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RESEARCH ARTICLE

Ultrastructure and elemental composition of the eggshell of Reeve's Pheasant (*Syrmaticus reevesii*)

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Abstract The eggshell of Reeve's pheasant (*Syrmaticus reevesii*) collected from the Dongzhai National Nature Reserve in Henan Province, China was studied. By using scanning electron microscopy and inductively coupled plasma (ICP) spectrometry, the ultrastructure and elemental composition of the eggshell was determined. The study showed that the average thickness of the surface layer of crystals, the palisade and cone layer, and the eggshell membrane were 20.8, 220.8 and 62.5 μm, respectively, accounting for 6.8%, 72.6% and 20.6% of the total thickness of the eggshell. There were many vesicular holes in the palisade layer with an average diameter of 0.32 ± 0.08 μm ($n = 30$). The function of these holes might be significant to air exchange. The shape of the eggshell pore on the surface layer of crystals is round or elliptical. The fracture surface of the pore is funnel-shaped. Some granules filled the upper part of the eggshell pores. The content of 21 elements in the eggshell of wild and captive Reeve's pheasants was compared and presented. It indicated that among the elements that made up the eggshell of the wild pheasant, the content of Ca, Mg, P and S was much higher, $\omega > 1$ mg/g, with ω (Ca) being higher than 40% of the eggshell. The contents of Na, Si, Sr, K and Al were $\omega = 0.1$–1 mg/g, while Fe, Zn, Pb, Mn, Cu, V and Ti had lower concentrations ($\omega = 1$–100 μg/g). The ω of Ni, Cr, Co, Se, Cd were lower than 1 μg/g. The elemental composition in the eggshell of the captive Reeve's pheasant kept in the Dongzhai National Natural Reserve was significantly different from that of the wild species, with a difference of over 20% on S, Cu, Fe, Al, Mn, Si, Sr, Se and Cr. The lower intake of Fe, Mn, Si and Sr on the one hand and the higher intake of S, Cu, Al and Cr on the other hand might be responsible for the low fertility of captive Reeve's pheasants in the Dongzhai National Nature Reserve. In order to ensure that the pheasants are receiving the proper amount of nutrition and to improve their breeding success, the amount of certain elements in the food should be adjusted.

Keywords threatened species, Reeve's pheasant (*syrmaticus reevesii*), eggshell, ultrastructure, elemental composition

1 Introduction

As the starting point of reproduction and development, egg-laying and hatching are basic characteristics of avians (Zheng, 1995). The shape, structure and function of eggs have been studied for a long time by scientists starting in the early 19th century. Later, many monographs on eggs were published successively, and then a new branch of ornithology—the Oology (Gill, 1994)—gradually came into being.

The outer layer of eggs is made of hard calcic materials, which provide protection for the content of the eggs. Researchers began to study eggshells with microscopes in the 1940s and they found that the structure of the eggshell was unique for each species, and useful for bird classification and evolution studies (Heyn, 1963; Becking, 1975; Sai et al, 1996; Chang et al, 2000; Rodriguez-Navarro et al, 2002). Chemical components in eggshells, especially some essential elements, are not only closely connected with the development of the embryo, but also reflect the status of female nutrition and environmental contamination. Since some heavy metal elements in eggshells can indirectly reflect the habitat quality of birds, detecting and analyzing the chemical components of eggshells has become one of the focuses for scientists in recent years (Li et al, 1990; Chang et al, 2001; Larison et al, 2001; Tilgar et al, 2002; Cusack et al, 2003).

Reeve's pheasant (*Syrmaticus reevesii*) is endemic to China, and has been listed as a China National Class II protected animal, IUCN (International Union for the

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Conservation of Nature and Natural Resources) vulnerable species and CITES (Convention on International Trade in Endangered Species) appendix I species, due to their small population in the wild. It has been recommended to be added to the China National Class I protected animal list in the *China Red Data Book of Endangered Animals* (*Aves*) (Zheng and Wang, 1997), in consideration of its decreasing range and population in the country compared with other animals in the list of Class I (Zheng and Wang, 1997). Biological studies on Reeve's pheasant will provide valuable data for improving the captive breeding of this species. However, most of the previous studies on this bird have focused on their habitat, habits, population, and management strategies (Wu, 1979; Hu and Wang, 1981; Xu et al, 1996; Sun et al 2001). The eggshell structure of Reeve's pheasant was first reported in the 1980s (Xu and Wu, 1985), but the study was not satisfactory owing to limited techniques and equipment at that time. Based on the findings of the previous studies, the present study analyzed the ultrastructure and chemical ingredients of eggshells of Reeve's pheasant with materials collected from the field. The objectives of this study are to supplement the basic data of biology studies on Reeve's pheasant, and provide a scientific basis for captive breeding of this rare species.

