Articles

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# Avian eggshell pigments are not consistently correlated with colour measurements or egg constituents in two *Turdus* thrushes

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The colourful appearance of avian eggshells is a prominent aspect of maternal reproductive effort in birds. Some differences in eggshell coloration have been reported to co-vary with various measures of maternal condition and these patterns support the hypothesis that, in some bird species, several aspects of eggshell colour (i.e. primary chroma and brightness) function as a signal of maternal and offspring quality to induce greater paternal investment. We directly quantified eggshell pigment concentrations of blackbird *Turdus merula* and song thrush *T. philomelos* eggshells and tested how the two key pigments (protoporphyrin IX and biliverdin) co-varied with other eggshell traits and egg constituents as measures of maternal reproductive investment, including total yolk carotenoid concentration, total lipid concentration, yolk mass, and shell thickness. Contrary to predictions, we detected few statistical patterns overall. We found that protoporphyrin IX concentration was negatively associated with blue-green chroma in blackbirds but not in song thrush. The concentration of protoporphyrin IX was significantly greater in blackbirds and also showed different patterns of association with total yolk lipids and yolk carotenoid concentrations between these two species (significant species interaction terms). Our results reveal that it is not appropriate to simply assume in these two avian species that reflectance-based eggshell colour measures are a suitable proxy for eggshell pigment concentrations or can be used as consistent predictors of maternal reproductive investment. These results highlight the need to assess and validate the strength and direction of the statistical relationships between eggshell colour measures, pigment concentrations, and maternal resource deposition in the egg for other species of birds.

Eggshell pigments are the conspicuous products of avian metabolism (Lang and Wells 1987), and studying their variability and functions is an increasingly dominant subject in modern ornithology (Cassey et al. 2011). The evolution of colourful eggshells has been reviewed extensively (Underwood and Sealy 2002, Kilner 2006, Cherry and Gosler 2010), and characterising the physiological and heritable correlates of colour production and function is vital for our understanding of how selection molds avian phenotypes (Hubbard et al. 2010). Notably, no single signaling or structural hypothesis adequately explains the adaptive significance of variability in eggshell appearance among wild birds (Kilner 2006, Cassey et al. 2010).

Individual female birds generally lay eggs of a 'constant' type relative to other conspecific females (Gosler et al. 2000, Moksnes et al. 2008, Morales et al. 2010, Honza et al. 2012), although laying date and order too can affect physical and perceivable inter- and intraclutch variation in eggshell colour (Avilés et al. 2007, Cassey et al. 2008). Several studies have found that differences in eggshell colour are related to various measures of maternal quality (Moreno et al. 2004, 2006, 2008, Krist and Grim 2007, Hanley et al. 2008, Hargitai et al. 2008; but see Hanley and Doucet 2009). These patterns have been used, in part, to support the sexually selected eggshell colour (SSEC) hypothesis that eggshell colour may be an adaptive signal of maternal reproductive quality used to induce greater paternal investment (Moreno and Osorno 2003).

Previously, we showed that maternally controlled traits of egg quality (egg size, laying order, yolk carotenoid concentrations) were not consistently correlated with metrics of eggshell colour (neither blue-green chroma nor relative photon capture for each avian photoreceptor class) in two related species of *Turdus* thrushes (Cassey et al. 2008). However, in that study we did not assess whether pigment concentrations and physical metrics of coloration, or pigment concentrations and egg quality traits were themselves correlated. This is an extremely important research step that is generally missed from most quantitative studies of eggshell colour (Siefferman et al. 2006, Avilés et al. 2011, Navarro et al. 2011; but see Moreno et al. 2006, Lopez-Rull et al. 2008). In particular, the only two previous studies to quantify the relationship between pigment concentration and eggshell appearance (Moreno et al. 2006, Lopez-Rull et al. 2008) were both conducted on the immaculate eggs of cavity nesting species for which clutches are arguably exposed to lower predation rates (Martin 1995), and very different light environments (Maurer et al. 2011a) from open-nesting species. Both of these studies also examined only a single pigment (biliverdin) for which it is known that cavity nesting species have, on average, a significantly lower concentration than open-nesting species (Cassey et al. 2012). It is thus important to examine how different eggshell pigment concentrations (biliverdin and protoporphyrin; Gorchein et al. 2009) vary in non-cavity nesting species, and particularly in closely related species with eggs that vary in their visible maculation.

In the present study, we quantified eggshell pigment concentrations from two related species of European thrushes, blackbird *T. merula* and song thrush *T. philomelos*, and tested how the eggshell pigments protoporphyrin IX and biliverdin covaried with physical metrics of reflected eggshell coloration as well as other maternally controlled putative traits of egg quality. As potential measures of the diverse mechanisms and molecules of physiologically costly maternal investment in eggs, including the lipid-rich yolk and the calcium-rich shell, we quantified yolk mass, yolk carotenoid concentrations, total lipid concentration, and eggshell thickness. Together these traits reflect the best known set of egg quality traits currently available to us for comparisons with prior work (Pilz et al. 2003, Navara et al. 2006, Safran et al. 2008). In particular, larger yolk mass (Ardia et al. 2006), higher yolk carotenoid concentrations (Newbery and Reed 2011), and greater total lipids (Royle et al. 1999, Nager et al. 2000) are all assumed to be costly for females to provide, and evidence suggests that these traits represent a form of adaptive maternal investment in offspring. While eggshell thickness may also be considered an egg quality trait (but see Bennett et al. 1988) we are more interested in its relationship with pigment concentration given that the eggshell may mediate the transmission of harmful light (Lahti 2008, Maurer et al. 2011a), and that it has been proposed that pigment (particularly protoporphyrin) is incorporated in the shell principally to strengthen it (Higham and Gosler 2006).

