation may be the strategy used by females to potentially optimize egg camouflage.

Keywords Body condition · Camouflage · *Coturnix coturnix japonica* · Eggshell maculation · Protoporphyrin

Zusammenfassung

Maternaler Einfluss auf die Befleckung der Eierschale: Folgen für kryptische getarnte Eier

Die Tarnung von Eiern könnte die adaptive Bedeutung der Pigmentierung der Eierschale bodenbrütender Vögel erklären. Die Befleckung der Eierschale ist hauptsächlich auf Protoporphyrin zurückzuführen, aber sowohl Biliverdin (ein Antioxidant) als auch Protoporphyrin (ein Prooxidant) können in gefleckten Eierschalen enthalten sein. Aufgrund der Rolle von Eierschalenpigmenten bei oxidativem Stress könnte ihre Einlagerung in die Eierschale konditionsabhängig sein. Aufgrund der Fitnessvorteile einer Färbung der Eierschale sollte ein kryptisches Aussehen der Eier bei Bodenbrütern jedoch hochkonserviert sein, unabhängig von der Kondition der Weibchen und der Konzentration der Eierschalenpigmente. Wir haben untersucht, ob Japanwachteln (*Coturnix coturnix japonica*) die Befleckung ihrer

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✉ Camille Duval
camille.duval07@gmail.com

¹ School of Psychology and Neuroscience, University of St Andrews, South Street, St Andrews KY16 9JP, UK

² School of Earth and Environmental Sciences, University of Adelaide, Adelaide, SA 5005, Australia

³ Division of Psychology, Social and Health Sciences, The University of Abertay, Dundee DD1 1HG, UK

⁴ Institute of Physiology, Academy of Sciences of the Czech Republic, Videnská, 1083, 14220 Prague, Czech Republic

⁵ Centre for Ornithology, School of Biosciences, College of Life and Environmental Sciences, University of Birmingham, Birmingham B15 2TT, UK

⁶ 57 rue du General Chapelle, 07300 Tournon Sur Rhône, France

Eierschale bei Futterknappheit beibehielten. Wir haben die Eierschalenbefleckung (d.h. den Anteil der Abdeckung mit Flecken) mit Hilfe digitaler Fotografie quantifiziert sowie die Protoporphyrin- und Biliverdin-Konzentrationen der Eier von Weibchen ermittelt, die entweder eingeschränkt oder ad libitum Futter erhielten. Weibchen mit hochwertiger Kost, die bekannterweise die Einlagerung von Protoporphyrin in die Eierschale reduzieren, reduzierten die Befleckung der Eierschale im Vergleich zu Weibchen mit eingeschränkter Kost, welche sie beibehielten. Zum ersten Mal liefern wir eine experimentelle Studie ab, die darauf hindeutet, dass die Befleckung der Eierschale von der Kondition des Weibchens abhängt und dass Weibchen die Befleckung der Eierschale beeinflussen könnten, als Strategie, um die Tarnung der Eier potenziell zu optimieren.

Introduction

To maximize their reproductive success, female birds are able to modulate their investment not only through the size of their clutch but also through the quality of their eggs (Bernardo 1996). Besides egg size (Amat et al. 2001; Arnold 1991; Birkhead and Nettleship 1982; Hill 1993), many other egg traits determine chick quality, including hormones (testosterone, corticosterone; Loyau et al. 2007; Mazuc et al. 2003; Petrie et al. 2001), antibacterial (lysozyme) agents (Saino et al. 2002) and antioxidants (e.g., carotenoids, vitamins; Costantini 2010; McGraw and Ardia 2003), all of which may be deposited by adults into their eggs. Females also modulate quantities of these components in response to breeding parameters such as female rearing capacity (Verboven et al. 2003), male attractiveness (Loyau et al. 2007; Sheldon 2000), but, initially, in response to their body condition (Williams 2012). Far less is known about the role of maternal investment in eggshell-specific components. The eggshell is vital in protecting the embryo from multiple mechanical and biological threats (Board 1980; Board and Halls 1973; Handrich 1989) and providing it with a source of calcium (Reynolds and Perrins 2010). Females adjust their allocation of resources such as calcium to the eggshell depending upon their physiological condition during a breeding attempt (Reynolds et al. 2004). Therefore, many eggshell-specific traits can be considered to depend heavily on the body condition of the reproductive female (Solomon 1997).