2 Materials and methods

The eggshells of *Syrmaticus reevesii* from the Dongzhai National Nature Reserve in Henan Province, China (114°18′–114°30′ E, 31°28′–32°09′ N) were used. Meanwhile, we collected eggshells from the same species that were caged at the same place.

Eggshell fragments were examined with scanning electron microscopy and inductively coupled plasma (ICP) spectrometry. The ultrastructure and elemental composition of the eggshell of *Syrmaticus reevesii* were studied.

3 Results

3.1 Ultrastructure

The *Syrmaticus reevesii* eggshell is composed mainly of four parts, i.e. the surface layer of crystals; the palisade layer; the cones and basal caps layer; and the eggshell membrane layer. The eggshell membrane layer is divided into the outer and inner eggshell membranes. We called the palisade and cone layers as such since the two had no obvious delimitation. There was some eggshell pore opening up to the air on the surface layer of crystals. We measured the thickness of every layer of the eggshell (Table 1).

Our measurement results showed that the thickness of the surface layer of crystals was the thinnest and it was only 6.8% (Table 1) of the shell. The thickness of the palisade and cone layer was the thickest and its percentage was 72.6% (Table 1). The ultrastructure of the three layers is described below.

Table 1 Thickness of the three layers in the eggshell of *Syrmaticus reevesii*

Layer	Thickness /μm	Percentage /%
Surface layer of crystals	20.8 (16.7–25.0)	6.8
Palisade and cone layer	220.8 (200.0–245.8)	72.6
Eggshell membrane	62.5 (50.0–87.5)	20.6

3.1.1 Surface layer of crystals

The surface layer of crystals of hatched individuals was rather smooth (Fig. 1(1) and (2)), but that of the unhatched individual was rough, and there was some substance (Fig. 1(3) and (4)) like short sticks and some pits. Irregular splits were distributed on it. The shape of the pores was round or oval. Some edges of the pore openings had some splits.

3.1.2 Palisade and cone layer

This layer was located under the surface layer of the crystals. It was made up of two parts, the palisade and the cone layer. The structure of the palisade layer was thick and hard. From SEM results, some lamellate pattern could be seen (Fig. 1(8)) and a great number of vesicular holes could also be seen at the middle of this layer (Fig. 1(9)) (diameter $= 0.32 \pm 0.08$ μm, $n = 30$). The vesicular holes connected with pores forming an air-hole net, which was useful for the exchange of air in the eggs. From the fractured eggshell, the cone layer was made of large cones arranged closely in order. From the inner surface, the mammillar cores were shown to adhere to the eggshell membrane. The mammillar cores of unhatched individuals looked like flowers (Fig. 2(1)), and was close to the fine fibers of the outer eggshell membrane. It was difficult to separate them, while that of the hatched individuals separated from the eggshell membrane easily. At the same time, the structure of the mamillar cores changed from the flower pattern into an irregular crystal body (Fig. 2(2) and (3)).