As a direct test of the SSEC hypothesis of maternal reproductive investment in eggshell coloration (Moreno and Osorno 2003, Moreno et al. 2004), we predicted positive relationships between pigment concentrations and measures of maternal deposition of resources in eggs because high quality females, relative to low quality females, should deposit more resources into all aspects of costly reproductive traits that might increase offspring fitness (Pilz et al. 2003). However, it is important to recognize that individual egg traits are likely to have different functions and therefore to be regulated by different mechanisms (Safran et al. 2008). In addition, we note that aspects of the SSEC hypothesis may similarly predict a trade-off (Morales et al. 2008) whereby the investment in signaling pigments is costly, and, consequently, resources canalized in pigment production cannot be invested into other reproductive components. It is therefore possible, that negative relationships between pigment concentrations and measures of maternal investment in egg constituents may also be observed.

## Material and methods

### Egg collection

We collected a single egg from 24 song thrush clutches, and 21 blackbird clutches from introduced wild populations nesting in blueberry *Vaccinium* sp. orchards near the outskirts of Hamilton in the Waikato, New Zealand (37°47′S, 175°20′E) during the 2005 austral breeding season. These eggs are a sub-sample of previously studied nests and details of the nest finding protocol and study site are provided elsewhere (Cassey et al. 2008, 2009a). Specifically, 25 nests were chosen for each species for which an early laid (first or second) egg was collected at clutch completion and prior to the onset of full-time incubation (i.e. no visible embryo). Eggs were chosen for which all clutch and egg specific data (see below) were available and which were laid in the inter-quartile range of the breeding season. In five cases (one song thrush and four blackbirds) individual eggs were removed from the analyses because one or more variables were incomplete (not measured).

After collection, eggs were measured with precision vernier calipers (length by width to 0.1 mm) and weighed with calibrated electronic scales with precision to 0.001 g. Eggs were subsequently transported to the field laboratory, washed in laboratory grade water, and split to remove yolk and albumen, to be stored at −80°C. Eggs were split by hand using a thumb-nail to 'cut' around the long axis of the shell. Albumen was collected directly into a 50 × 100 mm ziplock laboratory plastic bag and the whole yolk separately deposited in a 10 ml canonical volumetric flask and weighed. In both species, yolk mass was strongly positively correlated (all $r > 0.92$, $p < 0.001$) with both egg volume and egg mass. The eggshell halves were then washed again, air-dried, and stored in individual dark airtight containers at room temperature.

Thickness of the larger, better preserved half of each eggshell was measured, with the inner membrane intact, to an accuracy of 1 μm using a Mitutoyo Series 227–203 constant measurement force micrometer (Maurer et al. 2011b). Both anvils of the micrometer were custom-fitted with 6 mm aluminium pins (diameter 1.35 mm) with rounded tips of 0.675 mm radius. The single shell-half was placed in the micrometer so that it was at a 90° angle to the pin and measured at three different equator locations a measurement force of 1.5 N. We previously quantified the very high repeatability of these measurements (Igic et al. 2010b).

### Reflectance spectrophotometry

Both studied species of *Turdus* thrushes lay predominantly blue-green eggs but vary considerably in their degree, colour, and size of maculation (Supplementary material Appendix 1, Fig. A1). Eggshell reflectance was measured in situ at the field laboratory, using an Ocean Optics USB2000 Miniature Fiber Optic Spectrometer with illumination by a DT mini-lamp. Before the measurements were taken the lamp was turned on for 30 min to ensure consistent light production. A custom-built light-proof cap was fitted over the probe to maintain a consistent angle (90°) between the eggshell and the measuring fibre optics (as recommended in Andersson and Prager 2006). All of the measurements were conducted by the same person (PC) and were highly repeatable (Cassey et al. 2009b). Spectra were recorded in ~0.4 nm steps and were expressed relative to a white Ocean Optics WS-1 diffuse reflectance standard. Two measurements of the background shell colour were taken at each of the equator, pointy and blunt ends (6 measurements per egg) of the larger, better preserved half of each eggshell. For the measurements, specific care was taken to avoid visible maculation and to quantify the background shell colour only. All spectra were visually inspected prior to analysis. To minimize instrument error, dark and white standard reflectance calibration measures were taken regularly during sampling.

Reflection curves (Fig. 1a, b) were truncated across the avian visible wavelength; between 300 and 700 nm (Cherry and Bennett 2001). An interpolated average was used to calculate an average reflectance value at 5 nm steps. Brightness (following Montgomerie 2006) was calculated as the total area under the reflectance curve divided by the total length of the wavelength. Song thrush and blackbird eggs are known to reflect maximally in the short- and medium-wavelength sensitive (blue-green) region (Cassey et al. 2009b). Blue-green chroma was calculated, following Siefferman et al. (2006), as the proportion of the wavelength that represents the region of least absorbance for the blue-green pigment biliverdin ($\Sigma\lambda_{400-575}/\Sigma\lambda_{300-700}$).