Females also transfer pigments into their eggshells but their function remains hotly debated. Avian eggshell pigmentation has been studied repeatedly in the context of mimicry, egg recognition, female signalling, maternal inheritance and eggshell strength (Cassey et al. 2011; Kilner 2006), and embryo development and protection

(Maurer et al. 2011). One of the first hypotheses proposed to explain the adaptive significance of eggshell pigmentation was egg camouflage (Wallace 1889). To make their eggs cryptic and undetectable by predators, birds have evolved different strategies depending on their nesting environment. In ground-nesting species that do not always cover their eggs, matching egg colour with the colour of the nest background (i.e., background-matching) may reduce egg visibility (Collias and Collias 2014; Tinbergen et al. 1962). There is currently mixed support for egg crypsis (Castilla et al. 2007; Götmark 1992; Montevercchi 1976; Underwood and Sealy 2002; Weidinger 2001; Westmoreland 2008; Yahner and Mahan 1996); only sporadic studies have confirmed the survival advantage of cryptic eggs (Solis and De Lope 1995). One typical ground-laying species which lays heavily spotted eggs is the Japanese quail (*Coturnix coturnix japonica*). Japanese quail eggshells exhibit dark brown spots on the top of a greenish background, and are pigmented by both biliverdin and protoporphyrin (Duval et al. 2013). Recently, a study on laying substrate choice in this species suggested that in captivity, female quails are able to lay on the substrate that enhances egg crypsis through disruptive colouration (i.e., matching maculation colour and visually breaking the egg contour; Lovell et al. 2013). Thus, quails, which are able to recognize their own eggs (Pike 2011), may have some awareness of their own eggshell colouration and egg crypsis may be the main function for eggshell pigmentation in the species.

It has been suggested that eggshell pigment concentrations may be related to female condition due to the properties of biliverdin as an antioxidant (McDonagh 2001) and protoporphyrin as a pro-oxidant (Shan et al. 2000), the two main pigments responsible for colouring bird eggs. In brown-spotted eggs, protoporphyrin, which is the main pigment responsible for eggshell maculation, may, for instance, have structural properties and could compensate for a lack of calcium in the eggshell as it is structurally similar to phthalocyanine, a lubricant commonly used in solid-state engineering (Gosler et al. 2005; Solomon 1997). In great tits (*Parus major*) and blue tits (*Cyanistes caeruleus*), eggshell pigmentation (“maculation”) has been related to factors such as body condition and stress (Martínez de la Puente et al. 2007), body mass (Stoddard et al. 2012), breeding performance (Sanz and García-Navas 2009), eggshell strength (Gosler et al. 2005), female’s ability to cope with anaemia (De Coster et al. 2012), egg-immune compounds (Holveck et al. 2012), and has been tested for its heritability (Gosler et al. 2000) and intra-clutch variation (De Coster et al. 2013). In the house sparrow (*Passer domesticus*), eggshell pigmentation is related to clutch size and laying order (López-de-Hierro and Moreno-Rueda 2010). Although most of those studies

are correlative but measure a large range of female and egg characteristics, one crucial issue is the lack of pigment quantification, especially considering the poor relationship between egg appearance and its pigment content (Brulez et al. 2014). Thus, the relationship between eggshell maculation and female body condition remains poorly understood and needs some manipulative approach to advance our knowledge beyond being purely descriptive.

Recent manipulative work has tested the relationship between eggshell pigmentation and female condition, although the methodology is questionable, as we mentioned above. For instance, in the Eurasian kestrel (*Falco tinnunculus*), female food supplementation did not influence eggshell pigmentation (Martínez-Padilla et al. 2010). In the great tit, anaemic females showed an increased eggshell pigmentation with laying order (De Coster et al. 2012), however calcium supplementation did not affect eggshell maculation (Mägi et al. 2012). In a very recent paper, Giordano et al. (2015) used a food supplementation design in order to increase female condition during egg laying and investigated the effects on egg antioxidant and eggshell maculation (Giordano et al. 2015). The authors reported correlative results between eggshell maculation and some female and yolk characteristics. However, eggshell protoporphyrin concentration, as in most of the above studies, was not quantified. Thus, there has been some attempt to experimentally investigate the relationship between female condition and eggshell pigmentation; but there is a crucial need for extensive experimental and quantitative studies.

