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ORIGINAL ARTICLE

The pattern of distribution and interaction of metals and calcium in eggshells and egg contents in relation to the embryonic development of eggs in a small passerine bird

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Abstract Differences in element concentrations have been observed between embryonated and non-embryonated eggs of the Eurasian Reed Warbler *Acrocephalus scirpaceus*, presumably due to the resorption of calcium and other elements from the shell. To investigate this difference, we tested the hypotheses that (1) the distribution of elements in eggshells and egg contents, (2) the individual element–element relationships between shells and contents, and (3) the relationships among the elements in eggshells and egg contents vary between the two egg samples. Based on the levels of 11 elements (Cr, Cu, Ni, Cd, Pb, Mn, Fe, Co, Zn, Mg and Ca) in Reed Warbler eggs, our results confirmed the second and third hypotheses. Importantly, we found that embryonic development tended to blur the lines between two types of relationships: (1) between elements in eggshells and egg contents through an increase in the number of significant correlations, some of which may be enhanced by high levels of certain elements (Cd, Co, Pb, Zn are indicative of trace metal pollution in fish ponds); (2) among the various elements in eggshells and egg contents, as demonstrated by the differential grouping of elements in principal components analysis (PCA). The consistent positive direction of relationships for almost all elements in both the shells and contents of eggs is presumed to result from the initial parallel co-sequestration of all the elements which accompanies intensive calcium transfer from the female body into the incipient egg. Our study highlights the fact that only limited inferences can be drawn from the interactions of metals and calcium in eggshells and egg contents if the fertility status of the eggs is unknown.

Keywords Maternal transfer · Inorganic contaminants · Amniotic eggs · Eggshell thinning · Calcium resorption · Fish ponds · Trace element contamination

Communicated by K. C. Klasing.

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Zusammenfassung

Verteilungsmuster und Wechselwirkungen von Metallen und Calcium in Eischale und Eiinhalt in Bezug zum embryonalen Entwicklungszustand der Eier eines kleinen Singvogels

Bebrütete und nicht bebrütete Eier des Teichrohrsängers *Acrocephalus scirpaceus* scheinen sich in den Konzentrationen der Elemente zu unterscheiden—vermutlich aufgrund der Resorption von Calcium und anderen Elementen aus der Schale. Vor diesem Hintergrund überprüften wir die Hypothesen, dass sich (1) die Verteilung der Elemente in den Eischalen und Eiinhalten, (2) die individuellen Element–Element-Verhältnisse zwischen Eischalen und Eiinhalten und (3) die Beziehungen zwischen den Elementen in Eischalen und Eiinhalten bei Proben der zwei Eitypen unterscheiden. Basierend auf dem Gehalt an elf

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Elementen (Cr, Cu, Ni, Cd, Pb, Mn, Fe, Co, Zn, Mg und Ca) in Teichrohrsängereiern bestätigten unsere Ergebnisse die zweite und die dritte Hypothese. Eine wichtige Erkenntnis war, dass die Embryonalentwicklung zwei Typen von Beziehungen tendenziell verschleiert: (1) zwischen den Elementen in den Eischalen und den Eiinhalten durch die Zunahme signifikanter Korrelationen, von denen manche durch den hohen Gehalt an einigen Elementen noch verstärkt werden können (Cd, Co, Pb und Zn weisen auf Spuren von Metallbelastung in Fischteichen hin); (2) zwischen den verschiedenen Elementen in den Eierschalen und Eiinhalten, wie durch die differenzielle Gruppierung der Elemente bei der Hauptkomponentenanalyse (PCA; principal components analysis) veranschaulicht wird. Die durchgehend positive Ausrichtung der Beziehungen für fast alle Elemente, sowohl in den Eischalen als auch in den Eiinhalten, ist vermutlich auf die ursprünglich parallele Zuteilung aller Elemente zurückzuführen, welche den intensiven Calciumtransfer aus dem Körper des Weibchens in das entstehende Ei begleitet. Unsere Studie unterstreicht die Tatsache, dass sich aus den Wechselwirkungen zwischen Metallen und Calcium in Eischale und Eiinhalt nur begrenzte Schlüsse ziehen lassen, wenn der embryonale Entwicklungszustand der Eier unbekannt ist.

Introduction

The ecotoxicology literature abounds in studies on the variable levels of different elements, including indispensable micronutrients (like calcium or magnesium) and essential and/or non-essential/toxic metals (metalloids), in the shells and contents of bird eggs. Consequently, the results of these studies are treated as reliable indicators of environmental pollution or the exposure of birds to contaminants (Burger 1994, 2002; Jopek et al. 1995; Morera et al. 1997; Dauwe et al. 1999; García-Hernández et al. 2006; Klein et al. 2012; Hashmi et al. 2013; and the references therein). During their embryonic development, however, avian (and also reptilian) eggs undergo dramatic structural and anatomical changes in both the calcareous shell (resulting in progressive embryo-induced eggshell thinning due to calcium removal; e.g., Balkan et al. 2006; Castilla et al. 2010a) and the egg contents (where the developing embryo resorbs calcium and other elements, initially from the yolk and subsequently from the shell). This process fundamentally alters the concentrations of certain essential elements in both parts of the egg (Packard and Packard 1991; Uni et al. 2012; reviewed in Van Dyke et al. 2013, 2014).

The general pattern of mobilisation and deposition of elements—principally calcium, magnesium and phosphorus—in bird eggs (henceforth "eggs") has been documented for only a few species. Overall, this pattern appears to be similar between altricial and precocial birds (Romanoff 1967; Simkiss 1961; reviewed in Packard and Packard 1991; Deeming 2002; Uni et al. 2012). Avian embryos have three sources of calcium, magnesium and phosphorus to support embryogenesis. The shell is the major source of both calcium and magnesium, while most phosphorus is drawn from the yolk. The albumen contains very little calcium but constitutes a substantial supply of both phosphorus and magnesium (Packard and Packard 1991; reviewed in Richards 1997). In addition, some of the calcium mobilised from the shell is stored in the yolk, and hatchlings emerge from incubation provided with an ample reserve of calcium from that compartment (Packard and Packard 1991). However, the subtle differences in where these elements are deposited in the embryo, yolk or albumen are of less significance in ecotoxicology studies, because these focus on the elements in the egg contents as a whole.

