Eggshell colour is more strongly affected by maternal identity than by dietary antioxidants in a captive poultry system

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Summary

1. Biologists have long puzzled over the apparent conspicuousness of blue-green eggshell coloration in birds. One candidate explanation is the 'sexual signalling hypothesis' that the blue-green colour of eggshells can reveal an intrinsic aspect of females' physiological quality, with only high-quality females having sufficient antioxidant capacity to pigment their eggs with large amounts of biliverdin. Subsequent work has argued instead that eggshell colour might signal condition-dependent traits based on diet.

2. Using Araucana chickens that lay blue-green eggs, we explored (i) whether high levels of dietary antioxidants yield eggshells with greater blue-green reflectance, (ii) whether females differ from one another in eggshell coloration despite standardized environments, diets and rearing conditions, and (iii) the relative strength with which diet vs. female identity affects eggshell coloration.

3. We reared birds to maturity and then placed them on either a high- or low-antioxidant diet, differing fourfold in Vitamin E acetate and Vitamin A retinol. After 8 weeks, the treatments were reversed, such that females laid eggs on both diets in an order-balanced design. We measured the reflectance spectra of 545 eggs from 25 females.

4. Diet had a very limited effect on eggshell spectral reflectance, but individual females differed strongly and consistently from one another, despite having been reared under uniform conditions. However, predictions from avian visual modelling suggest that most of the egg colour differences between females, and nearly all of the differences between diets, are unlikely to be visually discriminable.

5. Our data suggest that eggshell reflectance spectra may carry information on intrinsic properties of the female that laid the eggs, but the utility of this coloration as a signal to conspecifics in this species may be limited by the sensitivity of a receiver to detect it.

Key-words: antioxidants, biliverdin, diet, eggshell colour, sexual signalling hypothesis

Introduction

Visual signals provide useful information for assessing other individuals, whether as potential mates, rivals, predators or prey. Consequently, there has been an extensive effort to understand what types of traits can function as signals and the mechanisms by which such traits can be informative to receivers (Maynard Smith & Harper 2003; Searcy & Nowicki 2005). Recently, researchers have

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explored the signalling potential of eggshell coloration in some species of birds, specifically asking whether the antioxidant contribution to blue-green eggshell pigmentation might signal the quality of the female that laid those eggs (Moreno & Osorno 2003). This 'sexual signalling hypothesis' (SSH) has received empirical support (see below) but has also been challenged on both theoretical and empirical grounds (Kilner 2006; Reynolds, Martin & Cassey 2009; Riehl 2011).

According to the SSH, males should provide heightened parental care when females lay intensely blue-green eggs, because this eggshell colour signals that the female

– and thus her offspring – should be of high quality. Many studies have now shown a positive relationship between blue-green eggshell colour and subsequent male parental care, with either correlational data (Moreno *et al.* 2004; Hanley, Heiber & Dearborn 2008; but see Lopez-Rull, Celis & Gil 2007; Lopez-Rull, Miksik & Gil 2008) or experimental data (Moreno *et al.* 2006b; Soler *et al.* 2008; but see Krist & Grim 2007; Hanley & Doucet 2009).

Despite data on the potential link between eggshell colour and male parental care, much less is known about a key underpinning of the SSH and other, alternative hypotheses (Hanley, Doucet & Dearborn 2010) about blue-green eggshell coloration: the mechanism for intraspecific variation in blue-green colour (Morales, Velando & Torres 2011). In species in which only some individuals lay blue-green eggs, the difference between those laying blue-green eggs and those laying white or brown eggs seems to be typically created by one or two autosomal loci (village weavers, *Ploceus cucullatus*: Collias 1993; chickens, *Gallus gallus domesticus*: Zhao *et al.* 2006; Japanese quail, *Coturnix japonica*: Ito *et al.* 1993). However, the more critical issue for the SSH is the source of eggshell colour variation among individuals that lay only blue-green eggs, and this variation could be driven by a mix of genetic and nongenetic factors. Biliverdin is the pigment causing blue-green eggshell coloration in birds (Kennedy & Vevers 1976; Wang *et al.* 2009), and biliverdin may have antioxidant properties in the adult female (Stocker, Yamamoto & McDonagh 1987; McDonagh 2001; Kaur *et al.* 2003). The SSH proposes that high-quality females have better antioxidant defence systems and are able to deposit higher amounts of biliverdin in their eggshells, leading to greater blue-green eggshell reflectance (Moreno & Osorno 2003; Morales, Velando & Moreno 2008). Fundamentally, this variation in antioxidant defences and in colour could be due to properties of the environments that females have experienced or to intrinsic properties of individual females (McCormack & Berg 2010), including genotypes and epigenetic factors such as maternal effects.