In their study on Japanese quail (*Coturnix coturnix japonica*), Duval et al. (2013) experimentally reduced female body condition through food restriction and quantified colouration using spectrophotometry and pigment content of eggshells. Females on a high quality (ad libitum) diet were in higher body condition and increased relative biliverdin deposition compared to protoporphyrin (see Table 1 for a summary of the results). However, eggshell spot and eggshell background reflectance were not affected by this change in relative pigment deposition. Reflectance spectrophotometry focuses on a single small point (≤ 2 mm in diameter) on an eggshell and allows precise quantification of wavelengths at specific locations. Thus, Duval et al. (2013) concluded that eggshell reflectance was strongly conserved

in the species because optimization of egg crypsis may be strongly adaptive. They also suggested that a strong selection for eggshell crypsis might explain why studies to date have failed to detect a direct relationship between colouration and pigment content of eggshells. In the current study, we quantified eggshell maculation (i.e., spot coverage) from digital photographs of eggs laid by quails that had experienced food restriction. If eggshell maculation is assumed to be mainly due to the presence of protoporphyrin, females depositing more protoporphyrin should lay more maculated eggshells. To the best of our knowledge, ours is the first study to experimentally quantify the condition dependence of eggshell maculation in a species where egg crypsis might be compromised if eggshell patterning changes.

Methods

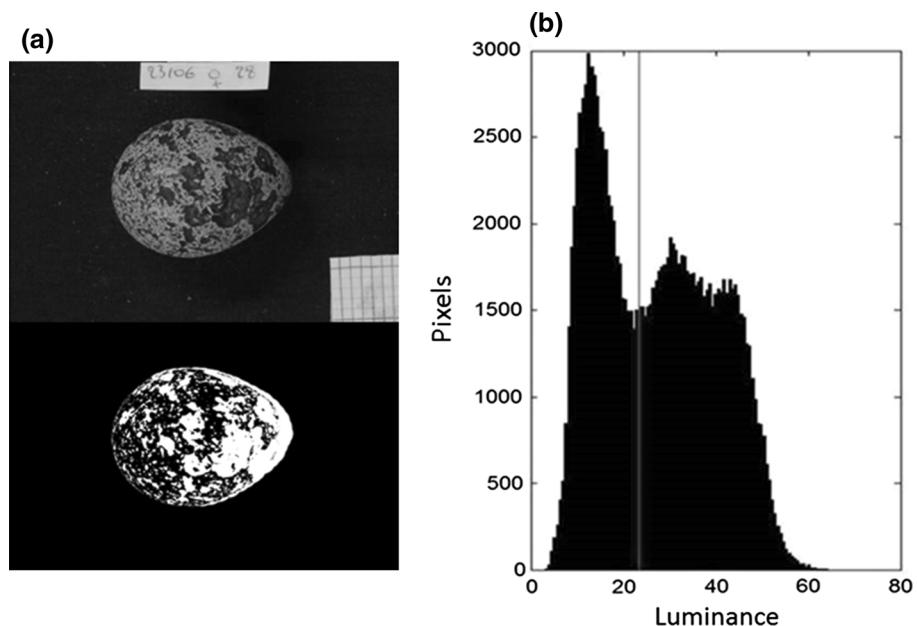
The experiment was conducted in 2010 in a captive population of outbred Japanese quails at Cochno Farm at the University of Glasgow. Following a pilot study that determined female daily food requirement (Duval et al. 2012), 24 females were individually identified with a unique numbered white leg ring and housed in individual cages ($61.0 \times 44.5 \times 50.8$ cm) for 1 week of habituation. All females were fed ad libitum with a standard commercial diet (Layers pellets, BOCM Ltd, Ipswich, UK), and randomly allocated to one of three treatment groups: HQ (i.e., high quality, fed ad libitum), MQ (i.e. medium quality, 90 % of daily food requirement) and LQ (i.e., low quality, 75 % of daily food requirement) food diet. The female-specific quantity of food (as described in Duval et al. 2012) was provided every morning at the same time for the entire feeding trial. Fertile egg collection (daily) began after 10 days of mating with a random male, and the food restriction lasted for 14 days. Ambient temperature was maintained at 18–18.9 °C and the light regime was 14 h:10 h (light:dark).

