Diversity of the Cuticle Layer of Avian Eggshells

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The structure and mineral composition of eggshell cuticles were studied in 5 species of birds. The approximate thickness of the cuticle layer at the top of shell columns was about 1µm in the Red Junglefowl (Gallus gallus), about 130µm in the White Pelican (Pelecanus onocrotalus), about 10µm in the Japanese quail (Coturnix japonica), about 110µm in the Greater Flamingo (Phoenicopterus ruber roseus) and about 45µm in the Humboldt Penguin (Spheniscus humboldti). The matrix of the cuticle layer decalcified with EDTA was composed of vesicles in a variety of sizes in all birds. Major elements in cuticle materials detected by X-ray microanalysis were O, C, Ca and P, and their percentage numbers of atoms decreased in this order. The concentration of P was significantly higher in the cuticles of the quail, flamingo, and penguin than in those of the junglefowl and pelican. Ca mapping on electron-microscopic images showed strong signals in the shell layer and weaker ones in the cuticle layer, whereas P mapping showed that signals were mostly confined in the cuticle layer. X-ray diffraction analyses on the inside of the shell layer showed a profile of calcite crystals of calcium carbonates in all birds. In the cuticle materials, the profile was of calcite in the junglefowl, and a mixture of calcite and vaterite in the pelican. The profiles in cuticle materials of the quail, flamingo and penguin showed no specific signals, indicating that mineral compounds are amorphous in these forms. It was suggested that the diversity of mineral structures in the cuticle layer is caused by the presence of phosphorus, in addition to the structure of the cuticle matrix.

Key words: calcite, cuticle layer, eggshell, phosphorus, vaterite

Introduction

The avian eggshell is almost completely solid and consists of a thick calcified layer and a thin cuticle layer that coats its outside (Burley and Vadehra, 1989). With the calcified shell layer, many studies have been done on the composition of the organic matrix that determines the crystal polymorphism of calcium carbonates and other characteristics of the eggshell (Gautron et al., 1996; Dominguez-Vera et al., 2000; Hernández-Hernández et al., 2008; Hinchke et al., 2008; Iwasawa et al., 2009), and on the composition of inorganic materials (Romanoff and Romanoff, 1949; Burley and Vadehra, 1989). The structure of the shell layer is thought to provide protection against physical damage (Burley and Vadehra, 1989).

Studies concerning the cuticle layer mostly focused on the physiological function that controls the passage of water and gases (Board and Halls, 1973; Deeming, 1987; Thompson and Goldie, 1990). In penguins, the cuticle reduces loss of water during the early part of egg incubation, and its erosion during incubation increases conductance of gases needed for normal embryonic development (Thompson and Goldie, 1990). Since the cuticle prevents evaporation by covering the external pore openings, it is also expected that the cuticle acts as a physical barrier to microbial infection (Board and Halls, 1973). Recently, we proposed a new function for the cuticle in quail eggs (Rahman et al., 2009). Egg rotation in the uterus is a prerequisite for the determination of embryonic body-axis in the aves (Kochav and Eyal-Giladi, 1971). The critical period for this determination is believed to be the second half of the uterine period (Kochav and Eyal-Giladi, 1971), and it is in this period that the cuticle layer forms on the shell layer (Rahman et al., 2009). Therefore, we reported that the cuticle may function as a lubricant that facilitates egg rotation in the uterus.

Since the cuticle makes a significant contribution to eggshell thickness, it is a priori believed that the cuticle is important in maintaining eggshell strength (Belyavin and Boorman, 1980). However, how the cuticle influences shell strength has not been established with certainty (Belyavin and Boorman, 1980). Avian eggs are incubated in widely different environments such as rocks, soil, sand, grass and so on, and are pressured by the parent’s body weight during incubation. Thus, there may be diversity in the structure of the cuticle layer which is directly related to the nest materials and parent’s body. The present study...
reports on the diversity in mineral crystals in the cuticle layer of five avian species.