In the context of these physiological processes in eggs, the timing of their collection is critical for assessing levels of minerals in the shell and contents (Packard and Packard 1991; Yalçin-Özdilek et al. 2011; Uni et al. 2012; Ikonomopoulou et al. 2013; Van Dyke et al. 2013, 2014). For instance, the total amounts of calcium and magnesium in the egg contents of the altricial Yellow-headed Blackbird *Xanthocephalus xanthocephalus* increase ca. 8-fold and 1.8-fold, respectively, between days 0 and 12 of incubation (Packard and Packard 1991). A similar increase is found in the concentrations of certain essential and non-essential trace elements, including copper (Cu), zinc (Zn), arsenic (As), selenium (Se) and chromium (Cr), measured in the eggs and embryos of the Green Sea Turtle *Chelonia mydas* (Ikonomopoulou et al. 2013); conversely, the concentrations of most micronutrients and trace elements in the shells reportedly decrease during embryonic development (Yalçin-Özdilek et al. 2011; Uni et al. 2012).

The interpretation of mineral levels in egg contents is limited by the substantial embryo-induced changes in the levels of various elements in eggshells and egg contents (cf. Gonzalez and Hiraldo 1988; Packard and Packard 1991; Richards 1997; Yalçin-Özdilek et al. 2011; Uni et al. 2012; Ikonomopoulou et al. 2013; Van Dyke et al. 2013, 2014). Researchers undertaking ecotoxicology studies of eggs, however, usually pay scant attention to the developmental stage of the eggs they are studying, often unwittingly pooling different egg samples that likely vary in embryonic advancement (e.g., Burger 1994, Burger 2002; Morera et al. 1997; Mora 2003; García-Hernández et al. 2006; Mora et al. 2011; Tsipoura et al. 2011; Hashmi et al. 2013). To the best of our knowledge, studies have rarely explored the relationships among the various

elements in the shells and contents (or within one compartment) of avian and/or reptilian eggs in the context of their embryonic development. Although some studies have provided information on the relationships between the concentrations of various elements measured in eggs, these authors did not distinguish between embryonated ($\approx$ fertile) and non-embryonated eggs. Analyses of interactions between certain elements relate predominantly to eggshells (e.g., Burger 2002; Morera et al. 1997; Dauwe et al. 1999; Rodriguez-Navarro et al. 2002; Ayas 2007; Ayas et al. 2008; Hashmi et al. 2013). The results of studies of elemental interactions have important implications for the physiological processes occurring within developing eggs, because some trace elements can interact with one another. For instance, orally administered dietary Ca or Cr can reduce the negative impact of Cd by increasing the thickness and reducing the brittleness of eggshells (Scheuhammer 1996; Skalicka et al. 2008). Therefore, understanding the sources of variation in the maternal transfer of elements is fundamental to determining which species inhabiting a contaminated area are likely to suffer reproductive consequences. Moreover, in systems contaminated with multiple trace elements, maternal transfer and embryonic assimilation are element- and species-specific, and may be inconsistent even among closely related species (Van Dyke et al. 2013).

Our recent study of the concentrations of several elements in the eggs of a population of the Eurasian Reed Warbler *Acrocephalus scirpaceus* (hereafter Reed Warbler) revealed that thinner-shelled eggs with embryonic development (on average 8.0 % thinner shells than eggs with no embryonic development; Orłowski et al. 2016a) contained distinctly and significantly higher levels ($\geq$22 %) of all 11 measured elements: cobalt (Co), 22.7 %; copper (Cu), 28.2 %; iron (Fe), 28.6 %; manganese (Mn), 29.9 %; nickel (Ni), 46.0 %; chromium (Cr), 46.1 %; cadmium (Cd), 47.4 %; magnesium (Mg), 49.3 %; zinc (Zn), 56.6 %; lead (Pb), 87.5 %; and calcium (Ca), 98.8 % (the most pronounced difference) (Orłowski et al. 2016b). Furthermore, the shells of embryonated eggs had significantly higher levels of copper (28.0 %), lead (32.8 %), iron (56.5 %) and zinc (104.8 %), and significantly lower levels of cobalt (8.9 %) as well as a smaller concentration of calcium (8.8 %), than the shells of non-embryonated eggs (Orłowski et al. 2016b; see Table S1). Because these two groups of eggs were found to contain different levels of most elements, in the present work we wanted to explore the distribution and interaction of metals and calcium in the shells and contents of Reed Warbler eggs—using the same data set of 11 elements (Cr, Cu, Ni, Cd, Pb, Mn, Fe, Co, Zn, Mg and Ca)—in relation to embryonic development.

Given the apparent differences in the concentrations of elements between embryonated and non-embryonated eggs

(presumably due to the resorption of calcium and other elements from the eggshell), the main objectives of this study were to determine (1) the distribution of elements in eggshells and egg contents, (2) the individual element–element relationships between eggshells and contents, and (3) whether relationships among the elements in eggshells and contents would vary between the two egg samples. In general, these hypotheses concur with our earlier tested theory that the pattern of relationships among trace elements in shells would vary with the growing load of contaminants measured in shells (for instance, resulting from a pollution gradient) (Orłowski et al. 2014a). At the outset, we do not predict any clear directions of relationships between the elements in the egg contents. Nonetheless, given the superior role and intensive sequestration of Ca, initially from the female oviduct (to the forming egg), and the subsequent intensive Ca resorption from the shell to the contents (Packard and Packard 1991; Richards 1997), the relationship between Ca and other elements seems physiologically justified. With detailed information on the direction of interactions between the various chemical elements present in eggshells and contents, we can assess potential inhibitory or synergistic effects resulting from the sequestration and/or mobilisation (from the shell into the egg contents) of an individual element in both these inseparable egg parts. Finally, we discuss the potential biotransfer of some essential and non-essential trace elements occurring in particularly high concentrations in the Reed Warbler eggs we examined and in other species inhabiting intensive inland fish-farming areas in Europe (Witkowski and Orłowska 2010).

Materials and methods

We used data on element concentrations measured in 161 Reed Warbler eggs sampled on the Słoneczny fishpond (51°32′N, 17°20′E) in the Stawy Milickie Nature Reserve (SW Poland) in May through August during the period 2010–2013 (Orłowski et al. 2016b).

The Reed Warbler is a small (12 g) passerine that breeds in the reed beds of the Palaearctic and winters in sub-Saharan Africa (Cramp 1998). It has a relatively long breeding season (May–August), and because of high nest losses, it frequently re-nests, laying up to five clutches in a season (Scheuhammer 1996; Halupka et al. 2014a, b). Most clutches comprise 3–5 eggs (occasionally 2 or 6). While both parents participate in incubation duties, the female's share is greater, both in our population and elsewhere (Cramp 1998; Klimczuk et al. 2015).

