Rock Pigeon eggshell study by SEI/BEI/EDX: Ultrastructure and elemental characterization

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Abstract

This study was aimed to examine ultrastructure, and qualitative and quantitative elements of Rock Pigeon by scanning electron microscope (with SEI/BEI/EDX). Nine samples of Rock Pigeon eggshells were collected in Amphoe Mueang, Nakhon Pathom Province, Thailand. All samples were determined for their ultrastructure and performed elemental characterization using secondary electron imaging detector (SEI), Back-scattered electron imaging detector (BEI), and energy dispersive x-ray microanalysis detector (EDX) with smiling program. The results showed that all eggshells had 3 layers; an outer cuticle layer, a middle palisade layer, and an inner mammillary layer at the thickness of 35.91±9.45, 94.29±10.02 and 11.92±2.31 micrometers, respectively. EDX detected 10 elements containing in the eggshells, including oxygen (O), carbon (C), calcium (Ca), magnesium (Mg), aluminum (Al), silicon (Si), phosphorus (P), sodium (Na), sulfur (S) and chlorine (Cl). The percentage of all elements was found in the following order: O (50.89±17.00) > C (24.73±8.54%) > Ca (23.31±19.72%) > Al (0.50±1.23%) > Mg (0.19±0.59%) > Si (0.14±0.60%) > P (0.03±0.08%) > Na (0.13±0.36%) > S (0.02±0.10%) > Cl (0.08±0.20%). This finding can be used as the model for further study of bird eggshells in case of classification and evolution among birds.

Keywords: back-scattered electron imaging (BEI), bird eggshell, energy dispersive X-ray analysis (EDX), secondary electron imaging (SEI)

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Introduction

Wild avian eggshells provide evidence on ornithology and ecological behavior study, leading to basic information e.g. nest structure or size, color and patterns of eggshells including behavior of the animals during nesting season. These data are important for taxonomic classification (Mikhailov, 1997; Nys *et al*., 2004). Moreover, based on morphological and elementally compositional data, they can be effectively used as environmental indicator of the animals' habitats e.g. heavy metal contamination in environment (Nys *et al*., 2004; Lundholm, 1995; Mora, 2003). Besides, the thickness of eggshells is also employed in implication of ages (Cusack *et al*., 2003) and health and disease status of the pairs (Ridler, 2000).

The essential of eggshells in propagation of all avian species leads into the importance of understanding eggshell structure and compositions. The avian eggshells have sophisticated structures, whose properties reflect perfectly their crucial functions in reproduction. These functions are basically to protect the contents of egg from microbial and physical environment, to control the exchange of water and gasses through pores during embryo development, and to provide the source of skeletal calcium for developing embryo (Nys, *et al*., 2004).

Although valuable data can be obtained from wild avian eggshells, performance of eggshell study is very difficult, particular sample collection. This is because birds naturally build a nest in hidden place where it is hard for predators to reach (Peterson, 1978). Sample collection is directly collecting either unhatched eggs or hatched eggshells where the parents usually remove debris far away from the nests (Snodderly and Max, 1978; Sordahl, 1994; Sandercock, 1996). However, most eggshells after hatching appeared in fragments so the study used hatched eggshells as materials may obtain incomplete information. The difficulty of sample collection results in few paper studies on wild avian eggshells.

In this study, wild Rock Pigeon eggshells were examined because they are easily to find. It also can be used as indicator of environment in sampling area. Thus the aims of this study were to examine the thickness, morphology and ultrastructure of Rock Pigeon using scanning electron microscope with secondary electron imaging detector, and to determine qualitative and quantitative elements of eggshell structure by energy dispersive x-ray analysis (EDX). The results will be used as information in establishing a database and a model of Thai avian eggshells. Moreover, the obtained data is taxonomic evidence in identification of avian species and in comparison of avian eggshells with other animal eggshells, which may imply the evolution between animals.