3.1.3 Eggshell membrane

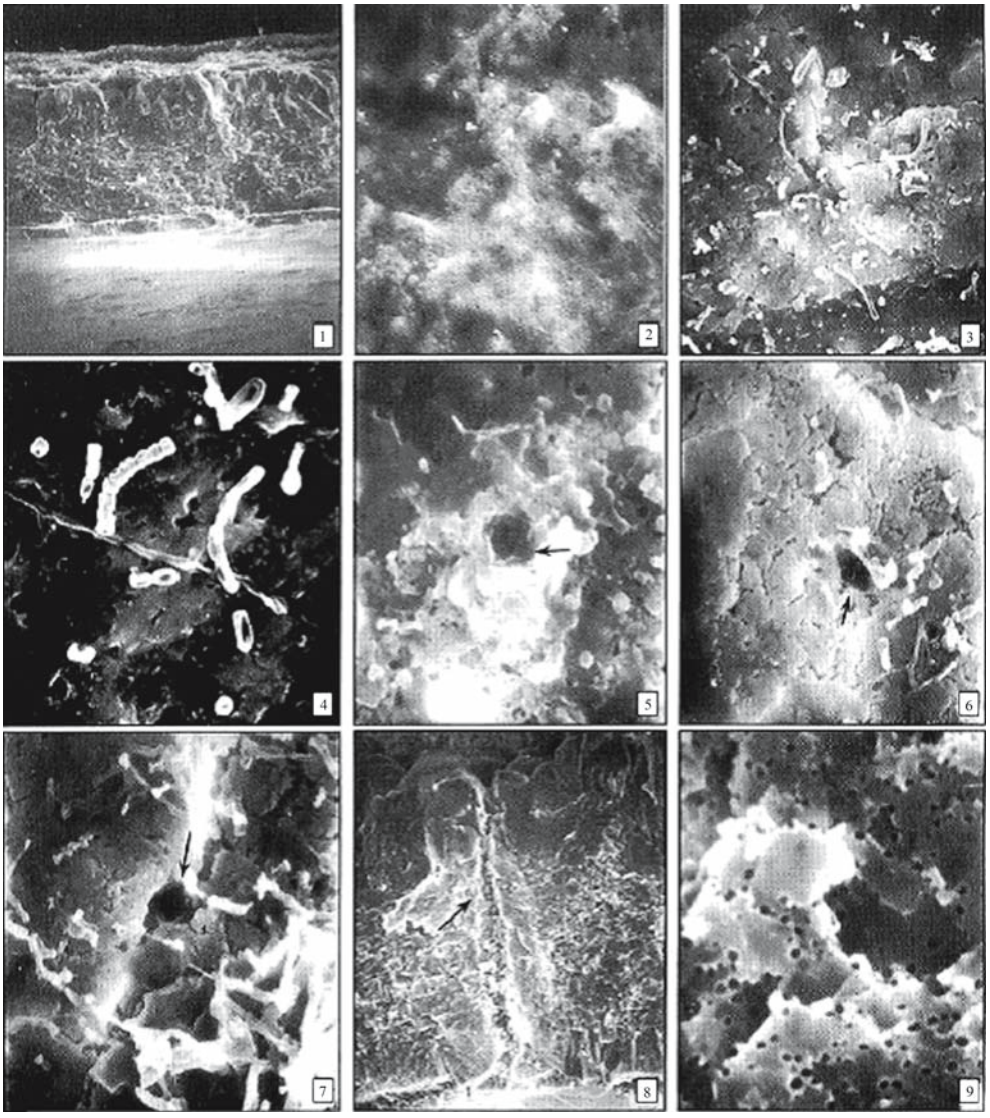
The eggshell membrane is located inside the eggshell, and has two layers made of many fibers. The fibers of the outer membrane are coarse with wide meshes, while the fibers of the inner membrane are fine fibers with fine meshes (Fig. 2(4–7)). The diameters of the inner and outer membranes are (1.18 ± 0.23) μm and (1.69 ± 0.48) μm, respectively (Table 2).

3.1.4 Eggshell pore

The shape of the eggshell pore on the surface layer of the crystals is round or elliptical. The fracture surface of the pore is funnel-shape and some granules fill the upper part of the eggshell pore.

3.2 Content of eggshell elements

The content of elements in the eggshell of wild and captive individuals was measured respectively (Table 3). In the



1 Fractured surface of eggshell, ×200, 2 Outer surface of eggshell, ×2500, 3 Outer surface of eggshell, ×3500, 4 Outer surface of eggshell, ×8000, 5 Outer surface of eggshell, pore ×6000, 6 and 7 Outer surface of eggshell, pore ×4000, 8 Fractured surface of pore, ×400, 9 Vesicular hole ×8000