Egg sampling was a part of an extensive project involving various aspects of the breeding biology of this species, which began in 2005 (for details see Halupka et al.

2008, 2014a, b). Clutches on average consisted of 3.97 (SD ± 0.74) eggs. We found most nests (about 85 %) at the construction stage, subsequently visiting them daily during the egg-laying period, and thereafter every 2 days. Before the expected day of hatching (generally 11 days after the last egg had been laid), we visited the nests daily to determine the hatching date of the first nestling(s). We typically took unhatched eggs 5 days after the hatching of the first nestlings (the maximum hatching spread found in our population was 54 h; L. Hałupka, unpublished data). Of the total 1354 eggs laid, we collected 161 eggs from 97 nests. The eggs were stored in small plastic bags and frozen at −20 °C.

Subsequently, we opened each egg after it had thawed, placed its contents in a glass dish and examined it for the presence of an embryo. We then classified the eggs as embryonated (a visible embryo was present in the yolk) or non-embryonated (no visible embryo present in the yolk, presumed infertile) (Orłowski et al. 2016a, b).

Chemical analysis

The chemical analysis and validation of certified reference material is described elsewhere (Orłowski et al. 2016b). Briefly, the entire contents of an egg (with an embryo if present) and the lower half of the eggshell with the shell membrane were analysed chemically, using flame atomic absorption spectroscopy (SpectrAA FS220; Varian Medical Systems, Palo Alto, CA, USA) to determine metal concentrations. This process was validated using reference material DORM-3 (fish protein) provided by the National Research Council of Canada Institute for National Measurement Standards. In most analyses, element concentrations are expressed in milligrams per kilogram (mg kg^{-1} or parts per million; ppm) of dry mass (d.w.) for shells and wet mass (w.w.) for contents, accurate to two decimal places. However, for pairwise comparisons of the distribution of elements in the shell and contents of the same eggs, we multiplied the concentrations of the wet mass of the contents by 5.05 to obtain the dry mass; this conversion was based on the average 80.16 % moisture content of eggs calculated from data given by Mora (2003) for 11 species of small passerines. Almost the same value (×5) for converting element concentrations from w.w. to d.w. was applied in earlier studies on the chemical composition of contents in passerine eggs (Jopek et al. 1995).

Statistical analyses

The primary objective of our analysis was to test whether the pattern of relationships between calcium and ten other elements (measured in the eggshell and egg contents, and between these egg parts) was fixed or variable as their levels changed. To test this hypothesis, we performed two separate principal analyses, one for embryonated eggs ($n = 62$) and the other for non-embryonated eggs ($n = 86$), which differ fundamentally in the levels of most elements (see Table S1 and the results of earlier analyses using the same data set in Orłowski et al. 2016b). As background, we carried out a similar analysis for all the eggs examined ($n = 161$).

Statistical analyses were performed using Statistica ver. 7.0 (StatSoft 2006) and Excel software; the statistical significance level was 0.05. The data are presented as arithmetic means ±1 SE. Prior to the analyses, we log-transformed the elemental concentration data to meet the assumptions of normality.

We applied pairwise comparison (first prediction) using the t test for dependent samples to assess the distribution of elements in the shells and contents of embryonated and non-embryonated eggs (both expressed as d.w.).

In addition, we used Pearson's correlation coefficient (second prediction) to analyse the relationships between the log-transformed concentrations of calcium and the other elements measured in the shells (d.w.) and contents (w.w.) of three egg samples (embryonated, non-embryonated, all eggs).

To test the differences in the pattern of relationships (third prediction) among the various elements in the shells and contents, we performed principal components analysis (PCA) using Statistica 7.0 on standardised log-transformed concentrations in shells and contents representing three egg samples (embryonated, non-embryonated, all eggs); the matrix of correlations between the various elements in the shells and contents of these three samples is given in the Supplementary Data (Table S2 and Table S3). In PCA we applied varimax normalised factor rotation, using factor loadings to interpret PC patterns across three different egg samples. We regarded principal components (PCs) with an eigenvalue >1 as representing a significant contribution to the total variance according to the latent root criterion (Hair et al. 1998).

Results

Overall, the concentrations of all eggshell elements decreased in consistent order in embryonated eggs, non-embryonated eggs and all eggs: Ca ≫ Mg > Fe > Zn > Co > Ni > Cu > Mn > Pb > Cr > Cd (Table S1). The analogous distribution of elements measured in the contents of non-embryonated eggs was Ca > Mg > Fe > Zn > Cu > Mn > Co > Pb > Cr > Ni > Cd. For embryonated eggs and all eggs there was only a slight difference in the rank of Co/Pb: Ca > Mg > Fe > Zn > Cu > Mn > Pb > Co > Cr > Ni > Cd (Table S1).

