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Speckles of cryptic black-headed gull eggs show no mechanical or conductance structural function

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Abstract

The structural-function hypothesis provides an alternative to signalling-based predictions to explain the remarkable diversity observed in avian eggshell colour. According to the hypothesis, protoporphyrin, the common pigment of visible speckles, lubricates and thus strengthens the shell and simultaneously moderates gas transfer across it. Correlational evidence for the structural-function hypothesis in form of a coincidence of both shell thinning and reduced evaporation with eggshell speckles comes from a restricted set of species with limited calcium supply or little nest predation and no need for camouflage of the eggs. Here, we investigate whether protoporphyrin-based pigmentation similarly affects a species with cryptically marked eggs and ample dietary calcium, the black-headed gull, *Larus ridibundus*. Although shell thinning of speckles occurred, this effect was minimal compared with thinning through embryonic growth. Furthermore, speckled and plain areas of the shell did not differ in water vapour conductance through the shell. We conclude that protoporphyrin speckling does not fulfil a structural function in gull eggs. Instead, during shell formation where the protoporphyrin of speckles is deposited in place of calcite it could inflict a structural cost. We propose that the mechanical and water vapour conductance functions of shell speckling need to be evaluated as separate hypotheses and that both functions could, in fact, negatively affect each other.

Introduction

The widespread pigmentation of their eggshells, both as background colour and as maculation, sets birds apart from all other egg-laying vertebrates and most remarkably from their closest relatives, the reptiles, with their pure white shells (Patent, 1977). Avian eggshell pigmentation is as diverse as it is puzzling, especially as it is believed that the entire palette of shell colouration is generated mainly by two tetrapyrrole pigments, protoporphyrin and biliverdin (Kennedy & Vevers, 1976; Gorchein, Lim & Cassey, 2009). By contrast other pigmented calcite structures such as mollusc shells, show a great diversity of pigments and pigment classes to match their wide variation in appearance (Comfort, 1951; Bandaranayake, 2006).

Despite this very conservative chemical basis of shell pigmentation, confirmed for more than 100 species (Kennedy & Vevers, 1976), finding support for a single hypothesis to explain the evolution of eggshell pigmentation across all avian taxa has proven difficult. More importantly, it has become increasingly clear that a single hypothesis is unlikely to explain sufficiently the complexity of shell pigment functions (Reynolds, Martin & Cassey, 2009). Consequently, a number of competing ideas have been proposed, which by themselves, or in combination, succeed in explaining shell maculation in some strictly defined groups (reviewed by Underwood & Sealy, 2002; Kilner, 2006). Many of these hypotheses focus on the visual signal provided by the shell, for example, predator avoidance (Wallace, 1889), mimicry by brood parasites (Davies & Brooke, 1989) or soliciting parental care (Moreno & Osorno, 2003; Hanley, Doucet & Dearborn, 2010). Alternatively, an intriguing hypothesis that focuses instead on the structural function of eggshell pigments has been formulated to explain maculation in an ecologically diverse range of species, where no need for crypsis or egg recognition is apparent (Solomon, 1987; Gosler, Higham & Reynolds, 2005; Higham & Gosler, 2006). The hypothesis is significant in three aspects: firstly,

it highlights a possible new selective mechanism for eggshell pigmentation not based on the signalling function of shell coloration. Secondly, the structural benefits and costs of eggshell pigmentation could greatly clarify the interaction of birds with their environment during reproduction. Finally, based on the structural-function hypothesis, shell pigmentation has been proposed as a potential bio-monitor of environmental pollution (Jagannath *et al*., 2008) and Graveland and colleagues used it as an indicator for shell quality of eggs laid on calcium depleted soils (Graveland *et al*., 1994; Graveland & Drent, 1997). Clearly with these important applications the structural-function hypothesis merits an assessment of its taxonomic and conceptual scope.

The structural-function hypothesis invokes two distinct properties of the avian eggshell, which may be influenced by protoporphyrin maculation. One is the mechanical function of the molecule as a shock absorbing lubricant (Solomon, 1987; Solomon, 1991; Gosler *et al*., 2005), which helps to maintain the structural integrity of the shell. The second aspect is its potential influence on gas conductance across the shell either by causing localized variation of infrared reflectance and temperature induced evaporation (Gosler *et al*., 2005), or by physically blocking pathways through the shell to gas transfer (Higham & Gosler, 2006). Both effects of protoporphyrin (lubrication and reduced evaporation) may be linked indirectly via shell thickness, which can determine shell strength (Ar, Rahn & Paganelli, 1979), as can eggshell density and shape (Picman, 1989; Picman & Pribil, 1997) and influences water vapour conductance across avian eggshells (Ar *et al*., 1974). Both effects have also gained limited observational and experimental support (Jagannath *et al*., 2008; Sanz & García-Navas, 2009, but see Berg, McCormack & Smith, 2009). In particular, birds which potentially experience a shortage of dietary calcium, for example, by breeding on calcium poor soils or because their calcium metabolism is disrupted (Gosler *et al*., 2005; Jagannath *et al*., 2008), seem to display higher levels of protoporphyrin pigmentation.

Protoporhyrin is a tetrapyrrole pigment derived from haem (Wang *et al*., 2009), which is present in the speckles of shells across a wide range of bird species and considered responsible for 'brown' and 'red' colour tones (Kennedy & Vevers, 1976). Its properties include the ability to bind metal ions in the centre of the tetrapyrrole ring (e.g. iron in red blood cells), and strong light reflection in the near infrared (Bakken *et al*., 1978). Furthermore, a certain structural similarity to phthalocyanine, a high temperature ($>800\,^{\circ}C$) lubricant (Krause, Cosgrove & Allen, 1961), has led to the suggestion of a potential 'greasing' function of protoporphyrin that could improve shell strength (Solomon, 1991). Additionally, eggshell speckles are likely to contain biliverdin, a 'blue-green' pigment and, to date, the only other pigment found in the shell. Its functional and visual interactions with protoporphyrin in the eggshell are still unclear.

