Characteristics of global organic matrix in normal and pimpled chicken eggshells

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ABSTRACT The organic matrix from normal and pimpled calcified chicken eggshells were dissociated into acid-insoluble, water-insoluble, and facultative-soluble (both acid- and water-soluble) components, to understand the influence of shell matrix on eggshell qualities. A linear correlation was shown among these 3 matrix components in normal eggshells but was not observed in pimpled eggshells. In pimpled eggshells, the percentage contents of all 4 groups of matrix (the total matrix, acid-insoluble matrix, water-insoluble matrix, and facultative-soluble matrix) were significantly higher than that in normal eggshells. The amounts of both total matrix and acid-insoluble matrix in individual pimpled calcified shells were high, even though their weight was much lower than a normal eggshell. In both normal and pimpled eggshells, the calcified eggshell weight and shell thickness significantly and positively correlated with the amounts of all 4 groups of matrix in an individual calcified shell. In normal eggshells, the calcified shell thickness and shell breaking strength showed no significant correlations with the percentage contents of all 4 groups of matrix. In normal eggshells, only the shell membrane weight significantly correlated with the constituent ratios of both acid-insoluble matrix and facultative-soluble matrix in the whole matrix. In pimpled eggshells, 3 variables (calcified shell weight, shell thickness, and breaking strength) were significantly correlated with the constituent proportions of both acid-insoluble matrix and facultative-matrix. This study suggests that mechanical properties of normal eggshells may not linearly depend on the organic matrix content in the calcified eggshells and that pimpled eggshells might result by the disequilibrium enrichment of some proteins with negative effects.

Key words: acid-insoluble matrix, water-insoluble matrix, facultative-soluble matrix, pimpled eggshell, eggshell breaking strength

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INTRODUCTION

Eggshells are of great importance to the poultry industry, not only protecting the egg against physical damage and against bacterial contamination, but also providing a source of calcium and a regulator of gas interchange for embryonic chick development (Nys et al., 1999). Chicken eggshell is a highly ordered porous ceramic comprised of bilayered membranes, calcified extracellular matrix, and cuticle. The calcified extracellular matrix, also designated as calcified shell, ultrastructurally forms mammillary cones, palisade, and a vertical crystal layer (Arias et al., 1993).

The calcified shell, comprised of $CaCO_3$ calcite crystals and a pervading organic matrix, is the major portion of chicken eggshell, and is a predominant contributor to the mechanical properties of eggshell. It is reported that the size, shape, and orientation of the calcite crystals can significantly influence the structural organization of eggshell (Rodríguez-Navarro et al., 2002). The size and orientation of calcite crystals are positively correlated with not only thickness but also the breaking strength of an eggshell (Dunn et al., 2012). Chicken eggshells consisting of highly oriented crystals with abnormal sizes are significantly weaker than eggshells with smaller and less-oriented crystals (Rodríguez-Navarro et al., 2002; Ahmed et al., 2005).

In chicken eggshells, the organic matrix contains proteins (70%) and polysaccharides (11%) as major constituents (Baker and Balch, 1962; Heaney and Robinson, 1976). In the last decade, abundant proteomic studies were carried out to identify the proteins

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distributed in shell matrix extracted by various strategies. Acetic acid-soluble matrix from the chicken calcified eggshells was found to contain 520 proteins (Mann et al., 2006). From the whole chicken eggshell matrix, 466 proteins have been described, after 20% acetic acid decalcification (Sun et al., 2013). After decalcification by 10% acetic acid, proteomic analysis yielded 697 proteins in the whole turkey eggshell matrix (Mann and Mann, 2013). In a whole shell matrix of quail after 50% acetic acid decalcification, 622 proteins were identified (Mann and Mann, 2015). These results suggest that a variety of proteins distribute in not only chicken but also other avian calcified shells. Using the 0.6 M ethylenediaminetetraacetic acid- (EDTA-) insoluble shell matrix, 16 proteins were determined in the palisade layer and 23 in the mammillary layer (Mikšík et al., 2010). Following 0.1 N HCl decalcification, 18 mammillary cone-specific proteins and an additional 18 proteins, enriched in the mammillary cones, were identified (Rose-Martel et al., 2015). Moreover, 216 shell matrix proteins were identified at 4 key mineralization stages during shell formation, among which 91, 132, 178, and 184 proteins were respectively identified at 5, 6, 7, and 16 h after previous oviposition (Marie et al., 2015). These results further suggest that abundant proteins spatiotemporally participate in the eggshell calcification. Various glycosaminoglycans and proteoglycans, such as hyaluronic acid, chondroitin sulfate/dermatan sulfate, keratan sulfate, and heparan sulfate/heparin have also been detected within the mineralized region of eggshells (Arias et al., 1992; Liu et al., 2014; Liu et al., 2016).

