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RESEARCH ARTICLE

Speckled and plain regions of avian eggshells differ in maternal deposition of calcium and metals: A hitherto overlooked chemical aspect of egg maculation

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ABSTRACT

Although it has been strongly implied that studies on structural function might resolve certain conflicting theories regarding the huge variability of coloration in avian eggs, including maculation, the fundamental physiological question about the functional role of eggshell speckling in the maternal deposition of micronutrients into maculated (pigment spots) and plain (unpigmented) eggshell regions remains largely unanswered. We measured (within the same egg) the concentrations of 2 micronutrients, the staple components of avian eggshells (calcium and magnesium), and 6 trace elements (copper, manganese, iron, cobalt, cadmium, and lead) in maculated (a superficial layer of brown pigment) and unpigmented areas of eggshells in 2 groups of Japanese Quail (*Coturnix coturnix japonica*) eggs representing 2 extremes of eggshell coloration: those with darker eggshells and more extensive pigment spots vs. those with bright eggshells and small, more clearly demarcated pigment spots. We found evidence that the concentrations of calcium and magnesium and of 2 trace elements (copper and cadmium) in both types of egg were significantly higher in speckled eggshell areas (where the shells were also significantly thicker) than in unpigmented ones. Conversely, lead (a toxic element) peaked markedly in the plain areas of eggshells compared with the speckled ones, whereas the concentrations of the 3 other trace elements (manganese, iron, and cobalt) measured in the speckled and unpigmented eggshell regions were variable and dependent on egg coloration. Our results give a clear indication that egg maculation can play a functional role in the chemistry of avian eggs, one that enables females to distribute micronutrients and trace elements into pigment spots and unpigmented regions of an eggshell to varying extents. This presumably represents a mechanism permitting prompt physiological adjustment to varying levels of maternal resources. It is possible that elements sequestered into protoporphyrin speckles are not further utilized by developing embryos; this might be a form of physiological chemoprotection through the disposal or deactivation of certain elements in pigment speckles, which do not (or only marginally) contribute to the biochemical processes of embryogenesis.

Keywords: calcium, egg maculation, eggshell thickness, maternal resources, micronutrient allocation, pigments, protoporphyrin pigment spots, trace elements

Las regiones manchadas y no pigmentadas de las cáscaras de los huevos de aves difieren en la sedimentación materna de calcio y metales: un aspecto químico hasta ahora ignorado del proceso de machado de los huevos

RESUMEN

Aunque se ha sugerido fuertemente que estudios sobre la función estructural podrían resolver algunas teorías contradictorias sobre la enorme variabilidad en la coloración de los huevos de aves, incluyendo el manchado, la pregunta fisiológica fundamental sobre el papel funcional de las manchas de las cáscaras de los huevos en la sedimentación materna de micronutrientes en regiones manchadas (áreas pigmentadas) y sin manchas (áreas no pigmentadas) aún no ha sido resuelta. En este estudio medimos (dentro del mismo huevo) las concentraciones de dos micronutrientes, los principales componentes de las cáscaras de huevos de aves (calcio y magnesio) y seis elementos traza (cobre, manganeso, hierro, cobalto, cadmio y plomo) en áreas manchadas (con una capa superficial de pigmento café) y no manchadas de cáscaras de huevo. Hicimos las mediciones en dos grupos de *Coturnix coturnix japonica* que representan dos extremos de coloración de las cáscaras: un grupo con cáscaras más oscuras y manchas de pigmento más extensas vs. un grupo con cáscaras claras y manchas de pigmento más pequeñas y definidas. Encontramos evidencia, en ambos tipos de huevos, de que las concentraciones de calcio y magnesio y de dos elementos traza (cobre y cadmio) fueron significativamente más altas en las áreas manchadas (en donde las cáscaras también fueron

significativamente más gruesas) que en áreas sin pigmentos. Por el contrario, el plomo (elemento tóxico) fue marcadamente más abundante en las áreas sin pigmentos de la cáscara que en las áreas manchadas, mientras que las concentraciones de los otros tres elementos traza (manganeso, hierro y cobalto) variaron dependiendo de la coloración de los huevos. Nuestros resultados dan una indicación clara de que el manchado de los huevos puede jugar un papel funcional en la química de los huevos de aves ya que permite a las hembras distribuir micronutrientes y elementos traza en las áreas pigmentadas y no pigmentadas de las cáscaras en grados variables. Presumiblemente, esto representa un mecanismo que permite un ajuste fisiológico rápido a los niveles variables de recursos maternos. Es posible que los elementos secuestrados en manchas de protoporfirinas no sean utilizados más adelante por los embriones en desarrollo: esta podría ser una forma de quimioprotección fisiológica mediante el desecho o desactivación de ciertos elementos en las manchas de pigmento, que no contribuyen a los procesos bioquímicos de la embriogénesis o lo hacen solo marginalmente.