Pairwise comparisons of the concentrations of the different elements measured in eggshells and egg contents showed quite a consistent pattern across the three groups of eggs. There were two exceptions, however: Cr and Pb concentrations were significantly higher in shells than in contents only in the non-embryonated eggs (analogous comparisons of the levels of these two elements in embryonated eggs and all eggs did not yield any significant results; Fig. 1). Across the three groups of eggs, the concentrations of three elements (Cu, Fe and Zn) were
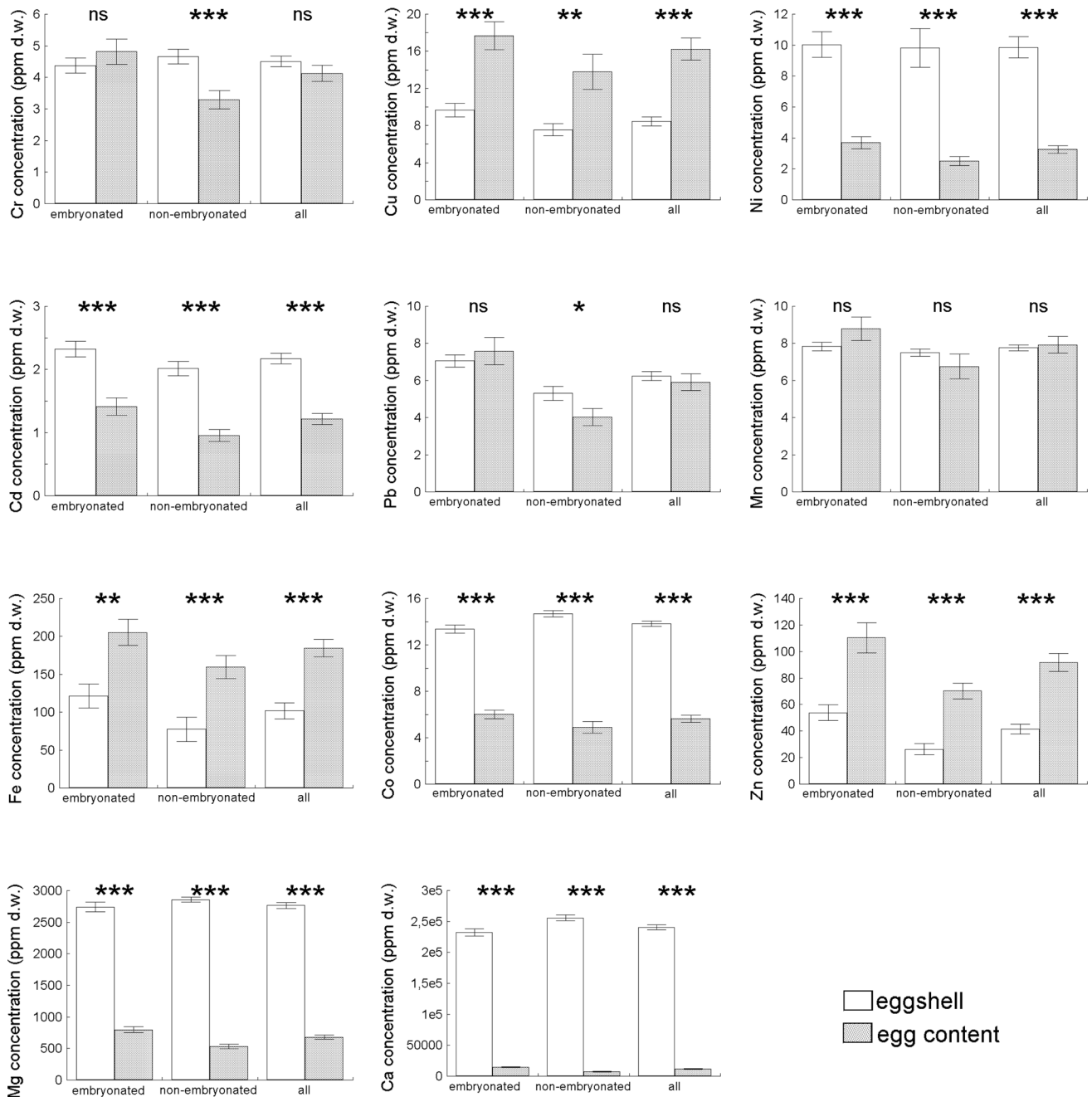

Fig. 1 Pairwise comparison (average ±1 SE) using the *t* test (for dependent samples) of concentrations (ppm d.w.) of various elements in the shells and contents of embryonated eggs (*n* = 62), non-embryonated eggs (*n* = 86) and all eggs (*n* = 161) of the Eurasian Reed Warbler *Acrocephalus scirpaceus* from the Milicz Fishponds Nature Reserve, SW Poland, sampled in 2010–2013. To obtain dry weights, the metal levels in the eggs (see Table S1) were multiplied by 5.05 (based on the average 80.16 % moisture content of eggs of small passerines (see "Materials and methods" for details); statistically significant differences are indicated as follows: *$p \leq 0.05$, **$p < 0.01$, ***$p < 0.001$

significantly and consistently higher in the contents than in the shells. Five other elements—Ni, Cd, Co, Mg and Ca—displayed the opposite pattern, i.e., a significantly higher level in the shells than in the contents. Lastly, only the Mn level did not differ between the shells and contents for any group of eggs (Fig. 1).
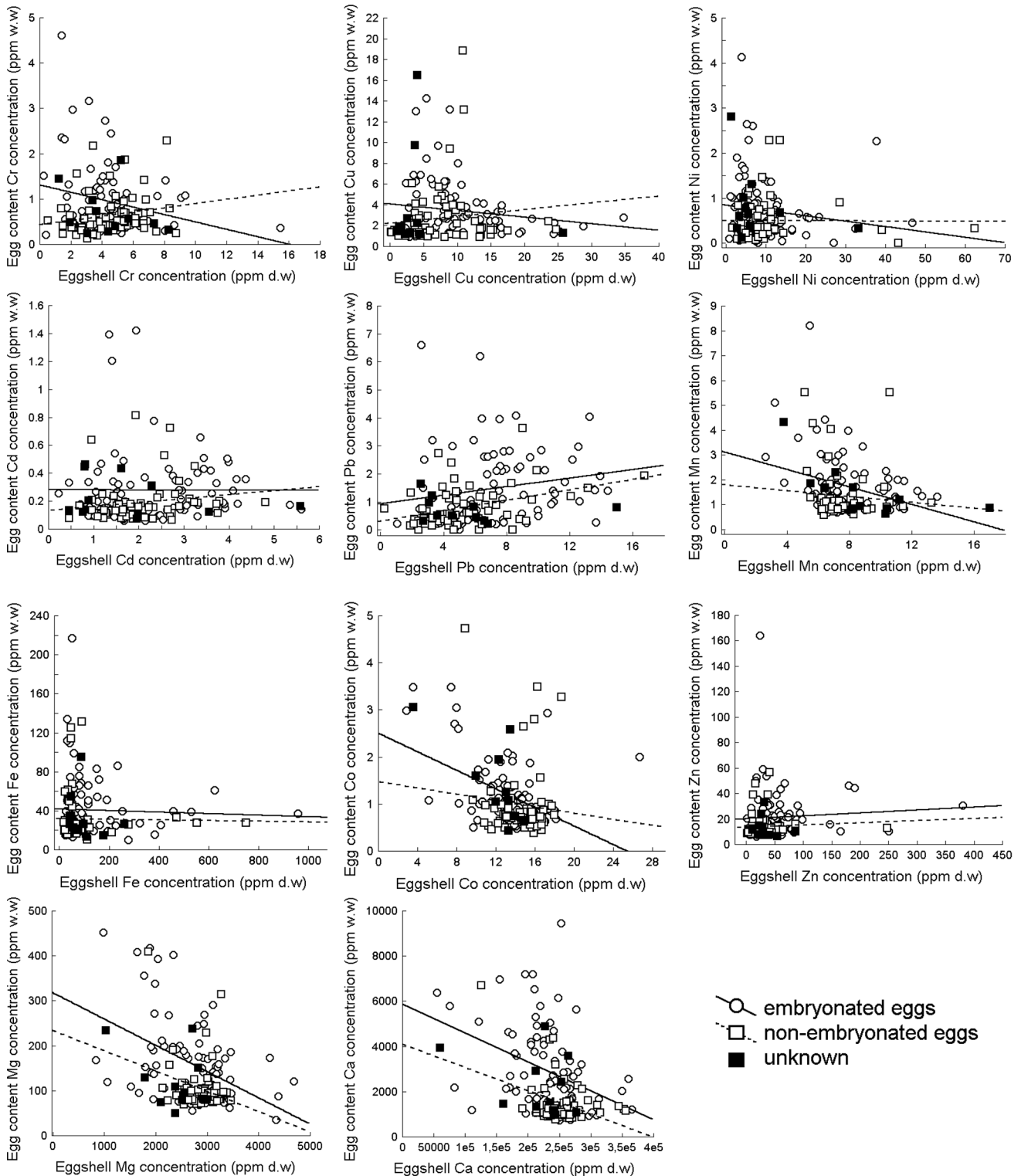