Over the years, the organic components present in eggshells have been demonstrated to control the nucleation, growth and shape of calcite crystals (Fernandez et al., 2004; Hernandez-Hernandez et al., 2008). For instance, genetic association studies have shown that ovalbumin and ovotransferrin correlate with crystal size, while ovocleidin-116 and ovocalyxin-32 are associated with crystal orientation (Dunn et al., 2012). Extracted eggshell matrix can also promote the formation of calcite crystals in vitro (Iwasawa et al., 2009), and uterine fluid, present at various phases of eggshell formation, can significantly control crystal size or morphology in vitro (Dominguez-Vera et al., 2000). Furthermore, in vitro mineralization experiments also suggest that the individual matrix proteins can participate in the nucleation and growth of calcite crystals, such as osteopontin (Chien et al., 2008) and ansocalcin (the goose homologue of chicken ovocleidin-17 (OC-17)) (Lakshminarayanan et al., 2002; Lakshminarayanan et al., 2005). Highly sulfated glycosaminoglycans and proteoglycans have high calcium affinity and can modify the precipitation of calcium carbonate in vitro through electrostatic interactions (Wu et al., 1994; Arias et al., 2002). The sulfate content of these polyanionic macromolecules may also significantly affect the morphology of calcite crystals in vitro (Arias et al., 2002).

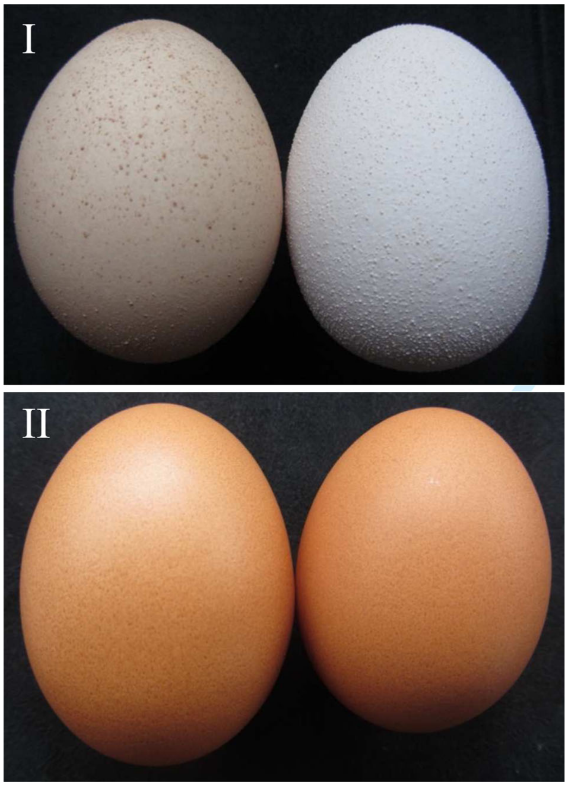

Figure 1. Egg samples. Panel I shows pimpled eggs, panel II shows normal eggs.

Overall, it is well known that there is an abundant organic matrix pervading the calcified eggshells, and many in vitro studies have verified that this matrix may regulate calcification. However, to our knowledge, there is sparse information about the association of global matrix in calcified eggshell with the eggshell qualities. In the present study, we dissociate the eggshell matrix into acid-insoluble, water-insoluble, and facultative-soluble (both acid- and water-soluble) components, analyze these matrix components with respect to eggshell qualities, and compare these matrix components between normal eggshells and pimpled eggshells. The pimpled eggshell is an eggshell abnormality usually characterized as many redundant calcified granules scattered on one or both eggshell apexes and even spreading to the equator of shells, along with a lighter-color cuticle (Figure 1), and exhibiting especially weak breaking strength.

MATERIALS AND METHODS

Experimental Eggs

Eggs were from about 65-week of Hy-line Brown commercial layers (Aige Layer Company, Lin'an, Zhejiang, China). The eggs were directly sampled from the layer cages on laying day. Two hundred twenty eggs with normal shape and cuticle color were collected as normal eggs (Figure 1), and 40 eggs with pimpled area more than 40% of the eggshell surface were selected as pimpled eggs (Figure 1).

For normal eggs, 220 eggs with normal shape and cuticle color were originally collected (Figure 1), and 60 eggs were finally selected for experimental eggshells according to following methods. Eggs were selected with an egg weight ranging from 62.5 to 69.5 g to minimize the size difference. And the egg shape index (length / width) ranged from 1.28 to 1.37 (length: 56.6 to 61.4 mm; width: 43.6 to 45.4 mm) to minimize the shape difference. The thickness of the 5 areas was determined to minimize the thickness heterogeneity in the same calcified eggshell (shell without membranes and cuticle). Six samples were collected from 3 areas including blunt, sharp, and equator areas, while 3 samples were collected from both apexes. The maximum thickness difference in the same area was less than 0.03 mm and the maximum difference among the 5 area average thickness was less than 0.03 mm.

For pimpled eggs, 40 eggs with pimpled area more than 40% of the eggshell surface were directly selected from the layer farm (Figure 1). The weight of these eggs ranged from 61.61 to 71.31 g, and shape index ranged from 1.28 to 1.40 (length: 56.3 to 65.0 mm; width: 41.3 to 47.2 mm). The thickness detection of pimpled eggshells was performed just as described above for normal eggshells; however, because of the redundant calcified granules scattering on eggshell, to the greatest extent possible, the shell locations without calcified granules were determined, and the thickness heterogeneity of pimpled eggshells was not considered.