Palabras clave: asignación de micronutrientes, calcio, elementos traza, espesor de las cáscaras de huevo, manchado de los huevos, manchas de pigmentos de protoporfirina, pigmentos, recursos maternos

INTRODUCTION

There is a growing body of evidence suggesting that no single signaling or structural hypothesis can adequately explain the adaptive significance of eggshell pattern variability among birds, including the presence of maculation (reviewed in Reynolds et al. 2009, Cassey et al. 2011, Deeming 2011, Gosler et al. 2011, Brulez et al. 2016). However, the contents of chemical elements in speckled (pigment spots) and plain (unpigmented) areas of avian eggs, as well as the relationship between eggshell pigmentation and trace-element content, remain virtually unknown (see Mikšík et al. 1996). This is surprising, given the fundamental physiological importance of eggshell micronutrients such as calcium (Ca) and a variety of trace elements, which are maternally transferred into eggs and are indispensable for developing avian embryos (Simkiss 1961, Reynolds 1997, Richards 1997, Miles 2000). It is assumed that eggshells provide female birds an excretion pathway for some trace elements (Burger 1994); consequently, avian eggshells (often only their fragments of various sizes) are used as a tool in biomonitoring studies of environmental pollutants, including some trace elements (Mora 2003, Espín et al. 2014, Hashmi et al. 2015). However, no studies have yet assessed whether Ca and other metals are distributed evenly between pigmented and plain eggshell regions, an aspect hitherto wholly overlooked in methodological recommendations for studying the chemistry of birds' eggs (see Klein et al. 2012, Espín et al. 2014).

Eggshell coloration in birds involves a background base color that is due to 2 pigments (Gorchein et al. 2009), namely protoporphyrin (responsible for "brown" and "red" colors and maculation) and biliverdin (responsible for "blue-green" colors), incorporated into the calcitic layer, and maculation (formed primarily as a layer or layers of protoporphyrin pigment located in different shell areas) that may lie among Ca-carbonate crystals or be associated with accessory shell material (Kennedy and Vevers 1976, Deeming 2011, Brulez et al. 2016). Protoporphyrin (a cyclic

tetrapyrrole) is an immediate precursor of the heme molecule, whereas biliverdin (a linear tetrapyrrole) is a product of hemoglobin breakdown. Protoporphyrin and biliverdin pigments can bind ions of trace metals, primarily iron (Fe) or zinc (Zn), but almost all other metals can be incorporated into their molecules (Ostfeld and Tsutsui 1974, Mikšík et al. 1996, Casini et al. 2003, Williams et al. 2016). Protoporphyrin and biliverdin occur at varying levels in the shells of most birds' eggs, irrespective of their coloration (Mikšík et al. 1996, Cassey et al. 2011, Wang et al. 2011, Brulez et al. 2016), and their distribution varies between the speckled and plain areas of the shells (Cassey et al. 2012). The presence of some trace elements at high levels, such as lead (Pb), copper (Cu), or Fe, can inhibit the biochemical synthesis of both calcite (a staple mineral component of eggshells; cf. Rodríguez-Navarro et al. 2002) and heme (Casini et al. 2003). Female birds in poor physiological condition and suffering a high level of oxidative stress, such as those exposed to pollution, may lay eggs with higher concentrations of protoporphyrin (Moreno and Osorno 2003; see Hargitai et al. 2016a, 2016b, and references therein) or biliverdin, or both these pigments simultaneously (Jagannath et al. 2008, Hanley and Doucet 2012), which translates into the darker pigmentation and greener background color of such eggs, respectively.

Several previous studies have shown that the bioaccumulation of various macrominerals and microminerals, in particular Ca, increases with the intensity of pigmentation (derived from various porphyrin and melanin pigments) of avian feathers (Niecke et al. 1999, Chatelain et al. 2016) and of human hair and skin (reviewed in McGraw 2003). It is therefore assumed that reservoirs of melanin pigments serve as a sink for potentially harmful transition metal ions, adsorbing and harboring the very minerals that helped produce them and thereby offering chemoprotection to adjacent cells and tissues (Larsson 1993, McGraw 2003). A recent investigation into the composition of pigments present in the shells of molluscs and birds' eggs revealed that protoporphyrin pigments occurred in both

these evolutionarily distant groups of animals but that biliverdin was present only in birds' eggshells (Verdes et al. 2015). Another recent investigation of molluscs showed that the more intensive dark pigmentation due to the presence of protoporphyrin increased the content of some trace elements (measured only by scanning electron microscope) like Cu (Williams et al. 2016). We therefore hypothesize that an analogous relationship between the presence of protoporphyrin pigments and the content of macrominerals and microminerals (trace elements) should also apply to the maculation of birds' eggs, as suggested earlier by Mikšík et al. (1996). To date, this presumed relationship between the chemical composition and the role of egg color and presence of maculation appears to tally with the structural function of eggshell coloration (cf. Hanley 2012, Hanley and Doucet 2012). Assuming this relationship to have been generally overlooked to date, we explore it in an attempt to resolve some of the conflicting theories regarding the variation in eggshell coloration in birds (cf. Reynolds et al. 2009, Gosler et al. 2011, Maurer et al. 2011b, Sparks 2011). Of equal importance, although there is a fairly substantial body of knowledge on variation in overall eggshell thickness in different species and orders of birds (Maurer et al. 2012; see Appendix), including embryo-induced eggshell thinning (reviewed in Orłowski and Hałupka 2015), there is no comprehensive information available on how the presence of speckling affects eggshell thickness (including regional variation in a single egg) in different bird species, especially in those whose eggs have a thick surface (= cuticular) layer of pigmentation (Baird et al. 1975).