In ecology and evolutionary biology, there is little agreement on the meaning of an individual's 'quality' (Wilson & Nussey 2010). The original formulation of the SSH viewed female quality as an intrinsic, heritable property of the individual (Moreno & Osorno 2003). However, egg colour could reveal a more extrinsic trait such as environmentally derived nutritional condition (Reynolds, Martin & Cassey 2009; Morales, Velando & Torres 2011) or potentially be impacted by maternal effects (Gorman & Nager 2004). To date, our understanding of whether eggshell coloration is driven by intrinsic vs. extrinsic factors is generally limited to separate studies that have tested either for effects of diet or for consistency of egg colour within females. With regard to diet, two experiments have looked for dietary effects on blue-green eggshell colour. One study found a small increase in blue-green chroma among pied flycatchers, *Ficedula hypoleuca*, given a dietary treatment aimed at increasing overall nutritional level (i.e. supplemental provisioning with mealworms; Moreno *et al.* 2006a), while another study found that blue-green chroma of second-laid eggs increased when blue-footed boobies, *Sula nebouxii*, were provisioned with supplementary carotenoids (Morales, Velando & Torres 2011). Other studies have tested for possible intrinsic effects by examining the consistency of egg colour in individual females. At the smallest temporal scale, several populations have been shown to exhibit moderate-to-high within-clutch similarity of eggshell colour (pied flycatchers: Moreno *et al.* 2005; ring-billed gulls, *Larus delawarensis*: Hanley & Doucet 2009; Mexican jays, *Aphelocoma ultramarina*: McCormack & Berg 2010; blue-footed boobies: Morales, Velando & Torres 2011; but see Sanz & Garcia-Navas (2009) for a counter-example based on protoporphyrin-derived eggshell spotting rather than eggshell ground colour). At a larger temporal scale, a longitudinal approach has been used to measure repeatability between clutches: a captive study of village weavers did indeed find much greater variation in turquoise eggshell coloration between females than within females, across individuals' breeding lifespans (Collias 1993), but in a wild population of blue tits, *Cyanistes caeruleus*, individual parents were not consistent across years in the extent and distribution of eggshell maculation (Sanz & Garcia-Navas 2009). One study has made the critical advance of estimating heritability of blue-green egg colour (Morales *et al.* 2010), finding a significant heritable component to four of nine measures of eggshell coloration (including blue-green chroma).

These studies are important steps towards evaluating the SSH and related hypotheses, but we also need studies that explore both diet manipulation and female consistency within the same system, so that we can gauge the relative strength of the effect of these two factors. A captive system can make an important contribution in this regard, because it allows repeated measures of females over time, the standardization of females' environmental histories, and the control of diet and other environmental factors during egg laying. In this study, we used a captive poultry system to conduct a repeated measures experiment in which all individuals lay eggs under both a high-antioxidant and low-antioxidant diet. We had three goals: (i) to test whether high levels of dietary antioxidants yield eggshells with greater blue-green reflectance, (ii) to test whether there are consistent differences between females in eggshell coloration despite standardized environments, diets and rearing conditions and (iii) to compare the relative strength with which diet vs. female identity affect eggshell coloration. In this framework, we analysed eggshell coloration with colorimetric estimates and also with an avian visual model to test whether differences in eggshell coloration are likely to be discriminable by a bird's visual system.

Materials and methods

STUDY ORGANISM, HOUSING AND DIET

To test these predictions, we measured the colour of multiple eggs per female under two different diet regimes – high and low antioxidants – in a repeated measures design that balanced for order effects. The Araucana chicken is a variety of *Gallus gallus domesticus* Linnaeus that is known for its blue eggs. Araucana chickens are derived from Chile, where blue-egg chickens (called 'collonca') were living in semidomestication in the subsistence agricultural society of indigenous Mapuche peoples from at least the early 1500s (Berdichewsky 1975; Langdon 1989; Gongora *et al.* 2008).

We obtained 48 female Araucana chicks from a commercial hatchery (McMurray Hatchery, Webster City, Iowa). The chicks hatched on 2 October 2006 and arrived at our facility at the age of 2 days. For the next 8 weeks, chicks were housed in a brooder and fed commercial broiler starter feed *ad libitum* (Clark's Feed Mills, Shamokin, PA, USA). At 8 weeks, birds were moved as a group to a larger indoor/outdoor enclosure and were switched from a diet of *ad lib* starter feed to a diet of *ad lib* laying mash. At 15 weeks, birds were moved to randomly assigned individual nesting boxes (61 × 46 × 46 cm) to ensure that eggs would be attributable to individual females. The nest boxes were partially outdoors, protected from wind and precipitation, illuminated with natural daylight supplemented with heat lamps and stacked in a ten-by-five grid that was used to systematically alternate the ensuing treatments. For the next 5 weeks, birds were housed individually but were all still receiving the same diet of *ad lib* laying mash. Because light and temperature were affected by the ambient environment, these factors changed over the course of the experiment; however, all birds were housed the same way and entered the experiment at the same date and age, such that birds did not differ from one another in the basic environmental conditions that they experienced.

