

RESEARCH ARTICLE

Condition-dependent strategies of eggshell pigmentation: an experimental study of Japanese quail (*Coturnix coturnix japonica*)

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SUMMARY

A relationship has been suggested between eggshell colour and female body condition based on the opposing antioxidant properties of the two main eggshell pigments: the antioxidant biliverdin (blue–green) and the pro-oxidant protoporphyrin (brown). We hypothesized that experimentally food-restricted females with low antioxidant capacity would deposit more protoporphyrin and less biliverdin in their eggshells, resulting in eggshells of reduced brightness but increased colour intensity. Two eggs were collected at the beginning and two at the end of a 2 week period from each of 24 female Japanese quails that were either food restricted or receiving *ad libitum* food (i.e. controls) during that time. Reflectance spectra were recorded and analysed using spectral shape descriptors, chromatic and achromatic contrasts were computed accounting for avian visual sensitivities, and eggshell pigments were quantified. We examined both spot and background pigmentation and found no significant effect of food restriction on eggshell reflectance. However, food-restricted females in lower body condition increased the deposition of protoporphyrin and decreased the amount of biliverdin invested in their eggshells. We hypothesize that in species laying brown-spotted eggshells, females modulate eggshell pigment investment in response to their body condition. According to this hypothesis, we predict that females maintain eggshell colour to limit visible changes that could be detected by predators and thereby conceal their eggs, although this work has yet to be conducted. We suggest that further experimental work on egg camouflage under different environmental conditions will elaborate on the process of pigment deposition and the physiological costs to females of laying heavily pigmented eggshells.

Key words: body condition, eggshell colouration, food restriction, pigment allocation.

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INTRODUCTION

Avian eggshells are diverse in their patterns of pigmentation and many adaptive hypotheses have been proposed culminating in a revived interest in the subject during the last decade (reviewed in Underwood and Sealy, 2002; Kilner, 2006). Across a wide range of species, the variation in eggshell colouration and patterning has been explained, among others, in the context of crypsis (Wallace, 1890; Tinbergen et al., 1962), mimicry and defence against brood parasitism (Dawkins and Krebs, 1979; Brooke and Davies, 1988; Rothstein, 1990), and protection of the developing embryo against solar radiation (Lahti, 2008). More recently, it has been proposed that eggshell colouration could be strongly related to female physiological condition and, in particular, antioxidant capacity (Moreno and Osorno, 2003; Soler et al., 2005; Siefferman et al., 2006; Hanley et al., 2008) (but see Riehl, 2011). This new assertion is founded on the investment of two main pigments: biliverdin, a blue–green antioxidant pigment, and protoporphyrin, a brown pro-oxidant pigment (Gorchein et al., 2009). Both may reflect the antioxidant capacity of the female and both are involved in the vertebrate haem metabolic pathway (Bloomer, 1988). Their concentrations are highly correlated in the avian eggshell (Wang et al., 2009). Thus, it is proposed that only females with an increasingly efficient antioxidant system are able to allocate more biliverdin into

their eggshells in the face of accommodating their oxidative stress (Moreno and Osorno, 2003). Moreover, because of its pro-oxidant properties, protoporphyrin causes a physiological oxidative stress in the liver (Shan et al., 2000) and females in lower body condition and under elevated stress may passively deposit more protoporphyrin into their eggshells to facilitate reduced oxidation (Moreno and Osorno, 2003; Martínez-de la Puente et al., 2007).

The ‘sexually selected eggshell colouration’ (SSEC) hypothesis (Moreno and Osorno, 2003) has provoked many experimental and correlative studies that have demonstrated positive relationships between eggshell colouration and female and/or chick body condition and immune capacity (e.g. maternal antibodies, yolk testosterone, yolk lutein) (Moreno et al., 2005; Hargitai et al., 2008). However, findings from an increasing number of studies are now in conflict with the predictions of this hypothesis. Many studies have failed to find a significant relationship between eggshell colouration and female and/or egg characteristics (Cassey et al., 2008; Honza et al., 2011; Riehl, 2011). Most of these have focused on species that lay blue–green eggs but substantially less attention has been paid to brown-spotted eggshells (Riehl, 2011; Dearborn et al., 2012). High eggshell concentrations of protoporphyrin have been positively related to thinner eggshells as a result of calcium deficiency (García-Navas et al., 2011) and pesticide contamination (Jagannath

et al., 2008). Thus, the brown colouration of maculated eggshells could reflect both egg quality and female body condition. This hypothesis has been examined in domestic chickens (*Gallus domesticus*), where older females laid lighter and less-coloured (i.e. red) eggs because of an increase in egg size, but there was no proportionate change in the quantity of pigment deposited over the eggshell surface (Odabaşı et al., 2007). In the house sparrow (*Passer domesticus*), pigment deposition would decrease with age and through the laying sequence (López de Hierro and De Neve, 2010). Furthermore, a cross-fostering experiment in house wrens (*Troglodytes aedon*) showed that less-pigmented eggshells indicated heavier eggs and higher female body condition (residuals from a regression of body mass on tarsus length) (Walters and Getty, 2010).