Based on the above framework, the present study addresses the fundamental physiological question, unexplored in avian biology, of the functional role of maculation or coloration in the maternal deposition of micronutrients and trace elements in eggshells. We aimed to explore 2 major potential effects associated with the presence of eggshell speckling: (1) How does the eggshell thickness in different egg regions vary when speckling is present? (2) Do differences exist in the contents of 2 micronutrients, staple components of avian eggshells (Ca and magnesium [Mg]), and 6 trace elements (Cu, manganese [Mn], Fe, cobalt [Co], and 2 toxic metals, cadmium [Cd] and Pb) between the speckled and unpigmented areas of an eggshell? To attempt to find an answer to these questions, we used the brown-spotted cryptic eggs of the Japanese Quail (*Coturnix coturnix japonica*; hereafter "quail"). Quail eggs represent a coloration typical of the egg crypsis of ground-nesting birds, with apparently distinct brown (protoporphyrin) cuticular pigment spots or blotches on the eggshells (Baird et al. 1975, Soh et al. 1993, Duval et al. 2013). It should be stressed that in quails, egg speckling is the result of a superficial layer of brown pigmentation (a

feature distinguishing other bird species studied in the context of the relationship between eggshell thickness and brown speckling; see above), which occurs immediately (~3 hr) before oviposition and is derived from pigment granules in the apical cells of the shell gland (Woodard and Mather 1964, Tamura et al. 1965, Baird et al. 1975). Quail eggs have been used as a model in many previous experimental investigations to examine the maternal and environmental effects of variation in egg coloration in the context of the deposition of 2 basic eggshell pigments and to analyze the sources of variation in egg coloration patterns and pigment spot distributions. Female quails can promptly adapt the coloration of their eggs both to body condition (more heavily maculated eggs are laid by females with a poorer body condition; Duval et al. 2013, 2016) and to darkly colored substrates (Lovell et al. 2013); such eggs are characterized by a greater hatchability and lower embryonic mortality (Taha 2011, Hassan et al. 2013). Very recently, Duval et al. (2016) reported some conflicting results on the role of speckling patterns in the accumulation of brown pigment in quail eggshells; they found that eggshell spot coverage was negatively correlated with the protoporphyrin concentration measured in entire eggshells, which suggests that protoporphyrin deposition does not increase the amount of visible brown spotting on the eggshell. It must be noted, however, that despite such multidimensional scientific interest in describing the causes or effects of variation in quail egg coloration, no data are available on variability in eggshell thickness in relation to the presence of maculation. This confirms our inference regarding the functional role of speckling, such as a presumed thickening of the shell at speckles due to accumulation of dark pigment.

METHODS

Study Bird Species, Housing, and Diet

The quail eggs we used were collected from a commercial flock bred at the Department of Poultry Breeding, Wrocław University of Environmental and Life Sciences. During the laying period, the females were kept indoors in a 3-tier colony cage, under controlled environmental conditions (temperature 21–22°C; 16 hr light:8 hr dark). There were 5 cages at each level, with 16 quails in every cage (240 birds in all). The 14% slope (8° below horizontal plane) of the cages' wire mesh floors allowed the eggs to roll down immediately after oviposition. Water and commercial feed for the laying quails were available ad libitum. The basic chemical composition of the feed was as follows: 88% dry matter; 190.1 g kg⁻¹ raw protein; 35.1 g kg⁻¹ crude fiber; 64.4 g kg⁻¹ raw fat; 11.4 MJ total metabolic energy; and mineral contents comprising Ca (33.1 g kg⁻¹), phosphorus (7.88 g kg⁻¹), selenium (0.103 g

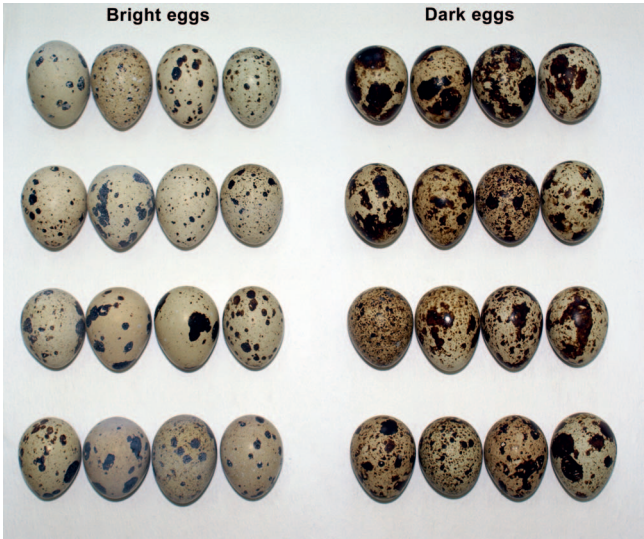


FIGURE 1. Japanese Quail eggs used in our analysis of the chemical composition of eggshells at pigment spots and in unpigmented areas. The eggs were classified into 2 groups based on (only) visual estimates of eggshell color and the size of the black–brown protoporphyrin pigment spots, representing 2 extremes of eggshell coloration: (1) “bright eggs,” those with light brown shells with relatively small and more sharply demarcated pigment spots; and (2) “dark eggs,” those with dark brown shells and more extensive pigment spots. Note that dark eggs were nearly 7% heavier and 2% wider, on average, at the equator than bright eggs (a significant difference; see Table 1).

kg^{-1}), and vitamin E (70.9 mg kg^{-1}). The daily consumption of feed was $25\text{--}30 \text{ g bird}^{-1}$.

Egg Selection

No special breeding or feeding (dietary) condition was applied before the eggs used in the study were collected. The eggs were selected from a large sample (~ 600) that had been laid by different females ($n = 240$) between July 3 and 5, 2015. The eggs were collected 3 or 4 times a day and immediately transferred to a refrigerator (at 3°C), where they were stored prior to further processing.