Materials and Methods

Sample collection

Eggs or eggshells of Rock Pigeons were collected and labeled from nests in Amphoe Mueang, Nakhon Pathom Province. The size and weight of each egg was measured at the laboratory (Fig. 1), and the thickness was measured under electron microscope.

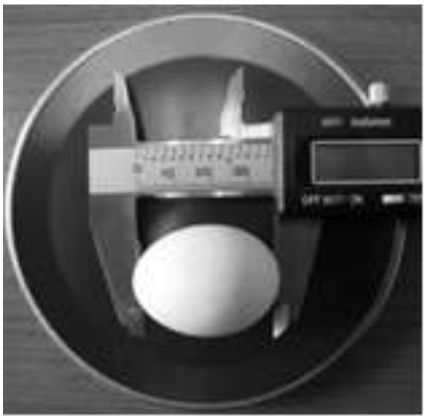

Figure 1: Measurement of length and width of Rock Pigeon eggs using sliding vernier calipers.

Preparation for scanning electron microscope

Side view, structure, and outer and inner morphology of eggshell were studied by scanning electron microscope (SEM). The samples were washed with distilled water several times and air dried. The eggshells were separated into two groups; the first group was mounted on stubs with conductive carbon tape, coated with gold particle at 20 nm thickness in an ion sputtering, and observed under SEM (CamScan Analytical, Maxim 2000S) by using secondary electron image and back-scattered electron imaging operating at 10 kV. The second group was mounted on stubs with conductive carbon tape and uncoated with gold particle for back-scattered electron imaging and then analyzed on side view at the apex, equator and bottom by using energy dispersive X-ray spectroscopy (EDX).

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Statistical analysis

Mean and standard deviation of the thickness of Rock Pigeon eggshell on 3 district layers was calculated. Statistical analysis at 95% significance level was determined using analysis of variance (ANOVA), and multiple comparisons were analyzed by Student-Newman-Keuls (SNK).

Results

Structure of eggshells

The Rock Pigeon eggshells were white and oval shape. Their weight were 14.58 ± 0.68 g (n=6) and their size were 33.01 ± 0.58 mm, 42.09 ± 1.67 mm (n=4). The results from scanning electron microscopy (using SEI and BEI) study demonstrated that Rock Pigeon eggshells composed of 3 layers which were the outermost part of shell, called cuticle layer, palisade layer and mammillary layer (Figs. 2, 3 and 5). The palisade layer is the thickest layer with numerous vesicular holes. The mammillary layer consists of dome shape which are numerous pore canals connecting from the outer cuticle layer to the inner mammillary layer for exchange of direct gases and water between bird’s embryo and its environment (Fig. 4). The finding will be useful for evaluating the success of hatching, survival rates of Rock Pigeon and reproductive potential of this animal.