Based on the physical attributes of protoporphyrin, the structural-function hypothesis has three core tenets: (1) protoporphyrin is deposited specifically in areas where the calcium-carbonate layer is thin, perhaps because it shares a carrier protein with calcium during shell formation (Gosler *et al*., 2005; Jagannath *et al*., 2008); (2) these deposits lubricate the shell and thus increase its ability to withstand external pressure (Solomon, 1987; Gosler *et al*., 2005); (3) they counteract the rise in gas conductance that accompanies shell thinning (Ar *et al*., 1974).

Localized eggshell thinning is likely to reduce structural strength and increase gas conductance (Ar *et al*., 1974). Thus, the observed coincidence between shell thinning and protoporphyrin deposits, that may counteract these effects is intriguing (but see Sanz & García-Navas, 2009) and forms the basis for the structural-function hypothesis (Gosler *et al*., 2005). Previous attempts to confirm the structural-function hypothesis either supported or contradicted earlier findings (Jagannath *et al*., 2008; Berg *et al*., 2009; Sanz & García-Navas, 2009), emphasizing the need for comparative and experimental research to investigate the generality of the hypothesis and its interaction with visual signalling-based explanations of eggshell pigmentation (Cherry & Gosler, 2010).

Here, we study the cryptic eggs of black-headed gulls, *Larus ridibundus* (Tinbergen *et al*., 1962), assessing the concentration of the biliverdin and protoporphyrin pigments in shells with different intensity of speckling. Furthermore we compare the physical structure of speckled areas and their surrounding plain areas. The structural-function hypothesis predicts that speckled areas are thinner and have a higher gas conductance than surrounding plain areas. Previous studies assessed the effect of maculation by comparing measurements taken on whole, intact eggs that differed in their patterns of overall speckling or intensity of speckles. This means it is unclear whether the effects found were caused by the maculation or by other differences between the eggs. By contrast, we were able to assess differences in thickness and water vapour conductance between speckled and plain areas of the same eggshell directly with each other. Taken together, these data highlight the possible functions and costs of eggshell maculation in birds.

Methods

Species

The black-headed gull is a small (200–400 g) gull species breeding in loose colonies on the ground, often in low vegetation, using a shallow scrape lined with some vegetation as a nest (Cramp & Simmons 1983). In these nests, the eggs (mean length $52 \times$ width 37 mm and usually 2–3 per clutch) have been documented previously to be camouflaged (Tinbergen *et al*., 1962), although a large variation in egg colouration exists ranging from a light green to a dark brown base colour with little to intense maculation. Black-headed gulls have a diet rich in calcium as they feed predominantly on terrestrial and aquatic invertebrates and occasionally vertebrates (Cramp & Simmons 1983).

Samples

Eggs were collected under license (Natural England license 20092237 to S. J. P.) in early May 2009 from a black-headed

gull colony of more than 100 pairs in Cambridgeshire, UK, after flooding in heavy rains destroyed all the nests in the colony and caused the parents to abandon their clutches before hatching. No reliable clutch information was available for the eggs to allow specific sampling of clutches. Instead, we selected the eggshells for our analysis from the total of over 300 eggs in a manner that ensured we sampled across multiple clutches: Based on their contents, the eggs were first classified into four developmental stages: (1) fresh; (2) developing yolk; (3) naked embryo; (4) feathered embryo ready to hatch. Ten eggs of each stage were randomly chosen for further analysis. All eggs were cleaned by removing the blunt end with surgical scissors and washing out their contents under laboratory grade running water. Afterwards sample eggs were soaked in de-ionized water to remove any remnant egg contents or external dirt, and dried at room temperature (25 °C) for 2 weeks.

Chemical pigment analysis

Clean fragments (1 cm × 1 cm) of 10 stage 1 black-headed gull eggshells containing both plain and speckled areas were analysed for their protoporphyrin IX and biliverdin content using high-performance reversed-phase liquid chromatography following the protocol of Mikšík *et al.* (1994), to confirm their presence in the eggshells used.

Maculation measurements

Speckles and neighbouring plain areas were determined visually during the course of the thickness measurements but no distinction was made between external and internal pigmentation, that is, following Gosler *et al.* (2005) rather than Jagannath *et al.* (2008). We used this approach because in black-headed gull eggs with their brown base colour it is impossible to determine the vertical location of pigment deposits visually, as seems possible in the white eggs of sparrowhawks. In addition, for 10 eggs each at stage 1 and stage 3 we also quantified the difference in eggshell reflectance between speckled and plain areas in order to confirm our visual choice by an objective measure of their optical properties, using an Ocean Optics USB 4000-UV-VIS Miniature Fiber Optic Spectrophotometer with illumination by a PX2 pulsed xenon light source (Ocean Optics Inc. Dunedin, FL, USA). 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. The opening of the probe was ~1 mm in diameter. Spectra were recorded in ~0.4 nm steps and were expressed relative to a white Ocean Optics WS-1 diffuse reflectance standard. Three independent measurements were taken each from an equatorial speckle and from its surrounding plain area. To minimize instrument error, dark and white standard reflectance calibration measures were taken regularly during sampling.

Reflection curves were truncated across the avian visible wavelength; between 300 and 700 nm (Cherry & Bennett, 2001). An interpolated average was used to calculate an average reflectance locus at 5 nm steps. Brightness (sensu Montgomerie, 2006) was calculated as the total area under the reflectance curve divided by the total wavelength (Fig. 1). Due to the curvature of the eggshell some spectra suffered from the intrusion of external light, which is clearly visible as extreme peaks in the plotted spectra. These spectra were excluded from statistical analysis.