The experimental variation in diet was begun when the birds were 20 weeks old. In brief, each of two groups was housed for 8 weeks on a low-antioxidant diet and 8 weeks on a diet of elevated antioxidants. Females were systematically assigned to the two groups ($n_1 = 24$, $n_2 = 24$) based on their random allocation to the grid of nest boxes. The groups differed only in the order in which they experienced the two treatments. Thus, all birds experienced common rearing conditions and common diets up until 20 weeks old. Group 1 then spent weeks 20–27 on the high-antioxidant diet followed by weeks 28–35 on the low-antioxidant diet, while Group 2 spent weeks 20–27 on the low-antioxidant diet followed by weeks 28–35 on the high-antioxidant diet. Birds that did not lay in both treatments were removed from the experiment, leaving a final sample of 13 females in Group 1 and 12 females in Group 2, for a total of 25 females that laid eggs under both dietary treatments.

In the experimental treatments, birds were provided 130 g per day of feed that was custom formulated for the experiment (Grove's Feed Mill, Lewisburg, PA, USA). Our low-antioxidant feed was designed to be approximately half of the recommended level of antioxidants for adult chickens (Scott, Neshei & Young 1982; National Research Council 1994), containing *c.* 5 IU Vitamin E per kg of feed (vs. recommendation of 10 IU Vitamin E per kg feed) and 750 IU Vitamin A per kg of feed (vs. recommendations of 1500 IU Vitamin A per kg feed). Our high-antioxidant diet used powdered supplements of Vitamin E acetate and Vitamin A retinol added to each individual serving of food, providing four times as much Vitamin E (20 IU kg^{-1} feed) and Vitamin A (3000 IU kg^{-1} feed) as in the low-antioxidant diet. Because both of these vitamins are fat soluble, care was taken to use levels that were low enough to avoid toxicity.

Although the diets differed in the antioxidants Vitamin A and Vitamin E, we did not expect females to transfer these particular antioxidants into eggshells. Rather, the SSH predicts that a female with high total-antioxidant capacity (achieved here by dietary supplementation of Vitamins A and E) could afford to deposit more biliverdin into eggshells, yielding eggshells with greater blue-green chroma.

ANALYSIS OF EGGSHELL COLOUR

Eggs in this study exhibited variation in eggshell coloration (as detailed in the Results), but in general, these eggs could be described as unspotted and blue-green. We measured eggshell colour with a spectrophotometer (USB4000; Ocean Optics, Dunedin, FL, USA) using an external deuterium halogen light source (DH2000 UV-VIS NIR), with reference to a Spectralon white standard (WS-1). Six measurements were taken for each shell, and these values were averaged to obtain one spectrum per egg.

Reflectance scores were initially recorded in 10-nm increments from 300 to 700 nm and were subsequently summarized with two methods. First, we used principal components analysis (Montgomerie 2006) to create three uncorrelated varimax-rotated colour scores that had eigenvalues >1. Second, we calculated blue-green chroma as the proportion of total reflectance between 400 and 570 nm relative to the reflectance across the full range of 300–700 nm (Siefferman 2006).

MODELLING AVIAN VISION

To determine the extent to which eggs in our study might be visually discriminable to a chicken or other avian visual system, we used a receptor-noise-limited opponent model of vision (Endler 1993; Vorobyev & Osorio 1998). Avian colour vision can be represented by a four-dimensional colour space, in which the axes are maximum quantum catches for each of the four single-cone types (Vorobyev 2003). A bird's ability to discriminate between two colours is based on the distance between the two colours within this colour space, and the amount of noise inherent in the receiver's visual system (Wyszecki & Stiles 2000; Vorobyev 2003). We calculated quantum catch and detectability with the program SPEC (Hadfield 2004) in R (R Development Core Team 2010), under average ambient daylight conditions; similar results but with slightly lower values of discriminability (not shown) were obtained using average filtered forest light conditions (Endler 1993). We used the cone sensitivity data for chicken vision provided in the SPEC program. Quantum catch was calculated as

$$Q_i = \frac{\int_\lambda R_i(\lambda) S(\lambda) I(\lambda) d\lambda}{\int_\lambda R_i(\lambda)} \tag{1}$$