It is noteworthy that there is currently little agreement in the literature about the relationship between eggshell colour and female body condition. It is common for researchers to speculate on female investment in terms of eggshell pigments (e.g. Poole, 1965; Walters and Getty, 2010; García-Navas et al., 2011) without measuring pigment concentrations. Therefore, the aim of our study was to examine the relationship between female body condition (through residuals from a regression of body mass on tarsus length), eggshell physical reflectance (brightness, UV chroma and chroma) or perceived discrimination by an avian visual system (chromatic and achromatic contrasts), and maternal investment in egg quality. We studied the Japanese quail (*Coturnix coturnix japonica*), a species laying heavily maculated brown-spotted eggs, where the male does not provide post-laying care towards eggs or chicks (Cooper, 1987). This study is the first to use an experimental approach that mimics a naturally challenging environment, by restricting food availability to captive laying birds to investigate the relationship between female body condition, and eggshell colouration and pigmentation. If eggshell spot and background colouration indicate female body condition, we predicted that, compared with control females, food-restricted females in lower body condition would exhibit quantifiable changes in eggshell colouration. We predicted that they would deposit more protoporphyrin and less biliverdin in their eggshells, resulting in a decreased achromatic (brightness) and an increased chromatic (UV chroma and chroma) colouration. We also predicted females in lower body condition would invest less in egg quality as measured through egg mass, egg volume and yolk/albumen proportion.

MATERIALS AND METHODS

Study species and experimental procedure

All experimental procedures were carried out under Home Office Project Licence 60/4068 (KAS), and Personal Licences 30/8939 (CD) and 70/13463 (KAS). Experiments were conducted on a captive population of outbred Japanese quail (*C. coturnix japonica*, Temminck and Schlegel 1849) at the University of Glasgow (Cochno Research Centre and Farm, Scotland). Twenty-four adult females and nine adult males were randomly selected from an outbred wild-type population and were housed in single sex groups in indoor 15 m² aviaries for 2 weeks to allow habituation to housing conditions before the start of the experiment. Birds were fed *ad libitum* with a standard commercial diet (Layers Pellets, BOCM Ltd, Ipswich, UK) during habituation. Each female was individually identified with a uniquely numbered white leg ring and moved to an individual cage (61.0×44.5×50.8 cm) for 1 week of further habituation prior to the start of food manipulation. All birds were in visual and acoustic contact with each other at all times. Each male was individually identified with a uniquely numbered coloured leg ring and males were group-housed in a single enclosure in the

same room as the females under *ad libitum* feeding conditions. Ambient temperature was maintained at 18–18.9°C and the light regime was 14h:10h light:dark.

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 calliper. All birds were returned to single sex group housing after the last egg collection.

Food intake measurement and manipulations

To determine daily food requirements for the treatment groups, a pilot study was conducted. Pre-treatment female body mass was highly variable (range 245–380 g, s.d. 36 g) (Duval et al., 2012) and, therefore, we determined individual food intake during four consecutive days for all females in the study. On the first day, all food and loose bedding were removed from individual cages and each food bowl was weighed. Japanese quails weighing between 120 and 160 g have an estimated daily requirement of 20–30 g of food (Shousha et al., 2007). However, our heaviest female weighed 380 g, which is more than twice the published body mass for Japanese quail. Consequently, we provided 70 g of food each day to each bird. We monitored each cage at 09:00 h daily, and collected all uneaten food to determine the quantity of food eaten in the previous 24 h. Japanese quails are natural ground feeders and thus we did not worry about food spillage as the majority of it would have been consumed by the birds. This procedure was repeated for three consecutive days. We used a previously published method (Lessells and Boag, 1987) to calculate repeatability estimates and found that food intake was significantly repeatable within-female across the 3 days of measurement ($r=0.81$, $P<0.001$). We then calculated a female-specific mean quantity of food that represented 100% of her daily intake.

Dietary manipulation commenced 1 week after the last day of the pilot experiment described above. This delay allowed us to confirm that female behaviour (e.g. feather pecking, routine feeding or drinking) was not adversely affected by the food manipulation treatment or by individual housing. Females were randomly allocated to one of three treatment groups: control (C: fed *ad libitum*, i.e. 100% daily requirements, $N=8$); medium quantity [MQ: 90% daily requirements, $N=8$ (one bird had to be removed from the experimental design for health reasons)]; and low quantity (LQ: 75% daily requirements, $N=8$). To control for possible ‘cage’ or ‘ceiling’ effects, individuals from each experimental group were housed in such a way that across all groups equivalent numbers of birds were in cages on the floor and close to the ceiling. The respective quantity of food calculated from the pilot study for each subject was then provided every morning at the same time for the entire feeding trial.

Egg collection

We only analysed fertile eggs. A male was randomly paired with one female of each treatment group (i.e. three females in total). Sexual activity in males is highest within the first 5 min of presentation to a female, averaging approximately three copulations before satiation (Schein et al., 1972). Therefore, a male was placed in a focal female’s cage for 5 min per day before being removed and allowed a 1 h resting period before presentation to a subsequent female. In this way, each male was exposed to three females each day, one from each treatment group. The order of presentation to females of different experimental groups was randomly assigned each day. Egg collection began after 10 days of mating, the period required to obtain fertile eggs (Adkins-Regan, 1995). Each cage was visited every morning and eggs were collected and placed in