Thickness measurements

For each of the 40 eggs three thickness measurements were taken from maculated and neighbouring plain areas (as judged by a human observer; Fig. 2) at each of five regions along the long axis of the eggs. This represented a total of 30 measurements per egg. The five measurement regions were in sequence: (1) the blunt end; (2) the shoulder area, halfway between the blunt end and the equator; (3) the equator of the egg; (4) the knee, halfway between the equator and the pointed end; (5) the pointed end (Fig. 2). The exact measurement location within these regions was chosen depending on

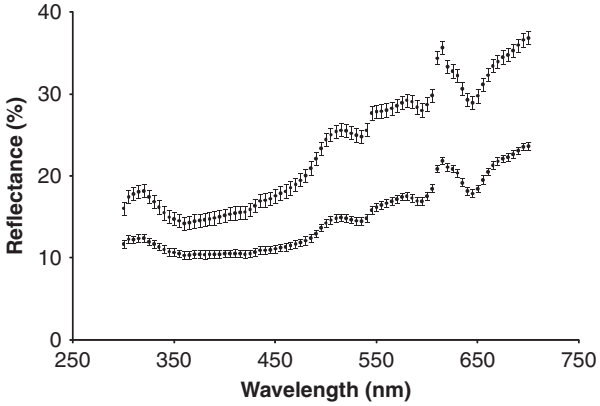

Figure 1 Reflectance spectra of speckles (■) and neighbouring plain areas (□) in the equator region of 10 stage 1 and stage 3 samples each. Error bars represent the ± 1 × SE The squares represent average reflectance (in per cent of total reflectance) for the 20 samples, calculated as interpolated averages in 5 nm steps.

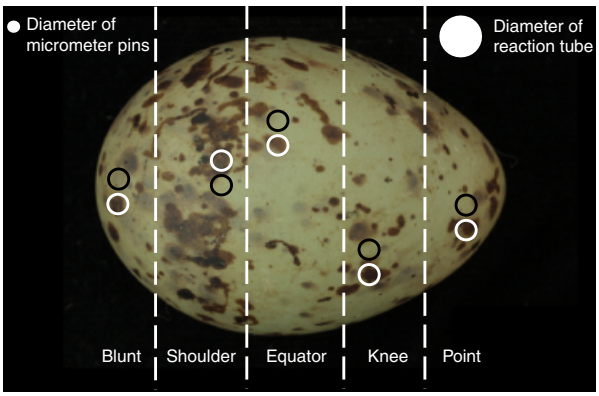

Figure 2 Regions of eggshell thickness measurements and examples of visually chosen maculated (white circles) and neighbouring plain areas (black circles) in a typical black-headed gull, *Larus ridibundus*, egg.

Table 1 Comparison of shell thickness of maculated and neighbouring plain areas measured at five different regions along the long axis of black-headed gull, *Larus ridibundus*, eggshells measured with and without membrane and cuticula (Fig. 2)

	With membrane			Without membrane		
Regions	Maculation	Stage	Maculation × Stage	Maculation	Stage	Maculation × Stage
Blunt	−0.3 ± 0.5	−1.5 ± 1.5	0.4 ± 0.5	0.5 ± 0.4	0.1 ± 1.6	−0.2 ± 0.4
160.1 ± 11.9	$F_{1, 199}$ = 0.31, P = 0.58	$F_{1, 199}$ = 0.94, P = 0.33	$F_{1, 198}$ = 0.73, P = 0.40	$F_{1, 199}$ = 1.41, P = 0.24	$F_{1, 199}$ = 0.01, P = 0.93	$F_{1, 198}$ = 0.16, P = 0.69
Shoulder	0.4 ± 0.6	0.5 ± 0.5	−0.2 ± 0.5	2.0 ± 0.5	−3.8 ± 1.4	−0.6 ± 1.2
171.8 ± 12.1	$F_{1, 199}$ = 0.37, P = 0.54	$F_{1, 199}$ = 0.11, P = 0.74	$F_{1, 198}$ = 0.22, P = 0.64	$F_{1, 194}$ = 16.11, P < 0.01	$F_{1, 194}$ = 6.91, P < 0.01	$F_{1, 193}$ = 1.76, P = 0.19
Equator	0.6 ± 0.6	−1.0 ± 1.9	0.3 ± 0.5	1.3 ± 0.4	−4.1 ± 1.6	0.6 ± 0.4
169.9 ± 13.9	$F_{1, 194}$ = 0.92, P = 0.28	$F_{1, 194}$ = 0.28, P = 0.60	$F_{1, 193}$ = 0.27, P = 0.61	$F_{1, 199}$ = 8.16, P < 0.01	$F_{1, 199}$ = 6.66, P = 0.01	$F_{1, 198}$ = 2.50, P = 0.12
Knee	−2.0 ± 1.6	−5.2 ± 2.6	1.4 ± 0.6	1.4 ± 0.5	−3.5 ± 1.9	0.4 ± 0.4
170.5 ± 14.8	$F_{1, 183}$ = 1.50, P = 0.22	$F_{1, 183}$ = 2.90, P = 0.09	$F_{1, 183}$ = 5.17, P = 0.02	$F_{1, 199}$ = 8.51, P < 0.01	$F_{1, 199}$ = 3.33, P = 0.07	$F_{1, 198}$ = 1.10, P = 0.24
Point	0.4 ± 0.6	−5.7 ± 2.6	−0.8 ± 0.6	0.8 ± 0.5	−5.8 ± 2.0	−0.2 ± 0.4
168.4 ± 11.5	$F_{1, 179}$ = 0.47, P = 0.50	$F_{1, 179}$ = 4.76, P = 0.03	$F_{1, 178}$ = 1.72, P = 0.19	$F_{1, 189}$ = 3.16, P = 0.08	$F_{1, 189}$ = 8.76, P < 0.01	$F_{1, 188}$ = 0.14, P = 0.71

Ten eggs each collected at four different stages of embryonic development were used (see text for details). Least square mean model estimates ± SE of effect on shell thickness are given, followed by their test statistics. The variance explained by the random effect (individual egg ID) was < 0.01 for all locations.