where R_i represents the spectral sensitivity of cone type *i*, *S* represents the spectral reflectance of the object and *I* represents the normalized irradiance spectrum. We calculated receptor noise with a flexible function accounting for the inherent noise-to-signal ratio for all cone types relative to the proportion of receptor types in the eye (Vorobyev *et al.* 1998; Vorobyev 2003; Cheney & Marshall 2009) as

$$e_i = \sqrt{\frac{\left(1/\left(\log\left(T\left(\frac{Q_{i,a}+Q_{i,b}}{2}\right)\right)\right)^2\right) + w_i^2}{n_i}} \tag{2}$$

where *T* is a scaling factor for luminance level (set to 10 000), w_i is the Weber fraction accounting for differences in response sizes based on magnitude of stimuli (Wyszecki & Stiles 2000) (set to 0·05 for all cone types) and n_i represents the relative number of receptor cells for each receptor type. The relative proportion of receptor types for ultraviolet, short-wave, medium-wave and

long-wave sensitive cone types vary according to the ecology and behaviour of the organism. Here, we used relative cone abundance from Kram *et al.* (2010); similar results but with slightly higher values of discriminability (not shown) were obtained with a model in which the four cones types are assumed to occur in equal abundance. We incorporated receptor noise with the following function (Vorobyev & Osorio 1998):

$$(\Delta S)^2 = \frac{\begin{bmatrix} (e_1^2 e_2^2)(\Delta f_4 - \Delta f_3)^2 + (e_1^2 e_3^2)(\Delta f_4 - \Delta f_2)^2 + (e_1^2 e_4^2)(\Delta f_3 - \Delta f_2)^2 + \\ (e_2^2 e_4^2)(\Delta f_3 - \Delta f_1)^2 + (e_3^2 e_4^2)(\Delta f_2 - \Delta f_1)^2 + (e_3^2 e_2^2)(\Delta f_1 - \Delta f_4)^2 \end{bmatrix}}{(e_1 e_2 e_3)^2 + (e_1 e_2 e_4)^2 + (e_1 e_3 e_4)^2 + (e_2 e_3 e_4)^2} \quad \text{(eqn 3)}$$

where Δf_i is the difference in quantum catch between two stimuli. Discriminability (ΔS) approximates units of just noticeable differences, where a value of one represents the smallest discriminable difference between two colours.

DATA ANALYSES

We tested for an effect of diet and female identity on egg colour, separately analysing principal components scores and blue-green chroma. First, we conducted a MANOVA on the three principal components, using each egg as a data point and using predictor variables of diet (high or low antioxidant), individual female identity (as a random factor) and time (as a categorical factor, with one value for first diet treatment and another value for second diet treatment). A significant MANOVA was followed by a similarly structured univariate mixed-model ANOVA for each principal component separately. As an alternative way of quantifying colour, we conducted an identical univariate analysis using blue-green egg chroma as the dependent variable.

We also calculated repeatability to yield a traditional measure of individual chickens' consistency in egg coloration over the course of the experiment. To measure consistency of females within diet treatments, we calculated the intraclass correlation coefficient (ICC; Lessells & Boag 1987) for each of the four colour scores (PC1, PC2, PC3 and blue-green chroma) for the eggs laid by each female on a particular diet; we also calculated the ICC for all eggs laid by a female, pooling across the diets. Lastly, we assessed consistency of females' egg colour across the two diet treatments by averaging the scores on a colour variable for all eggs laid by one individual during one diet treatment and then testing for correlations of individuals' mean scores between the two treatments (e.g. mean value of PC1 from a female's eggs during the low-antioxidant diet vs. mean value of PC1 during the high-antioxidant diet).

We made two types of comparisons with the predictions of visual discriminability of eggshell colours. First, we tested whether the average differences in egg colour within and between females were detectable to a chicken visual system. For each female, we calculated mean discriminability between all pairwise comparisons of her own eggs and then between all pairwise comparisons of each of her own eggs with all eggs laid by other females. Second, we tested whether the dietary antioxidant treatment had an effect that was detectable to an avian visual system. To do this, we calculated mean discriminability between all pairwise comparisons of each female's own eggs across the two diet treatments, to yield a single value per female.

Results

We measured the colour of 545 eggs from 25 females that laid under both the low- and high-antioxidant food treatments (overall mean = 21.8 ± 6.4 SD eggs per female, range 7–37). The earliest egg was laid 2 days after the first diet treatment began, and the average first lay date for females was approximately midway through the first diet treatment. In a repeated measures analysis, individual females laid more eggs in the second treatment that they experienced ($F_{1, 518} = 35.30$, $P < 0.0001$; mean 8.0 ± 4.3 for first treatment and 13.8 ± 3.9 for second treatment) and showed a tendency to lay more eggs while on the high-antioxidant diet when controlling for treatment order ($F_{1, 518} = 4.23$, $P = 0.0513$; 11.8 ± 5.1 for high-antioxidant diet and 10.0 ± 4.9 for low-antioxidant diet).