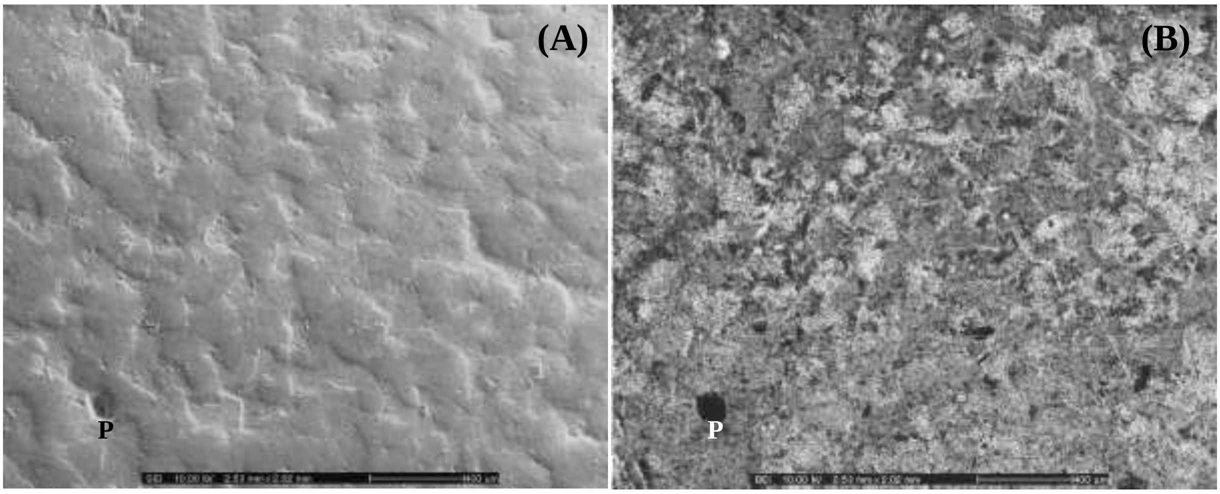

Figure 2: Scanning electron micrographs of top view of external surface of Rock Pigeon eggshell (A) micrograph from SEI and (B) micrograph from BEI showing pore (P) on outer cuticle layer.

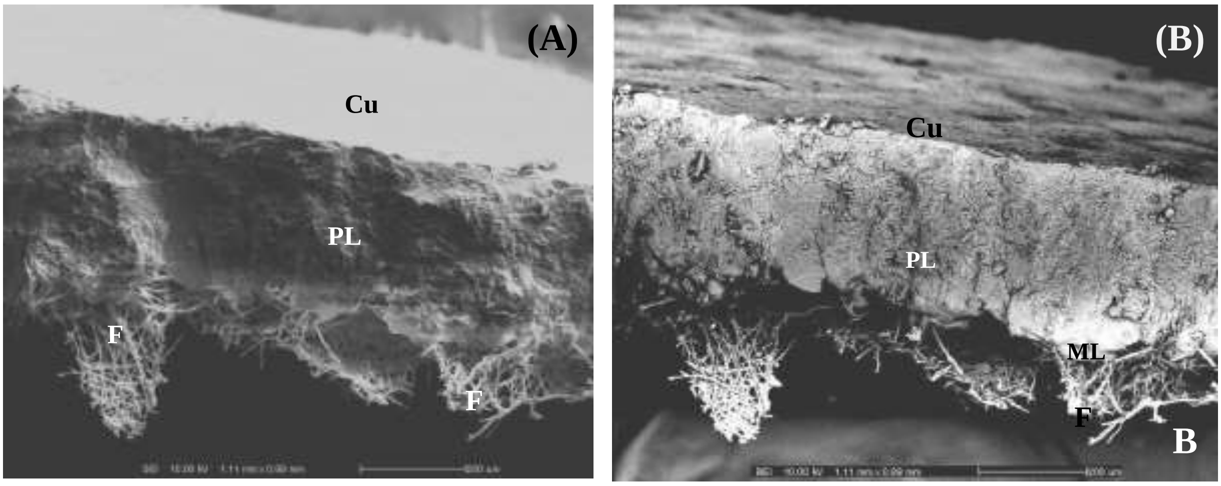

Figure 3: Scanning electron micrographs of side view structure of Rock Pigeon eggshell (A) micrograph from SEI and (B) micrograph from BEI showing outer cuticle layer (Cu), middle palisade layer (PL) , and fibrous (F) which connect to shell membrane (SM).