## Eggshell pigments

The larger and/or better preserved (entire) 'half' of the eggshell was used for chemical analysis. Protoporphyrin IX and biliverdin concentrations were determined in the form of their dimethylesters. The procedure was based on a previously established method for determination of eggshell porphyrins (Mikšík et al. 1996). Eggshells pigments were extracted (and esterified) by 15 ml absolute methanol (LiChrosolv, gradient grade for chromatography,

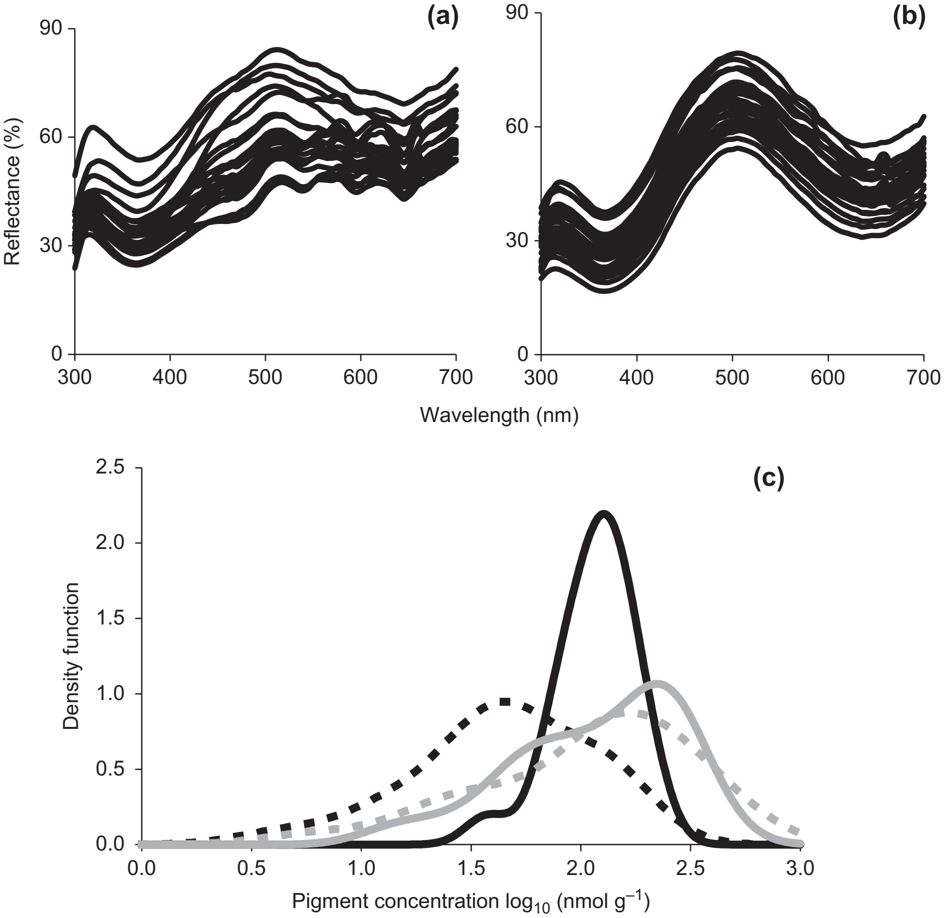

Figure 1. Reflectance spectra of (a) blackbird (n = 21) and (b) song thrush (n = 24) eggs (each egg from a different female). Each reflectance spectrum is an average of six measurements per egg. Bounded kernel density functions (c) of the smoothed distributions of the concentration of eggshell pigments biliverdin (grey lines) and protoporphyrin IX (black lines) for song thrush (broken lines) and blackbird (solid lines) eggs. The estimated kernel density function was produced in Proc KDE (SAS ver. 9.2) and is more effective, for small sample sizes, than using a histogram to identify features that might be obscured by the choice of histogram bins or sampling variation.

Merck, Darmstadt, Germany) containing 5% concentrated sulphuric acid at room temperature in the dark under $N_2$ for 24 h. Extracts were decanted and 10 ml chloroform (Merck; chloroform GR, ISO) and 10 ml distilled water were added, then shaken. The lower (chloroform) phase was collected, and the higher (water) phase was again extracted with chloroform (chloroform phases from both extractions were collected). These phases were washed with 5 ml 10% NaCl, followed by distilled water until the washing was neutral. Extracts were evaporated to dryness and reconstituted in 1 ml chloroform with an internal standard (5,10,15,20-tetra(4-pyridyl)-21H,23H-porphine, Aldrich, Sigma-Aldrich, St Louis, MO, USA; 0.01 mg $ml^{-1}$). Standards for quantification (protoporphyrin IX and biliverdin, MP Biomedicals, LLC, Eschwege, Germany) were treated by the same procedure.