Measurement of Egg or Eggshell Variables

After collection from the farm, the egg weight and egg shape index (length/width) were measured immediately using balance and caliper; then the eggshell breaking strength was measured by eggshell strength gauge (FHK, Fujihara Co., Tokyo, Japan). Following shell breaking strength testing, each egg was broken into halves, the egg content was discarded, and the shell was repeatedly washed by tap water, then rinsed into 5% EDTA for 25 min to facilitate mechanical removal of cuticle and membranes to obtain calcified eggshells. The calcified eggshells were dried at room temperature. Thickness of calcified shell was measured with a digital micrometer. The pimpled eggshells and finally selected normal eggshells were stored at −70 ° C.

Extraction of Organic Matrix Components

After removal of membranes and cuticle, the organic matrix components were extracted from each calcified eggshell. Briefly, the eggshell was individually powdered using mortar and pestle, then decalcified by stirring with 10% acetic acid at 20 ° C for about 18 h. The amount of 10% acetic acid used was about 22 to 25 mL/g shell powder, and the acetic acid was added stepwise as the proportion of 35:35:30, the first 2 steps of decalcification were individually carried out over 3 to 4 h, and the last time decalcification was performed overnight.

After decalcification, the suspension was centrifuged (fixed-angle rotor) at 23,500 × *g* for 18 min and the deposit was washed 2-times with distilled water and centrifuged, the pellet was freeze-dried and designated as acid-insoluble matrix (expressed as M1). The supernatant (referred as acid-soluble matrix) was repeatedly dialyzed 4 times against 25-volumes of distilled water at 20 ° C using a Spectra/Por 6 dialysis tubing bag (molecular weight cutoff (MWCO) 8 kDa; Spectrum Labs, Rancho Dominguez, CA, USA). The sample was then centrifuged (swinging-tube rotor) at 3,500 × *g* for 40 min to obtain water-insoluble matrix (deposit) (expressed as M2) and (both acid- and water-soluble) facultative-soluble matrix (supernatant) (expressed as M3). The water-insoluble matrix (M2) was freeze-dried, and the facultative-soluble matrix (M3) was concentrated using a Millipore spin column (MWCO 8 kDa) and freeze-dried. After freeze-drying, each matrix component was individually weighed using digital microbalance (total weight of EP tube with dried matrix minus the empty EP tube).

Statistical Analysis

The software of one-way ANOVA in SPSS 19.0 was used to analyze the difference of matrix and other variables between normal egg and pimpled eggs. The software of Bivariate Correlations in SPSS 19.0 was used to determine the strength and direction of Pearson correlation coefficients between pairs of variables, including matrix variables and other egg or eggshell variables. Statistical values expressed as mean ± standard deviation (**SD**), and the threshold of significant difference chosen for all analyses was set as $P < 0.05$.

RESULTS

Eggshell and Matrix Variables of the Normal and Pimpled Eggshells

Six samples were determined in multiple areas, such as blunt, sharp, and equator zones, and 3 samples were determined in each apex, moreover, the thickness difference was restricted to 0.03 mm. This served to keep the thickness homogeneity and reduce the strength heterogeneity in the same eggshell. Finally, 60 shells were selected, from original 220 eggs, as normal specimens (Table 1), the selection ratio was about 27%, which was much higher than previous study of about 10% (Liu et al., 2016).

The statistical testing results showed there were no significant differences in egg weight, egg shape index, egg length, egg width, and shell membrane weight ($P > 0.05$) between the normal and pimpled eggs

(Table 1). However, other variables (such as calcified shell weight, breaking strength, overall calcified shell thickness, and the thickness in 5 parts of the calcified eggshells) showed significant differences ($P < 0.0001$) (Table 1): pimpled eggshells were observably characterized with less calcified shell weight (4.46 g vs. 5.70 g), thinner calcified shells (2.287 mm vs. 0.347 mm), and weaker breaking strength (1.37 kgf vs. 3.47 kgf) (Table 1).

Proteins in an eggshell can be divided into insoluble and soluble proteins. The insoluble proteins are thought to be inter-mineral matrix and acts as a structural framework, while the soluble proteins are thought to be intra-mineral matrix that are embedded within the crystal during calcification (Guru and Dash, 2014). Since quantification of matrix fractions in eggshells, especially in pimpled eggshells has seldom been carried out, and we systemically measured the amounts or yields of various matrices extracted from individual calcified shell, the percentage contents of various matrices in per gram of shell, and the constitutive proportions of 3 matrix fractions in the total matrix.