We selected 2 smaller subsamples of eggs for the chemical analysis of the pigment spots and unpigmented areas of the eggshells, reflecting the 2 extremes of color and extent of maculation of the quail eggs: (1) poorly pigmented eggs ($n = 30$), defined as those with light brown shells, with relatively small, better-demarcated pigment spots (hereafter “bright eggs”); and (2) heavily pigmented eggs ($n = 30$), defined as those with dark brown shells and more extensive pigment spots (hereafter “dark eggs”). The rationale for selecting these 2 distinct groups was based on the possibility that more marked differences (both in morphological features and chemical composition) existed between them (Figure 1).

General description of size-related traits of eggs. In September 2015, each egg ($n = 60$) was weighed to the nearest 0.001 g (Ohaus No. CT600-S); in addition, the length, the breadth at the equator (or at the shoulder, the broadest part of the shell), and the breadth at the geometric halfway point between the sharp and blunt poles were measured. Then each egg was opened and its contents poured out. Using a micrometer gauge, one of the authors (P.P.) measured (to the nearest $10 \mu\text{m}$) the thickness of the eggshells along with the adjacent shell membrane at randomly chosen point(s) in 3 egg regions: the sharp pole and the blunt pole (each measured only once) and the equator (4 measurements, subsequently averaged for further calculations). These measurements are listed in Table 1.

Eggshell thickness at pigment spots and in adjacent unpigmented areas. Further measurements were made (by P.P.) to precisely assess the relationship between the presence of pigment spots and eggshell thickness. The eggshell thicknesses at the pigment spot and in the adjacent unpigmented area (the latter always located in the immediate vicinity up to $\sim 1 \text{ mm}$ from the pigment spot) were measured in pairwise fashion in 3 egg regions: the sharp pole, the blunt pole (2 paired measurements for each, i.e. at the pigment spot and the adjacent unpigmented area), and the equator (4 paired measurements, subsequently averaged for further calculations). Figure 2 and Table 1 summarize these measurements.

TABLE 1. Comparison of size-related traits (means \pm SE) of dark eggs ($n = 30$) and bright eggs ($n = 30$) of Japanese Quail used in the chemical composition of eggshell maculation (pigment spots) and adjacent unpigmented areas.

Egg-size trait (unit)	Dark eggs	Bright eggs	Test of difference ^a	
			<i>t</i>	<i>P</i>
Mass (g)	11.16 ± 0.13	10.47 ± 0.19	2.94	0.005
Length (mm)	32.70 ± 0.19	32.16 ± 0.24	1.78	0.081
Breadth at equator (mm)	25.46 ± 0.13	24.98 ± 0.16	2.38	0.021
Breadth in geometric middle (mm)	24.95 ± 0.12	24.58 ± 0.16	1.87	0.067
Eggshell thickness at equator (mm)	0.234 ± 0.003	0.229 ± 0.005	0.98	0.333
Eggshell thickness at sharp pole (mm)	0.261 ± 0.007	0.248 ± 0.008	1.22	0.227
Eggshell thickness at blunt pole (mm)	0.249 ± 0.005	0.252 ± 0.008	−0.32	0.747

^a Independent-samples *t*-test.

Eggshell Treatment

For the chemical analysis, each eggshell (with the adjacent membrane) was air dried to constant mass, and from each one all the areas of pigment spots were accurately cut off from the remaining unpigmented area. This laborious task was performed by the technical staff of the Department of Hydrobiology and Aquaculture, Wrocław University of Environmental and Life Sciences, using surgical instruments (tweezers, scissors, and scalpel). We tried to separate all visible pigment spots larger than ~1 mm in diameter. In the case of the less strongly maculated (i.e. bright) eggs, such a procedure in practice yielded homogeneous and distinct eggshell samples containing unpigmented areas and dark, pigmented areas (spots). With regard to some heavily maculated (dark) eggs, however, the samples from the unpigmented eggshell region also contained some tiny pigment spots less than ~1 mm in diameter. Consequently, from each group of eggs (i.e. dark eggs and bright eggs; $n = 60$), we obtained 2 paired samples of eggshells from the pigmented and unpigmented regions ($n = 120$ in total). These were then chemically analyzed.

Chemical Analysis

Two of the authors (W.D. and P.P.) performed the chemical analysis at the Department of Hydrobiology and Aquaculture, Wrocław University of Environmental and Life Sciences. The eggshell samples were mineralized in nitric acid in a high-pressure microwave digestion system (MARS-5; CEM, Matthews, North Carolina, USA). Flame atomic absorption spectroscopy (SpectrAA FS220; Varian, Palo Alto, California, USA) was then used to determine the content of 2 micronutrients (Ca and Mg) and 6 trace elements (Cu, Mn, Fe, Co, Cd, and Pb). The measurement process was validated using reference material DORM-3 (fish protein) provided by the National Research Council of Canada Institute for National Measurement Standards (Ottawa, Ontario, Canada). The mean recoveries in 6 replicates of certified material (0.9 g) expressed the precision of the method, or the difference between the mean value obtained by analyzing a certified reference material and its certified value for Cu, Fe, Cd, and Pb performed on the same sample; this was within 5% of the concentrations stated for the reference material. All metal concentrations were expressed in milligrams per kilogram (mg kg^{-1}) or parts per million (ppm) of dry mass.