Three varimax-rotated principal components accounted for 98.4% of variance in the original reflectance variables. PC1 accounted for 33.4% of variation in the original variables and had strong, positive loadings for reflectance measures between 450 and 600 nm, such that a large value would correspond to high reflectance in the blue to yellowish-green part of the spectrum. PC2 accounted for 32.9% of variation and had strong, positive loadings for reflectance measures below 450 nm, such that a large value would correspond to high reflectance in the UV and violet. PC3 accounted for 32.1% of variation and had strong, positive loadings for reflectance measures above 590 nm, such that a large value would correspond to high reflectance of yellow, orange and red (Fig. 1).

In a MANOVA of the three principal components ($n = 545$ eggs), there was a small effect of diet ($F_{3, 516} = 4.42$, $P = 0.0044$, partial $\eta^2 = 0.025$) and a large effect of individual female ($F_{72, 1543} = 40.75$, $P < 0.0001$, partial $\eta^2 = 0.655$); there was also a time effect, such that egg colour changed from the first treatment to the second treatment regardless of which treatment was first ($F_{3, 516} = 19.00$, $P < 0.0001$, partial $\eta^2 = 0.099$). In follow-up univariate mixed-model ANOVAs, there were strong effects of female identity on all three dependent variables (PC1: $F_{24, 518} = 58.10$, $P < 0.0001$, partial $\eta^2 = 0.7292$; PC2: $F_{24, 518} = 15.33$, $P < 0.0001$, partial $\eta^2 = 0.4153$; PC3: $F_{24, 518} = 38.01$, $P < 0.0001$, partial $\eta^2 = 0.6378$) and weak effects of diet on all three dependent variables (PC1: $F_{1, 518} = 9.33$, $P = 0.0024$, partial $\eta^2 = 0.0177$; PC2: $F_{1, 518} = 4.34$, $P = 0.0377$, partial $\eta^2 = 0.0083$; PC3: $F_{1, 518} = 2.12$, $P = 0.1459$, partial $\eta^2 = 0.0041$). There were also weak effects of time, with some measures of egg colour differing between the first diet and the second diet, controlling for the balanced order of the high- and low-antioxidant diets; specifically, eggs laid in the second treatment were more strongly reflective in UV-violet (PC2: $F_{1, 518} = 23.37$, $P < 0.0001$, partial $\eta^2 = 0.0432$) and in yellow-orange-red (PC3: $F_{1, 518} = 23.36$, $P < 0.0001$, partial $\eta^2 = 0.0431$), but not significantly different in blue to yellowish-green (PC1: $F_{1, 518} = 2.78$, $P = 0.0958$, partial $\eta^2 = 0.0053$).
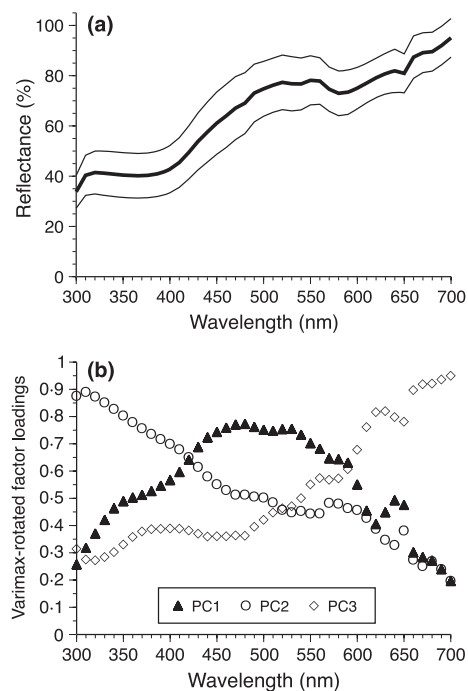
Fig. 1. Eggshell reflectance. (a) Average (thick line) ± SD (thin lines) for 300–700-nm reflectance spectra of 545 eggs, pooled across high- and low-antioxidant diets. (b) Factor loadings for three varimax-rotated principal components generated from the set of 41 variables that measured eggshell reflectance every 10 nm from 300 to 700 nm.

In a univariate linear mixed model of blue-green chroma ($n = 545$ eggs), blue-green egg chroma varied among females (as a random factor; $F_{24, 518} = 69 \cdot 1$, $P < 0 \cdot 0001$, partial $\eta^2 = 0 \cdot 762$) and increased from the first treatment to the second (time; $F_{1, 518} = 5 \cdot 2$, $P = 0 \cdot 023$, partial $\eta^2 = 0 \cdot 010$, with slightly bluer eggs in the second treatment), but there was not a significant effect of diet ($F_{1, 518} = 3 \cdot 4$, $P = 0 \cdot 067$, partial $\eta^2 = 0 \cdot 006$).