Two eggs laid prior to (egg 1 and egg 2), and two following (egg 13 and egg 14), 14 days of the food restriction were obtained. Eggs were placed within a dark box at 4 °C to avoid photo-degradation of eggshell pigments (Cassey et al. 2012a). Pigment concentration was analyzed in two eggshells per female (eggs 1 and 14) and quantified using

Table 1 Summary of previous findings on the effects of food manipulation on eggshell appearance in Japanese quail

| Trait | High quality diet (control) | Medium quality diet | Low quality diet | Evidence |
|------------------------------------|-----------------------------|---------------------|------------------|------------------------------|
| Female body condition | Increase | Constant | Decrease | Fig. 1, Duval et al. (2013) |
| Eggshell reflectance | Constant | Constant | Constant | Table 2, Duval et al. (2013) |
| Eggshell protoporphyrin proportion | Decrease | Constant | Increase | Duval et al. (2013) |
| Eggshell biliverdin proportion | Increase | Constant | Decrease | Fig. 4, Duval et al. (2013) |

Fig. 1 **a** One photograph taken of 25 % of a Japanese quail egg on a black velvet surface (*top*) and the corresponding egg mask (*bottom*). **b** Example of a histogram of luminance values of a Japanese quail egg. The vertical line shows the cut-off between *dark areas* to the left where luminance is low (corresponding to maculated areas), and light areas to the right where luminance is high (corresponding to non-maculated areas)



high-performance liquid chromatography (HPLC; Mikšík et al. 1996). Each female was weighed (to the nearest 0.01 g) on an electronic balance before the feeding trial and, again, after her last egg had been collected. Right tarsus length was measured (to the nearest 0.01 mm) with a digital caliper. Body condition of each female was calculated as the residual from a linear regression of body mass on tarsus length. We then calculated the difference in body condition between the beginning and the end of the dietary manipulation. All birds were returned to single-sex group-housing after the last egg collection.

Using calibrated digital photography, we characterized eggshell spot coverage by quantifying pixels corresponding to the spots and background areas for each photograph. Constant lighting and long exposures, rather than flash photography, were used to protect the eggshell pigments from light degradation. A calibrated Canon EOS 450D camera with a 105-mm Sigma autofocus (AF) lens was used and was activated remotely using a Canon RC1 infrared control. Eggs were placed beneath the camera on a black velvet photographic standard background with a white graph-paper as a white standard, with egg ID for identification. Before each photographic session, a picture of a color chart and a grey standard (Colour Confidence, Spectrum Point, Birmingham, UK) were obtained for calibration. Four eggs per female were photographed. For each photograph, the camera was adjusted on its stand so that the egg filled the entire frame. The picture of the egg was taken including a label with the date and female identity and a size standard. For each egg, four images

were taken with a 90°-rotation to the right between successive images. The overlap between the four images was minimized and over-sampling gives a more accurate ratio than under-sampling. For each photograph, the focus was maintained. All digital egg images were saved in standardized RAW format that is beneficial for color analyses (Cassey et al. 2012b). The characterization of the camera's spectral sensitivities and the calibration process were as described in Lovell et al. (2005). The linear RAW images were converted to XYZ (CIE XYZ color-space coordinates; CIE, 1986), and subsequent conversion from XYZ to CIELAB space was implemented using the MATLAB image processing toolbox (2008a, The MathWorks, Natick, MA, USA). Variations in the illumination of the photographed scene were controlled for by normalizing the luminance values (the L channel) to 0 for the darkest area of the black velvet background and to 60 for the white graph-paper.

The area of the photograph occupied by the egg was identified (Fig. 1a), and for pixels in this area, a histogram of the spread of luminance values was plotted, giving a bi-modal distribution of luminance values corresponding to spots and background (Fig. 1b). We then visually selected the cut-off between the maculation and background areas for each photograph; all eggs were analyzed with the researcher blind to the treatment. Finally, the spot coverage percentage was calculated as the number of pixels in the brown spots region divided by the total number of pixels constituting the egg in the photograph, multiplied by 100 (average: 73.7 %; range: 48.7–90.6 %).