Table 2 Comparison of shell thickness of black-headed gull, *Larus ridibundus*, eggshells collected at an early and late stage of their development (N = 10 eggs each, see text for details) and measured with and without their inner membrane and cuticula at maculated and neighbouring plain areas across five regions of the egg

Locations	Membrane	Stage 1–Stage4	Maculation	Stage × Maculation	Stage × Membrane
Blunt	−28.8 ± 0.9	5.7 ± 3.8	−0.5 ± 0.6	−0.1 ± 1.3	−5.2 ± 1.3
	$F_{1, 217}$ = 2427.14, P < 0.01	$F_{1, 217}$ = 0.66, P = 0.42	$F_{1, 217}$ = 0.70, P = 0.40	$F_{1, 216}$ = 0.01, P = 0.94	$F_{1, 217}$ = 17.02, P < 0.01
Shoulder	−54.7 ± 1.2	−1.3 ± 3.7	1.1 ± 0.8	−0.7 ± 1.7	12.6 ± 1.7
	$F_{1, 217}$ = 3377.37, P < 0.01	$F_{1, 217}$ = 1.92, P = 0.17	$F_{1, 217}$ = 1.59, P = 0.21	$F_{1, 216}$ = 0.18, P = 0.68	$F_{1, 217}$ = 57.14, P < 0.01
Equator	−52.6 ± 1.4	4.1 ± 4.1	0.9 ± 0.9	−1.1 ± 1.9	9.7 ± 1.9
	$F_{1, 211}$ = 2469.59, P < 0.01	$F_{1, 211}$ = 5.09, P = 0.32	$F_{1, 211}$ = 0.93, P = 0.34	$F_{1, 210}$ = 0.33, P = 0.57	$F_{1, 211}$ = 25.70, P < 0.01
Knee	−49.4 ± 1.9	19.5 ± 5.2	1.3 ± 1.2	−2.6 ± 2.3	−7.1 ± 2.5
	$F_{1, 199}$ = 1837.36, P < 0.01	$F_{1, 199}$ = 10.26, P < 0.01	$F_{1, 199}$ = 1.21, P = 0.27	$F_{1, 198}$ = 1.22, P = 0.27	$F_{1, 199}$ = 8.27, P < 0.01
Point	−51.6 ± 1.5	16.5 ± 5.9	0.6 ± 1.4	1.2 ± 2.8	−2.5y3.0
	$F_{1, 188}$ = 1209.67, P < 0.01	$F_{1, 188}$ = 7.85, P < 0.01	$F_{1, 188}$ = 0.17, P = 0.68	$F_{1, 186}$ = 0.19, P = 0.66	$F_{1, 187}$ = 0.70, P = 0.40

Estimates ± SE of effect on shell thickness are given, followed by their test statistics. The variance explained by the random effect (individual egg ID) was < 0.007 for all locations.

the position of suitable maculation. All measurements were first conducted on eggs with the shell membrane attached to the inside of the egg (the so-called outer membrane) intact and then repeated the measurements after the removal of that shell membrane and the external cuticular layer (generally much thinner than the shell membrane) through soaking in a 5% bleach (NaClO) solution overnight. In some samples, the removal of the shell membrane led to the disintegration of already cracked parts of the calcite shell, which made reliable measurements impossible and explains the variation in degrees of freedom in Tables 1 and 2. A small effect of the treatment on the cuticle or the organic component of the calcium layer itself cannot be ruled out but any effect should be similar for maculated and plain areas of the same fragment unless systematic differences in the organic matrix between speckled and plain areas are assumed. Before eggshells were measured, eggs were cut into two equal halves using a Microtorque 2 dental drill (Milnes Bross., Croydon, UK) with an 817T diamond head (Intensive Swiss Dental, Grancia, Switzerland). All measurements were taken to an accuracy of 1 μm using a Mitutoyo Series 227–203 (Mitutoyo, Kawasaki, Japan) constant measurement force micrometer. To enable precise point measurements 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. Shells were placed in the micrometer so that they were at a 90° angle to the pin and measured on three slightly different locations within a speckle or plain area at a measurement force of 1.5 N.

Water vapour conductance

Water vapour conductance of the eggshell strongly correlates with other gas exchange functions, for example, oxygen consumption, but can be determined more simply and reliably on eggshell fragments than conductance of other gases (Paganelli, 1991). In our study, water vapour conductance was measured for 10 samples chosen randomly from all eggs still available of developmental stage 1 and 3 eggs each, to obtain a comparison between early and late stage

eggs. Stage 4 eggs frequently showed partial loosening of the shell membrane and were thus deemed potentially unsuitable for the conductance comparison. Using the dental drill, a maculated and neighbouring plain fragment (*c.* 1×1 cm total size and < 5.5 mm diameter of speckle/plain area covering the tube opening) were removed from the equator region and glued tightly on top of a 250 μL mini test tube (Eppendorf AG, Hamburg, Germany) filled with 200 μL of de-ionized water. The maculated fragment was chosen so that a single speckle or several overlapping speckles covered the opening of the test tube. To measure vapour conductance we used a highly repeatable method (correlation coefficient of subsequent measures > 0.95), developed by Booth & Seymour (1987) and Portugal, Maurer & Cassey (2010), where samples were placed in desiccators in standardized conditions, with a constant amount of desiccant (500 g Silica in a 16.5 L desiccator), and a constant temperature of 25 °C while measuring barometric pressure to allow us to control for its effects when calculating conductance. Over 3 consecutive days they were removed briefly every 24 h for weighing and any mass loss was assumed to be a result of water vapour permeating out of the eggshell. All conductance samples were checked for cracks in the eggshell fragment and discarded if a defect was found, resulting in seven and eight valid samples for stage 1 and stage 3 eggs, respectively. Conductance was calculated from mean daily mass loss as mg day^{-1} $\times$ torr (Ar *et al.*, 1974). Thickness of each fragment was measured as described above.