In individual calcified eggshells, the weight of pimpled eggshell was observably less than that of normal shell (4.46 g vs. 5.70 g), only the amount of facultative-soluble matrix (M3) in pimpled shell was significantly less than that in normal eggshell (8.20 mg vs. 9.34 mg) ($P < 0.05$), while the amount of water-insoluble matrix (M2) was similar ($P > 0.05$) (Table 1). It is noteworthy that the amounts of total matrix and acid-insoluble matrix (M1) in individual pimpled shell were much higher than that in normal eggshell (126.37 mg vs. 104.33 mg, and 98.03 mg vs. 73.48 mg, respectively) ($P < 0.0001$) (Table 1).

After normalization based on calcified shell weight, the amounts of matrix were converted to matrix contents. The results show that contents of various matrix in pimpled shells, including total matrix, M1, M2 and M3, were all higher than that in normal shells, 2.86% vs. 1.84%; 2.23% vs. 1.30%; 0.45% vs. 0.38%; 0.18% vs. 0.16%, respectively (Table 1).

As for the constituent proportions of 3 matrix components in the total matrix, the results showed that, in both types of eggshells, the total matrix was dominantly comprised by acid-insoluble matrix (M1, the proportion was > 70%), while the facultative-soluble matrix (M3) was a minor constituent, with its proportion <10% (Table 1). Moreover, in pimpled eggshells, the ratio of M1 to total matrix was significantly higher than that in normal eggshells (77.09% vs. 70.41%, $P < 0.0001$). The constituent proportions of both M2 and M3 in pimpled eggshells were significantly less than that in normal eggshells (16.40% vs. 20.62%, $P < 0.0001$; 6.51% vs. 8.97%, $P < 0.0001$, respectively).

Table 1. Eggshell and matrix variables of the normal and pimpled eggshells.

Variables	Sample size	Egg weight (g)	Egg shape index	Egg length (mm)	Egg width (mm)	Membrane weight (mg)	Calcified shell weight (g)	Shell breaking strength (kgf)	Calcified shell thickness (mm)
Normal	60 eggs	65.24 ± 3.02 $^{N.S.}$	1.32 ± 0.05 $^{N.S.}$	58.54 ± 2.21 $^{N.S.}$	44.33 ± 0.66 $^{N.S.}$	151.5 ±19.2 $^{N.S.}$	5.70 ± 0.53**	3.47 ± 0.89**	0.347 ± 0.027**
Pimpled	40 eggs	64.84 ± 5.40 $^{N.S.}$	1.31 ± 0.22 $^{N.S.}$	57.99 ± 9.85 $^{N.S.}$	43.04 ± 7.30 $^{N.S.}$	143.4 ±21.7 $^{N.S.}$	4.46 ± 0.75**	1.37 ± 0.85**	0.287 ± 0.047**
Variables	Sharp zone thickness (mm)		Equatorial thickness (mm)	Blunt zone thickness (mm)	Sharp apex thickness (mm)	Blunt apex thickness (mm)	Total matrix amount (mg)	M1 amount (mg)	M2 amount (mg)
Normal	0.351 ± 0.030**		0.348 ± 0.027**	0.342 ± 0.028**	0.348 ± 0.029**	0.343 ± 0.029**	104.3 ±13.1**	73.5 ± 9.7**	21.5 ± 3.6$^{N.S.}$
Pimpled	0.287 ± 0.049**		0.296 ± 0.048**	0.289 ± 0.046**	0.270 ± 0.070**	0.293 ± 0.054**	126.4 ± 27.9**	98.0 ± 25.8**	20.1 ± 6.0 $^{N.S.}$
Variables	M3 amount (mg)		Total matrix content (%)	M1 content (%)	M2 content (%)	M3 content (%)	Ratio of M1 to total matrix (%)	Ratio of M2 to total matrix (%)	Ratio of M3 to total matrix (%)
Normal	9.3 ± 1.9*		1.84 ± 0.23**	1.30 ± 0.17 **	0.38 ± 0.06**	0.16 ± 0.03*	70.41 ± 2.71**	20.62 ± 2.48**	8.97 ± 1.44**
Pimpled	8.2 ± 3.0*		2.86 ± 0.56**	2.23 ± 0.60**	0.45 ± 0.09**	0.18 ± 0.05*	77.09 ± 5.97**	16.40 ± 4.72**	6.51 ± 1.78**

Notes: 1) M1: acid-insoluble matrix; M2: water-insoluble matrix; and M3: water and acid facultative-soluble matrix.

2) "matrix amount" means the amount of matrix possessed in whole individual calcified shell; "matrix content" means the percentage content of matrix in per gram calcified shell, i.e., the matrix amount normalized by calcified shell weight.

3) N.S. represents no significant difference between values in the same column, i.e., $P > 0.05$; *represents the difference between values is at the level of $0.01 < P < 0.05$; **means the difference is at $P < 0.0001$.

Correlations between 3 Matrix Components

Correlation analysis was carried out based on the contents of various matrices to study the interrela-

Table 2. Bivariate Pearson correlations between contents of 3 matrix components.