Pigments were quantified by reversed-phase high-performance chromatography using an Agilent 1100 LC system (Agilent, Palo Alto, CA, USA) consisting of a degasser, binary pump, auto-sampler, thermostat column compartment, and multi-wavelength and fluorescence detectors. Chromatographic separation was conducted in a Gemini 5u C18 110A column (250 × 2.0 mm I.D., Phenomenex, Torrence, CA, USA). The sample (10 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 $min^{-1}$ and temperature 55°C). The gradient started at A/B 80:20 reaching 10:90 rations after 15 min and after 10 min it reached 100% B. For the next 10 min the elution was isocratic at 100% B. Elution was monitored by absorbance at 410 nm and by fluorescence at 405ex/620em nm.

Analyses were confirmed by the same HPLC coupled to an ion-trap mass spectrometer (Agilent LC-MSD Trap XCT-Ultra; Agilent, Palo Alto, CA, USA). The elution was achieved using a linear gradient (A = water with 0.1% formic acid, and B = acetonitrile with 0.085% formic acid), flow rate 0.35 ml $min^{-1}$ and temperature 55°C. The gradient started at A/B 80:20 reaching 10:90 rations after 15 min and reaching 100% B after 5 min. For the next 10 min the elution was isocratic. Atmospheric pressure ionization-electrospray ionization (API-ESI) positive mode ion-trap mass spectrometry at MRM (multiple reaction monitoring) mode was used when precursor ions were 619 (internal standard), 611 (biliverdin) and 591 (protoporphyrin IX). Operating conditions were as follows: drying gas ($N_2$), 12 l $min^{-1}$; drying gas temperature, 350°C; nebulizer pressure, 30 psi (207 kPa).

Following previous studies the concentration of the eggshell pigments were standardized by the mass ($g^{-1}$) of the sample eggshell fragments (Mikšík et al. 1994, Moreno et al. 2006). Fragments were weighed to a precision of 0.001 g on a Mettler PC 440 digital scale. This measure of concentration (nmol $g^{-1}$) will be reliable, for example, if pigmentation occurs throughout the entire depth of the shell matrix (Nys et al. 2004, Jagannath et al. 2008). At least one study, however, has reported that the majority of pigmentation may occur in the outermost layer (Wang et al. 2007), and therefore a more suitable measure of pigment concentration might be standardized by the surface area of the sample fragment ($mm^{-2}$) (Igic et al. 2010a). Surface area (A) of the shell was calculated following Schönwetter (1960–1992) and Maurer et al. (2010) as:

$$A = B^2 \times (0.914 + 2.228 \times (L/B))$$

where L and B are the length and breadth of the egg respectively. Surface area of the fragment used for pigment extraction was estimated from the percentage of the dried shell mass. This measure of concentration (nmol × 1000 $mm^{-2}$) will be reliable, for example, if pigmentation mostly occurs in the outermost cuticle layer of the eggshell independent of shell thickness (Wang et al. 2007).

## Yolk carotenoids

Yolk samples were weighed (on the same portable scales as above) and their volume measured in a 10 ml canonical volumetric flask. Yolk samples (ca 200 μg) were homogenized with 0.7 ml NaCl 5% and 1 ml ethanol, and carotenoids were extracted adding 2 ml hexane and further homogenization, centrifugation and collection of the hexane phase (extractions were repeated twice). Hexane extracts were pooled and evaporated at 60–65°C under nitrogen flow, and the residue was dissolved in 0.1 ml dichloromethane and 0.1 ml methanol. Total carotenoid concentration was determined using high-performance liquid chromatography (HPLC) with a Spherisorb type $S5NH_2$ reverse-phase column, 25 cm × 4.6 mm (Phase Separation, Clwyd, UK) with a mobile phase of methanol-distilled water (97:3), at a flow rate of 1.5 ml $min^{-1}$ as described by Hõrak et al. (2002). Lutein (Sigma, Poole, UK), the dominant individual carotenoid (Cassey et al. 2008), was used to calibrate samples.

## Lipid extraction and quantification

Frozen egg yolk samples were stored at −80°C, and approximately 10 mg was transferred into a tared 1 ml glass V-vial (Wheaton), before returning the remainder of the sample to the −80°C freezer. The mass of the yolk sample in the V-vial was recorded after 5 min at room temperature. This process was repeated so that three replicate samples of yolk were obtained from each egg. Lipid content from yolk subsamples was extracted as described in Sewell (2005) with the exception that samples were sonicated for 20 min in a Unisonics (Brookvale, Australia) ultrasonication bath.

Lipid classes were quantified using an Iatroscan Mark Vnew TLC/FID system and silica gel S-III Chromarods. Briefly, the lipid extract was dried immediately before use in a stream of instrument grade N2 gas and re-suspended in 500 μl of chloroform. Subsamples (1 μl ) of four different egg extracts were quantified in each run (2 replicate Chromarods of each extract; total 8 rods), with the remaining 2 Chromarods blanks to test for contamination of the developing solvents. The Chromarods were developed for 28 min in 60 ml of Hexane, 6 ml of Diethyl-ether and 0.1 ml of Formic Acid, dried for 5 min in a Rod Dryer TK-8 (Iatron Laboratories) at 60°C, then run in the Iatroscan with a 30 s and settings of 2000 ml $min^{-1}$ $O_2$ and 160 ml $min^{-1}$ $H_2$. Quantification of the total lipids in each sample was based on multilevel calibration curves generated for separate lipid class. Rods were calibrated with a 5-component composite standard made from highly purified lipid standards (99%) in HPLC-grade chloroform [aliphatic hydrocarbon (AH) = nonadecane; ketone (KET) = 3-hexadecanone; triglyceride (TG) = tripalmitin; free sterol (ST) = cholesterol; phospholipid (PL) = L-α-phosphoditylcholine].