Statistical analysis

We tested for differences in eggshell brightness between maculated and plain shell areas and stage of development using generalized linear mixed models (GLMM) with individual as a random effect (controlling for replicate measurements per egg). We analysed the difference in thickness for matched (neighbouring) maculated and plain shell areas measured with and without membrane and cuticula, respectively, using a GLMM with thickness as the response variable; maculation, developmental stage and their interaction as explanatory variables and individual egg as a random factor. These analyses were conducted separately for each shell region since initial analysis had shown a strong interaction of egg region with maculation and stage, likely due to differential calcium resorption of the eggshell regions (Castilla *et al.*, 2007; Maurer *et al.*, 2011). The effect of the shell membrane and cuticula on absolute shell thickness in the course of the embryonic development was assessed separately for each shell region in a GLMM with thickness as the response variable; membrane presence or absence, maculation and stage (1 and 4 only) as explanatory variables; and individual as a random effect. The correlation between water vapour conductance and thickness was calculated using Pearson's correlation coefficients. The significance level for all analyses was $\alpha = 0.05$, data are presented $\pm$ SE and analyses were conducted in SAS v9.2 (SAS Institute Inc., Cary, NC, USA).

Results

The black-headed gull eggs used in this study were highly maculated across the length of the egg (e.g. Fig. 2). Visible speckles differed from neighbouring plain areas in that they had lower overall brightness (plain vs. speckled 4.79 ± 0.21 vs. 3.01 ± 0.21; GLMM, $t_{62} = 9.65$, $P < 0.01$; Fig. 1).

Pigment content of shell fragments

The protoporphyrin and biliverdin content of black-headed gull eggshells showed a strong and significant positive correlation ($r = 0.78$, $n = 10$, $P = 0.008$). Furthermore, regardless of the absolute concentration of pigments eggs contained approximately twice as much protoporphyrin as biliverdin (Fig. 3).

Thickness comparison of speckled and neighbouring plain areas

Eggshell thickness, measured with the inside shell membrane and the cuticula in place, did not differ between maculated and neighbouring plain areas for 10 eggshells of each of the four developmental stages, at any stage of embryonic development (Table 1, Fig. 4). However, without shell membrane and cuticula, the eggshell in maculated areas was minimally, but significantly, thinner than in neighbouring plain areas for the three central regions of the eggshell: shoulder, equator and knee (Table 1, Fig. 2, Fig. 4). This difference between plain and speckled areas of the same egg after removal of the membrane and cuticula was greatest in the shoulder area with an average of 2.0 μm (or 1.18% of total

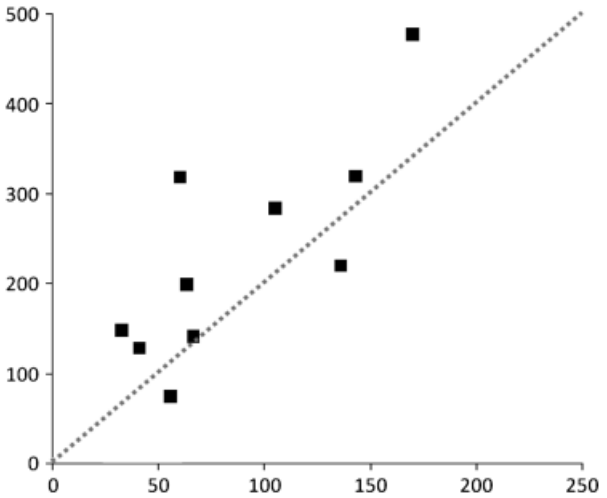

Figure 3 Positive association between Protoporphyrin (*y*-axis) and Biliverdin (*x*-axis) for 10 black-headed gull, *Larus ridibundus*, eggshells (nmol g^{-1} eggshell), extracted from fragments containing speckled and plain areas following the protocol of Mikšík *et al.* (1994). Dotted line is the 2:1 bisect (rather than the line of best fit) indicating that eggs contain more than twice as much, on average, protoporphyrin than biliverdin). The positive correlation is significant ($r = 0.78$, $n = 10$, $P = 0.008$).

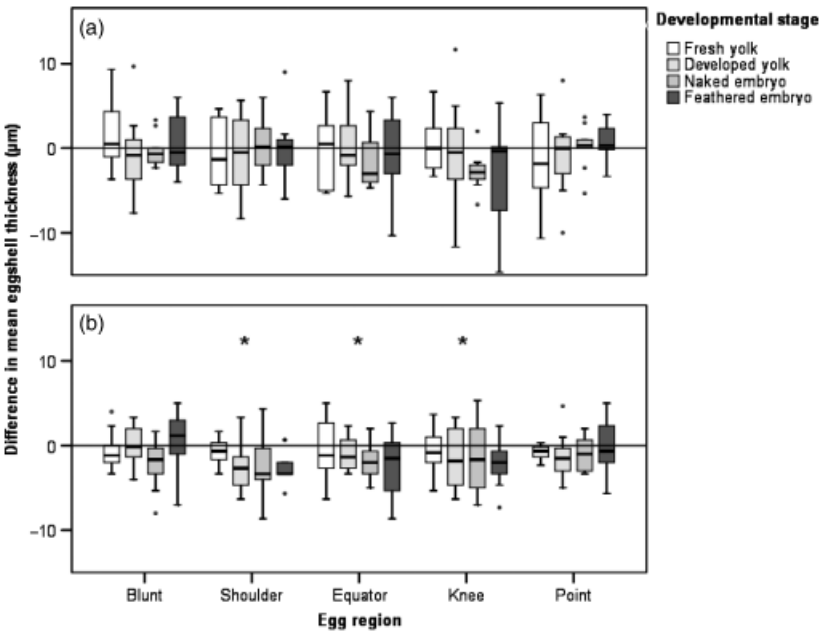

Figure 4 Differences in thickness between maculated and neighbouring plain areas measured at five different locations of black-headed gull, *Larus ridibundus*, eggshells (see Fig. 2) with the membrane and cuticula intact (a) and removed (b). Box plots (with outliers shown as dots) illustrate the mean difference in μm between maculated and plain areas for 10 eggs of each of four embryonic stages from fresh yolk to feathered embryo (see text for details). An asterisk (*) indicates the egg region at which maculated and plain areas differed significantly ($P<0.05$).

shell thickness). Shell thickness, measured on samples without membrane and cuticula, also decreased over the course of embryonic development at all regions except the blunt end and the knee. This decrease occurred across maculated and plain areas similarly as demonstrated by their non-significant interaction terms (Table 1), except the knee area when measured with the membrane and cuticula.