Matrix	Statistics	M1 content	M2 content	M3 content
M1 content	r		0.52	0.44
	P−value		**<0.0001**	**0.001**
M2 content	r	−0.53		0.27
	P−value	**0.001**		**0.040**
M3 content	r	−0.03	0.40	
	P−value	0.855	**0.014**	

Note: 1) The values in the upper triangle represent the results of normal calcified eggshells, and values in the lower triangle are the results of pimpled calcified eggshells.

2) M1: acid-insoluble matrix, M2: water-insoluble matrix, and M3: water and acid facultative-soluble matrix.

3) "matrix content" means the percentage content of matrix in per gram calcified shell, *i.e.* the matrix amount normalized by calcified shell weight.

tion between the 3 matrix fractions. The results on normal eggshells showed that the correlations between the 3 fractions were all significant and positive, among which, acid-insoluble matrix (M1) content was moderately correlated with both water-insoluble matrix (M2) (r = 0.52, $P < 0.0001$) and facultative-soluble matrix (M3) (r = 0.44, $P = 0.001$), while the correlation between water-insoluble matrix (M2) and facultative-soluble matrix (M3) was weaker (r = 0.27, $P < 0.01$) (Table 2). However, the correlations between matrix fractions in pimpled shells were much more incongruous, particularly the correlations between M1 and other 2 fractions, among which M1 actually negatively correlated with M2 (r = –0.53, $P = 0.001$), and showed almost no correlation with M3 (r = –0.03, $P = 0.855$) (Table 2). These results suggest that 3 matrix fractions in normal eggshells showed a linear relation to one another, but no linear relation was observed between the 3 matrix components of pimpled eggshells.

Correlations between Eggshell Variables and Matrix Amounts in Individual Calcified Eggshell

The egg weight, shell membrane weight, egg shape index, egg length, and egg width, regardless of normal or pimpled eggshells, all had no significant correlation with the amount of the various matrix components of individual calcified shell, including total matrix, acid-insoluble matrix (M1), water-insoluble matrix (M2), and facultative-soluble matrix (M3) ($P > 0.05$) (Table 3). Calcified eggshell weight and thickness were significantly and positively correlated with the amounts of various matrix in both normal eggshells and pimpled eggshells ($P < 0.05$) (Table 3). This means that the more calcified the shell or the thicker the calcified shell, the more matrix components it contained.

The results also showed that, in normal eggshells, shell breaking strength was positively and moderately correlated with the total matrix amount (r = 0.36, $P = 0.006$), and weakly correlated with M1 amount (r = 0.33, $P < 0.05$) and M2 amount (r = 0.28, $P < 0.05$),

Table 3. Correlations between eggshell variables and matrix amounts in individual calcified eggshell.

Eggshells	Matrix	Statistics	Egg weight (g)	Membrane weight (mg)	Calcified shell weight (g)	Calcified shell thickness (mm)	Shell breaking strength (kgf)	Shape index	Egg length (mm)	Egg width (mm)
Normal eggshells	Total matrix amount	r	0.07	0.08	0.38	0.45	0.36	−0.06	−0.02	0.11
		P−value	0.615	0.556	**0.003**	**<0.0001**	**0.006**	0.669	0.899	0.434
	M1 amount	r	0.07	0.18	0.32	0.39	0.33	−0.05	0.00	0.13
		P−value	0.587	0.180	**0.015**	**0.003**	**0.011**	0.727	0.990	0.350
	M2 amount	r	0.03	−0.11	0.32	0.40	0.28	−0.04	−0.03	0.01
		P−value	0.831	0.432	**0.015**	**0.002**	**0.032**	0.790	0.814	0.917
	M3 amount	r	0.04	−0.17	0.38	0.40	0.25	−0.09	−0.07	0.06
		P−value	0.768	0.200	**0.004**	**0.002**	0.060	0.502	0.618	0.664
Pimpled eggshells	Total matrix amount	r	0.15	0.05	0.55	0.55	0.34	−0.01	0.02	0.04
		P−value	0.362	0.770	**<0.0001**	**<0.0001**	**0.035**	0.944	0.886	0.802
	M1 amount	r	0.11	0.07	0.33	0.34	0.15	−0.05	−0.02	−0.02
		P−value	0.516	0.680	**0.040**	**0.040**	0.381	0.776	0.890	0.921
	M2 amount	r	0.19	−0.09	0.74	0.71	0.66	0.09	0.13	0.17
		P−value	0.253	0.585	**<0.0001**	**<0.0001**	**<0.0001**	0.608	0.436	0.307
	M3 amount	r	0.10	0.05	0.78	0.79	0.63	0.13	0.16	0.19
		P−value	0.539	0.788	**<0.0001**	**<0.0001**	**<0.0001**	0.438	0.330	0.242

Note: 1) M1: acid-insoluble matrix; M2: water-insoluble matrix; and M3: water and acid facultative-soluble matrix.

2) "matrix amounts" represents the amounts of various matrix possessed in whole individual calcified shell.

Table 4. Correlations between eggshell variables and matrix contents in calcified shells.