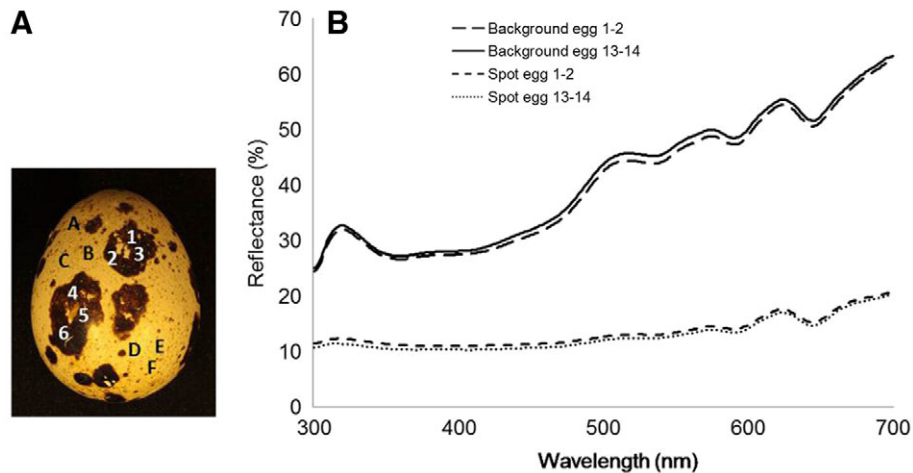


Fig. 1. (A) Zones on a typical Japanese quail eggshell where reflectance spectra were measured by spectrophotometry. From each half of the egg, three measurements were taken from two spots (1–3 and 4–6) while six measurements (A–F) were taken from the background. (B) Mean representative reflectance spectra from the spots and background of Japanese quail eggs (egg numbers 1 and 2, and 13 and 14 in the laying sequence).

a dark box in a cold room (4°C) to avoid degradation of eggshell pigments by exposure to light (Cassey et al., 2011).

Four eggs per female were collected. Specifically, they were egg numbers 1 and 2, and 13 and 14 within a clutch, with the modal clutch size of free-living birds being 14 eggs (Shousha et al., 2007). The eggs were analysed to assess the effect of dietary treatment on their mass, volume, eggshell colouration and pigment concentrations (Hoyt, 1979). The first and last eggs (i.e. 1 and 14) were carefully opened along the longitudinal axis using dissecting scissors on the day of laying. Yolk and albumen were separated and weighed (to the nearest 0.01 g) on a digital balance to determine relative egg components by mass as a proxy for egg macronutrient content (Baumgartner et al., 2008). Eggshells were washed with distilled water and kept in a dark box to dry at room temperature and to avoid direct exposure to light that could cause pigment degradation (Cassey et al., 2011).

Measurement of eggshell reflectance by spectrophotometry

The colouration of Japanese quail eggs varies considerably across a population with a background colour varying from white to blue–green to light yellow–brown, upon which darker speckles or spots of variable size, shape and colour occur (Sezer and Tekelioglu, 2009) (see Fig. 1A and repeatability estimates in ‘Data analysis’ for further details). Eggshell reflectance was measured between 300 and 700 nm in the laboratory using an Ocean Optics USB4000 Miniature Fibre Optic spectrophotometer with a DH-2000-FHS deuterium–halogen light source (Ocean Optics, Eerbeek, The Netherlands). A 90 deg probe with a black plastic extension was used to ensure stability for measurement and to maintain a consistent angle and distance between the eggshell and the measuring fibre optics (Cassey et al., 2010). Two spots were randomly chosen from each half of an egg (one from the apex and one from the blunt) and three replicates of reflectance were measured from each spot (Fig. 1A). For eggshell background, three reflectance spectra were randomly measured in each area (apex and blunt, as shown in Fig. 1A). Spectra were expressed relative to a white Ocean Optics WS-1 and a black standard (see Fig. 1B for representative spectra from spots and background of Japanese quail eggs).

Shape model

From these spectral measurements, brightness, UV chroma, blue–green chroma and red chroma were extracted as spectral shape descriptors using Avicol software (Gomez, 2006; Doutrelant et al., 2008). Brightness was estimated as the total reflectance (R) between

the wavelengths 300 and 700 nm. UV chroma was calculated as $R_{320-400\text{ nm}}/R_{300-700\text{ nm}}$, which is the proportion of the reflectance in the UV zone (320–400 nm) (Pérez-Rodríguez et al., 2011). Then, we calculated blue–green chroma (BGC) (Siefferman et al., 2006) as $R_{400-575\text{ nm}}/R_{300-700\text{ nm}}$, and red chroma as $R_{595-655\text{ nm}}/R_{300-700\text{ nm}}$.

Vision model

To account for the avian visual system, we used the protocol of Loyau and colleagues (Loyau et al., 2007) to compute two types of chromatic (ΔS ; colour) and achromatic (ΔQ ; brightness and forms) contrasts (Vorobyev and Osorio, 1998) using Avicol software (Gomez, 2006) as described by Osorio et al. (Osorio et al., 1999). We used the photoreceptor spectral sensitivities and relative densities data available for the domestic chicken as it is the closest species to Japanese quail in terms of photoreceptor characteristics (Hart and Hunt, 2007). Chickens have tetrachromatic colour vision based on single cones containing visual pigments with specific absorption maxima of 570 nm ($\lambda_{\text{max,red}}$), 508 nm ($\lambda_{\text{max,green}}$), 455 nm ($\lambda_{\text{max,blue}}$) and 419 nm ($\lambda_{\text{max,violet}}$) (Bowmaker and Knowles, 1977). They also possess double cones that mediate luminance, pattern and texture detection (Bowmaker and Knowles, 1977; Vorobyev and Osorio, 1998).

Determination and quantification of eggshell pigments

Eggshell pigment content was quantified in eggs 1 and 14, the same eggshells as used for the spectrophotometric measurements. Pigments were identified and their concentrations calculated using high-performance liquid chromatography (HPLC) (Mikšik et al., 1996). Briefly, each eggshell was weighed, and washed with distilled water and then solubilized (and esterified) in the dark for 2 days at room temperature in 15 ml of methanol containing 8.5% concentrated sulphuric acid. The resulting solution was filtered (to remove shell membranes), 7.5 ml of chloroform and 5 ml of distilled water were added and then the solution was shaken. The lower chloroform phase was washed with 5 ml of 10% sodium chloride solution, followed by distilled water until the washing water had neutral pH (typically after two washes). The extract was evaporated to dryness and reconstituted in 1 ml of chloroform. Standards for the quantification of protoporphyrin IX and biliverdin (Sigma, St Louis, MO, USA) were treated using the same procedure. Porphyrins were analysed by reversed-phase HPLC using Agilent 1100 LC system (Agilent, Palo Alto, CA, USA) consisting of a degasser, binary pump, autosampler, thermostatically controlled column compartment and multi-wavelength and fluorescence detectors.