### Statistical analyses

We calculated the Pearson correlation coefficient for pigment concentration standardised for fragment mass and surface area. We constructed generalized linear models accounting for species as a fixed effect to model the relationships between the eggshell pigment concentrations ($\log_{10}$ transformed) and all of the putative maternal traits. It has become increasingly popular in ecological disciplines to adopt an information theoretic (IT) approach (Burnham and Anderson 2002, Garamszegi 2011). However, in the absence of clear a priori candidate models, 'one cannot really learn anything' from IT-based inference (Mundry 2011, p. 64). We therefore constructed full models from a small set of explanatory variables and individually tested first order interactions between species and all of the maternal traits. The predicted values for all variables in the models were visually inspected for the distribution and homoscedasticity of their errors. All analyses were conducted in SAS ver. 9.2.

## Results

The two measures of concentration (nmol) of the two eggshell pigments (biliverdin and protoporphyrin IX) standardized by either sample mass ($g^{-1}$) or sample surface area (1000 $mm^{-2}$) were strongly and positively correlated with each other (all p-values $< 0.001$) in both species (blackbird, $n = 21$: protoporphyrin IX $r = 0.982$, biliverdin $r = 0.995$; song thrush $n = 24$: protoporphyrin IX $r = 0.992$, biliverdin $r = 0.991$). Given that sample mass was precisely measured (Methods), without any further calculation and does not rely on the assumption whether pigments are deposited in a uniformly thin layer on the surface of the eggshell, independent of shell thickness, we report all further analyses based on pigment concentrations by eggshell sample mass ($g^{-1}$).

Average eggshell pigment concentrations (± 1 SE) are provided in Table 1. The average concentration of eggshell protoporphyrin IX was significantly greater in blackbirds, compared with song thrush (Welch–Satterthwaite unequal variance t-test; $t_{31.6} = 4.48$, $p < 0.001$), whereas the concentration of biliverdin was not significantly different between the two species ($t_{42.7} = 0.30$, $p = 0.763$; Fig. 1c). The concentrations of the two eggshell pigments were positively correlated (Fig. 2a), although only the

Table 1. Average eggshell pigment concentrations (± 1 SEM) for two open-nesting European *Turdus* species. Note in all subsequent analyses pigment concentrations (nmol $g^{-1}$) were $\log_{10}$ transformed.

| Pigments | | Biliverdin | Protoporphyrin IX |
|---|---|---|---|
| Blackbird (*T. merula*) | ($n = 21$) | 152.96 (22.49) | 118.92 (8.84) |
| Songthrush (*T. philomelos*) | ($n = 24$) | 159.98 (27.12) | 61.54 (9.81) |