Statistical Analysis

Our first aim was to assess the differences between the concentrations of elements measured in the pigment spots and unpigmented areas of eggshells in the 2 predefined egg types representing 2 extremes in eggshell coloration: those with dark shells and more extensive pigment spots vs. those with bright shells and small but more sharply demarcated pigment spots. We used the dependent-samples t -test for

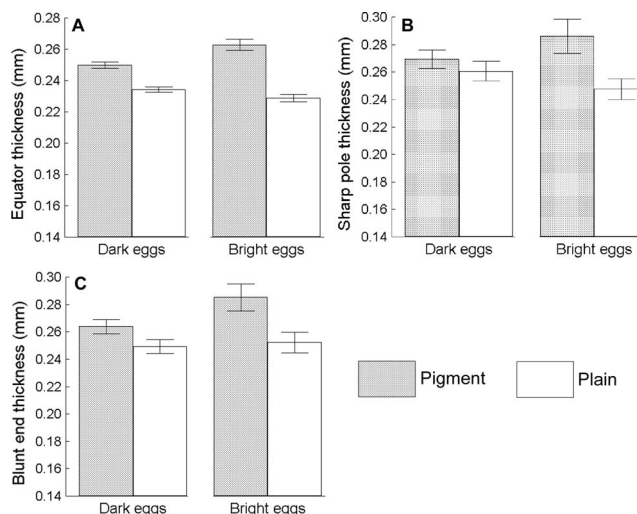


FIGURE 2. Results (means \pm SE) of statistical analysis (dependent-samples t -test) comparing eggshell thickness at pigment spots and in adjacent unpigmented (plain) areas of “dark eggs” ($n = 30$) and “bright eggs” ($n = 30$) of Japanese Quail, measured in 3 egg regions: (A) equator (dark: $t = 12.1$, $P < 0.0001$; bright: $t = 6.74$, $P < 0.0001$); (B) sharp pole (dark: $t = 0.86$, $P = 0.398$; bright: $t = 1.95$, $P = 0.064$); and (C) blunt pole (dark: $t = 3.68$, $P < 0.001$; bright: $t = 3.95$, $P < 0.001$). No significant differences were found between dark and bright eggs in eggshell thickness at the pigment spots or in the unpigmented eggshell areas in the 3 egg regions (independent-samples t -test, $P \geq 0.068$).

pairwise comparisons of the concentration differences of the 8 elements in the pigment spots and the unpigmented areas in each of the 2 egg types, as well as in pooled samples of dark and bright eggs. We also performed an analogous pairwise comparison for the eggshell thickness data determined for pigment spots and adjacent unpigmented areas measured in 3 egg regions: equator, sharp pole, and blunt pole.

Secondly, using the independent-samples t -test, we assessed the differences between dark eggs and bright eggs with respect to (1) the concentrations of elements measured in eggshell pigment spots, (2) the concentrations of elements measured in unpigmented eggshell areas, and (3) general size-related morphological traits of eggs.

All the concentrations of elements, eggshell thickness data, and size-related traits of eggs met the assumption of normality; the use of parametric statistics was therefore justified. The statistical analyses were done using Statistica 7.0 (StatSoft, Tulsa, Oklahoma, USA) and Excel software. The statistical significance level was 0.05.

RESULTS

The shells of the dark eggs (Figure 1) were significantly (7% and 6% on average, respectively) thicker at pigment spots than in adjacent unpigmented areas at their equators and blunt poles (Figure 2). In bright eggs (Figure 1), however,