Stage and membrane effects in freshly laid versus ready to hatch eggshells

To explore further the role of embryonic development and the potential of calcium resorption to affect the thickness difference between plain and maculated areas, we directly compared the developmental extremes sampled in this study. This involved a comparison of eggshells from the earliest and the latest stages of embryonic development (stage 1 vs. stage 4), which can be clearly identified even by lay observers inexperienced in avian embryonic development. Furthermore, in future studies, these stages could be sampled without sacrificing the embryo if the eggshells of infertile and newly hatched eggs are sampled. We measured the thickness stage 1 and stage 4 eggshells with and without membrane and cuticula revealed that stage 4 eggs had significantly thinner shells at the knee and pointed end areas (Table 2). The analysis also confirmed and quantifies the expectation that eggshells measured without the membrane should be significantly thinner than the same eggshells with their shell membrane and cuticula intact. These data suggest a smaller effect of membrane removal at the blunt end than in other eggshell areas. The significant interaction between incubation stage and membrane for all regions except the pointed end suggests that overall eggshell thinning over the course of embryonic development is not independent of the membrane thickness. However, the direction of this effect, as indicated by the signs of the estimates (Table 2), is inconsistent across the egg regions. The direct comparison between stage 1 and stage 4 eggs, does not reveal any differential calcium resorption and thus shell thinning between plain and maculated areas in the course of embryonic development. The eggshell thinning observed between the two ages also allows a comparison to published data on embryonic calcium resorption.

Water vapour conductance

A mixed model comparison including eggshells of both stages showed that water vapour conductance of stage 1 (fresh yolk) eggs was lower than that for stage 3 (naked embryo) eggs, but no significant effect of maculation or the interaction between stage and maculation on the water vapour conductance was found (stage: $F_{1,26}=3.03$, $P=0.09$; maculation: $F_{1,26}=0.3$, $P=0.86$; stage $\times$ maculation: $F_{1,26}=1.56$, $P=0.22$). Water vapour conductance in mg day^{-1} $\times$ torr was 0.312 ± 0.049 and 0.249 ± 0.0245 for plain and maculated stage 1 eggs, respectively, and 0.340 ± 0.068 and 0.423 ± 0.070 for plain and maculated stage 3 eggs (Fig. 5). As in the larger sample of eggshells measured with the membrane and cuticula intact above, we found no difference in shell thickness between plain and speckled areas (stage 1, mean thickness difference plain minus maculated fragment: 244.0 ± 7.2–$243.0\pm6.0=1$ μm; $t_6=-0.43$, $P=0.15$, $N=7$; stage 3, mean thickness difference plain minus maculated fragment: 226.4 ± 5.8–$226.2\pm7.5=0.2$ μm; $t_7=-0.04$, $P=0.97$, $N=8$). Shell thickness was not significantly correlated to water vapour conductance for either plain (Pearson's correlation coefficient

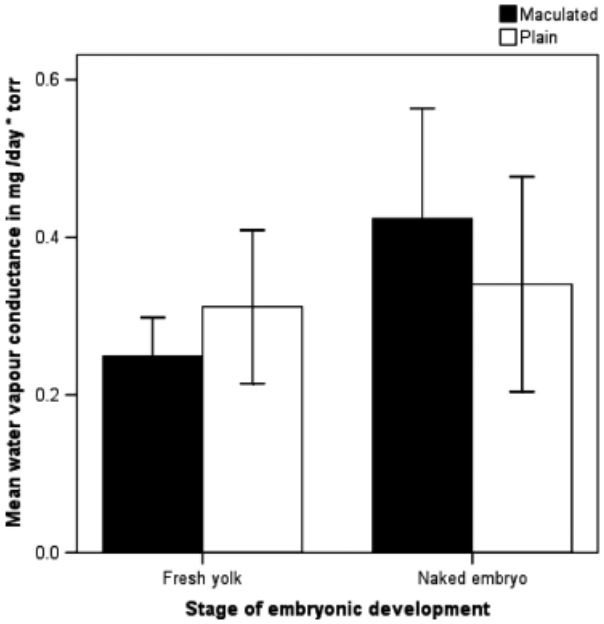

Figure 5 Mean water vapour loss (mg day^{-1} × torr) of maculated and neighbouring plain eggshell fragments at two different stages of embryonic development of black-headed gulls, *Larus ridibundus* (fresh yolk, $N=7$ and naked embryo, $N=8$). Error bars represent ± 2 SE.

$r = 0.39$, $P = 0.15$, $N = 15$) nor maculated fragments of eggshells (Pearson's correlation coefficient $r = -0.09$, $P = 0.75$, $N = 15$).