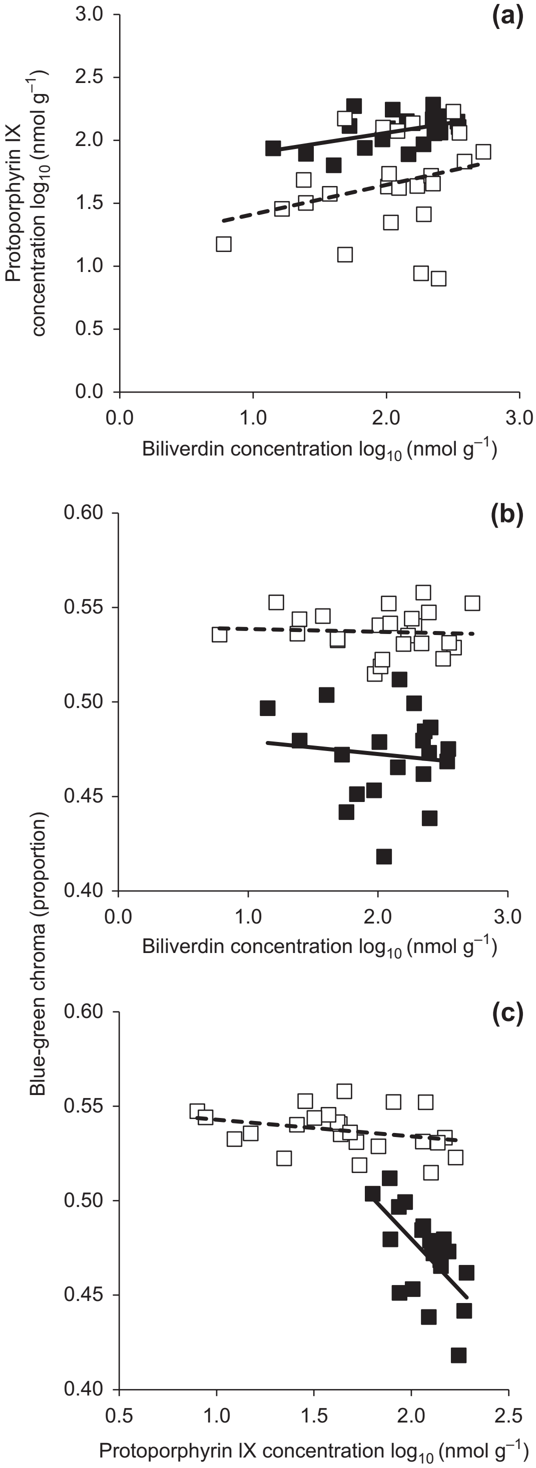

Figure 2. Bivariate relationship between (a) pigment concentrations of biliverdin and protoporphyrin IX from song thrush (hollow loci) and blackbird (solid loci) eggs; (b) proportions of blue-green chroma and biliverdin concentration in song thrush and blackbirds; and (c) proportions of blue-green chroma and protoporphyrin IX concentration in blackbirds and song thrush. Best fit linear regression lines are plotted for comparison.

Table 2. Least square mean estimates (and standard errors) for fixed effects from generalized linear models testing the putative association between the concentration of the two eggshell pigments biliverdin and protopophyrin IX and two physical metrics of eggshell reflectance (blue green chroma and brightness) in a full-model with first order species interactions. Estimates, and their test statistics, in bold text were statistically significant ($\alpha = 0.05$).

| Reflectance | Blue-green chroma | t-value (DF = 39) | Brightness | t-value (DF = 39) |
|---|---|---|---|---|
| Intercept (full model) | **0.57 (0.02)** | 32.51*** | **38.28 (6.53)** | 5.86*** |
| Species (*T. merula*) | **−0.06 (0.02)** | −2.13* | **71.96 (17.26)** | 4.17*** |
| Biliverdin ($\log_{10}$(nmol g$^{-1}$)) | −0.01 (0.01) | −0.06 | 1.78 (2.66) | 0.67 |
| Protoporphyrin ($\log_{10}$(nmol g$^{-1}$)) | **−0.02 (0.01)** | −3.01** | 2.86 (3.12) | 0.92 |
| Species × biliverdin (interaction) | 0.01 (0.01) | 0.88 | **−10.41 (4.74)** | −2.20* |
| Species × protoporphyrin (interaction) | **−0.09 (0.02)** | −3.56*** | **−23.14 (9.43)** | −2.46* |

*p < 0.05; **p < 0.01; ***p < 0.001.

correlation in blackbirds was statistically significant (blackbirds: r = 0.486, n = 21, p = 0.025; song thrush: r = 0.248, n = 24, p = 0.241).

Eggshells of both species reflected maximally in the blue-green region of the wavelength (Fig. 1a, b). Pigment concentrations were not consistently associated with physical metrics (blue-green chroma and brightness) of eggshell coloration in the two thrush species (Table 2). The proportion of blue-green chroma was not positively associated with increasing biliverdin concentration in either blackbird or song thrush (Fig. 2b, Table 2). Significant interactions between species and pigment concentrations (Table 2) were due to the decreasing concentration of both pigments and increasing brightness in blackbirds (but not song thrush), and a significant decrease in the proportion of blue-green chroma with increasing protoporphyrin concentration in blackbirds only (Fig. 2c).

Blue-green chroma was significantly lower in blackbirds (Table 3, Fig. 2c) and associated with a significant decrease in yolk mass. Eggshell brightness was not associated with any egg constituent traits (Table 3). The concentration of biliverdin was not associated with any egg traits (Table 3). The concentration of protoporphyrin IX was significantly greater in blackbirds and was associated with an increase in total yolk lipids (Table 3). There were significant species interactions between the concentration of protoporphyrin IX and both total yolk lipids and total yolk carotenoids (Table 3, Fig. 3a, b). For total yolk carotenoids the significant species interaction was due to the significant negative relationship with eggshell protoporphyrin in song thrush but not in blackbirds (Fig. 3a). For total lipid concentrations the significant species interaction was due to the significant negative relationship with eggshell protoporphyrin in blackbirds and the significant positive relationship in song thrush (Fig. 3b).

## Discussion

This study represents the first detailed concurrent analysis of the two known colourful pigments present in avian eggshells (following Gorchein et al. 2009), and their natural range of co-variation with eggshell traits and egg yolk constituents in two open-nesting European thrush species. We acknowledge that the interpretation of our data is based on correlations, and not experimental manipulations, and that our results may be species and/or habitat specific. However, correlative analyses from wild systems are essential for the evaluation of critical assumptions of the prevalent hypothesis that avian eggshell appearance functions in a signaling context between reproductive partners (Lopez-De-Hierro and Moreno-Rueda 2010,

Table 3. Least square mean estimates (and standard errors) for fixed effects from generalized linear models testing the putative influence of maternal traits on two physical metrics of eggshell reflectance (blue green chroma and brightness) and the concentration of the two eggshell pigments (biliverdin and protopophyrin IX). Traits in bold text were significant ($\alpha = 0.05$) in a full model that included all putative traits. Models were also conducted, individually, with each first order 'species × trait' interaction. Traits for which an interaction with species was significant ($\alpha = 0.05$) are highlighted in grey.