We tested the effect of food restriction on the change in female body condition over the experiment using a Kruskal–Wallis test. We then calculated intra-class correlation coefficient (r) repeatability estimates (Lessells and Boag 1987), and compared between- and within-clutch variation in spot coverage at the beginning ($r = 0.78$, $P < 0.001$, $n = 48$) and at the end of food restriction ($r = 0.69$, $P = 0.004$, $n = 48$), and then used the mean spot coverage per female in the analyses. We compared spot coverage between groups before the treatment using an independent samples Kruskal–Wallis test. We used Pearson's correlation to investigate the relationship between eggshell spot coverage and its protoporphyrin and biliverdin concentrations at the beginning of food restriction. We tested the effect of food restriction on spot coverage over the experiment by calculating the difference between the pre- and post-treatment eggs. We used a generalized linear model (GLM) with the difference in spot coverage as the dependent variable, the treatment-group as fixed factor and the difference in protoporphyrin proportion [i.e., (protoporphyrin concentration/total pigment concentration)/100] as a covariate to account for variation between groups. We used the proportion of protoporphyrin as a covariate instead of its concentration as it reflects the switch in pigment deposition reported in a previous study (Duval et al. 2013) (i.e., an increased biliverdin proportion on the high quality diet and an increased protoporphyrin proportion on the low quality diet). *Post hoc* analyses for main effects were performed using a Bonferroni method (Holm 1979). Model residuals were found to be normally distributed. All statistical analyses were performed in SPSS Statistics 19.0.0 (SPSS, Inc., Chicago, IL).

Results

Female body condition was not significantly different between groups at the start of dietary treatment (Kruskal–Wallis test, $H = 0.48$, $P = 0.78$), but was significantly affected by the treatment with LQ females being in lower body condition than C or MQ females, whose body condition increased throughout the manipulation ($F_{2,22} = 8.05$, $P = 0.003$).

Eggshell spot coverage did not differ between birds before (Kruskal–Wallis: $H = 0.58$, $P = 0.74$, $n = 24$) and after ($H = 2.20$, $P = 0.33$, $n = 24$) the dietary treatment. However, the dietary treatment significantly influenced eggshell spot coverage (GLM: group: $F_{2,22} = 6.24$, $P = 0.008$; post hoc: HQ vs. LQ: $P = 0.006$; HQ vs. MQ: $P = 0.22$; MQ vs. LQ: $P = 0.17$). Females on the HQ diet decreased spot coverage (pre-treatment mean \pm 1 standard deviation (SD): $74.77 \pm 7.11\%$; post-treatment: $70.23 \pm 7.17\%$), compared to food-restricted females (MQ

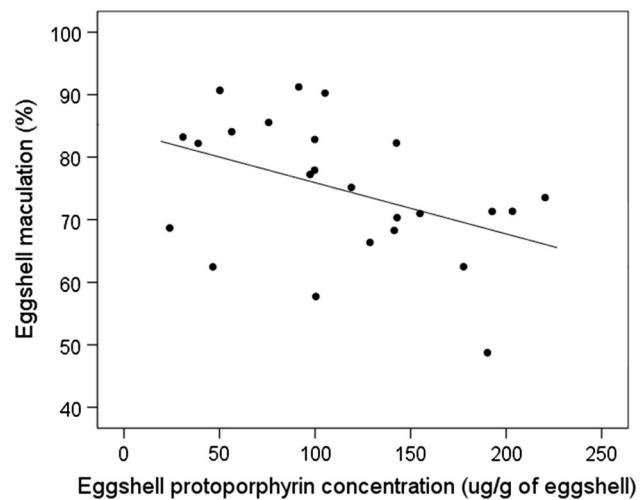


Fig. 2 The bivariate relationship between eggshell maculation (i.e., spot coverage) and eggshell protoporphyrin concentration in all eggs laid before the start of food restriction ($n = 24$)