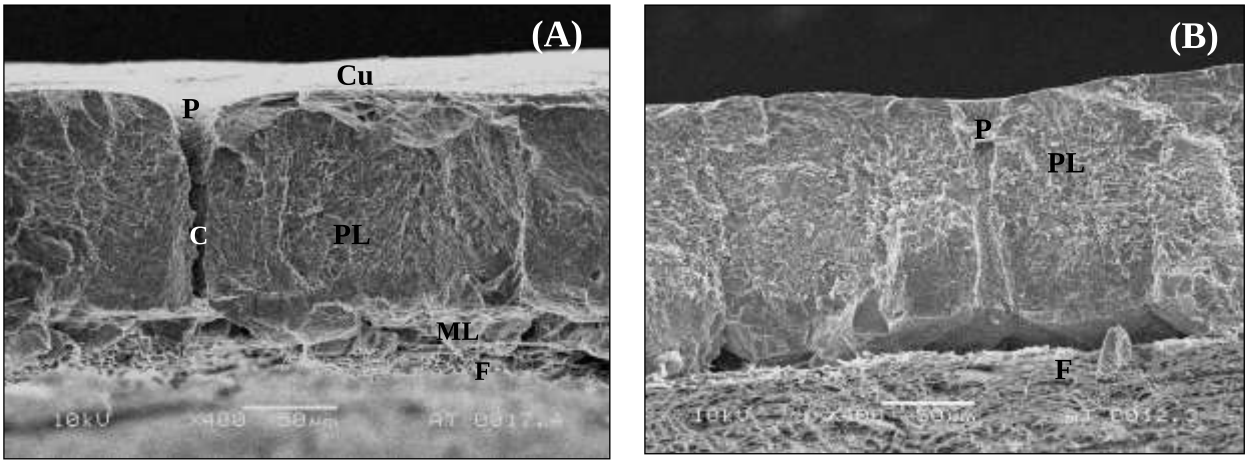

Figure 4: Scanning electron micrographs of structure of lateral side view of Rock Pigeon eggshell from SEI showing outer cuticle layer (Cu), middle palisade layer (PL) and an inner mammillary layer (ML) and pores (P) and funnel- shaped cannel (C).

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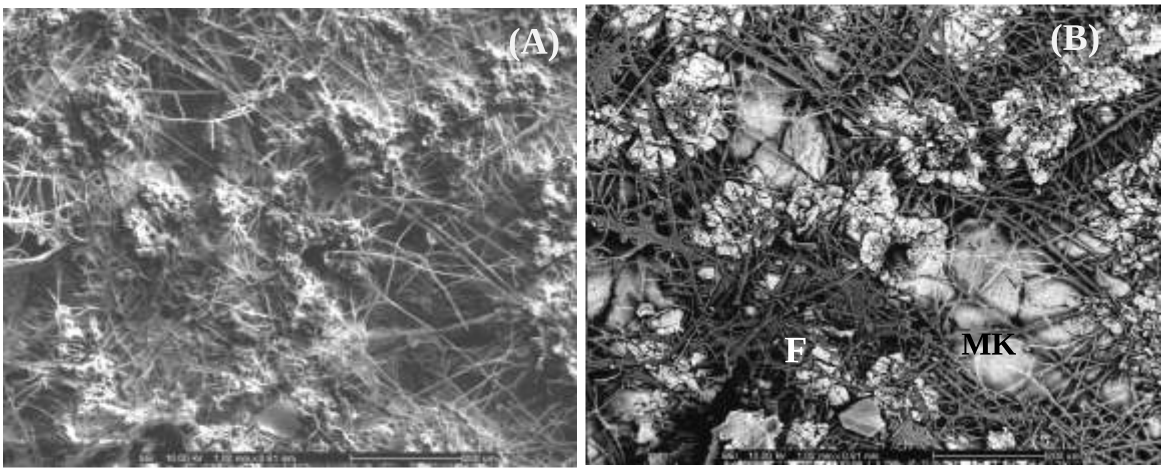

Figure 5: Scanning electron micrographs of lower side view of Rock Pigeon eggshell (A) micrograph from SEI and (B) micrograph from BEI showing of circular shape of mammillary knobs (MK) and fibrous (F) of shell membrane.