Discussion

The thinning of speckled areas of the eggshell found in this study on the camouflaged eggs of black-headed gulls appears to match the results previously reported for speckles of great tit and sparrowhawk eggs (Gosler *et al.*, 2005; Jagannath *et al.*, 2008). Eggshell thickness of gull eggs was significantly reduced at the location of visible speckles compared with neighbouring plain areas when the shell membrane and possibly cuticula were removed. Our ability to distinguish speckles from plain areas by human vision alone was confirmed through spectrophotometric measurements. Furthermore, our study confirms that shell thinning persists through different stages of the embryonic development and is found in most regions across the length of the egg, except the blunt end. However, the reduction of shell thickness found in the speckles of black-headed gull eggs was minimal both compared with absolute shell thickness (average 1.2% thinner shell at speckles) and to shell thinning in the course of embryonic growth (average 6.5–11% shell thinning through development; Finnlund *et al.*, 1985; Castilla *et al.*, 2007). Similarly, the effect of maculation on water vapour conductance, apparent in great tit eggs kept in the little controlled conditions of a fridge (Gosler *et al.*, 2005; Higham & Gosler, 2006), could not be detected in our black-headed gull eggs in a more rigorously controlled environment.

Our results thus highlight potential taxonomic or ecological limitations of the structural-function hypothesis. Based on these results, we propose that the protoporphyrin deposits visible in the speckles of black-headed gulls are unlikely to serve a structural function. Instead, thinning of the calcite layer may be a consequence of, rather than the cause for, the observed maculation, if protoporphyrin and calcium competed for the deposition pathway. This effect may occur regardless of the adaptive (e.g. crypsis as shown for gull eggs) or non-adaptive reasons for protoporphyrin deposition in bird eggs. In bird species with underlying calcium shortages and already thin shells, such thinning may even pose a structural cost. The observation of an effect of maculation on shell thickness but not water vapour conductance (maybe driven by the very small absolute values of shell thinning of maculated areas) also leads us to propose that these two aspects of the structural-function hypothesis may not always be linked and should be addressed separately.

Mechanical function (Gosler *et al.* 2005)

We found a correlation between reduced shell thickness and shell maculation. The average reduction, however, never exceeded 2 μm (*c.* 1.2%) compared with the plain neighbouring areas, and clearly is much less than that found in sparrowhawks and great tits (*c.* 8 μm, 3.3% and 6 μm, 7.5%, respectively, Gosler *et al.* 2005; Jagannath *et al.*, 2008) making a mechanical function of shell maculation in black-headed gull eggs unlikely. Interestingly, the small difference in shell thickness is masked (or perhaps compensated for) entirely by the variation in the thickness of the shell membrane and cuticula (Fig. 4). If only internal pigment deposits were related to shell thinning, as in the sparrowhawk (Jagannath *et al.*, 2008), our visual selection of shell speckles may have sometimes missed the speckles, where such internal thinning occurred. However, in great tits, where such a distinction between internal and external maculation is also difficult and has not been made, shell-thinning was still apparent at a much greater scale than in our study. We therefore consider our finding to represent the scale of shell thinning at speckles in black-headed gull eggs accurately. Microscopic analysis of the relationship between shell thinning and location of pigment deposits in this and other species could contribute to the understanding of the structural role of protoporphyrin. Finally, it is important to keep in mind that the assessment of the contribution of pigmentation to eggshell strength, depends on the assumption that in great tits and black-headed gulls shell strength was determined by shell thickness and not other shell characteristics (Picman, 1989; Picman & Pribil, 1997).

In addition to the effect of maculation, our sample allowed us to calculate an estimate of the relative degree of eggshell thinning that occurs between laying and hatching as a consequence of the avian embryo's use of eggshell calcium during its development (Deeming, 2002; Karlsson & Lilja, 2008). Independent of maculation, developmental stage coincided with a reduction of eggshell thickness of up to 19.5 μm (*c.* 11%) suggesting substantial calcium resorption

from the shell by the developing embryo. Less developed eggs, however, also would mostly have been laid later in the season than those containing older emryos. It is thus conceivable that a similar pattern of shell thickness variation may have arisen, if the less developed eggs were laid by females producing eggs with thicker shells due to food availability or their intrinsic shell production capacity. We consider this an unlikely explanation, because of the small time (*c.* 20 days that would have passed between the laying of undeveloped and freshly laid eggs and the probable individual variation in egg production capacity. Finally, in support of developmental shell thinning in black-headed gulls we observe that our values of eggshell thinning are well within the range of those found for other non-passerines (*c.* 7–15%, Finnlund *et al.*, 1985; Castilla *et al.*, 2007; Castilla *et al.*, 2010).

The significant interaction between stage and membrane effects highlights the dynamic changes the calcium shell, the inner membrane and possibly the cuticula undergo in the course of embryonic development, which we discuss in more detail elsewhere (Maurer *et al.*, 2011). Although the differences observed here are between different eggs and vary in their direction, their extent exceeds that of the thickness difference between maculated and neighbouring plain areas. This further supports our argument that differences between these areas may have a negligible effect on egg stability and gas conductance in black-headed gulls.

Gas conductance function (Gosler *et al.* 2005; Higham & Gosler, 2006)

A function of protoporphyrin in the conductance of respiratory gases, based on it blocking the interstices between the calcite crystals of the shell particularly of well-vesiculated passerine eggshells, assumes that it reduces the amount of water vapour that passes through the shell. The dependence of conductance on the distance the gas travels through the eggshell that is the shell thickness (Ar *et al.*, 1974) implies that plain and speckled areas of similar thickness should differ in conductance, if protoporphyrin impedes the progress of gases. In turn this implies that increased conductance due to shell thinning could in part be compensated for by pigment deposits (Higham & Gosler, 2006). We measured water vapour conductance in the equator region, where the reduction in the calcite shell of speckled areas was small (on average $<1\%$) compared with its plain surroundings, and we predicted reduced conductance in speckled areas. However, our measurements detected no difference between plain and speckled areas in fresh eggs or after embryonic development (and further shell thinning). Given the minimal thickness difference between the neighbouring areas, we consider it unlikely that the lack of a difference in gas conductance represents 'perfect' compensation of the effect of shell thinning through pigmentation. Instead we argue that no structural blockage (sensu Higham & Gosler, 2006) of the main routes of gas transfer occurs in black-headed gull eggs for maculated areas of the shell. Our results were obtained on freshly dried and washed rather than fresh eggshells after a 24 h period of acclimatization. While this ensured that no egg white obstructed water vapour conductance and the humidity within the test tube-eggshell setup was constant, small random variation of water vapour conductance as a result of a previously dried inner shell membrane cannot be ruled out and may have affected the power of the statistical analysis.