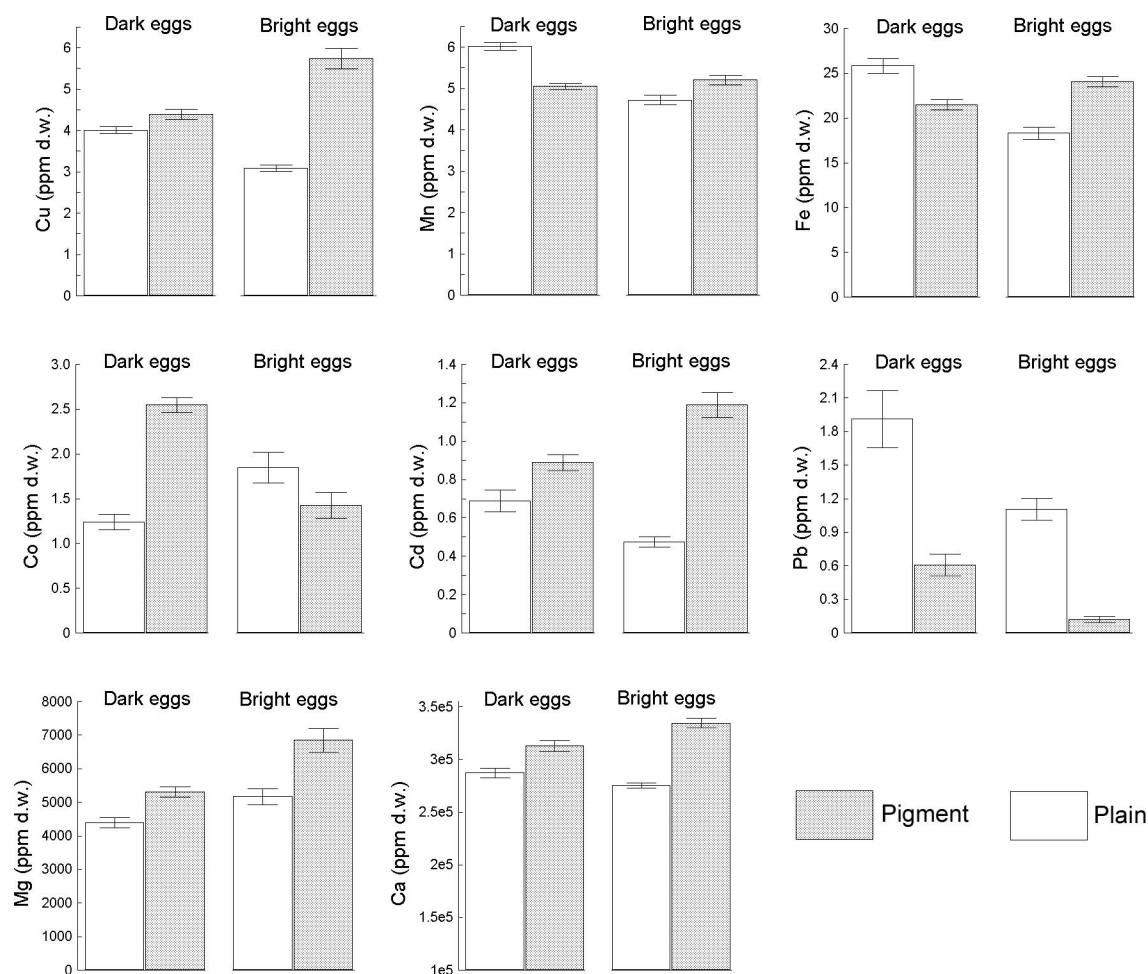


FIGURE 3. Concentrations (means \pm SE) of 8 chemical elements measured in the pigment spots of eggshells and unpigmented (plain) eggshell areas of “dark eggs” ($n = 30$) and “bright eggs” ($n = 30$) of Japanese Quail. See Figure 1 for examples of eggs in both groups; Appendix Table 3 lists the concentrations for both types of eggs.

these differences were even more pronounced; eggshells were, on average, 14% and 13% thicker at pigment spots than in adjacent unpigmented areas at their equators and blunt poles, respectively (Figure 2).

Chemical analysis showed that the concentrations of Ca and Mg (and also of Cu and Cd) in the shells of both dark and bright eggs were significantly higher in the pigment spots than in unpigmented areas (Figure 3; Appendix Table 3). Specifically, Cu levels were 10% and 85% higher, on average, in the pigment spots than in unpigmented areas of dark and bright eggs, respectively; the corresponding average values for the other 3 of these elements in each group of eggshells were 30% and 148% for Cd, 21% and 33% for Mg, and 9% and 21% for Ca (Figure 3). The opposite pattern was observed in the case of Pb, which appeared to prevail in the plain areas of eggshells compared with the pigment spots: 213% and 817% lower in the pigment spots of dark and bright eggs, respectively (Figure 3).

The concentrations of the other 3 elements (Mn, Fe, Co) measured in pigment spots and unpigmented areas were variable and depended on the egg type. In dark eggs, Mn and Fe levels were 19% and 20% lower, respectively, at pigment spots than in unpigmented regions; but in bright eggs, Mn and Fe levels were 10% and 31% higher, respectively, at pigment spots than in unpigmented areas (Figure 3). Co levels varied significantly only in dark eggs, pigment spots containing 106% more Co than unpigmented areas (Figure 3; Appendix Table 3).

Furthermore, we found significant differences between dark and bright eggs with regard to the concentrations of all the elements measured in unpigmented regions (Table 2). The levels of 6 (Cu, Mn, Fe, Cd, Pb, and Ca) were higher in the dark eggs than in the bright ones, whereas the concentrations of the other 2 (Co and Mg) peaked in bright eggs (Figure 3).

Similarly, there were significant differences between dark and bright eggs in the concentrations of all elements

TABLE 2. Results of the statistical comparison (independent-samples *t*-test) of concentrations of 8 chemical elements (ppm dry mass) between pigment spots (maculation) on the shells of dark and bright eggs, and between unpigmented shell areas of dark and bright eggs determined for 60 eggs of Japanese Quail. For the element concentrations, see Figure 3 and Appendix Table 3.

Element	Plain area (<i>n</i> = 60)		Pigment spot (<i>n</i> = 60)	
	<i>t</i>	<i>P</i>	<i>t</i>	<i>P</i>
Cu	7.83	<0.0001	−4.83	<0.0001
Mn	8.96	<0.0001	−1.16	0.251
Fe	6.91	<0.0001	−3.12	0.003
Co	−3.18	0.002	6.81	<0.0001
Cd	3.39	0.001	−3.90	<0.001
Pb	2.97	0.004	4.84	<0.0001
Mg	−2.73	0.008	−4.05	<0.001
Ca	2.21	0.031	−3.08	0.004

except Mn measured at pigment spots (Table 2). Thus, the pigment spots of bright eggs had higher Cu, Fe, Cd, Mg, and Ca concentrations but lower Co and Pb levels in comparison with dark eggs (Table 2). Notably, we found that the plain areas in dark eggs contained 4% more Ca than the same areas in bright eggs, a significant difference; conversely, the pigment spots of bright eggs contained 7% more Ca than pigment spots of dark eggs (Figure 3), also a significant difference (Table 2).