Eggshell thickness

The mean ± SD of Rock Pigeon egg thickness of 3 layers were measured and calculated under SEM. The results showed eggshells had 3 layers; an outer cuticle layer, a middle palisade layer, and an inner mammillary layer at the thickness of 35.91±9.45, 94.29±10.02 and 11.92±2.31 micrometer. The palisade layer was the thickest layer, while the mammillary layer had the middle thickest and the cuticle layer was the thinnest layer.

Energy dispersive X-ray Analysis (EDX)

EDX analysis detected 10 elements containing in Rock Pigeon eggshells which were oxygen (O), carbon (C), calcium (Ca), magnesium (Mg), aluminum (Al), silicon (Si), phosphorus (P), sodium (Na), sulfur (S) and chlorine (Cl). The percentage of all elements was found in the following order: O (50.89±17.00) > C (24.73±8.54%) > Ca (23.31±19.72%) > Al (0.50±1.23%) > Mg (0.19±0.59%) > Si (0.14±0.60%) > P (0.03±0.08%) > Na (0.13±0.36%) > S (0.02±0.10%) > Cl (0.08±0.20%).

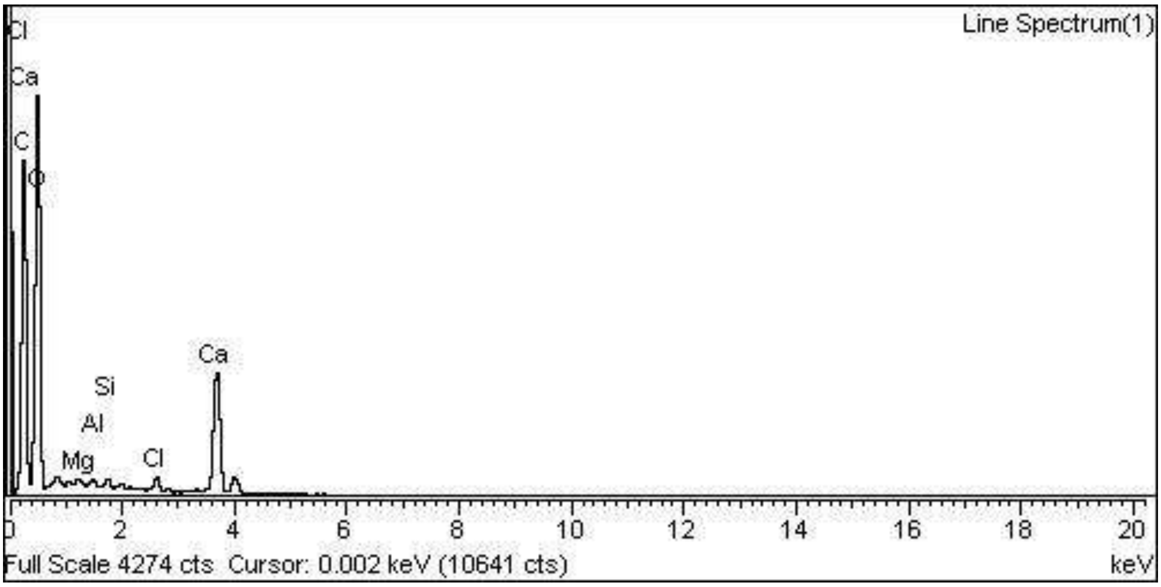

Figure 6: EDX showed contents of oxygen (O), carbon (C), calcium (Ca), magnesium (Mg), aluminum (Al), silicon (Si), and chlorine (Cl).

Discussion

The difficulty of wild avian eggshell study is a sampling step because birds always build their nests in safe places hiding from predators (Peterson, 1978). There are 3 sampling methods that have been recommended for eggshell study, which are collecting from fresh laid eggs, using eggs that are failed to hatch in nests and lastly using broken shells after hatching which can be found under nests (Snodderly and Max, 1978; Sordahl, 1994; Sandercock, 1996). The last choice may have problem of cracking after hatching and cannot rule out exactly for what parts of egg they are. Moreover, the most important problem of the last method is a removal behavior, in which most birds will remove the eggshell away from nest. As a result, in order to collect the eggshell properly, the nest will have to be continuously observed.

