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Evaluation of Calcium Carbonate Content in Eggshells of Avian, Turtle, Snail, and Ostrich Using Chemical Analysis and Scanning Electron Microscopy

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Abstract - **In the present study, different eggs were collected and analyzed from five various animal species: European pond turtle (*Emys orbicularis* (Linnaeus, 1758)), giant African land snail (*Achatina fulica* (Bowdich, 1822)), common ostrich (*Struthio camelus* (Linnaeus, 1758)), white, light-brown, and dark-brown laying hen *(Gallus gallus domesticus* (Linnaeus, 1758) and European quail (*Coturnix coturnix* (Linnaeus, 1758). The typical mineral shell mainly composed of the calcite polymorph of $CaCO_3$ but the eggshell consists of membranes, that composed mainly of proteins. The shell quality also could be assigned by several external and internal factors such as oviposition time, animal genotype and age, housing system (for poultry), and mineral nutrition complex. The $CaCO_3$ content was determined by the standard titration method, coz the titration could provide a reliable method for evaluation of $CaCO_3$ content in different types of eggshells. The structural surface characterization of eggshells was performed by scanning electron microscopy (SEM) with a field emission gun. In terms of chemical composition, ostrich eggshells generally did not differ much from those of laying hen, turtles, giant snails, or quail eggs, but the concentration of calcium carbonate was the highest. The average calcium carbonate content of various eggshells is between 84 and 98%. The thickness of the eggshell ranges from 0.08 to 1.89 mm, and it is not the same over the entire surface of the egg. At the sharp end of the egg, the shell is slightly thicker than at the blunt end. The purpose of this study was to study the quantitative content of calcium carbonate in various eggshells of different animals to draw further conclusions in which animals the eggshell contains the maximum amount of biological calcium carbonate**.

Keywords - **Avian Eggshells; *Achatina fulica*; *Emys orbicularis*; *Gallus gallus domesticus; Struthio camelus*; *Coturnix coturnix*; bio calcium carbonate ($CaCO_3$)**

I. INTRODUCTION

Determination of calcium carbonate (CaCO3) composition between different eggshells is an important step towards the better understanding of detection and characterization of shell components of various avian and animal's species. The shells of eggs are the perfect complex bio-mineral that superposes functionality, mechanical stiffness, and aesthetic appearance and contains all that an embryo requires for safe development through to hatching in harsh environmental conditions. The chicken eggshell mainly consists of calcium carbonate (95% w/w), hydroxyapatite crystals (1% w/w) in the outermost

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calcified layer of the chicken eggshell, organic matrix (2 % w/w of the palisade layer), pigments as colorants (0.15–1200 nmol/g) and more than 10% (v/v) void cavities with about 0.4% (w/w) of adsorbed water [1], [2]. The surface of the inner shell consists of two membranes followed by interstitial calcite columns, a so-called mammillary layer that is a regular array of cones, each with a core of concentration of organic matter that had been described as neutral mucopolysaccharide [1]. The eggshell of the European pond turtle consists mostly of calcium carbonate in aragonite form, while eggshells of other reptiles and birds contain mostly calcium carbonate in calcite form [3]. Compared to chicken eggs, the ostrich eggs weigh approximately 1.5 kg and 97% of the ostrich shell is of mineral origin allocated among calcium carbonate (97.4%), magnesium phosphate (1.9%), and tricalcium phosphate, TCP (0.7%) [4]. Moreover, it is worth mentioning that a female ostrich is able to produce approximately 60 eggs per year. However, the shell of quail eggs consists of 90% calcium carbonate and contains a lot of essential minerals, like zinc, copper, iron, potassium, calcium, magnesium, sodium, sulfur, phosphorus, and others. In addition, quail egg weighs about 10 g and their eggshell colors vary from white to blue and green which have brown or reddish-brown patterned areas on a light background [5]. It is noteworthy that the weight of giant African land snail eggs ranges from 1.54 – 2.45 g and they are capable of laying 4 -18 eggs in 1- 2 minutes [6].