Chromatographic separation was carried out on a Gemini 5u C18 110A column (250×2 mm i.d.; Phenomenex, Torrance, CA, USA). The sample (20 µl) was injected into the column and eluted with a gradient consisting of (a) methanol–water–pyridine 35:65:0.25 v/v and (b) methanol–acetonitrile–pyridine 90:10:0.25 v/v (flow rate 0.3 ml min⁻¹ at a temperature of 55°C). The gradient started at a–b 80:20 reaching 10:90 ratios after 15 min. For the next 10 min, the elution was isocratic (the composition of the mobile phase is unchanged during the entire elution process) followed by another 10 min isocratic elution at 100% b. Protoporphyrin was detected by fluorescence at 405 nm excitation/620 nm emission, whereas biliverdin was detected by absorbance as it has no fluorescence response. The two detectors were connected in tandem. We used LC-MS (i.e. liquid chromatography was directly coupled to mass spectrometry).

Data analysis

Shape model

For all four colour variables (i.e. brightness, UV chroma, blue–green chroma and red chroma), mean spot and background reflectance values were calculated for each egg per eggshell area (apex and blunt areas) (Fig. 1A). Univariate generalized linear models (GLMs; SPSS Statistics 19.0.0) were conducted to test for the effect of eggshell area (apex or blunt) on spot and background reflectance, with colour variables as dependent variables, and egg area (apex or blunt) as a fixed factor. Female identity was included as a random factor. There was no effect of egg area on any dependent variable in the analysis (spot: $0.09 < P_s < 0.38$; background: $0.27 < P_s < 0.79$). Therefore, all subsequent analysis was carried out on data averaged (i.e. on eggshell means) across the whole egg.

Pearson's correlations were performed on the four colour variables, between eggs 1 and 2, and then between eggs 13 and 14, for each female to test whether eggshell reflectance was similar between eggs from the same female. As eggshell reflectance was significantly correlated between eggs 1 and 2, and between eggs 13 and 14 ($0.42 < R_s < 0.97$, all $P_s < 0.001$), mean reflectance values for each female at the beginning (mean of the two first eggs) and at the end (mean of the two last eggs) of the manipulation were calculated for the four colour variables.

Vision model

All spectra were interpolated to obtain a reflectance value every 1 nm from 300 to 700 nm for the first two and the last two eggs per female. We first investigated egg discriminability by calculating the chromatic and achromatic contrasts within and between females. We computed for all pairwise comparisons contrasts or just-noticeable differences (JNDs) within a female's eggs (i.e. JND within). Then, we calculated contrasts between females by computing all pairwise comparisons between a female's eggs and all those laid by other females (i.e. JND between).

To assess whether food restriction had a perceptible effect on eggshell spot and background chromatic and achromatic variations, we then calculated contrasts between beginning/end eggs for each female. We also calculated a mean spot/background contrast for each female (Holveck et al., 2010) at the beginning and at the end of the manipulation to assess the effect of the treatment on this perceived contrast.

Within each change (ΔS or ΔQ), contrasts were compared between first and last eggs with 1 as the discrimination threshold, below which chromatic or achromatic differences are not detectable and above which they become more detectable for larger JND values (Dearborn et al., 2012). We assumed that light (neural noise) did

not limit visual performance (Holveck et al., 2010). We also tested whether the average differences in egg colour within and between females were detectable by a domestic chicken's visual system by comparing the within- and between-female contrast values to the threshold 1 using one-sample *t*-tests (all JNDs were normally distributed). Paired *t*-tests were performed to test whether the within and between contrasts were significantly different for each type of contrast computed.

Effect of dietary manipulation on eggshell colour

As we observed a significant difference in background red chroma between the three experimental groups before the start of the dietary manipulation (Kruskal–Wallis *H*-test: $H_2=5.63$, $P=0.04$), we examined the effect of dietary treatment on the change in body condition and on eggshell reflectance over the trial. 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 and in eggshell reflectance parameters between the beginning and the end of the dietary manipulation. Univariate GLMs were performed to test whether dietary treatment influenced the change in egg traits (i.e. mass, volume, yolk proportion), eggshell reflectance, pigment quantities, and chromatic and achromatic contrasts, as dependent variables, with the pre-dietary manipulation colour variables as covariates. One-sample *t*-tests were performed to test whether differences in colour variables differed from 0, and whether the contrasts differed from the discrimination threshold 1. All residuals were normally distributed.

Finally, Pearson correlations examined whether female body condition at the start of the feeding trial was correlated with egg traits (i.e. mean mass, volume and yolk proportion), eggshell colouration and pigment content.

RESULTS

Effect of food restriction on females and their egg characteristics

Female body condition was not significantly different between groups at the start of food manipulation (Kruskal–Wallis test, $H_2=0.48$, $P=0.78$). Food restriction, however, significantly affected female body condition with LQ females being in lower body condition than C or MQ females, whose mass and body condition increased throughout the manipulation (Δ body condition: $F_{2,22}=8.05$, $P=0.003$; Fig. 2).