Fig. 2 Relationships between concentrations of individual elements measured in the shells and contents of embryonated eggs ($n = 62$) and non-embryonated eggs ($n = 86$) of the Eurasian Reed Warbler *Acrocephalus scirpaceus*; see Table 1 for Pearson correlation coefficients. Eggs of unknown embryonic developmental status ($n = 13$) were not used for fitting the regression line

Analysis of the relationships between element concentrations measured in shells and contents (Fig. 2) yielded only three significant correlations for non-embryonated eggs: one positive (Pb) and two negative (Mg and Ca) (Table 1). Much more significant relationships (for 7 elements) were found for embryonated eggs: two positive (Pb and Zn) and five negative (Cr, Mn, Co, Mg and Ca) (Table 1; Fig. 2). Analogously, all eggs exhibited two positive (for Pb and Zn) and four negative relationships (Mn, Co, Mg and Ca) (Table 1; Fig. 2). Only three elements (Pb, Mg and Ca) showed a consistent and statistically significant dependence across the three groups of eggs (Table 1).

PCA of the 11 elements measured in the egg contents (based on the correlation matrix in Table S2) consistently yielded three components with eigenvalues >1.47 across the three egg groups, which explained from 13 to 34 % of the variance, albeit with slight differences in the grouping of some elements and the direction of their association (Table 2). For non-embryonated eggs, the first extracted principal component (PC1) consisted of Cu, Cd, Pb, Fe and Zn, which were loaded in a positive direction; Cr was loaded in the opposite direction as PC2; and Mn and Ca were loaded as PC3 in a positive direction. For embryonated eggs (only a positive association obtained), PC1 consisted of Fe and Zn, PC2 consisted of Mn, Co, Mg and Ca, and PC3 consisted of Cr and Cd. Similarly, for all eggs (only a positive association obtained), PC1 consisted of Cu, Pb, Fe and Zn, PC2 consisted of Co, Mg and Ca, and PC3 consisted only of Cr (Table 2).

Compared to the relationships between the various elements in the contents (Table S2), element–element interactions in the shells were much more intense and, as in the extreme case of non-embryonated eggs and all eggs, all possible correlations were statistically significant and exclusively (!) positive (Table S3). Consequently, PCA of the 11 shell elements yielded only two components with high eigenvalues >3.24 for non-embryonated eggs; PC1 consisted of eight elements (Cr, Cu, Ni, Mn, Co, Zn, Mg and Ca), while PC2 consisted of two non-essential metals (Pb and Cd) (Table 2). For embryonated eggs, only one component was obtained with an extremely high eigenvalue and an explained variation consisting of ten elements (Cr, Ni, Cd, Pb, Mn, Fe, Co, Zn, Mg and Ca) (Table 2). Lastly, one component comprising all the elements was obtained for all eggs (Table 2).

Discussion

Our results confirmed our second and third hypotheses: the individual element–element relationships between the eggshells and egg contents, and the relationships among the various elements in the shells and contents, varied between embryonated eggs and non-embryonated eggs. The first important finding was that embryonic development tended to blur two patterns. One concerns the relationships between individual elements in shells and contents through the greater number of significant correlations (relating in particular to four additional elements occurring at higher levels in embryonated eggs—Cr, Mn, Zn, Co—for which additional significant relationships compared to non-embryonated eggs are reported). The other applies to the relationships among the various elements in shells and contents as demonstrated by the variable grouping of elements in PCA (presumably a consequence of large variations in element concentrations translated into their different groupings in various PC scores).

The consistent grouping of the elements in shells into three different PC scores (PC1–PC3) may represent a general picture of considerable complexity and/or variability of the chemical composition of avian eggs, or even of naturally occurring biological material (e.g., Hashmi et al. 2013). Very surprisingly, however, we found an

Table 1 Pearson correlation coefficients between (log-transformed) concentrations of individual metals measured in the shells (ppm d.w.) and contents (ppm w.w.) of three categories of eggs (non-embryonated, embryonated and all) of the Eurasian Reed Warbler *Acrocephalus scirpaceus* (* $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Element pair	Eggs		
	Embryonated ($n = 62$)	Non-embryonated ($n = 86$)	All ($n = 161$)
Cr–Cr	0.189	−0.228*	−0.122
Cu–Cu	0.192	0.024	0.111
Ni–Ni	0.086	−0.200	−0.134
Cd–Cd	0.185	0.036	0.072
Pb–Pb	0.354**	0.278*	0.338***
Mn–Mn	−0.133	−0.333**	−0.305***
Fe–Fe	0.033	0.067	0.079
Co–Co	−0.114	−0.517***	−0.449***
Zn–Zn	0.200	0.226*	0.274**
Mg–Mg	−0.279*	−0.429***	−0.407***
Ca–Ca	−0.337**	−0.354**	−0.401***

Table 2 Component values and factor loadings of the principal components analysis (PCA) of the (log-transformed) concentrations of different elements measured in the shells (ppm d.w.) and contents (ppm w.w.) of three categories of eggs (embryonated eggs, $n = 62$; non-embryonated eggs, $n = 86$; and all eggs, $n = 161$) of the Eurasian Reed Warbler *Acrocephalus scirpaceus*