Materials and Methods

Materials
The eggshells used were obtained from various sources. Eggs with the wild-type color of Japanese quail (Coturnix japonica) were obtained from the farm of Gifu University. Eggshells of the Red Junglefowl (Gallus gallus), Greater Flamingo (Phoenicopterus roseus) and White Pelican (Pelecanus onocrotalus) were supplied by Tama Zoological Park, Tokyo, Japan, and those of the Humboldt Penguin (Spheniscus humboldti) were supplied by Yagiyama Zoological Park, Sendai, Japan. Since the Humboldt Penguin is designated in the International Endangered Species of Wild Fauna and Flora in the Law for the Conservation of Endangered Species of Wild Fauna and Flora by the Ministry of the Environment, Japan, transfer of the eggshell was done with due process for the law.

Interior materials of eggs were removed by suction through a hole made on the eggshell, and the eggshell was air-dried and kept in a plastic bag. Special attention was paid for the protection of shell surface during the transportation.

Electron Microscopy
For scanning electron microscopy, eggshells were coated with a layer of osmium in a Neoc-ST ion sputter (Meiwa, Tokyo, Japan), and examined by using an S-3000 N scanning electron microscope (Hitachi, Tokyo, Japan). To demonstrate the elements in the eggshells, the specimens were examined with an energy dispersive X-ray micro analyzer, the EMAX EX-220 (Horiba, Tokyo, Japan), attached to the microscope. With the same equipment, quantitative determination of element concentration was done for the cuticle materials which were scraped from the eggshells with a file. Determination of each element’s concentration was done by calculating the average of three specimens in each bird.

For transmission electron microscopy, eggshells were treated with 150 mM EDTA in 0.1 M cacodylate buffer (pH 7.2) for 1 d. Decalcified shell-matrices were fixed in 2.5% glutaraldehyde (vol/vol) in 0.1 M cacodylate buffer for 3 h at 4°C, rinsed in cacodylate buffer, postfixed with 1% osmium tetroxide (wt/vol) in the same buffer, and dehydrated in acetone. Specimens were then embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined by using an S-7000 transmission electron microscope (Hitachi).

X-Ray Diffraction
To identify the crystalline phase, the eggshells and the scraped cuticle materials were subjected to X-ray diffractometry in an X-ray diffractometer, the Rad-2R (Rigaku, Tokyo, Japan). Crystal planes were identified by referring to 85–1108 for calcite and 74–1867 for vaterite in the 2009 JCPDS-International Centre for Diffraction Data.

Statistical Analysis
Data were analyzed with Fisher’s protected least-significant difference in the software of Microsoft Excel 2007. Significance was based on P<0.01. Values reported under Results are means±SD.

Results

Electron Microscopy of Eggshell Cross Sections
The shell cuticle was a relatively thin layer on the outside of the calcified columns. Its thickness varied depending on the location; the thinnest part at the top of the columns and the thickest part between the columns. The approximate thickness at the top of the columns was about 1 μm in the junglefowl, about 130 μm in the pelican, about 10 μm in quail, about 110 μm in the flamingo and about 45 μm in the penguin (Fig. 1). Their relative thicknesses in the whole calcified eggshell were about 0.3, 14.7, 5.6, 18.9 and 7.5%, respectively (Fig. 3).

In intact eggshells, the cuticle layer had a similar appearance to that of the calcified shell in junglefowls and quail, whereas it had an appearance of granular accumulation clearly distinct from the shell in the pelicans, flamingoes and penguins (Fig. 1). In decalcified eggshells, the matrix of the cuticle layer was composed of vesicles in all birds (Fig. 2). The sizes of the vesicles varied in a single cuticle of each species, but they tended to be smallest in junglefowls and largest in pelicans, while those of quail, flamingoes and penguins are the middle. A large space between the vesicles was characteristic in the cuticle layer of pelicans, flamingoes and penguins.