There was no significant effect of food manipulation on any egg characteristics (Δ egg mass: $F_{2,22}=0.69$, $P=0.51$; Δ egg volume: $F_{2,22}=0.35$, $P=0.71$; Δ yolk proportion: $F_{2,22}=1.10$, $P=0.35$). However, we found that heavier females in higher body condition at the start of the food manipulation laid heavier and bigger eggs (egg mass: $r=0.44$, $P=0.03$; egg volume: $r=0.49$, $P=0.02$).

Effect of food restriction on eggshell reflectance

Shape model

We found no significant effect of food manipulation on eggshell colour variables (Table 1). None differed significantly from 0 (one sample *t*-tests, $-1.32 < t_s < 1.64$, all $P_s > 0.05$, $N=24$), suggesting that there was no natural variation in eggshell colouration during the dietary manipulation. Eggshell colour was not correlated with egg mass (spot: $-0.28 < r_s < 0.21$, all $P_s > 0.05$; background: $-0.24 < r_s < 0.16$, all $P_s > 0.05$, $N=24$), nor with egg volume (spot: $-0.28 < r_s < 0.20$, all $P_s > 0.05$; background: $-0.30 < r_s < 0.20$, all $P_s > 0.05$, $N=24$). However, females in higher body condition at the start of the experiment laid eggs that displayed bluer backgrounds ($r=0.45$, $P=0.03$, $N=24$).

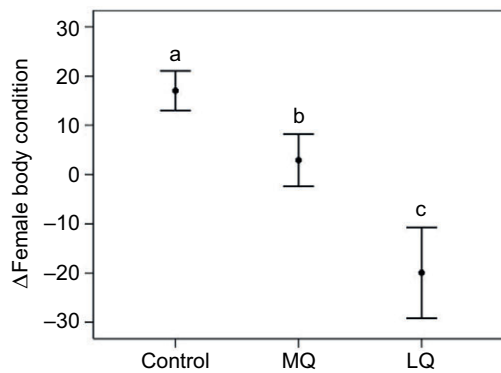


Fig. 2. Effect of dietary manipulation on female body condition (mean \pm 1 s.e.m.) of Japanese quail that were on *ad libitum* (control), medium quantity (MQ) or low quantity (LQ) food diets. The difference in body condition (residuals from regression of body mass on tarsus length) was calculated as the difference between that before the dietary manipulation and that after the last egg was laid. Different lowercase letters reflect statistically significant differences.

Vision model

The results from our avian vision model suggested that some of the variation measured with reflectance spectrophotometry would be detectable by the avian visual system. For each female, the mean visual contrast for eggshell background was greater when comparing eggs between females than within females (paired *t*-test, ΔS background: $t_{22} = -2.19$, $P = 0.04$; ΔQ background: $t_{22} = -2.99$, $P = 0.007$; Fig. 3). However, we did not find any significant difference between spot contrasts within females compared with those between females (paired *t*-test, ΔS spot: $t_{20} = -0.90$, $P = 0.38$; ΔQ spot: $t_{20} = 0.005$, $P = 0.99$; Fig. 3), suggesting that differences in background contrasts would be more detectable by an avian vision model than differences in spots.

Visual (chromatic and achromatic) contrasts were not correlated with any of the eggshell reflectance variables computed with the descriptive shape model (all $P_s > 0.05$). We did not find any significant effect of dietary manipulation on eggshell spot and background contrast, and on spot/background difference of reflectance (Table 2).

Eggshell pigments

Pigment analyses revealed that eggshells of Japanese quail contained high concentrations of protoporphyrin IX (mean \pm s.d., $113.75 \pm 57.18 \mu\text{g g}^{-1}$ eggshell) and biliverdin ($91.03 \pm 48.40 \mu\text{g g}^{-1}$ eggshell), with the two concentrations positively correlated at the start of the food manipulation ($r = 0.40$, $P = 0.05$, $N = 24$). We found that dietary manipulation significantly affected both protoporphyrin ($F_{2,22} = 3.48$, $P = 0.05$) and biliverdin ($F_{2,22} = 3.67$, $P = 0.04$) deposition in eggshells. Compared with control females, food-restricted females invested more protoporphyrin and less biliverdin in their eggshell contents (Fig. 4).

No significant correlation was found between eggshell pigment contents and any measure of female body condition or egg quality (biliverdin: $-0.07 < r_s < 0.36$, all $P_s > 0.05$; protoporphyrin: $-0.09 < r_s < 0.28$, all $P_s > 0.05$, $N = 24$). However, eggshells containing more biliverdin exhibited bluer spots with higher blue chroma values ($r = 0.50$, $P = 0.01$, $N = 24$) and those containing more protoporphyrin displayed backgrounds of lower brightness ($r = -0.50$, $P = 0.01$, $N = 24$).

DISCUSSION

Our results reveal that an experimental manipulation of female body condition through food restriction induced a change in eggshell

Table 1. Effect of dietary manipulation on eggshell colour variation of Japanese quail

Parameter	Factors	<i>F</i>	<i>P</i>
Spot reflectance			
	Brightness	Treatment	0.24
	Initial value	0.25	0.13
UV chroma	Treatment	1.03	0.37
	Initial value	5.75	0.03
Blue–green chroma	Treatment	0.60	0.56
	Initial value	11.00	0.003
Red chroma	Treatment	0.96	0.40
	Initial value	4.32	0.05
Background reflectance			
	Brightness	Treatment	0.17
	Initial value	0.22	0.64
UV chroma	Treatment	0.21	0.81
	Initial value	17.16	0.001
Blue–green chroma	Treatment	0.53	0.60
	Initial value	29.28	<0.001
Red chroma	Treatment	0.223	0.80
	Initial value	25.37	<0.001

The 24 female Japanese quails were exposed to *ad libitum*, medium quantity or low quantity food manipulations (for details of dietary manipulation, see Materials and methods).