In summary, approaches based on principal components or on blue-green chroma found at most a small effect of time and dietary antioxidant treatment on egg coloration; in contrast, a large amount of colour variation was attributable to consistent differences between females (Table 1). Additional analyses of egg colour (not shown) found small effects of laying order within the treatments, but these effects were not consistent in direction or pattern and did not meaningfully alter or clarify the primary effects of individual identity or diet.

Intra-class correlation coefficients were calculated for 24 females that laid at least twice under the low-antioxidant diet and 25 females that laid at least twice under the high-antioxidant diet.

These analyses revealed moderate-to-strong within-female repeatability for all measures of colour (Table 2). There were also extremely strong between-treatment correlations of all four colour variables based on averages of eggs laid by an individual on a particular diet treatment (Table 2). Together, these results indicate that individual birds exhibit egg colour consistency both within and between dietary treatments, whether measured by blue-green chroma or by PCA across the spectrum of avian vision.

Predictions from our perceptual modelling suggested that much of the egg colour variation measured with reflectance spectrometry could not be detected by an avian visual system. For each bird, the average visual discriminability was greater when comparing self eggs to nonself eggs than when comparing self eggs to other self eggs (Fig. 2). However, most of these discriminability values were small: on average, birds would be unable to see a difference in colour between two eggs laid by the same female, as all of these averages were less than 1 JND, and differences in the colour of eggs laid by two different females would be discriminable only for a small subset of birds (Fig. 2). This limited discriminability was also true when comparing eggs laid by a particular female on the high- vs. low-antioxidant diet, with mean discriminability exceeding 1 JND for only 1 of the 25 birds (Fig. 3).

Discussion

The SSH for blue-green eggshell colour in birds proposes that this biliverdin-based colour reveals the quality of the laying female (Moreno & Osorno 2003). Previous explorations of this idea have varied in whether they viewed female quality as intrinsically or extrinsically derived (Moreno & Osorno 2003; Reynolds, Martin & Cassey 2009; Morales, Velando & Torres 2011) and perhaps consequently have not made explicit tests of both factors in the same system. In a repeated measures experiment with each bird experiencing high- and low-antioxidant diets, we found that eggshell colour was strongly affected by individual identity and only weakly affected by dietary antioxidants. However, it appears that birds might not be able to detect most of those differences in reflectance spectra.

Table 1. Partial η^2 as measure of effect of diet (high vs. low antioxidants) and individual identity on eggshell coloration, from analyses based on blue-green chroma or principal components of reflectance spectra

Analysis	Dependent variable	Partial η^2 for diet	Partial η^2 for identity
Univariate ANOVA	Blue-green chroma	0·006	0·762
MANOVA	PC1, PC2, PC3	0·004	0·655
Univariate ANOVA	PC1 (mainly blue to yellowish-green)	0·018	0·792
Univariate ANOVA	PC2 (mainly UV and violet)	0·043	0·415
Univariate ANOVA	PC3 (mainly yellow, orange, red)	0·043	0·638

Table 2. Consistency of eggshell colour measurements for different eggs laid by the same female

Comparison	PC1 (mainly blue to yellowish-green)	PC2 (mainly UV and violet)	PC3 (mainly yellow, orange, red)	Blue-green Chroma
ICC within the low-antioxidant diet (n = 248 eggs from 24 birds)	0·7971	0·4682	0·6127	0·8256
ICC within the high-antioxidant diet (n = 296 eggs from 25 birds)	0·7068	0·4013	0·6980	0·7489
ICC of all eggs, with no accounting for diet treatment (n = 545 eggs from 25 birds)	0·7185	0·3731	0·6155	0·7557
Pearson's r between average of low-antioxidant eggs and average of high-antioxidant eggs (n = 25 pairs of values)	0·739	0·905	0·876	0·915

PC1, PC2, and PC3 are the varimax-rotated principal components that capture the most variation in reflectance spectra; blue-green chroma is reflectance between 400 and 570 nm as a proportion of all reflectance from 300 to 700 nm. Within-diet repeatability is the intra-class correlation coefficient for eggs laid by a given female while on a given diet. Across-diet repeatability is the intraclass correlation coefficient for all eggs laid by a given female irrespective of diet. The last row is the Pearson correlation coefficient between the average egg colour of a female while on each of the two diets.
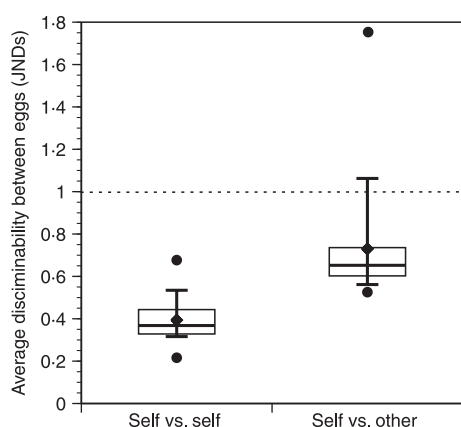