Element	Embryonated eggs			Non-embryonated eggs			All eggs		
	PC1	PC2	PC3	PC1	PC2	PC3	PC1	PC2	PC3
Eggshells									
Cr	−0.099	0.236	**0.754**	0.123	**−0.734**	−0.102	0.007	0.264	**0.721**
Cu	0.690	−0.091	0.586	**0.779**	0.191	−0.136	**0.778**	−0.106	0.346
	0.401	0.336	0.431	0.628	0.288	0.319	0.603	0.309	0.194
Ni	0.264	0.053	**0.812**	**0.864**	−0.135	−0.007	0.600	0.050	0.619
Cd									
Pb	0.587	0.141	0.620	**0.809**	−0.239	0.067	**0.750**	0.067	0.399
Mn	0.509	**0.743**	0.066	0.310	0.109	**0.787**	0.473	0.699	−0.183
Fe	**0.934**	0.050	0.011	**0.713**	0.459	0.376	**0.898**	−0.002	−0.277
Co	−0.173	**0.870**	0.252	−0.173	−0.677	0.076	−0.152	**0.839**	0.316
Zn	**0.886**	−0.055	0.151	**0.799**	0.216	0.064	**0.867**	−0.119	−0.084
Mg	0.003	**0.880**	−0.016	−0.147	−0.654	0.456	−0.063	**0.853**	0.087
Ca	0.022	**0.890**	0.204	−0.085	−0.181	**0.743**	0.038	**0.887**	0.161
Eigenvalues	3.01	3.08	2.27	3.72	1.92	1.67	3.70	2.91	1.47
Variation explained (%)	27	28	21	34	17	15	34	26	13
Egg content									
Cr	**0.915**	–	–	**0.769**	0.459	–	**0.910**	–	–
Cu	0.685	–	–	**0.734**	0.173	–	**0.722**	–	–
Ni	**0.816**	–	–	**0.824**	−0.019	–	**0.788**	–	–
Cd	**0.894**	–	–	0.654	**0.701**	–	**0.903**	–	–
Pb	**0.823**	–	–	0.009	**0.899**	–	**0.755**	–	–
Mn	**0.938**	–	–	**0.867**	0.414	–	**0.938**	–	–
Fe	**0.886**	–	–	0.607	0.644	–	**0.878**	–	–
Co	**0.912**	–	–	**0.881**	0.370	–	**0.898**	–	–
Zn	**0.864**	–	–	0.704	0.624	–	**0.889**	–	–
Mg	**0.882**	–	–	0.709	0.587	–	**0.889**	–	–
Ca	**0.766**	–	–	**0.790**	0.492	–	**0.804**	–	–
Eigenvalues	8.06	–	–	5.76	3.24	–	8.04	–	–
Variation explained	73 %	–	–	52 %	29 %	–	73 %	–	–

Factor rotation varimax normalised

Figures in bold indicate the variable for which each factor exhibited the greatest variability

apparent reduction in the number of PC scores for elements in the contents (a consequence of the unprecedented, very strong mutual relationships between all the elements; see Supplementary Data) to two (PC1 and PC2; non-embryonated eggs) or even one (PC1; embryonated and all eggs). We speculate that this unexpected finding might stem from the superior role of biochemical processes related to the sequestration and mobilisation of calcium within eggs. We found a generally consistent positive direction of relationships for almost all elements in both shells and contents. It seems, therefore, that the potential explanation for the very strong mutual and positive relationships among all the elements is their initial parallel co-precipitation and/or co-

sequestration accompanying the intensive transfer of calcium from the female body into the eggs produced in her oviduct (non-embryonated eggs) and the subsequent mobilisation of these elements (accompanying primarily intensive calcium resorption) from the shells to the contents (Richards 1997; Packard and Packard 1991; Uni et al. 2012). Calcium sequestration in the biochemical/physiological context is attributable to the same ion transporter(s) that bind(s) a few different elements that are simultaneously deposited in the eggshell (cf. Scheuhammer 1996; Rodriguez-Navarro et al. 2002; Jonchere et al. 2012). During oogenesis in female birds, the absorption of certain non-essential Ca-mimetic metals (like Pb or Cd) increases

during Ca sequestration into shells (Scheuhammer 1996). Generally, the elements analysed in our study are divalent cations: we assume that these are negatively associated with calcium and inhibit its precipitation in calcified tissues (Wada et al. 1995; Parsiegla and Katz 1999). Collectively, these findings suggest that such a generally superior role of Ca in the dynamics of elements in eggshells and eggs contents is valid.

Compared with physiologically/biochemically changed embryonated eggs, the direction of correlation and the much smaller number of significant relationships between the concentrations of individual elements in the shells and contents of non-embryonated eggs (containing the primary = maternally transferred levels of elements) is equally important for clarifying the maternal transfer and dynamics of elements in shells and contents during embryonic development. The negative direction (Cr, Mn, Co, Mg and Ca) and the overall larger number of significant relationships observed in the shells of embryonated eggs (cf. Table S2) presumably reflects the higher levels of elements resulting from embryonic depletion due to embryo-induced mobilisation from the shell into the egg content/embryo. However, the explanation for the positive relationships reported for Pb and Zn in both embryonated and non-embryonated eggs must be further scrutinised (cf. Table 1; Fig. 2). Presumably, these divergent results can be explained by the overall high Pb and Zn levels, which are a consequence of the large maternal input of both these trace metals into the eggs.

Levels of elements of major environmental concern: potential sources of variability in element–element interactions

In comparison to published data, the mean Pb, Zn, Cd and Co concentrations in our Reed Warbler egg sample were unusually high. For instance, the highest respective mean Pb and Zn concentrations in several species of small passerines from Arizona did not exceed 1.8 Pb ppm d.w. and 79.8 Zn ppm d.w. in the egg contents and 1.5 Pb ppm d.w. and 79.8 Zn ppm in shells (for two species) (Mora 2003). Similarly, the post-hatch eggshells of the Rook *Corvus frugilegus* (a focal bird species of European farmland especially vulnerable to high doses of inorganic contaminants derived from their invertebrate diet obtained from crop fields) from 43 rookeries in rural and urban areas of Poland contained a mere 3.29 (range = 1.72–11.53) Pb ppm d.w. and 13.81 (range = 0.52–117.96) Zn ppm d.w. (Orłowski et al. 2010, 2014b). The average Zn concentration in the egg contents of Tree Swallows *Tachycineta bicolor* from a wetland in Minnesota sampled between 1998 and 2001 ranged from 58.2 to 69.1 Zn ppm d.w. (Custer et al. 2006). In our sample of Reed Warbler eggs,

the respective average Pb concentrations in the shells of embryonated and non-embryonated eggs were 7.1 and 5.3 ppm d.w.; the corresponding values for Zn were 53.8 and 26.3 Zn ppm d.w. (see Table S1). The respective analogous concentrations in the contents of embryonated and non-embryonated eggs were 7.6 and 4.0 Pb ppm d.w., and 110.3 and 70.3 Zn ppm d.w. (Fig. 1).