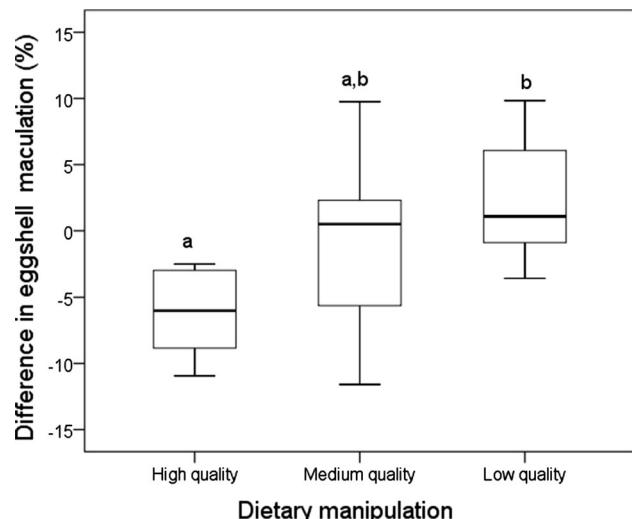


Fig. 3 Variation in eggshell maculation (spot coverage) calculated as the difference in mean spot coverage between pre- and post-treatment eggs ($n = 24$), in the three dietary treatment groups (Low quality, medium quality and high quality (ad libitum food)—see text for further details). The boxes represent the median, the 25th and 75th percentiles, and the minimum and maximum observed values. Different lowercase letters reflect statistically significant differences

and LQ), which maintained a spot coverage similar to their pre-treatment levels (MQ pre-treatment: $77.10 \pm 10.28\%$; MQ post-treatment: $76.16 \pm 8.33\%$; LQ pre-treatment: $72.47 \pm 14.80\%$; LQ post-treatment: 74.82 ± 11.32 ; Fig. 3), and this was positively influenced by the change in eggshell protoporphyrin proportion ($F_{2,22} = 6.89$, $P = 0.02$). In addition, spot coverage was negatively correlated with eggshell protoporphyrin concentration (Pearson correlation: $r = -0.43$, $P = 0.04$, $n = 24$) before the beginning of the food restriction (Fig. 2) but was not

correlated with eggshell biliverdin concentration ($r = 0.14$, $P = 0.51$, $n = 24$).

Discussion

Our data demonstrate that eggshell maculation is influenced by maternal body condition. A previous study found that eggshell reflectance in Japanese quail may have evolved to maintain eggshell crypsis in changing environments (Duval et al. 2013). However, eggshell maculation degree was not considered in that study. The surface of the eggshell will be the first egg trait that conspecifics and which predators will visually detect. Thus, eggshell patterning, such as its maculation, may be key (Stoddard and Stevens 2010) to understanding the functional significance of eggshell pigmentation. For instance, in the house sparrow (*Passer domesticus*), egg rejection increased significantly when spot patterns, rather than eggshell colour, were experimentally modified (López-de-Hierro and Moreno-Rueda 2010).

Here, we investigated eggshell maculation (i.e., spot coverage) under food-restriction. Before any manipulation, eggshell spot coverage was negatively correlated with eggshell protoporphyrin concentration but not related to its biliverdin content, suggesting that protoporphyrin deposition does not increase the amount of visible brown spots on the eggshell. In the same females, Duval et al. (2013) found that eggshells containing more protoporphyrin displayed darker backgrounds, but spot brightness was not correlated with eggshell protoporphyrin content. Interestingly, Brulez et al. (2014) recently tested whether eggshell spots scoring (i.e., intensity, distribution, size) could be used as a proxy for protoporphyrin concentration of an eggshell in great tits and blue tits. Although a tit's eggs have different patterning than a quail's eggs, and the methods used to quantify eggshell maculation were different from ours, the authors' main conclusion was that eggshell spot scoring is not a reliable proxy of its pigment content. This counter-intuitive and hotly debated finding is surprising but it might be due to a complex interaction between protoporphyrin and the eggshell matrix. Indeed, protoporphyrin in Japanese quail is deposited throughout the shell's structure from the inner shell membranes through to the outer cuticle (Tamura and Fujii 1967), and it remains unsolved how both biliverdin and protoporphyrin contribute to eggshell color and maculation in the different compartments of the eggshell (Butcher and Miles 2011).