Fig. 2. Average predicted discriminability in pairwise comparisons of self-laid eggs (left box) and in pairwise comparisons between eggs laid by self vs. nonself (right box). Discriminability is calculated for each of the 25 females, with values >1 JND representing differences that are likely detectable by an avian visual system. Horizontal lines in box are 25th, 50th and 75th percentiles; whiskers are 10th and 90th percentiles; diamonds show means; circles show minimum and maximum.

Birds in our experiment differed statistically from one another in eggshell reflectance spectra, despite the fact that all females were domesticated birds that experienced the same rearing conditions, the same housing conditions and the same dietary treatments. There were multiple lines of evidence for these statistical differences between individual birds. First, in analyses of egg colour using multiple predictor variables, the effect size for individual identity was large, with partial η^2 ranging from 0·415 to 0·792 for the different measures of eggshell colour. Second, we used correlation analyses to measure repeatability of the colour of multiple eggs laid by individual females. Within a given diet treatment, intraclass correlation coefficients were moderate to high, ranging from r = 0·401 for reflectance in the UV and violet (PC2) to r = 0·826 for blue-green chroma. Importantly, there was also evidence for individual consistency even when pooling eggs from different dietary

treatments. These intraclass correlation coefficients were high, including a value of r = 0·756 for blue-green chroma. Correlation coefficients were also very high when comparing a bird's mean egg colour on one treatment with its mean egg colour on the other treatment, such that a bird that laid intensely blue-green eggs (relative to other birds) on one diet also did so on the other diet. In all of these measures of the effect of individual identity, within-bird consistency was generally strongest for blue-green chroma and for the principal component corresponding to reflectance in the blue to yellowish-green part of the spectrum.

These findings of repeatable differences between females in eggshell coloration are consistent with the original formulation of the SSH – that blue-green eggshell colour might signal quality as an intrinsic property of the laying female (Moreno & Osorno 2003). Intrinsic differences between females could arise from three nonexclusive sources. First, genetic differences can exist between birds (Morales *et al.* 2010), affecting eggshell colour either directly or via an effect on antioxidant physiology. Second, maternal effects can influence antioxidant physiology (Saino *et al.* 2011) with possible consequences for eggshell colour. And third, antioxidant physiology can be influenced by posthatching environmental differences between individuals (Zhuang *et al.* 2007; although our birds were reared together from the age of two days). For any cause of intrinsic differences between females, the SSH is predicated on male birds being able to detect differences between females in eggshell colour, and our model of the avian visual system suggests that most of the statistical differences between females may not be visually discriminable by these birds. Of course, these models are simply predictive efforts to understand how eggshell reflectance is perceived; corroboration with behavioural assays would be a useful next step.

In contrast to the strong statistical effect of individual identity, dietary antioxidants had only a weak effect on eggshell colour. In the analysis based on principal components of the reflectance spectrum, diet had effects on all
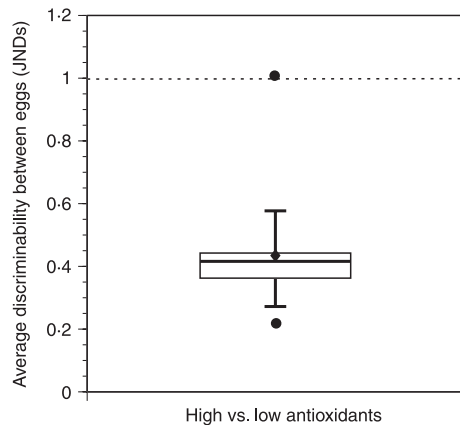
Fig. 3. Average predicted discriminability in pairwise comparisons of eggs laid by an individual female while on a high- vs. low-antioxidant diet. Mean discriminability between eggs on the two diets is calculated for each of the 25 females, with values greater than 1 JND representing differences that are likely detectable by an avian visual system. Horizontal lines in box are 25th, 50th and 75th percentiles; whiskers are 10th and 90th percentiles; diamonds show means; circles show minimum and maximum.