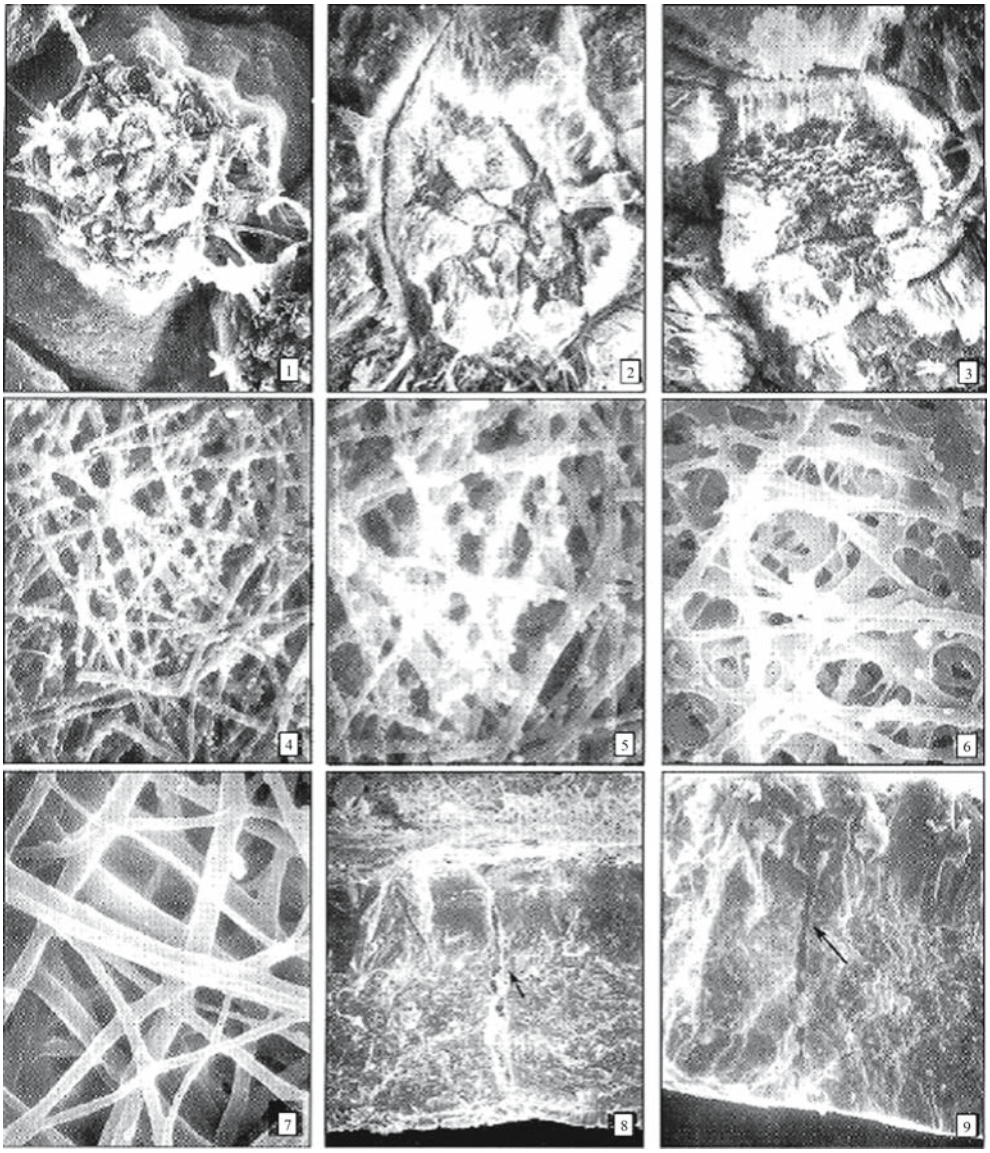
Fig. 1 Ultrastructure and elemental composition in eggshell of Reeve's Pheasant **(***Syrmaticus reevesii***)**

elemental composition of eggshells from wild *Syrmaticus reevesii*, the content of Ca, Mg, P, and S was extremely high, ($\omega > 1$ mg/g). The ω(Ca) was higher than 40% of the eggshell. The content of Na, Si, Sr, K and Al was $\omega =$ 0.1–1 mg/g, while Fe, Zn, Pb, Mn, Cu, V and Ti had lower concentrations ($\omega =$ 1–100 μg/g). The ω of Ni, Cr, Co, Se, Cd was lower than 1 μg/g. The elemental content in the eggshell of the captive species was significantly different from that of the wild, with a difference of over 20% on S, Cu, Fe, Al, Mn, Si, Sr, Se and Cr.

4 Discussion

The direction of the crystal alignment in the crystal layer is different. Vertical crystals are generally thicker than non-vertical ones. This finding agrees with Becking's report (Becking, 1975). The average thickness of the surface layer of crystals is 20.8 μm, accounting for 6.8% of total thickness of the eggshell, which is distinct with the counterpart of Tibetan eared-pheasant (*Crossoptilon harmani*), 9.3 μm and accounting for 2.4% of total eggshell thickness (Chang et al, 2000). In addition, the crystal shape of the eggshell of Reeve's pheasant is rectangular, while the *Crossoptilion mantchuricum* is spindle-shaped (Gan, 1992). These facts manifest the difference of bird species based on their eggshells.