Eggshell colour variation was judged as the difference in eggshell reflectance between eggs collected before the start of food restriction and after 15 days of treatment. Mean reflectance of egg numbers 1 and 2, and of egg numbers 13 and 14 was used in the statistical models.

Univariate generalized linear models (GLMs) were performed to test whether dietary treatment (d.f.=2) influenced eggshell reflectance, with the pre-dietary manipulation colour variables as covariates (Initial value, d.f.=1). Bold text indicates statistical significance.

pigment investment but not in the apparent colour of the eggshell. Previous studies on the function of eggshell colouration have experimentally manipulated female body condition through an increase in food availability or antioxidant content. For instance, Moreno and colleagues found that female European pied flycatchers (*Ficedula hypoleuca*) supplemented with mealworms (*Tenebrio molitor*) laid bigger and bluer eggs (Moreno et al., 2006). They suggested that this provided experimental evidence that blue–green eggshell colour and biliverdin content were an expression of female nutritional condition in birds. More recently, Dearborn and colleagues manipulated the antioxidant content of food provided to Araucana chickens and found that the differences in eggshell colouration between birds were due to female identity rather than to the food manipulation, and that variation in eggshell colour was unlikely to be perceived by the chicken (Dearborn et al., 2012). Nevertheless, in both studies, there was neither nutrient restriction nor measurement of protein and energy intake, female body condition, oxidative stress or eggshell pigment content. Our study represents the first to induce a decline in female body condition experimentally in order to investigate the effect on eggshell colouration in a species laying heavily maculated eggs (Fig. 1A).

We found that food restriction decreased female body condition but not measures of egg quality. Egg size or mass may be more sensitive measures of egg production than clutch size as food limitation is likely to operate initially on egg volume as opposed to egg numbers (Martin, 1987; Reynolds et al., 2003). Moreover, the deposition of hormones, immunological compounds (carotenoids and antibodies) and nutrients by females into their eggs influences offspring growth and development (Grootuis and Schwabl, 2008; Ho and Burggren, 2010). Thus, egg mass is a widely used measure of egg quality, with heavier eggs being more fertile

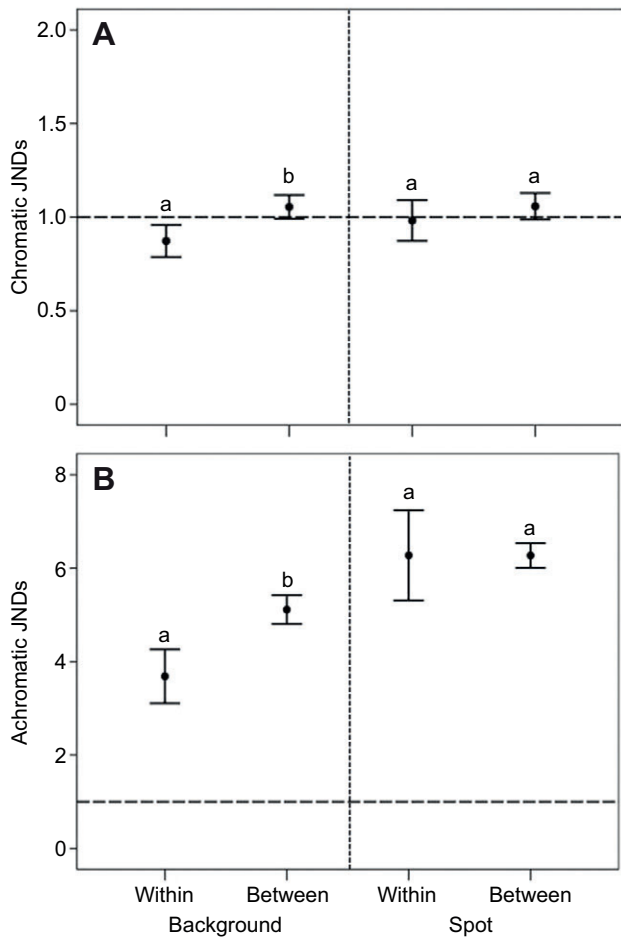


Fig. 3. Mean detectability (± 1 s.e.m.) of chromatic (A) and achromatic (B) contrasts in pairwise comparisons of self-laid eggs (Within) and in pairwise comparisons between eggs laid by each female *versus* eggs laid by all the other females (Between). Discriminability was calculated for each of the 24 Japanese quail females, with values >1 just-noticeable difference (JND; threshold shown by horizontal dashed line) representing contrasts that are likely to be detected by a bird's visual system. Visual contrasts between spots and background have not been statistically tested in the model so the different lowercase letters, which reflect statistically significant differences, have to be noted independently for spots and background.

and containing more nutrients and antibodies that are essential for chick survival (Galbraith, 1988; Farooq et al., 2003; Grindstaff et al., 2005). High 'quality' females may invest more in reproduction and deposit more resources into their eggs if they are to increase offspring fitness (Pilz et al., 2003). Accordingly, we predicted that food-restricted females would lose body mass and body condition, resulting in smaller and lighter eggs. Yet, we found that egg mass, volume and yolk proportion were not affected by food restriction of the layer, even if heavier females generally laid heavier and bigger eggs. Similarly, Giuliano and colleagues found that female northern bobwhites (*Colinus virginianus*) and scaled quails (*Callipepla squamata*) that were food restricted (i.e. quantity, protein and energy content) lost body mass through the feeding trial but did not modify their investment in egg mass or size (Giuliano et al., 1996). One explanation for our findings is that females would optimize the quality of their eggs and of their chicks by reducing their own body mass and activity when food was restricted, while maintaining the size and mass of their eggs similar

Table 2. Effect of dietary manipulation (d.f.=2) on the perceived eggshell colour variations through the use of an avian vision model

Parameter	<i>F</i>	<i>P</i>
Achromatic variations		
Spot/background contrast	0.03	0.97
Spot	2.71	0.09
Background	3.16	0.06
Chromatic variations		
Spot/background contrast	2.83	0.08
Spot	0.29	0.75
Background	0.20	0.83

The 24 female Japanese quails were exposed to *ad libitum*, medium quantity or low quantity food manipulations (for details, see Materials and methods).