The structure of the avian shells has mainly been investigated with imaging methods e.i. X-ray microscopy (XRM) [7], scanning electron microscopy (SEM) [8], transmission electron microscopy (TEM) [9] and optical light microscopy (LM) [10]. Despite the widely recognized importance of the chemical identification of various avian eggshell pigments and the detection of the pesticides on shells using various imaging methods, researching the morphology of the calcareous layer of eggshells as well as calcium carbonate content between different eggshells is an important step towards the better understanding of detection and characterization of shell components of avian eggs. Therefore, the present study was undertaken to evaluate the calcium carbonate content and the structural surface characterization of the calcareous layer of eggshells from five various animals species: European pond turtle (*Emys orbicularis* (Linnaeus, 1758)), giant African land snail (*Achatina fulica* (Bowdich, 1822)), common ostrich (*Struthio camelus* (Linnaeus, 1758)), white and brown laying hen (*Gallus gallus domesticus* (Linnaeus, 1758) and European quail (*Coturnix coturnix* (Linnaeus, 1758) using chemical analysis and SEM.

II. MATERIALS AND METHODS

Eggshell samples

Five different types of eggshells: European pond turtle (*Emys orbicularis* (Linnaeus, 1758)), giant African land snail (*Achatina fulica* (Bowdich, 1822)), common ostrich (*Struthio camelus* (Linnaeus, 1758)), white, light-brown, and dark-brown laying hen (*Gallus gallus domesticus* (Linnaeus, 1758) and European quail (*Coturnix coturnix* (Linnaeus, 1758) were analyzed with scanning electron microscopy (SEM) and chemical analysis.

The eggs were cracked by hand and their shells were separated from egg white, egg yolk, and the inner membranes.

Sample preparation and scanning electron microscopy (SEM)

For scanning electron microscopy (SEM), eggs were slowly air-dried at room temperature (20 °C ±2°C) over a period of several days and their surface structure was observed. We mounted dried, untreated eggshell fragments onto aluminium stubs, allowing the visualization of the shell surface, which we then sputter-coated with Ag (10.5 g/m3) conductive coating on the sample surface used turbomolecular pumped coater (Quorum EMS150T ES, UK). Scanning electron micrographs were taken at a working distance of 1.58‒2.38 mm with an accelerating voltage of 2.0 kV. For examination of non-ground fragments of eggshells TM-1000 (Hitachi Tabletop Microscope, Japan) and MAIA 3 (TESCAN, Czech Republic) were used at a magnification up to 15 kx. The main concern was a detailed investigation and analysis of the visibility of as many as possible eggshell structures in order to compare and determine differences among animal species. A small fragment of the different eggshells (at least 1000 × 1000 μm) was taken.

Determination of calcium carbonate composition of eggshells

The calcium carbonate ($CaCO_3$) content in the eggshell powder was determined by the titration method. For the measurement of $CaCO_3$ content, pieces of eggshells from seven different eggs were collected and the protein-membrane was removed on the inside of the eggshells. The pieces were air-dried and the eggshells were then grounded into fine powder by mortar and pestle. 1g of eggshell powder was weighed accurately by using an electronic balance and it was transferred to a conical flask by adding several drops of ethanol which acted as a wetting agent and helped the HCl dissolve the $CaCO_3$. 10mL of 1.0M HCl solution was pipetted to dissolve the eggshell powder. The solutions were heated until they begin to boil and then were allowed to cool. i.e. for complete digestion. Phenolphthalein indicator was added to each flask, using a funnel, a clean burette was partly filled 0.1M NaOH solution to rinse it and was empty into the sink then the burette was again filled with NaOH solution and run some solutions out to remove all of the bubbles from the tip. The initial volume of the burette was constituted ± 0.01mL. The samples were titrated with NaOH solution to the first persistent pink color, when it was close to the endpoint, the color faded slowly and the remaining NaOH was added drop-wise until the color remains for some seconds where the final volume was recorded. The percentage of $CaCO_3$ in each eggshell was calculated.

Statistical analysis

The content of $CaCO_3$ was calculated on the average values of three replicate samples. Data are expressed as means ± standard errors of means (SEM), and $p < 0.05$ was considered to be statistically significant.

III. RESULTS AND DISCUSSION

It is well established that the eggshell is a highly specialized mineralized structure, which comprises inner and outer membranes, composing mainly of proteins and glycoproteins, and the mineral shell mainly consisting of the calcite polymorph of $CaCO_3$ [11,28]. The quality of the shell is tightly linked to many internal and external factors including oviposition, age, and genotype as well as housing system, nutrition, microclimate, etc. [12]. The comparative analysis of $CaCO_3$ content was carried out for eggshells of laying hen (white eggshell $CaCO_3$ content: 93.72%, light-brown eggshell: 94.44%, and dark-brown: 95.48%), turtle ($CaCO_3$ content: 96.21%), giant African land snail ($CaCO_3$ content: 84.33%), ostrich ($CaCO_3$ content: 98.79%), and quail ($CaCO_3$ content: 96.51%) by using the back titration method (Fig.1).