Becking (1975) pointed out that the eggshells of tropical birds have more vesicular holes and bigger diameter. The average diameter of vesicular holes is between 0.6 μm and 1.2 μm. We once reported that the diameter of vesicular holes of the Tibetan eared-pheasant, which inhabits Lhasa, is about 0.23 μm. But in this study, we found the diameter of vesicular



1 Inner surface of eggshell, mammillary core ×1500, 2 Inner surface of eggshell, mammillary core ×1000, 3 Inner surface of eggshell, mammillary core ×2500, 4 Inner eggshell membrane ×2000; 5 Inner eggshell membrane ×3500, 6 Outer eggshell membrane ×2000, 7 Outer eggshell membrane ×3500, 8 Fractured surface of pore ×300, 9 Fractured surface of pore ×400,
Fig. 2 Ultrastructure and elemental composition in eggshell of Reeve's Pheasant (*Syrmaticus reevesii*)

Table 2 Diameter of vesicular holes in the palisade layer and fibers in eggshell membrane of *Syrmaticus reevesii* ($n = 30$)

	Diameter /μm
Vesicular holes	0.32 ± 0.08
Inner eggshell membrane	1.18 ± 0.23
Outer eggshell membrane	1.69 ± 0.48

holes of Reeve's pheasant to be around 0.32 μm, which inhabits the Dabie Mountain area. It suggests that the size of the vesicular hole is closely related to the bird's habitat. In conclusion, the size of vesicular holes becomes smaller as latitude increases. This change is probably related to the climate condition that the bird adapts to. Moreover, the fibers of the inner and outer eggshell membranes of Reeve's pheasant are all stronger than those of the Tibetan eared-pheasant. This may explain the differences between the two species, but it is also possibly a result of the way birds adapt to different climates in their habitat. Further studies are needed in this aspect.

The same elemental constitution and content of the eggshell may show the nutritional condition of the female pheasants, and also show the quality index of the surrounding environment (Li et al, 1990; Chang et al, 2001; Larison et al, 2001). There are 21 elements in the eggshell of the Reeve's pheasant. These are Ca, Mg, P, S, Na, Si, Sr, K, Al, $\omega >$ 100 μg/g, and Fe, Zn, Pb, Mn, Cu, V, Ti, Ni, Cr, Co, Se, Cd, $\omega < 100$ μg/g. The elemental constitution of the eggshell in the wild species is the same as the captured species. However, some elemental content are very different, such as the

Table 3 The elemental composition of eggshells of *Syrmaticus reevesii* (μg/g)

	S	P	Mg	Ca (mg/g)	Na	K	Zn
Wild species	1615	1716	5660	409.2	884.6	140.0	12.53
Caged species	2854	1988	5261	350.3	751.7	134.0	13.81
	Fe	Co	Al	Cu	Mn	V	Ti
Wild species	19.28	0.6023	125.7	1.685	6.347	7.349	2.118
Caged species	15.09	0.5895	164.7	3.182	1.021	6.956	1.778
	Si	Ni	Sr	Cd	Pb	Se	Cr
Wild species	331.8	0.9895	245.2	0.2403	9.179	0.2725	0.9048
Caged species	195.8	0.9846	76.24	0.2159	8.875	0.3478	1.702

elements of S, Cu, Fe, Al, Mn, Si, Sr, Se and Cr. We have further found that the percentage of the elements of S, Cu, Al, Se and Cr of the eggshell of the captured species is much higher than that in the wild. But the percentage of Fe, Mn, Si and Sr of the eggshell in the captured species is even lower than that in the wild. The content of Mn and Sr in captive Reeve's pheasant accounts for only 16.07% while that in the wild individuals is 31%. Researches already indicate that trace elements are rare in organisms, but they are necessary for animal growth and development. The lack of some elements, such as Fe and Mn, has a direct impact on animal growth, especially the development of body and brain of infants. In contrast, the excess of some elements may have a negative influence on the metabolism of organisms. For example, metabolic difficulty will occur if too much Al and Cr are consumed. Therefore, it is essential to enhance the breeding success by maintaining an appropriate nutrition level for captive animals.

The Dongzhai National Nature Reserve is one of the main ranges for Reeve's pheasant in China. High density and breeding success observed in the field indicate that the habitat here can meet the growing and breeding requirements of Reeve's Pheasant. However, pheasants raised in this reserve still have issues like high fertilization failure and nestling mortality. Results of this study show that the main reason for the low breeding success of Reeve's pheasants in captivity is malnutrition resulting from unreasonable food provision. Studies of the chemical components of the eggshell from captive birds revealed that the content of Fe, Mn, Si, and Sr consumed are far below the level required. On the contrary, the content of S, Cu, Al, Se, and Cr is far beyond the level in eggshells from the field. To enhance the breeding success of captive Reeve's pheasants, results of this study suggest adjusting the supply of certain elements in their food to ensure reasonable nutrition.

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