Eggshells	Matrix	Statistics	Egg weight (g)	Membrane weight (mg)	Calcified shell weight (g)	Calcified shell thickness (mm)	Shell breaking strength (kgf)	Shape index	Egg length (mm)	Egg width (mm)
Normal eggshells	Total matrix content	r	−0.37	0.11	−0.36	−0.18	−0.06	0.07	−0.05	−0.29
		P−value	**0.005**	0.428	**0.006**	0.185	0.672	0.609	0.723	**0.031**
	M1 content	r	−0.34	0.21	−0.38	−0.21	−0.07	0.07	−0.03	−0.24
		P−value	**0.010**	0.119	**0.003**	0.111	0.610	0.618	0.809	0.067
	M2 content	r	−0.27	−0.09	−0.20	−0.05	−0.01	0.07	−0.04	−0.26
		P−value	**0.040**	0.488	0.128	0.704	0.946	0.626	0.755	**0.047**
	M3 content	r	−0.24	−0.17	−0.10	−0.01	−0.02	0.00	−0.08	−0.20
		P−value	0.072	0.216	0.466	0.917	0.892	0.996	0.549	0.139
Pimpled eggshells	Total matrix content	r	−0.005	0.05	−0.27	−0.17	−0.14	−0.22	−0.22	−0.22
		P−value	0.978	0.772	0.105	0.306	0.405	0.188	0.182	0.179
	M1 content	r	−0.02	0.07	−0.33	−0.25	−0.24	−0.21	−0.21	−0.22
		P−value	0.908	0.677	**0.046**	0.124	0.148	0.216	0.202	0.185
	M2 content	r	0.10	−0.19	0.29	0.35	0.47	−0.03	−0.01	0.02
		P−value	0.541	0.263	0.080	**0.032**	**0.003**	0.847	0.940	0.914
	M3 content	r	−0.01	0.05	0.43	0.56	0.50	0.09	0.10	0.12
		P−value	0.960	0.763	**0.007**	**<0.0001**	**0.001**	0.606	0.564	0.480

Note: 1) "matrix content" means the percentage content of matrix in per gram of calcified shell, *i.e.* the matrix amount normalized by calcified shell weight.

2) M1: acid-insoluble matrix; M2: water-insoluble matrix; and M3: water and acid facultative-soluble matrix.

but had no significant correlation with M3 amount (r = 0.25, $P > 0.05$) (Table 3). In pimpled eggshells, the breaking strength was positively and moderately correlated with the total matrix amount (r = 0.34, $P < 0.05$), and strongly correlated with the M2 amount (r = 0.66, $P < 0.0001$) and M3 amount (r = 0.63, $P < 0.0001$), but had no significant correlation with M1 amount (r = 0.15, $P > 0.05$) (Table 3). Above all, the amount of total matrix in both calcified shells were positively correlated with shell breaking strength; but in normal eggshells, the significant contributing fractions were M1 and M2, while the significant contributing fractions in pimpled eggshells were M2 and M3.

Correlations between Eggshell Variables and Matrix Contents in Calcified Eggshells

In both types of eggshells, the shell membrane weight, egg shape index, and egg length were not significantly correlated with the contents of 4 matrix components, i.e., total matrix, acid-insoluble matrix (M1), water-insoluble matrix (M2) and facultative-soluble matrix (M3) ($P > 0.05$) (Table 4).

The egg weight of pimpled eggs showed no significant correlation with all 4 matrix components ($P > 0.05$) (Table 4). However, in normal eggs, the egg weight showed a negative and significant correlation with total matrix content (r = –0.37, $P = 0.005$), M1 content (r = –0.34, $P = 0.010$) and M2 content (r = –0.27, $P = 0.040$), but the negative correlation with M3 content was not significant (r = –0.24, $P = 0.072$) (Table 4). These results suggested that with an increase in normal egg weight, the contents of various matrix components in calcified shells tended to decline.

Similarly, calcified eggshell weight of normal eggshells showed negative correlations with all 4 matrix components, among which the correlations with both total matrix content (r = –0.36, $P = 0.006$) and M1 content (r = –0.38, $P = 0.003$) were significant, but the correlations with M2 content (r = –0.20, $P > 0.05$) and M3 content (r = –0.10, $P > 0.05$) were not significant (Table 4). These results reflect a trend that with the increasing of normal calcified shell weight the contents of various matrix components decreased, especially contents of total matrix and M1. However, the eggshell weight of pimpled eggshells was negatively and significantly correlated with M1 content (r = –0.33, $P = 0.046$), but positively correlated with M3 content (r = 0.43, $P = 0.007$) (Table 4).

Finally, in pimpled eggshells, the calcified shell thickness and shell strength showed no significant correlations with the contents of total matrix and M1 ($P > 0.05$), but were significantly correlated with both contents of M2 and M3 ($P < 0.05$ and $P < 0.01$) (Table 4). In normal eggshells, both calcified shell thickness and shell strength all showed no significant

Table 5. Correlations between eggshell variables and matrix constitutions.