X-Ray Microanalysis of Eggshells
Major elements in eggshells detected by X-ray microanalysis were Ca, P, C and O. The Ca mapping on electron-microscopic images showed strong signals in the shell layer and weaker ones in the cuticle layer (Fig. 3).

Fig. 1. Scanning electron micrographs of eggshells of the junglefowl (a), pelican (b), quail (c), flamingo (d) and penguin (e). Granular appearance in the cuticle layer (C) is distinct in the pelican, flamingo and penguin. Arrow indicates the cuticle layer of the junglefowl (a). External surface of eggshell is to the right in an image of pelican (b), whereas those are to the top in images of other birds. S, shell layer. Scale bar = 10 μm.
The cuticle matrix (CM) is constructed by an accumulation of vesicles in various sizes. Spaces among the vesicles are distinct in the pelican, flamingo and penguin. SM, shell matrix. Scale bar m.

Signals of Ca (a–e) are strong in the shell layer (S) and weaker in the cuticle layer (arrows) in all birds. Signals of P (a–e) are confined to the cuticle layer. They are strong in the quail (c), flamingo (d) and penguin (e), weak in the junglefowl (a), but absent from the pelican (b). a–e, ordinary electron micrographs. External surface of eggshell is to the right in images of pelican (b), whereas those are to the top in images of other birds. Scale bars m.

With the P mapping, the signals were largely absent in the shell layer in all species. On the other hand, specific strong signals for P were detected in the cuticle layer of quail, flamingoes and penguins, but weak signals in that of junglefowls. In the pelican cuticle layer, the P signal was not detected. The C and O mapping showed even distribution of signals throughout the eggshells in all species (not shown).

Cuticle materials isolated from eggshells were then examined quantitatively for elements by X-ray microanalysis (Fig. 4). The percentage number of atoms was highest in O, at around 50%, and lowered in C, Ca and P in this order in all species, although the percentage of P was almost negligible in junglefowls and pelicans. The ratios of Ca, C and O were 1.0:1.1:3.3 in junglefowls and 1.0:1.7:3.8 in pelicans, indicating that the mineral compound was CaCO₃. In quail, flamingoes and penguins, the percentage of Ca was lower but that of P was more than junglefowls and pelicans. There was a significant difference between the relative amounts of P (n = 3) of junglefowls (0.4% ± 0.0) and pelicans (0.1% ± 0.1), and those of quail (3.0%...
±0.1), flamingoes (4.7% ± 0.3) and penguins (3.2% ± 0.2).

**X-Ray Diffraction of Eggshells and Scraped Cuticle Materials**

When the X-ray diffraction analysis was done on the inside of eggshells, the profiles of all species were of calcite crystal of calcium carbonates (Fig. 5, line a); the main peaks were {012}, {104}, {006}, {110}, {113}, {202}, {018}, {116}, {122} and {0012}. However, the analyses of the outside of eggshells gave profiles that were basically of calcite form, but variously deformed; in the eggshells of junglefowls, their outside profiles were mostly the same as those on their inside, although the relative intensity of the peak signals was a little different (line b); the peaks {002}, {110}, {111}, {112}, {200}, {114}, {222} and {208} of vaterite crystals were added in eggshells of pelicans (line c); other peaks than {104} and {018} were reduced in quail eggshells (line d); {104} peak was very weak in eggshells of flamingoes (line e) and penguins (line f).

Since X-ray diffraction signals collected from the outside of eggshells may be a mixture of signals both from the shell layer and the cuticle layer, we next performed analyses directly in isolated cuticle materials (Fig. 6). The profiles of junglefowls (Fig. 6, line a) and pelicans (line b) were mostly the same as those of the eggshells measured on the outside. However, no peaks were obtained in specimens of quail (line c), flamingoes (line d) and penguins (line e).