DISCUSSION

Our results demonstrate for the first time that eggshells of maculated avian eggs are not chemically homogeneous and that there are significant differences between speckled and unpigmented eggshell regions in the maternal deposition of several chemical elements. In particular, we found evidence that the concentrations of 2 micronutrients (Ca and Mg) and 2 trace elements (Cu and Cd) were significantly higher in speckled eggshell regions (which were also significantly thicker) than in their unpigmented counterparts. Some earlier studies showed a strong association between the availability of dietary or environmental Ca and eggshell maculation, implying that Ca shortages resulted in localized eggshell thinning (see Cherry and Gosler 2010, Hargitai et al. 2016b), but no analyses of Ca concentrations in speckled eggshell regions have previously been undertaken. We found that the maculated eggshell regions were thicker, and they contained significantly more Ca than the unpigmented areas, which suggests parallel cellular transport of protoporphyrin at high Ca levels (but see Maurer et al. 2011a). Our results contradict those of previous investigations, probably because we selected a species with a different eggshell pigmentation structure—quail eggs have a thick superficial

layer, compared to some other birds' eggs that have a thin internalized or superficial layer (cf. Duval et al. 2016). However, it seems that a thick superficial pigment layer (such as in the quail) does not contradict the concept that maculation strengthens the eggshell (Gosler et al. 2005).

It is highly probable that higher levels of 4 elements (Ca, Mg, Cu, and Cd) in maculated eggshell areas indicate a close association of these metals with the presence of the protoporphyrin or dark pigmentation responsible for the brown coloration. On the other hand, our chemical analysis involved both the pigment layer (approximately 10–20% of the eggshell thickness) and the other inner layers of eggshell plus the shell membrane without visible brown pigmentation, showing that protoporphyrin pigment was present in different concentrations in all these layers (Tamura and Fujii 1967). Hence, this is probably itself a confounding factor and would have an influence on the levels of certain elements in an entire eggshell sample. This applies in particular to Ca and Mg, whose levels are not homogeneous throughout the shell thickness (Abdel-Salam et al. 2006). Conversely, another toxic element (Pb) peaked markedly in unpigmented eggshell areas (where its level was nearly 9× higher than in speckled areas), and this metal may well be more closely bound to biliverdin or, alternatively, to the deeper unpigmented and more calcified eggshell region.

We obtained conflicting results for 3 other trace metals (Mn, Fe, and Co), the concentrations of which were variable and dependent on egg coloration. In quail eggshells, a relatively large amount of protoporphyrin is also deposited in the unpigmented shell region (Tamura and Fujii 1967), particularly in those eggs with a darker background color (Duval et al. 2013), and/or is visible as a small pigment inclusion on the outer shell surface (Lovell et al. 2013). In females, the concentrations of both protoporphyrin (measured in the uterus) and biliverdin (measured in the shell gland) were independent of egg coloration (Baird et al. 1975, Zhao et al. 2006, Liu et al. 2010; see Sparks 2011). Hence, there may be an equilibrium (i.e. a balanced distribution of a female's resources) between speckled and plain eggshell regions. Such an explanation is supported by our findings that the maculation of bright eggs (significantly lighter and narrower at the equator than dark eggs) has disproportionately thicker eggshells (presumably due to the greater accumulation of pigment; Figure 2) than the speckles of dark eggs. This is a novel and important finding with regard to the developmental biology of the quail (and presumably other Galliformes), suggesting that protoporphyrin may also be accumulated by way of an increase in eggshell thickness. Presumably, therefore, the absolute pigment content cannot be evaluated correctly by any attempt at a visual description of an eggshell's external pigmentation (e.g., Brulez et al. 2014, Pike 2015). It thus

appears that this overlooked mechanism of pigment deposition through a thickening of its layer can better explain some conflicting results published earlier—for example, that the greater number of visible brown spots on quail eggshells is not due to enhanced protoporphyrin deposition (Duval et al. 2016). It should also be borne in mind that, apart from the elements or contaminants deposited in eggshells, a portion of these maternal resources is accumulated in the egg contents; a positive relationship has been reported between the level of pesticide residues measured in egg contents and the greenness of the ground color related to biliverdin pigment (Jagannath et al. 2008, Hanley and Doucet 2012).

Our findings imply that speckling can play a functional role in the chemistry of avian eggs by enabling females to variously distribute micronutrients and trace elements into pigment spots and unpigmented regions of eggshells; this presumably represents a mechanism by which they can make prompt physiological adjustments to varying levels of maternal resources. There is an urgent need to investigate how maculation affects eggshell thickness and the levels of micronutrients or trace elements in eggs varying in the location or thickness of the pigment layer, especially in the context of the embryonic depletion of eggshell micronutrients and trace elements. We strongly recommend studies aimed at resolving the still open question of how maternal resources (i.e. micronutrients, trace elements, and pigments) allocated in pigment speckles and in different eggshell layers contribute to embryonic development during their depletion (Orłowski et al. 2016), especially in view of the fact that embryo-induced shell thinning at pigment speckles is minimal compared to that in plain eggshell regions (Maurer et al. 2011c) and that the innermost (mamillary) layer of an eggshell is eroded or decalcified during embryonic development (Karlsson and Lilja 2008, Igić et al. 2017). This suggests that certain elements sequestered into eggshell pigment speckles (especially in those located in the upper eggshell layers not subject to decalcification) may not be further utilized by developing embryos. This might be a form of physiological chemoprotection through the disposal or deactivation of certain metals, transferred from females' bodies to egg speckles, which do not (or only marginally) contribute to embryogenesis. Finally, because the speckling or coloration of eggshells, a consequence of putative pigment deposition, affects Ca and metal levels in eggshells with an outer pigment layer, follow-up studies are necessary on another species that varies in the position of pigment layers. In this respect, we strongly recommend the use of analytical methods that enable elemental concentrations in eggshell micro-samples to be determined; isolated pigment spots or speckles or even powdered pigment scratched off an egg permits an unequivocal relationship to be found between maternal

allocations of co-occurring eggshell elements and pigments.