Eggshells	Matrix	Statistics	Egg weight (g)	Membrane weight (mg)	Calcified shell weight (g)	Calcified shell thickness (mm)	Shell breaking strength (kgf)	Shape index	Egg length (mm)	Egg width (mm)
Normal eggshells	Proportion of M1 in total matrix	r	– 0.03	0.36	– 0.21	– 0.20	– 0.04	0.04	0.05	0.02
		P–value	0.826	**0.006**	0.120	0.132	0.773	0.756	0.707	0.869
	Proportion of M2 in total matrix	r	0.02	– 0.23	0.11	0.13	0.03	0.00	– 0.02	– 0.05
		P–value	0.908	0.079	0.422	0.334	0.823	0.981	0.908	0.734
	Proportion of M3 in total matrix	r	0.03	– 0.28	0.20	0.15	0.01	– 0.08	– 0.07	0.04
		P–value	0.814	**0.036**	0.137	0.275	0.919	0.534	0.618	0.774
Pimpled eggshells	Proportion of M1 in total matrix	r	– 0.04	0.07	– 0.41	– 0.40	– 0.42	– 0.15	– 0.17	– 0.19
		P–value	0.806	0.658	**0.010**	**0.014**	**0.009**	0.357	0.319	0.262
	Proportion of M2 in total matrix	r	0.05	– 0.12	0.31	0.29	0.35	0.12	0.13	0.15
		P–value	0.761	0.485	0.055	0.081	**0.033**	0.477	0.432	0.364
	Proportion of M3 in total matrix	r	0.003	0.06	0.55	0.57	0.48	0.20	0.21	0.22
		P–value	0.984	0.711	**<0.0001**	**<0.0001**	**0.002**	0.231	0.209	0.177

Note: 1) "matrix constitutions" represents the constituent proportions of 3 matrix components in total matrix.
2) M1: acid-insoluble matrix; M2: water-insoluble matrix; and M3: water and acid facultative-soluble matrix.

correlations with any matrix content ($P > 0.05$) (Table 4). This suggests that both eggshell thickness and breaking strength do not linearly increase with the contents of any matrix component in normal calcified eggshells.

Correlations between Eggshell Variables and Constituent Proportions of 3 Matrix Fractions in Total Matrix

In normal eggs, although the shell membrane weight showed no significant correlation with both amounts and contents of various matrices (Tables 3 and 4), it significantly showed positive correlation with the constituent proportion of acid-insoluble matrix (M1) ($r = 0.36$, $P = 0.006$) and negative correlation with facultative-soluble matrix (M3) ($r = –0.28$, $P = 0.036$) (Table 5). However, except for shell membrane weight, the constituent proportions of all 3 matrix fractions had no significant correlations with any other variables, including egg weight, egg shape index, egg length, egg width, and even calcified shell weight, calcified shell thickness and breaking strength ($P > 0.05$) (Table 5).

In pimpled eggs, constituent proportions of all 3 matrix fractions showed no significant correlations with egg weight, shell membrane weight, egg shape index, egg length and width ($P > 0.05$) (Table 5). They did show significant correlations with calcified shell weight, calcified shell thickness and breaking strength, among which, the constituent proportion of acid-insoluble matrix (M1) was negatively correlated with calcified shell weight ($r = –0.41$, $P = 0.010$), calcified shell thickness ($r = –0.40$, $P = 0.014$), and breaking strength ($r = –0.42$, $P = 0.009$) (Table 5). The constituent proportion of facultative-soluble matrix (M3) was positively correlated with calcified shell weight ($r = 0.55$, $P < 0.0001$), calcified shell thickness ($r = 0.57$, $P < 0.0001$), and breaking strength ($r = 0.48$, $P = 0.002$) (Table 5). The constituent proportion of water-insoluble matrix (M2) was only positively correlated with calcified shell weight ($r = 0.35$, $P < 0.033$) (Table 5).

DISCUSSION

The present results show that, in normal eggshells, the total matrix content of the calcified shell was 1.84% (Table 1), which is slightly higher than our previous result of 1.67%, using similar extraction method (Liu et al., 2014), but lower than another result of 2.29% obtained by combustion at 580°C in muffle furnace (Wang et al., 2014). The difference between the first 2 studies may be caused by different egg sources or different processes of decalcification. In the present study, 10% acetic acid was added stepwise to decalcify, while the acetic acid was added one time in the previous study. Present pimpled calcified eggshells contained 2.86% of organic matrix (Table 1), which was even higher than previous study of 2.68% using combustion at 580°C in muffle furnace (Wang et al., 2014). The differences may result from different egg sources, as only eggs pimpled over 40% of eggshell surface were selected as specimens in the present study.