Duval et al. (2013) showed that females fed ad libitum were in higher body condition and contained a decreased proportion of protoporphyrin in the eggshell after the dietary treatment. This was not as a result of changes in protoporphyrin deposition, but to an increased deposition of

biliverdin over the clutch. This may suggest that females fed a high-quality diet might have had better antioxidant capacities at the end of the dietary manipulation, probably due to decreased activity as they do not need to forage as much for food. This might explain why they were able to afford the deposition of increased amounts of biliverdin, which is an antioxidant pigment (McDonagh 2001), contrary to protoporphyrin, which is a pro-oxidant (Shan et al. 2000). Our current study showed a decreased eggshell maculation under ad libitum food when biliverdin deposition increased. Manipulating eggshell maculation may allow females to deposit increased amounts of the antioxidant pigment into their eggshells without compromising eggshell crypsis. Interestingly, food-restricted females did not show any change in eggshell maculation in this study, despite a decreased deposition of biliverdin. We cannot exclude the hypothesis that they might have compromised their own health or some aspect of egg quality, such as eggshell structure or embryo protection against UV light (Maurer et al. 2011), to maintain their eggs cryptic appearance. In a parallel study, Giordano et al. (2015) investigated the relationship between female condition and her oxidative stress level, and eggshell pigmentation. They found that supplemented females had lower oxidative damage levels than control ones, the latter showing a negative correlation between their oxidative damage levels and egg yolk antioxidant. In addition, the authors claimed that eggshell pigmentation is an indicator of female quality (Giordano et al. 2015). Although our current study also suggests that better-quality females may possess enhanced antioxidant capacity, we cannot directly compare our results regarding eggshell pigmentation to Giordano's paper. Indeed, the authors did not find any significant effect of food supplementation on eggshell pigmentation. In addition, they quantified eggshell spot distribution, whereas we measured eggshell maculation (i.e., spot coverage). Thus, although we acknowledge that our sample sizes are small, and in line with Giordano et al. (2015), we experimentally demonstrated that eggshell maculation is a condition-dependent trait directly related to changes in eggshell pigments deposited by the female, at least in Japanese quails.

Our results have major implications for future evolutionary studies which will investigate eggshell pigmentation as a plastic trait. Indeed, the potential relationship between female immuno-competence and the crypsis of her eggs is a novel idea that has never been investigated to date. Here, we propose that a female might face a trade-off between fighting against oxidative stress, while preserving the appearance of the egg to keep it cryptic. Interestingly, we showed that eggshell maculation changed due to the dietary treatment, and this may compromise egg crypsis by changing eggshell surface patterning. However, we showed that females may vary eggshell maculation without

compromising egg camouflage, as only the degree of eggshell maculation is reduced by 4.54 (± 6.66) %, which is unlikely to be perceived by potential predators. Another hypothesis is that females may manipulate not only eggshell spot coverage but also the contrast between eggshell spots and eggshell background color to induce crypsis through disruptive coloration (Cuthill et al. 2005). Numerous questions remain unanswered, such as the role of eggshell pigments in chick development, or the characteristics of eggshell spots that aid camouflaging, such as the consistency of spot shape (Nguyen et al. 2007), particularly in quails. Knowing which parameter of eggshell pigmentation is crucial for egg crypsis would help to understand how a change in eggshell maculation adds or detracts from camouflage strategy (i.e., background matching or disruptive coloration). In addition, it has been suggested that quails have previous knowledge of their egg appearance (Lovell et al. 2013); however, how this is affected by sudden environmental changes and how this alters their behavior when choosing a laying substrate remain to be investigated. Further studies manipulating female environments and investigating laying substrate choice would help clarify whether the optimization of egg crypsis is dependent on female body condition.

In conclusion, eggshell camouflage could be an extended female phenotypic trait, in which case only females in higher body condition are able to maintain constant eggshell reflectance and maculate their eggs in order to optimize crypsis when pigments deposition changes, without compromising their health.

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Compliance with ethical standards

All of the procedures were agreed by the local ethics committee at the University of Glasgow and the experiment was conducted under the Animals (Scientific Procedures) Act 1986 (under PIL 30/8939 held by CD and PPL 60/4068 held by KAS).

Conflict of interest The authors declare that they have no conflicts of interest.

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