| Eggshell colour | Blue-green chroma | t-value (DF = 39) | Brightness | t-value (DF = 39) |
|---|---|---|---|---|
| Intercept (full model) | **0.51 (0.05)** | **10.92*** ** | 32.43 (19.35) | 1.68 |
| Species (*T. merula*) | **−0.06 (0.01)** | **−7.12*** ** | 1.91 (3.72) | 0.51 |
| Shell thickness (mm) | 0.01 (0.01) | 1.41 | 0.22 (0.17) | 1.29 |
| Yolk mass (g) | **−0.04 (0.02)** | **−2.21*** | −2.97 (7.63) | −0.39 |
| Total yolk carotenoids (μg g$^{-1}$) | 0.01 (0.01) | 0.44 | −2.03 (5.93) | −0.34 |
| Total yolk lipids (μg μg$^{-1}$) | 0.01 (0.06) | 0.18 | −5.75 (25.37) | −0.23 |
| Pigments | Biliverdin | | Protoporphyrin IX | |
| Intercept (full model) | 1.33 (1.15) | 1.16 | **1.91 (0.79)** | **2.41*** |
| Species (*T. merula*) | −0.05 (0.22) | −0.21 | **0.59 (0.15)** | **3.90*** ** |
| Shell thickness (mm) | 0.02 (0.01) | 1.60 | 0.01 (0.01) | 0.33 |
| Yolk mass (g) | −0.18 (0.45) | −0.40 | −0.13 (0.31) | −0.43 |
| Total yolk carotenoids (μg g$^{-1}$) | −0.61 (0.35) | −1.73 | **−0.65 (0.24)** | **−2.71** ** |
| Total yolk lipids (μg μg$^{-1}$) | 0.54 (1.51) | 0.36 | **2.28 (1.03)** | **2.20*** |

*p < 0.05; **p < 0.01; ***p < 0.001.

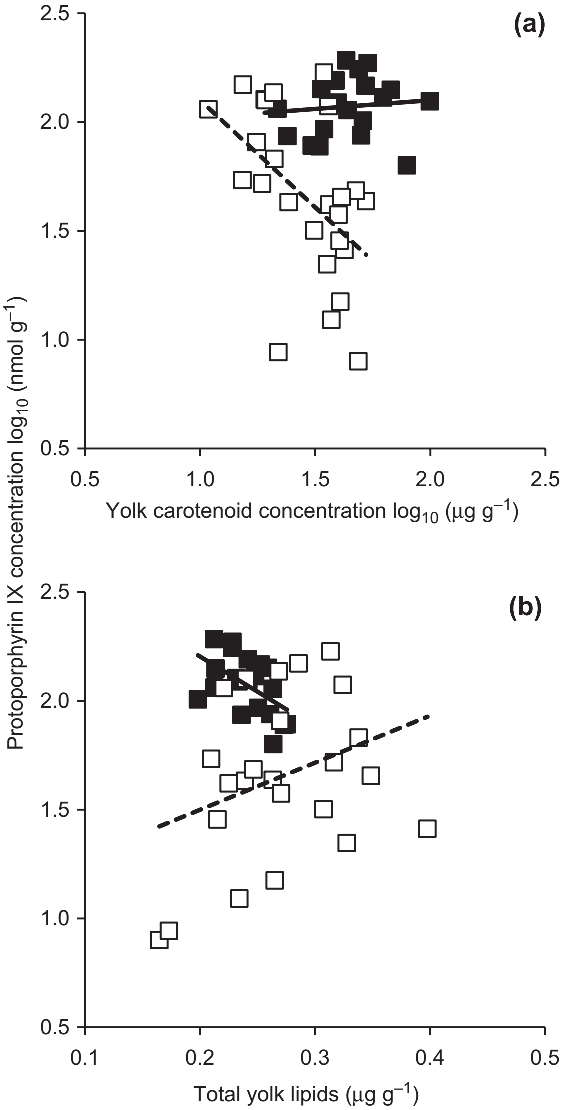

Figure 3. Bivariate relationship between protoporphyrin IX concentration and (a) total yolk carotenoids, and (b) total yolk lipids from song thrush eggs (hollow loci) and blackbirds (solid loci). In both cases, the species interaction is significant (Table 3).

Avilés et al. 2011). Importantly, our data reflect biological reality because any paternal visual assessment of female (or chick) quality based on egg phenotype is inevitably based on the same correlative information we collected and analysed in this study.