It is well established that abundant of organic matrix is distributed in calcified eggshells (Mann et al., 2006; Hernandez-Hernandez et al., 2008; Sun et al., 2013; Liu et al., 2014; Marie et al., 2015). Over the years, the organic matrix has been thought to play a major role in the calcification and hierarchical structures of the eggshell, and many in vitro experiments have been performed to verify this in the case of multi-components and individual proteins. In multi-component experiments, both the uterine fluid collected at key mineralization phases of shell formation (Dominguez-Vera et al., 2000) and the matrix extracted from eggshells (Iwasawa et al., 2009) all promote calcite crystal formation, which suggests that the global organic matrix is capable of promoting the assembly of eggshell and determining its mechanical properties. The pimpled egg is characterized by thinner calcified shell thickness and weaker shell strength (Table 1); however, in pimpled calcified eggshells, the contents of all 4 groups of matrix, i.e., total matrix, acid-insoluble matrix (M1), water-insoluble matrix (M2) and facultative-soluble matrix (M3), were actually and significantly higher than that in normal eggshells ($P < 0.0001$, $P < 0.0001$, $P < 0.0001$, and $P = 0.040$, respectively) (Table 1); in particular, the amounts of total matrix and acid-insoluble matrix (M1) extracted from individual pimpled calcified eggshells were unexpectedly and significantly higher than that from individual normal eggshell ($P < 0.0001$) even though the weight of individual pimpled calcified eggshells was much less than that of normal calcified shells ($P < 0.0001$) (Table 1).

The reason for above results may be that the shell matrix is comprised of abundant individual proteins, but the roles of individual proteins in eggshell mineralization may be quite variable. Based on the limited information on the in vitro crystallization of specific proteins, many proteins promote biomineralization, such as lysozyme (Wang et al., 2009), ansocalcin (Lakshminarayanan et al., 2002; Lakshminarayanan et al., 2005), OC-17 (Reyes-Grajeda et al., 2004). (In Lakshminarayanan's results, OC-17 had no significant effect on the nucleation of calcite (Lakshminarayanan et al., 2005)). However, osteopontin (**OPN**) shows inhibitory effects on calcite growth (Chien et al., 2008). Both quantification studies of individual proteins of the *in vivo* shell matrix also show that ovotransferrin and ovalbumin are inversely correlated with shell breaking strength (Panheleux et al., 2000; Ahmed et al., 2005). Therefore, it can be inferred that there might be other proteins, in the protein inventory of shell matrix, with negative effects on biomineralization. It has been speculated that normal shell calcification requires a precise equilibrium among the expression of various matrix proteins, any deviation from this equilibrium will cause a shell abnormality (Arazi et al., 2009). Our results also show that in comparison with normal eggshells the contents of the 3 matrix fractions in pimpled eggshells are disproportionate (Table 2). Moreover, both in vitro and in vivo investigations reported that serum albumin, a major protein in avian eggshell (Mann and Mann, 2015; Rose-Martel et al., 2015), promotes the growth rate of Ca-P precipitates at low concentrations but inhibits the growth rate at high concentrations (Combes et al., 1999; Combes and Rey, 2002; Wang et al., 2013). Overall, the pimpled eggshells may result from the disequilibrium enrichment of some proteins with negative effects on biomineralization.

Among the in vitro crystallization studies, most show the promotion effect of matrix or individual proteins on nucleation, morphology and size of calcite depends on the concentration of organic material, such as uterine fluid collected at the initial and terminal stages of shell formation (Dominguez-Vera et al., 2000), extracted eggshell matrix (Iwasawa et al., 2009), ansocalcin (Lakshminarayanan et al., 2002; Lakshminarayanan et al., 2005), OC-17 (Reyes-Grajeda et al., 2004), OPN (Chien et al., 2008), and lysozyme (Wang et al., 2009). Based on the above studies, we might speculate that the mechanical properties of eggshell would increase with the content of matrix in calcified shell. However, the present study shows that in normal eggs of similar weight, size and shape, the contents of 4 groups of matrix, i.e., total matrix, acid-insoluble matrix (M1), water-insoluble matrix (M2) and facultative-soluble matrix (M3), all unexpectedly show no significant correlations with calcified shell thickness and breaking strength ($P > 0.05$) (Table 4). These results imply that the organic matrix is crucial to regulating the interaction and deposition of matrix-mineral (Rodríguez-Navarro et al., 2015), but its effect on the ultrastructures and mechanical properties of eggshell may not linearly depend on the percentage content of organic matrix in each eggshell. Other physicochemical properties of organic matrix may impact the mechanical properties of eggshell. Many eggshell proteins undergo various post-translational modifications. For example, osteopontin is a phosphoprotein (Mann et al., 2007), OC-17 is a lectin-like phosphoprotein and can be glycosylated or nonglycosylated (Mann, 1999), and OC-116 is a dermatan/chondroitin sulfate proteoglycan (Hincke et al., 1999). Such modifications may influence biomineralization by electrostatic properties (Wu et al., 1994; Arias et al., 2002), resulting in various shell qualities.

CONCLUSIONS

Pimpled eggshells have much more matrix in individual calcified shells than normal eggshells, a possible consequence of a disequilibrium enrichment of proteins having a negative impact on eggshell quality. In normal eggshells, the organic matrix necessary to regulate the interaction and deposition of matrix-mineral, but the percentage content of organic matrix in calcified eggshell does not directly determine the mechanical properties of an eggshell.

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