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APPENDIX

The Structural Function of Eggshell Maculation: A Short Review and Research Problems

Previous studies have suggested that the function of eggshell maculation is, first and foremost, a structural one related to Ca availability (Solomon 1991, Gosler et al. 2005, 2011, García-Navas et al. 2010), in that pigment spots (i.e. the maculated eggshell area) demarcate thinner areas of the shell (reviewed by Cherry and Gosler 2010). Such localized eggshell thinning at pigment spots has been reported in a few species in the Passeriformes (Gosler et al. 2005), Charadriiformes (Maurer et al. 2011a, 2011c, Bulla et al. 2012), and Falconiformes (Jagannath et al. 2008). This phenomenon has been invoked as evidence for the structural function of maculation in the mechanism compensating for Ca deficiency and/or strengthening the eggshell (see Cherry and Gosler 2010). However, there is an important, hitherto overlooked, issue directly linked to this physiological association between eggshell thinning and the presence of maculation—namely, the distribution and volume or thickness of the protoporphyrin layer over the eggshell surface (see Harrison 1966, Tyler 1969; discussed in Maurer et al. 2011b, Sparks 2011), a generality that must be universally applicable to all birds (cf. Reynolds et al. 2009). Maurer et al. (2011b) summarized evidence for eggshell maculation in 7 orders of birds. However, the distribution of the protoporphyrin layer(s) is not uniform and varies between different orders or even within a single egg. The spots of dark pigment may be present in the surface layer of the eggshell and/or at varying depths within the eggshell; in the latter case, spots may not be visible on the surface (Harrison 1966, Tyler 1969, Jagannath et al. 2008). Hence, not all maculation may have a structural function (Cherry and Gosler 2010, Maurer et al. 2011b). For instance, the darker pigment in Eurasian Sparrowhawk (*Accipiter nisus*) eggs

occurs as a layer on the shell surface and as an internal one within the shell; but only the latter was found to demarcate the thinner eggshell area and to be correlated with the level of environmental contaminants or pesticide residues (DDT metabolites; Jagannath et al. 2008).

APPENDIX TABLE 3. Comparison of mean concentrations (95% confidence interval) of 8 chemical elements (ppm dry mass) measured in the pigment spots and unpigmented areas of eggshells reported in dark eggs, bright eggs, and all eggs of Japanese Quail, with results of pairwise comparisons (dependent-samples t-test) for the same eggs. For the differences between the concentrations of elements in plain areas and between the pigment spots of dark and bright eggs, see Table 2. Results in bold are insignificant.

Element	Dark eggs (n = 30)				Bright eggs (n = 30)				All eggs (n = 60)			
	Plain area	Pigment spot	t-test		Plain area	Pigment spot	t-test		Plain area	Pigment spot	t-test	
Cu	4.01 (3.83–4.19)	4.39 (4.14–4.64)	t = -3.2 P = 0.003		3.09 (2.93–3.25)	5.73 (5.22–6.25)	t = -10.5 P < 0.0001		3.55 (3.38–3.72)	5.06 (4.74–5.39)	t = -7.5 P < 0.0001	
Mn	6.02 (5.83–6.20)	5.05 (4.90–5.20)	t = 7.1 P < 0.0001		4.72 (4.49–4.95)	5.21 (4.98–5.44)	t = -3.0 P = 0.006		5.37 (5.15–5.59)	5.13 (4.99–5.27)	t = 1.7 P = 0.096	
Fe	25.8 (24.1–27.5)	21.5 (20.3–22.6)	t = 3.7 P = 0.001		18.3 (16.9–19.7)	24.0 (22.8–25.3)	t = -5.2 P < 0.0001		22.1 (20.6–23.5)	22.8 (21.9–23.6)	t = 0.7 P = 0.512	
Co	1.24 (1.06–1.42)	2.55 (2.38–2.72)	t = -10.5 P < 0.0001		1.85 (1.50–2.19)	1.43 (1.13–1.72)	t = 1.6 P = 0.121		1.54 (1.34–1.75)	1.99 (1.77–2.21)	t = -2.4 P = 0.018	
Cd	0.69 (0.57–0.81)	0.89 (0.80–0.97)	t = -2.4 P = 0.025		0.48 (0.42–0.53)	1.19 (1.06–1.32)	t = -9.7 P < 0.0001		0.58 (0.51–0.65)	1.04 (0.95–1.12)	t = -7.0 P < 0.0001	
Pb	1.91 (1.39–2.43)	0.61 (0.41–0.81)	t = 4.9 P < 0.0001		1.10 (0.91–1.30)	0.12 (0.06–0.18)	t = 9.8 P < 0.0001		1.51 (1.22–1.80)	0.36 (0.25–0.48)	t = 8.1 P < 0.0001	
Mg	4.389 (4.071–4.707)	5.312 (5.003–5.621)	t = -5.5 P < 0.0001		5.166 (4.678–5.655)	6.850 (6.138–7.562)	t = -5.6 P < 0.0001		4.778 (4.477–5.078)	6.081 (5.655–6.507)	t = -7.3 P < 0.0001	
Ca	287,196 (277,642–296,750)	312,926 (302,273– 323,580)	t = -4.1 P = 0.0003		275,541 (270,500–280,582)	334,567 (324,934– 344,199)	t = -10.6 P < 0.0001		281,368 (275,913– 286,823)	323,746 (316,232– 331,261)	t = -8.9 P < 0.0001	