The achromatic (brightness) or chromatic (colour) variations were calculated as the just noticeable differences (JNDs) between eggs from the beginning (mean spectrum between egg numbers 1 and 2) and those from the end of the food manipulation (mean spectrum between egg numbers 13 and 14).

to those of control birds, to compensate for their decreased body condition and loss of nutrients invested in their eggs (Meijer and Langer, 1995). According to the poultry science literature (e.g. Moula et al., 2009), an egg is composed of approximately 60% albumen, 30% yolk and 10% eggshell; this composition can vary with environmental factors such as breed, age, female health status, egg mass and female diet. Variation in the yolk compared with the albumen fraction is an index of egg quality that is used by the poultry industry to commercial ends. Thus, we predicted that food-restricted females would lay eggs with a reduced fraction of yolk/albumen compared with control females. While we successfully reduced female body condition through food restriction, we cannot attribute this to nutrient limitation as we only used an approximation of egg quality (egg and yolk mass). More detailed analyses of yolk constituents (e.g. proteins, carotenoids, hormones) and albumen (e.g. lysozyme) content would allow us, in the future, to verify whether the nutritional stress induced in the females influenced resource allocation into their eggs.

We compared between- and within-clutch variation in spot and background reflectance (i.e. brightness, UV chroma, blue-green chroma and red chroma) using repeatability estimates (Lessells and Boag, 1987), and found that between-female variance was markedly higher than within-female variance, suggesting that eggshell reflectance could be highly heritable and would constitute a female-specific phenotypic trait (Sezer and Tekelioglu, 2009). Any variation in eggshell colouration due to female body condition could constitute a signal of female quality towards conspecifics (Moreno and Osorno, 2003). We chose to analyse the reflectance data using a neural noise model (Holveck et al., 2010), assuming that light was not limiting visual performance. We did not compare eggshell reflectance to a background (e.g. nest) but, instead, we investigated within-individual variations (Cassey et al., 2009) that were subject to environmental modification such as food restriction. Under constant conditions in the laboratory, birds are thought to use chromatic aspects of colour for the detection of large targets and achromatic aspects (that are based solely on differences in the intensity of reflected light) for the detection of small objects and pattern (Osorio et al., 1999; Spaethe et al., 2001). In our experimental group of birds, eggshell background contrasts were greater when comparing eggs between females than within females, whereas we did not find any significant difference between spot colour contrasts within females compared with between females. This suggests that

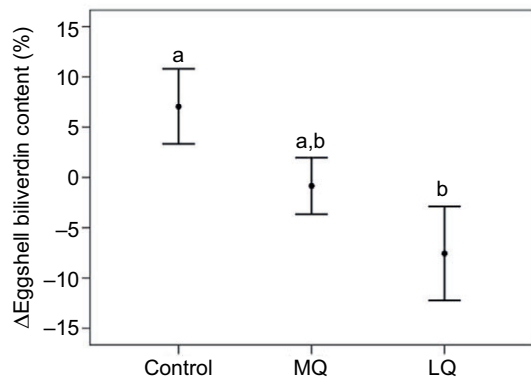


Fig. 4. The difference (mean \pm 1 s.e.m.) in biliverdin content (as a percentage of total pigment deposited) of eggshells collected between the start and the end of the laying sequence (see Materials and methods for details) of Japanese quails fed *ad libitum* (control), medium quantity (MQ) or low quantity (LQ) food diets. Different lowercase letters reflect statistically significant differences.

eggshell background colour contrasts would be more detectable by an avian model than spot colour differences (see also Holveck et al., 2010). Moreover, all the chromatic discriminability values were small (<1 JND) compared with achromatic contrasts (Dearborn et al., 2012), suggesting that differences in luminance and texture would be more easily detectable than differences in colour (Kelber et al., 2003; Avilés, 2008). Thus, our results suggest that even in species nesting on the ground in open environments with optimized visual acuity, birds would be able to detect eggshell brightness and form variations better than colours.

Female body condition at the start of the experiment predicted the intensity of eggshell background blue–green colouration, with females in higher body condition laying bluer eggshells (e.g. higher blue chroma values). However, female body condition was not correlated with any other parameter of eggshell colouration, suggesting that there may be a strong relationship between body condition and biliverdin investment, explaining why there is no direct relationship between female body condition and spot red chroma. This confirms previous findings (Cassey et al., 2012) showing that eggshell pigment concentrations were not consistently associated with eggshell colour parameters in two species of *Turdus*. Moreover, spot and background colour and darkness might indicate an aspect of female health such as antioxidant capacity (Hanley et al., 2008) or physiological stress (Martinez-de la Puente et al., 2007), which are not signalled by female body condition alone. However, contrary to our predictions, food restriction had no significant effect on either the physical properties of eggshell spectra or the achromatic and chromatic contrasts that could be perceived by birds themselves or by conspecifics.