It is possible that protoporphyrin influences the gas conductance of the shell through its reflective rather than its structural properties (Gosler *et al.*, 2005). Whether possible temperature differences between speckles and plain areas (Bakken *et al.*, 1978) are large or persistent enough to reduce vapour conductance has been questioned (Higham & Gosler, 2006). Consequently, we did not test for this effect specifically.

It may not seem surprising that we find no obvious structural function of maculation in the black-headed gulls, given the established role of pigmentation in eggshell crypsis (Tinbergen *et al.*, 1962). However, the effects of maculation on eggshell thickness and gas conductance in this species are particularly instructive for the understanding of the structural-function hypothesis and in suggesting alternative explanations for these coincidences of shell thinning and maculation.

Small, but probably structurally irrelevant, shell thinning of the speckles supports the idea that protoporphyrin and calcium deposition are not independent of each other, potentially due to competition for the same carrier protein (Solomon, 1987; Gosler *et al.*, 2005) but even in the best known system for the study of egg production, the domestic chicken, *Gallus gallus domesticus*, their exact interaction in the uterus remains to be studied. Furthermore, in domestic chickens only a set amount of time is available for shell formation and deposition periods of calcium and protoporphyrin overlap (e.g. Nys *et al.*, 1991, 1999). Pigment deposition may thus be at the expense of the construction of the calcite shell. This effect of competition could be exacerbated, when calcium for shell formation is in short supply and the ratio of calcium ions to porphyrins in the uterine fluid is skewed. The reasons for such calcium shortages are diverse and can include scarcity of soil calcium (Gosler *et al.*, 2005), environmental pollution (Graveland *et al.*, 1994; Graveland & Drent, 1997; Jagannath *et al.*, 2008), or perhaps individual variation in calcium sequestration in the shell gland. The latter might explain the heritable variation in pigment darkness (i.e. protoporphyrin concentration) in great tits (Gosler, Barnett & Reynolds, 2000), which correlates with shell thickness and conductance (Gosler *et al.*, 2005; Higham & Gosler, 2006). This scenario of protoporphyrin deposition leaves room to interpret maculation, either as an adaptive or a non-adaptive consequence of shell thinning.

The lack of a difference in conductance may reflect differences in the mechanisms of gas transfer between the eggs of black-headed gulls and those of great tits, where the intensity but not the spread of speckling was correlated with reduced conductance (Higham & Gosler, 2006). The eggs of small passerines, including great tits, are distinct in lacking the external layer of vertical calcite crystals, found in larger species and consist almost entirely of the porous

squamosum (Mikhailov, 1997; Gosler *et al.*, 2005). In great tit but not in gull shells, a large proportion of the gas exchange may occur through this porous layer and protoporphyrin deposition in this layer could then greatly influence conductance. Accordingly, the structural-function hypothesis was developed specifically from work on a passerine species (Gosler *et al.*, 2000). Our findings are not in conflict with the idea that the hypothesis may apply only to eggs with particular characteristics of the calcite shell. Rather, our data highlight the need to consider shell type and gas conductance explicitly, when studying the potential function of protoporphyrin speckling.

The basic assumption underlying the structural-function hypothesis is that speckles contain more protoporphyrin pigment than plain areas and has been argued in the original papers (Gosler *et al.*, 2005; Jagannath *et al.*, 2008) and earlier studies on eggshell pigments. The present study did not aim to test this assumption, instead we worked of this assumption and aimed to determine whether the conclusion made in the original papers regarding strength and conductance of speckled areas can be extended easily to species other than those studied originally. It is therefore important to note that the reflectance measurements of eggshell speckled and plain areas corroborate objectively the ability of a human observer to reliable distinguish maculated and plain areas. In particular, while the results of the spectrophotometry seem to suggest higher pigment concentrations in the speckled than in plain areas, they do note confirm this. Similarly, the persistently greater concentration of protoporphyrin than biliverdin and the strong positive correlation of the pigments confirm the presence and importance of protoporphyrin in black-headed gull eggshells but they do not reveal the location of the pigment in the shell. These observations highlight the need to refine the methods of pigment analysis so that the relationship between the reflectance spectra of a maculated or plain area of the shell and its pigment concentration can be determined. Confirming this basic assumption and quantifying the differences in pigment concentrations between maculated and plain areas would remove some of the uncertainty that has plagued conclusions drawn on the basis of the structural-function hypothesis from its outset.

In summary, we recommend that the effects of protoporphyrin postulated by the structural-function hypothesis are best recognized as two separate hypotheses – a *Mechanical Function Hypothesis* and a *Gas Conductance Hypothesis* – because the two may not always be linked. The mechanical-function hypothesis states that protoporphyrin lubricates and strengthens the shell (sensu Solomon, 1987), while the gas conductance hypothesis contends that protoporphyrin impedes the exchange of one or all of the respiratory gases across the shell. In addition, we want to emphasize that the benefits of protoporphyrin maculation for the structural properties of the calcite shell suggested so far (Gosler *et al.*, 2005; Higham & Gosler, 2006) could in other bird species, or contexts, represent a cost for the structural integrity or physiological function of the eggshell; and this aspect of the structural-function hypothesis should form another research focus. Therefore, future studies should use species from a broad taxonomic range of birds to gain an understanding of the extent of the correlation between shell thinning and maculation and the role of dietary calcium in shell pigmentation (Reynolds & Perrins, 2010). Such data are essential to clarify the interaction between structural and signalling functions of shell pigmentation and its influence on the evolution of characteristic patterns of maculation in different bird species.

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