The structure of avian eggshells can be studied by different techniques. The size and orientation of crystals forming eggshell can be determined directly by optical microscopy using thin-slices (<30 μm) of radial sections and cross-polarizers. Information from optical microscopy is visual and accurate, but is also two-dimensional. However, it is local-lacking statistical meaning and limited to about 1-μm resolution as well as requires tedious sample preparation. Recently, a combination of both optical and X-ray techniques has been used for systematic studies of three-dimensional eggshell textures in order to determine plausible correlation with mechanical properties and organic components. In addition to these techniques, scanning and

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transmission electron microscopy as well as electron diffraction have been used. In this report, determination of Rock Pigeon eggshell ultrastructure has been performed by SEM using 3 detectors; SEI, BEI and EDX. Comparisons of the results obtained from 3 detectors revealed that SEI method allowed us to observed morphology of eggshells clearly. BEI analysis demonstrated the location or area of element compositions present in the samples while the quality and quantity of metals can be analyzed by energy dispersive X-ray spectroscopy (EDX). The results indicated that Ca is the major component of Rock Pigeon eggshells with minor elements such as P, S, Na, Mg etc. Cusack *et al.* (2003) reported Mg distribution is not constant throughout the shell, decreasing from nucleation until after fusion of the mammillary caps and then increasing to termination in domestic fowl.

Observation of eggshell structure in 3 layers, cuticle layer, and calcareous layer and fibrous revealed a number of pores inside. In general, pores appear both on the apex and bottom sides. This porous texture is required for exchanging gases, water and small molecules between egg and environment and provides the source of skeletal calcium, which is essential for development of embryo (Nys *et al*. 2004). Moreover, the eggshell would protect the enclosed embryo from the weight of the parent’s body during incubation, and perhaps also serve as an adaptation for lack of parental care.

Ultrastructure, size and pore density of pore of avian eggshells were used for species or group identification or classification. Bird eggshell has different thickness and structure, such as mammillary layer-dome shaped in Rock pigeon (*Columba livia*) or V shaped in Ostrich (*Struthio camelus*) (Karlsson and LiUa, 2008). Chang *et al*. (2007) reported that eggshell of Reeves’s Pheasant (*Syrmaticus reevesii*) composted 3 layers: cuticle, palisade and mammillary -dome shaped thickness 20.8, 220.8 and 62.0 mm, respectively, accounting for 6.8%, 72.6% and 20.6% of the total thickness of the eggshell. There are many vesicular holes in the palisade layer with an average diameter of 0.32% ± 0.08 mm (n = 30). In addition, total pore area showed an isometric increase with egg mass and nest habitat (Deeming, 2006).

Many factors are known to have influence on eggshell quality, diets, female’s health and age, and contaminants in the environment. For examples, inadequate nutrition (calcium, vitamin A and D, trace elements, etc.) can also lead to inferior eggshell quality. At present, dietary manipulation is the primary means of trying to correct eggshell quality problems. Some diseases e.g. avian influenza and infectious bronchitis may produce severe effects on eggshell and internal quality. Whenever, there are some diseases infection on the uterus and ovary will cause the eggs passing through oviduct too quickly then result in the shell will not get the salts they require. Dirksen, *et al*. (1991) reported that the negative effect of organochlorine contaminants is the thinning of eggshell. This has been found in several species of fish-eating water birds and raptors. In addition, aluminum had been found as major effect on the eggshell defective formation (Nyholm, 1981). All reports mentioned above demonstrated the causes that can severely damage and hinder the success of reproductive development in birds. However, all samples in this study revealed no contamination of heavy metal which indirectly indicates that the environments of Amphoe Mueang, Nakhon Pathom Province are non-polluted by these substances.

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