We also found high concentrations of Cd (non-essential) and Co (essential elements) in our sample of Reed Warbler eggshells, regardless of whether the eggs were embryonated (Fig. 1; Table S1). Generally, cobalt concentrations have not been known to exceed 0.94 Co ppm d.w. in the eggshells of wild birds (Burger and Gochfeld 1988; Dauwe et al. 2005; Ikemoto et al. 2005; Al-Obaidi et al. 2012) or 0.87 Co ppm d.w. in the eggshells of hand-reared species (reviewed in Dobrzański et al. 2007; Abduljaleel et al. 2011). Our cobalt concentration appears to be the highest yet reported in avian eggshells: ca. 16 times greater than the highest previously reported value compared to the shells of non-embryonated Reed Warbler eggs (cf. Table S1). The highest overall levels of cobalt in biota (up to 5.63 Co ppm w.w. = ca. 28 Co ppm d.w.) have been reported in freshwater organisms, such as fish from Dutch polders, and extremely high levels, up to 860 Co ppm in *Myriophyllum verticillatum*, a species from lakes in central Ontario. These values generally reflect the intense accumulation of cobalt in bottom sediments and its subsequent biomagnification along the freshwater ecosystem food chain (ATSDR 2004). Interestingly, taking all the results into consideration, our analysis also revealed the highest correlation coefficients between Co concentrations in the shells and contents of embryonated eggs (but without an analogous correlation for non-embryonated eggs; Table 2). A nearly 9 % decrease in Co concentration in the shells of embryonated eggs (see Table S1) suggests that large amounts of Co are transferred from the shell to the contents. Most of the Co probably enters the yolk, which contains ca. 80 % of the entire cobalt stored in the contents, the remainder (20 %) being stored in the albumen (Richards 1997).

Cadmium levels in eggshells are low, <0.3 Cd ppm, across a range of bird species (reviewed in Hashmi et al. 2013). High average Cd concentrations in eggshells have been reported in Tree Swallows *Tachycineta bicolor* (1.8 Cd ppm d.w.; Kraus 1989) and Cattle Egrets *Bubulcus ibis* (1.23 [max = 2.8] Cd ppm d.w.; Hashmi et al. 2013). To the best of our knowledge, however, the highest Cd concentrations ever reported in avian eggshells from our study area, the Stawy Milickie Nature Reserve, were measured in the Eurasian Coot *Fulica atra* (up to 5.7 Cd ppm d.w.) and the Tufted Duck *Aythya fuligula* (4.5 Cd ppm d.w.; Marek 1990). This reflects the substantial accumulation of cadmium and other trace elements (Cu, Cd, Zn and Pb, and

presumably also Co) in the bottom sediments of these fish ponds and their subsequent biomagnification at successive trophic levels of the local food chain: macrophytes, zooplankton, zoobenthos, emerging insects, fish and aquatic birds (including their eggs) (Marek 1990; Pokorny et al. 2006, 2007; Binkowski and Sawicka-Kapusta 2015). Given this exposure to high levels of essential and non-essential trace elements in the Milicz fishponds (primarily bottom sediments, which accumulated up to ×4344 Cd, ×107,607 Pb and ×12,287 Ni more than the levels of these elements in pond water; Pokorny et al. 2007) and their subsequent bioaccumulation in freshwater organisms (including Reed Warbler eggs in particular, and the tissues of aquatic birds from this area in general; cf. Binkowski and Sawicka-Kapusta 2015; Binkowski and Meissner 2013; Binkowski et al. 2013a, b), the key concern lies in identifying the primary sources of trace element contamination in this system.

A detailed chemical characterisation of the components of the local environment, including fish-farming activity, found two main sources of contamination: water (containing both untreated domestic wastewater and surface runoff from crop fields), and fish feed and fish-farming fertilisers (occurring there in concentrations up to ×5400 Pb higher than in water) (Marek 1990). In general, only small quantities of trace elements are assimilated from the digestive tracts of fish, although this does also depend on their age and the concentrations of these elements in the food they consume (Baines et al. 2002). Between 1980 and 2000, the Cd and Pb concentrations in bottom sediments of the Milicz fish ponds increased 10 and 4 times, respectively, although much greater increases over this same time span were reported for the concentrations of these trace elements in the water: up to ×200 Cd and ×300 Pb (Pokorny et al. 2007). A direct link (including the dietary transfer of contaminants) between the products used in fish farming and the high load of trace metals in the various components of the fish pond environment thus seems highly probable. Consequently, comprehensive evaluations are needed to inform and enhance our understanding of the pathways of trace elements and other contaminants across the various levels of the food chain (including top consumers, such as birds) in the anthropogenic fish pond habitat.

Therefore, given the high levels of four trace elements (Pb, Zn, Cd and Co) in the Reed Warbler eggs, their initial (=maternally derived) level is presumably an additional factor governing the intensity of relations between some metals. It should be stressed that, since embryonic development generally increases the levels of elements in egg contents and reduces them in shells (cf. Supplementary data), the proper starting point for studies aiming to assess the *real maternal transfer* of elements into these two egg

compartments should be non-embryonated eggs (Packard and Packard 1991); otherwise, the physiologically altered levels of elements will not represent their 'maternal levels' (but see Bryan et al. 2003). The fact that we were unable to confirm our first prediction (that the distribution pattern of elements in the eggshells and egg contents varies between embryonated and non-embryonated eggs) suggests that the distribution patterns were similar among almost all the elements, irrespective of whether embryonic development had taken place (Fig. 1). The only exceptions were the concentrations of Cr and Pb, which were significantly lower in the egg contents than in the shells (non-embryonated eggs only). Similarly, with the exception of the Co and Pb concentrations (both very similar) in the contents of embryonated eggs, we found a consistent order of element concentrations in shells and contents, regardless of whether the eggs were embryonated.