Firstly, our analysis revealed that eggshell pigmentation and physical measures of colourful eggshell appearance were inconsistently, and at best weakly, correlated, so that patterns could not be generalized even across our two, closely related, congener bird species. To date, almost all studies examining variability in eggshell pigmentation implicitly assumed that the reflectance-based coloration of eggshells is a reliable correlate of pigment concentration (reviewed by Reynolds et al. 2009). The exceptions are two studies, from cavity nesting species, showing that blue-green chroma is positively associated with the concentration of biliverdin in the eggshells of the pied flycatcher *Ficedula hypoleuca* (Moreno et al. 2006) and spotless starling *Sturnus unicolor* (Lopez-Rull et al. 2008). These contradicting results, between our research and the prior two studies, are difficult to reconcile. A possible explanation may be that these previous studies have used bird species with immaculate eggshells compared to our two thrush species with sparsely (song thrush) and moderately (blackbird) maculated eggs. In both of these previous studies the authors also only considered the concentration of a single pigment, biliverdin. It is possible that the complex interaction between the maculation patterns underlying background versus spotting patterns by the two eggshell colour pigments masked any simple correlation between pigment concentration and colormetric variables in our analyses. However, this explanation does not account for the fact that the blue-green background chroma of the predominantly immaculate song thrush eggs (Supplementary material Appendix 1, Fig. A1) is essentially invariant with the concentration of the blue-green pigment biliverdin (Fig. 2b). In all cases we explicitly attempted to measure the reflectance of the blue-green background colour of the eggshell (see peak reflectances in Supplementary material Appendix 1, Fig. A1). In fact, the only significant intra-specific relationship between pigment and physical colour attributes is the decline in blue-green chroma with the increase in the concentration of the red-brown pigment protoporphyrin IX in the more densely maculated eggs of blackbirds. It remains unclear how pigment concentration varies across the eggshell, and further studies that examine the variability between specific reflected colours and pigment concentrations, across the shell, will be greatly rewarding.

Interspecific differences, between the two closely related thrush species, also included divergent patterns of the relative contributions of each of biliverdin and protoporphyrin IX to generating the physical (and contributing to the perceived) appearance of eggshell coloration. Specifically, the same range of pigment concentration of either pigment, between the two species, cannot be used to predict the physical reflectance of the eggshell, as estimated by either blue-green chroma or brightness in our analyses (see significant interactions in Table 2). For example, song thrush eggs have significantly higher blue-green chroma at the same range of biliverdin concentrations as blackbird eggs (Fig. 2b), whereas increasing protoporphyrin concentrations is associated with reduced blue-green chroma in blackbirds but not in song thrushes (Fig. 2c). Interestingly, this difficulty with obtaining simple visually based estimates of pigment concentration, from eggshell colour, is similar to what has been reported for feather colours, because visual and physical assessment of plumage colours and pigment quality and quantity, are not fully inter-predictable in birds (Mcgraw et al. 2004). This pattern has also been reported more generally for inter-specific comparisons of non-passerine eggshell pigment concentrations in British breeding birds (Cassey et al. 2012).

Second, comparisons between eggshell pigments and egg quality traits revealed inconsistent interspecific patterns of co-variation in these two species. For example, increases in eggshell pigments were associated with significant species interactions in the concentrations of both total yolk carotenoids and lipids (Table 3, Fig. 3a, b). It was previously shown that a trade-off might exist between a female's investment in pigmentation and other costly egg constituents (Morales et al. 2008). Alternatively, higher condition and quality females may be able to invest more in costly eggshell pigmentation and egg constituents used to enhance embryonic and hatchling functions (Morales et al. 2011). However, our results from two open cup-nesting thrush species in the same agricultural habitat suggest that any associations which do occur are not generally applicable to these two species and unlikely to reliably signal maternal investment or territory quality. This conclusion parallels our previous findings that intraspecific variation in reflectance-based eggshell coloration between eggs laid by the same or different females are difficult to discriminate by the avian perceptual system of thrush-like birds (Cassey et al. 2009b; but see Avilés et al. 2011), and do not predict intraspecific patterns of yolk carotenoid concentrations (Cassey et al. 2008; but see Navarro et al. 2011).

In summary, we have shown that it is not appropriate to simply assume that concentrations of avian eggshell pigments are well represented by (i.e. correlated with) different physical indices of eggshell coloration. This conclusion has critical implications against the broad comparative use of simple colorimetric variables (e.g. blue-green chroma) as a corollary measure of pigment composition, content, and concentration across different laying females (or across different bird species) without confirming the assumption first that physical reflectance metrics and pigment concentrations are predictably correlated (Moreno et al. 2006). Our data also counter the prevailing assumptions in the literature, as applied for the two species examined here, that eggshell pigment concentrations are strongly and predictably positively related to costly maternal investment through egg content and constituents, measured in the present study as egg yolk mass, yolk carotenoid and yolk lipid concentrations, and shell thickness. For example, we detected no consistent statistical patterns between biliverdin or protoporphyrin IX concentrations and any measured egg trait in blackbirds or song thrush. We note that this is the first time a study has explicitly evaluated the role of multiple and co-varying eggshell pigments as a component of egg quality in wild bird species. Critically, however, in the absence of detailed data on the conditions of the laying females, our study does not fully address assumptions of the predictions of the signaling function of costly maternal investment hypothesis. Future experimental studies on diverse avian species with colourful and maculated eggs are needed to demonstrate the causative nature of associations between ecological variables (Gosler et al. 2005, Avilés et al. 2007), maternal condition (Moreno et al. 2006), including antioxidant capacity and immune function (Morales et al. 2006), and investment into egg quality and eggshell structure and pigmentation (Hargitai et al. 2010).

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Supplementary material (Appendix JAB5576 at <www.oikosoffice.lu.se/appendix>). Appendix 1.