three principal components, but the effect sizes were very small; in the analysis based on blue-green chroma, there was not a significant effect of diet on colour. Not surprisingly, predictions from our model of the avian visual system indicate that nearly all effects of diet on eggshell colour were not visually detectable to birds. Thus, by any of these measures, the influence of diet was small, despite the fact that our diets were carefully formulated to widely bracket the recommended vitamin level for egg-laying chickens, with the two treatments differing fourfold in Vitamin A and Vitamin E. One possible explanation for a limited effect of diet on egg colour is that there is not an exact functional interchangeability of different types of antioxidants (Costantini *et al.* 2010); consequently, an input of one type of antioxidant may not afford an output of biliverdin. However, there are several examples of biliverdin output being tied to levels of other antioxidants. In pied flycatchers, for instance, an experimental increase in reproductive effort caused females to exhibit a trade-off between plasma antioxidant capacity and egg coloration (Morales, Velando & Moreno 2008). In blue-footed boobies, the blue-green chroma of the second-laid egg increased in response to a dietary supplement of the carotenoids lutein, zeaxanthin and canthaxanthin administered when the first egg was laid (Morales, Velando & Torres 2011). Although the clear trade-off between using a particular carotenoid for coloration vs. for free radical scavenging or immunostimulation (Clotfelter, Ardia & McGraw 2007) is perhaps more straightforward than the analogous argument for biliverdin, it still seems plausible that a meaningful trade-off could exist between a female's general antioxidant defence and her deposition of biliverdin into eggshells (Hanley, Heiber & Dearborn 2008).

In addition to the effects of identity and diet on eggshell colour in our experiment, there was also an effect of time.

When controlling for the balanced order of the two dietary treatments, there was a tendency for eggs laid during the second treatment to score slightly higher on all colour variables than eggs laid during the first treatment (with significant effects on PC2, PC3, and blue-green chroma, but not on PC1). This change in egg colour between the first and second part of the experiment could be driven by seasonal changes in temperature or photoperiod or by age-based changes in the chickens, as the first treatment period was mid-February to mid-April, when the birds were 20–27 weeks old, and the second treatment period was mid-April to mid-June, when the birds were 28–35 weeks old.

We used domesticated chickens to achieve a manageable captive system for this study, and it is important to consider the potential differences between our experimental system and wild birds. A captive study afforded a high degree of control, as we were able to standardize the environment, diet and history of the birds. In wild birds, by comparison, one could predict that the effect of individual identity might be reduced by uncontrolled (and typically unknown) environmental variation (Beaulieu *et al.* 2010), but is it possible that this could be offset by greater genetic differences between individuals in a wild population compared with the somewhat standardized Araucana poultry breed. Another consideration is the selection regime to which wild birds vs. Araucana fowl have been exposed. Perhaps, the selection regime in the Araucana lineage did not lead to a physiology that maximizes the contribution of antioxidants to blue-green eggs; if so, we would expect an effect of dietary antioxidants to be larger in another system where the SSH is operating more clearly. However, we do know that chickens in the Araucana lineage have laid blue eggs for at least four centuries in a semidomesticated setting with indigenous peoples in South America (Berdichewsky 1975; Langdon 1989; Gongora *et al.* 2008). A useful next step would be assessing the extent to which sexual selection acts on eggshell coloration in this system. Generally speaking, there is evidence of sexual selection still operating on some traits in a variety of *Gallus gallus* study systems. Of particular relevance are studies showing postcopulatory sexual selection in *Gallus gallus*, such as males providing more or better sperm to high-quality females (Pizzari *et al.* 2003; Cornwallis & O'Connor 2009), providing more sperm to females that have the most dissimilar major histocompatibility complex (Gillingham *et al.* 2009) and increasing their postmating investment by alarm calling more often (Wilson & Evans 2008). More broadly, evidence for sexual selection in *Gallus gallus* comes not only from captive populations of wild jungle fowl stock (e.g. Zuk 1990; Parker & Ligon 2007), but also from domesticated breeds (Pizzari, Froman & Birkhead 2002; Wilson *et al.* 2008; Tiemann & Rehkämper 2009). Thus, the use of domesticated fowl may still be informative about processes in natural populations.

Overall, our study showed the potential for birds with a shared environmental history to exhibit statistically strong, repeatable differences in eggshell coloration. However,

even if eggshell colour carries information on intrinsic properties of the female that laid the egg, much of that variation may not be discriminable to a chicken, which would be the intended signal recipient under the SSH. This highlights the importance of examining whether statistically significant differences in colour result in biologically meaningful differences in colour (Cassey *et al.* 2009). Such a distinction would be important when examining hypotheses concerning a bird's ability to distinguish self-laid eggs from intraspecific brood parasitic eggs (Øien, Moksnes & Røskaft 1995; Soler *et al.* 2011) or to use egg recognition to identify one's own nest within a colony (Birkhead 1978).

Acknowledgements

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