Our analysis of eggshell pigment concentration revealed that the eggshells of Japanese quails are pigmented with protoporphyrin IX and smaller amounts of biliverdin. The two pigments are part of the same biochemical pathway and the positive correlation that we found between their quantities in whole eggshells suggests that the processes of deposition of these pigments are not independent, and that the quantities of biliverdin and protoporphyrin should change proportionately (Wang et al., 2009). Indeed, Moreno and Osorno (Moreno and Osorno, 2003) suggested an adaptive significance of the relationship between deposition into the eggshell of such pigments and female body condition: the SSEC hypothesis proposed that eggshell colouration signals female quality to the

male. Pigment deposition into the eggshell would be modulated by female antioxidant capacity and males would subsequently adjust their care in response to the intensity of eggshell colour. Thus, according to our predictions, food restriction would modulate eggshell pigment investment by the female. Indeed, food-restricted females increased their investment in protoporphyrin and decreased the amount of biliverdin deposited into the eggshell. This result suggests that the decrease in female body condition could be associated with a decrease in antioxidant capacity or an increase in oxidative stress and, thus, that females with low antioxidant capacity in our study passively deposited more protoporphyrin into their eggshells to remove this pro-oxidant. The fact that females in lower body condition also decreased the deposition of biliverdin into their eggshells supports this hypothesis. It confirms that only females in higher body condition can face the trade-off between pigmentation of their eggshells with biliverdin and control of oxidative stress (Moreno and Osorno, 2003). Measurements of oxidative stress and antioxidant capacity in our subjects were not the focus of our investigation; therefore, we cannot confirm this hypothesis but our conclusions indicate fruitful directions for future research.

Our methodology for quantifying pigments did not allow determination of pigment content in different egg regions (Fig. 1A), but both pigments may be responsible for spot and background colouration in mixed quantities. The spot reflectance spectra of eggshells of Japanese quail exhibit a peak at ~ 630 nm (Fig. 1B), which is consistent with the presence of protoporphyrin IX (Sanz and García-Navas, 2009). However, background spectra show the same peak at 630 nm, and also two more peaks: one around 500 nm, similar to the reflectance spectra of blue–green eggshells (Siefferman et al., 2006), and one in the UV zone at 320 nm. This suggests that protoporphyrin would be mainly responsible for spot colour but both pigments may be responsible for background colour. Indeed, Poole described the eggshell background of Japanese quail as pale green and suggested that a strong genetic control for shell colour would act in that species (Poole, 1964). Moreover, eggshells would be superficially pigmented with red–brown or green–brown dots. Pigment masses would be deposited at first as dots and then spread into blotches on the shell surface by contractions of the shell gland and rotation of the egg (Tanaka et al., 1977). Yet, we found that eggshells containing higher concentrations of protoporphyrin displayed darker backgrounds. This could suggest that protoporphyrin, being a red–brown pigment, darker than biliverdin, might be responsible for the lightness of eggshell background rather than influencing the colour itself as suggested by the lack of relationship between protoporphyrin content and background red chroma. We also found that eggshells containing more biliverdin displayed bluer spots. These results confirm the complexity of eggshell pigment distribution in different areas of the eggshell (Sparks, 2011). Eggshell pigment synthesis and deposition are still hotly debated mechanisms in heavily spotted eggs, but the proposition that protoporphyrin IX and biliverdin are involved in the synthesis of haem is now gaining credence (e.g. Milgrom, 1997; De Coster et al., 2012). Both pigments circulate in the bloodstream, and are metabolized in the shell gland (Poole, 1965; Wang et al., 2009; Honza et al., 2012). Pigments are deposited a few hours before oviposition (Poole, 1965) and the two pigments could be differentially allocated on the eggshell according to female body condition. Future work should certainly examine ways to differentiate between pigments destined for the two eggshell ‘components’ of maculation and base colour as initial steps in investigation of their synthesis in relation to their deposition.

CONCLUSIONS

To the best of our knowledge, this is the first study in which female body condition has been modified to investigate spotted-eggshell colouration by quantifying not only eggshell reflectance of spots and background independently but also pigment concentrations in the eggshell. Many previous studies have examined avian species with post-natal paternal care and have focused on the SSEC hypothesis (reviewed by Riehl, 2011). However, males of species that do not invest care post-hatching could also be subject to selection. Where predation pressure is high and egg colouration is linked not only to female body condition but also to crypsis, the assumptions of the SSEC hypothesis might also apply. To date, no study has investigated the relationship between eggshell pigmentation and female body condition in such a species. In addition, few studies have experimentally manipulated female body condition to examine its direct effects on eggshell pigmentation (García-Navas et al., 2011; Morales et al., 2011). We suggest that eggshell colouration could be used in making eggs cryptic in Japanese quails. Moreover, females facing challenges from fluctuating environmental conditions (e.g. food availability) would face a trade-off between investing pigment in their antioxidant capacity and pigmentation of their eggshells. Yet, they would be able to maintain the apparent colour of their eggs in their chosen nesting area. We found that eggshell background contrasts can be perceived by an avian visual model and could be a signal of female body condition towards avian predators. We propose that maintaining eggshell colour despite a switch in pigment allocation to the eggshell could be an adaptive behaviour that has evolved to facilitate egg camouflage and decrease predation risk in species such as Japanese quail that lay spotted eggs and nest on the ground.

The independent quantification of pigments in eggshell spots and background, as well as image analysis of spot spread and size, both appear essential to understand more precisely the mechanisms of eggshell pigment deposition and the possible strategies of pigment allocation adopted by the female. Laying substrate choice experiments could allow us to understand how females maximize the crypsis of their individual eggs as well as of the complete clutch and how they choose the best location in which to nest, taking into account the fact that eggshell colouration is unlikely to change during the egg-laying period.

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