A possible weakness in our comparison of element levels in shells and contents is the conversion of element concentrations in the egg contents from wet to dry mass. But even as a result of this treatment, our data would only be partly skewed, as the potential bias appears to be distributed equally across all the eggs. Hence, the concentrations of elements in the contents of embryonated and non-embryonated eggs based on dry mass presumably represent proportionally changed values, the use of which is justified for our sample of eggs. However, a potential subtle deviation from the actual element concentrations in the egg contents may restrict comparison with analogous literature data, so these should be treated with caution. Generally speaking, apart from Ca and Mg—the levels of which are always many times lower in the egg contents—earlier studies showed no uniform distribution pattern of elements in eggshells and contents, which varied from species to species, and may partly result from the levels of elements within the egg itself (cf. Burger 1994; Morera et al. 1997; Dauwe et al. 1999; Mora 2003; Swaileh and Sansur 2006; Ikemoto et al. 2005).

For instance, Burger (2002) studied the eggs of Herring Gulls *Larus argentatus* and Roseate Terns *Sterna dougallii* and found that Pb and Cr concentrations were 2–8 times greater in the contents, Mn concentrations were up to 1.5 times greater in the shells, and Cd levels varied between the shells and the contents in both species. In freshly laid eggs of Audouin's Gull *Larus audouinii*, Morera et al. (1997) found a higher concentration of Zn (ca. ninefold), Mn (ca. sixfold) and Cu (1.2-fold) in the contents than in the shells. Roughly doubled levels of Cu and Zn in the contents were reported in eggs of Blue Tits *Parus caeruleus* and Great Tit *P. major* from Belgium, and Cd and Pb concentrations were up to ca. eightfold higher in the shells. Mora (2003) showed that Cu, Mn, Ni and Pb concentrations in the eggs of two species with generally low levels of

trace elements were 2–26 times greater in the shells than in the contents, although the distribution (the shell-to-contents ratio) of Zn varied, at 0.18 and 1.2 in the two species, respectively. In eggs of the Black-footed Albatross *Phoebastria nigriceps* from Japan, the Cr, Mn, Cu and Zn concentrations were 2–21 times greater in the contents, whereas Co, Cd and Pb concentrations were 4–29 times greater in the shells (Ikemoto et al. 2005). In eggs of House Sparrows *Passer domesticus* from urban regions of the West Bank (Palestinian Authority) Cu, Pb and Zn concentrations were 2–4 times greater in the contents, in contrast to the Cd level, which was approximately twice as high in the shells (Swaileh and Sansur 2006). Such results, demonstrating the heterogeneous distribution of trace elements in eggshells and egg contents, suggest that a detailed meta-analysis to reassess the pattern of the element composition in both egg compartments (including the differentiation of element concentrations) is needed.

To conclude, it should be assumed that, in general, the quantity of elements maternally transferred to whole eggs (i.e., shell and contents) do not change (or do so only minimally?) during embryonic development (some potential pathways include the transport of elements through the egg pores; e.g., Hui 2002). Thus a key aspect of studies aiming to assess the sources and dynamics of elements—including toxic ones—in developing embryos is to consider all the egg compartments, but above all the shell. Ignoring the shell as a source of elements or in the element budget of the embryo undoubtedly prevents proper inferences being made about the flux and bioaccumulation of contaminants in amniote embryos or the various egg compartments per se. It seems, therefore, that such a follow-up approach linking the quantity of elements in all egg compartments, as well as the assessment of embryonic stages (not done in our study, where we applied the simplified dichotomy of the description of embryo presence), is highly desirable in future studies of the maternal transfer of elements, including contaminants, into developing embryos. Ultimately, this will translate into a better understanding of the pattern of bioaccumulation of elements taking place in eggs at the various stages of embryogenesis (cf. Ikonomopoulou et al. 2013; Van Dyke et al. 2014).

An alternative explanation for the observed positive relationships between the Pb and Zn concentrations measured in the eggshells and egg contents could be derived from the differences in the allocation of some proportion of these elements in the various layers of the eggshell (and/or egg membrane) that are not subject to the physiological shell thinning and decalcification accompanying embryonic development (Richards 1997). This may well occur in the two outermost (not eroded in the course of embryonic development) layers of the avian eggshell, i.e., the palisade layer and cuticle. In contrast to eggshells, the shell membrane thickens during embryonic development (Castilla et al. 2010b) and exhibits a high binding affinity for a variety of heavy metals (Suyama et al. 1994; reviewed in Richards 1997). Furthermore, since phosphorus and magnesium are concentrated mainly in the external eggshell layer (Cusack et al. 2003; Rodriguez-Navarro et al. 2013), whereas calcium is deposited in the mammillary layer (substantially eroded during embryonic development; reviewed in Hincke et al. 2012), it may be assumed that some portion of Pb and Zn (their chemical properties are similar to those of Ca) could be deposited in the mammillary layer of a shell. Moreover, embryos partially, and perhaps even selectively, assimilate trace elements from yolk as indicated by the observation that the concentrations of Cu, Zn and Fe are not correlated with their levels measured in hatchlings and egg contents (Van Dyke et al. 2014). Analogously, in our case, this might be true for Cu and Fe, because in neither of our egg samples (embryonated and non-embryonated) did we find that the concentrations of these elements in the shells and contents were correlated. These assumptions imply the need for a detailed assessment of the part played by the various eggshell layers (including shell membranes) in the bioaccumulation of elements, especially in the context of physiological changes linked to embryonic development.

To conclude, the results of our analysis serve as a significant contribution to our understanding of the dynamics of chemical elements in bird eggs, and highlight the lack of stable levels of elements in eggshells and egg contents (a consequence of embryonic development): this has clear practical and critical implications for studies on the chemistry of avian eggs. In particular, our results confirm the relationship between the presence of embryos in eggs and the distribution patterns of metals and calcium in the shells and contents. Some of these relations may be intensified by high levels of Cd, Co, Pb and Zn, which are indicative of the trace metal pollution of the fish pond environment. Without reference to embryonic development, however, inferences regarding elemental interactions based on egg samples are necessarily limited. We emphasise the need for further holistic studies on the biotransfer and interactions of elements, including trace elements, as well as other environmental contaminants (pesticide residues), across various levels of the food chain (including bird eggs in the context of embryonic development) and variable levels of environmental pollution, especially in the fish pond habitat, which is subject to the intense pressure of aquaculture.

University of Wrocław (projects 1018/S/IZ/2010-11 and 1073/S/SORM/2012-13). All procedures in this study were conducted according to the relevant Polish legislation. The permits to conduct a study involving a protected species in the protected area of a nature reserve were issued by the Regional Directorate of Nature Protection in Wrocław (Poland), and study procedures were accepted by the Ethics Commission in Wrocław. Licenses for bird ringing were issued by the Ornithological Station of the Polish Academy of Sciences based on the decisions of the Polish Ministry of Environment. Peter Senn kindly corrected the English of the manuscript.

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