**Discussion**

In *in vitro* crystallization of calcium carbonates with no additives, reported ratios of calcite, aragonite and vaterite were 60:18:22 or 55:22.5:22.5 (Hernández-Hernández *et al.*, 2008). The crystal polymorphism in biological materials is principally determined by the matrix structure (Iwasawa *et al.*, 2009). The eggshell, with a matrix consisting of a meshwork of vesicles, is calcified by calcium...
The planes of calcite diffraction peaks are shown in regular font in line a, and those of vaterite are in italic font in line b. Line a, junglefowl; line b, pelican; line c, quail; line d, flamingo; line e, penguin. Deg. = degree.

Carbonates in the form of calcite (Burley and Vadehra, 1989), whereas the sperm-associated body, with a matrix consisting of a radiation of rod-shaped projections, is calcified in the form of aragonite in avian eggs (Iwasawa et al., 2009). The present study showed that the cuticle layer consists of an accumulation of vesicles of various sizes and has a significant amount of Ca, C and O in all bird eggshells observed. In the isolated cuticle materials of junglefowls, the crystal form of calcium carbonates was calcite and the crystals seemed to grow successively on the shell columns. The cuticle materials of domestic fowls gave the same crystal form as that of junglefowls (unpublished). In the cuticle materials of pelicans, however, the X-ray diffraction analysis showed signals of vaterite in addition to those of calcite, indicating that the crystals of calcium carbonates were a mixture of calcite and vaterite. These crystals buried the spaces among the matrix vesicles and were clearly separated from the shell columns. A unique matrix structure, however, has not yet been identified in the pelican cuticle layer, which supports the vaterite crystals.

X-ray diffraction analyses in isolated cuticle materials of quail, flamingoes and penguins showed no specific signals of any crystals, indicating that the minerals in the cuticle layer of these forms are in an amorphous body state. It is well known that the major mineral elements constituting the chicken eggshell are 98.2% Ca, 0.9% Mg and 0.9% P of the total mineral content in mass ratios, and that the compounds of these elements are calcium carbonate, magnesium carbonate and tricalcium phosphate (Romanoff and Romanoff, 1949; Burley and Vadehra, 1989). The present study showed that these phosphorous compounds comprised 3–4.7% of the atom numbers of cuticle elements and were mostly confined in the cuticle layer. Since the X-ray diffraction analyses on the inside of eggshells which consisted of Ca, C and O consistently gave profiles of calcite crystals, the crystallization in the cuticle layer, where these elements were also present sufficiently, may also be influenced by phosphorus. Possibly, the formation of crystals in the cuticle layer was disturbed by the mutual interference of the compounds in these species. The concentration of P seemed to be a key factor for the crystal formation of calcium carbonates; a lower P value allows crystal formation in jungle fowls and pelicans, but a higher P does not in quail, flamingoes and penguins, although the critical concentration of P has not yet been determined.

Two ideas have been proposed for the function of the cuticle layer in avian eggs. One is that the cuticle reduces loss of water during the early part of egg incubation (Thompson and Goldie, 1990), and another is that the cuticle functions as a lubricant that facilitates egg rotation in the uterus (Rahman et al., 2009). The present study revealed diversity in terms of the construction of mineral compounds in the cuticle layer. In avian incubation, eggs are kept between the parent’s body and the nest floor, which is made of rock, soil, grass or the parent’s feet. Although how the cuticle influences shell strength has not been established with certainty, Belyavin and Boorman (1980) suggested a third idea that the cuticle is important in maintaining eggshell strength. Since the structure constructed by an amorphous body or a mixture of crystals resists to pressure by dispersing a given force, the cuticle layer may function to reduce the pressure caused by the incubating parent. The diversity in the structure of the cuticle layer may be linked to the environment of the nest. Further studies are necessary in a variety of birds to assess this third idea about the function of the cuticle layer.

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References


Burley RW and Vadehra DV. The Avian Egg: Chemistry and