According to our experimental analysis of eggshell samples, the chemical composition of ostrich eggshells did not differ significantly compared to other eggshells, but the concentration of $CaCO_3$ was the highest, also significant morphological features were observed in the pore distribution and surface structure of the ostrich eggshell during SEM (Fig.2). The increased or decreased calcium levels in birds and animals might be depending upon their habitat. In addition, the calcium level was not influenced by the weight of eggs. Furthermore, the calcium levels of different eggshells change in pre-laying and laying phases [13,21,22]. The dynamics of changes in the eggshell properties in hens are related to physiological processes of mineral metabolism before, during, and after molting. During the pre-laying period, young hens create reserves of mineral substances especially calcium, which is stored in bones and other tissues [14]. However, during the laying period, the Ca reserves in medullar bones reduce, and an insufficient intake of Ca through the feed, the eggshell quality deteriorates, the incidence of nonstandard and broken eggs increased, and also shell-less eggs can be laid [14]. Moreover, it is worth mentioning that dark-colored eggshells contain a higher percentage of $CaCO_3$ when compared to white-colored eggshells and this may be due erence in hens' diet [15], indicating that brown egg has a higher strength than a white one. Similar results have been noticed by prior researchers [16].

The holes or micropores on the surface of eggshells which can be seen in this image (Fig.2) enable the gaseous exchange of the developing embryo. Most of the eggshell material is calcium carbonate which accounts for about a tenth of the egg's weight, this $CaCO_3$ presents in the eggshells as a calcite mineral and about 3.5% (by weight) organic material (including H_2O). Among hundreds of proteins identified by proteomics and various other means in the eggshell organic matrix, osteopontin is a major shell matrix protein and a member of a group of mineral-binding proteins. On the other hand, ostrich eggs are pitted with larger pores (Fig.2) and the uniformity of shell thickness contributes towards an overall increase in shell strength [17,23,24]. The eggshell of the African land snail, on the other hand, had a lower content of calcium carbonate in comparison with other samples, which was also noticeable in micrographs - the shell surface was not uniform, there were various irregularities and bulges on the surface (Fig.2). European pound turtle and European quail eggshells have almost the same concentration of calcium carbonate, a little less in a turtle, but an electron micrograph shows significant differences in the surface of the eggshell. The surface of the European quail (Fig.3) is more uniform, smooth, formed, which may indicate that its nutrition was more balanced, calcium carbonate was most likely added to the feed daily. The turtle eggshell (Fig.3) has small growths and depressions, cracks, this indicates that the nutrition was very different and this did not contribute to the formation of a uniform surface of the eggshell. It was noted that the average $CaCO_3$ content of various eggshells was between 84 and 98% and the thickness of the eggshell ranged from 0.08 to 1.89 mm, and it was not the same over the entire surface of the egg. At the sharp end of the egg, the shell is slightly thicker than at the blunt end. Eggshell thickness is considered as one of the major indirect parameters for the evaluation of eggshell quality [18].

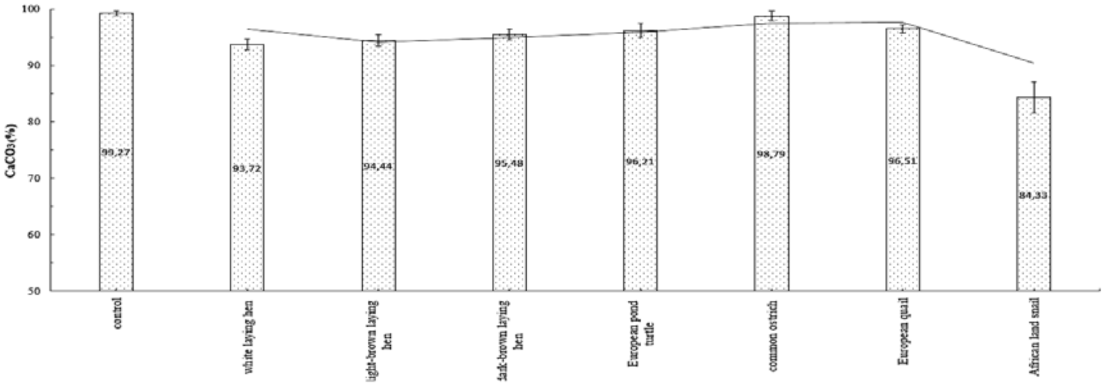
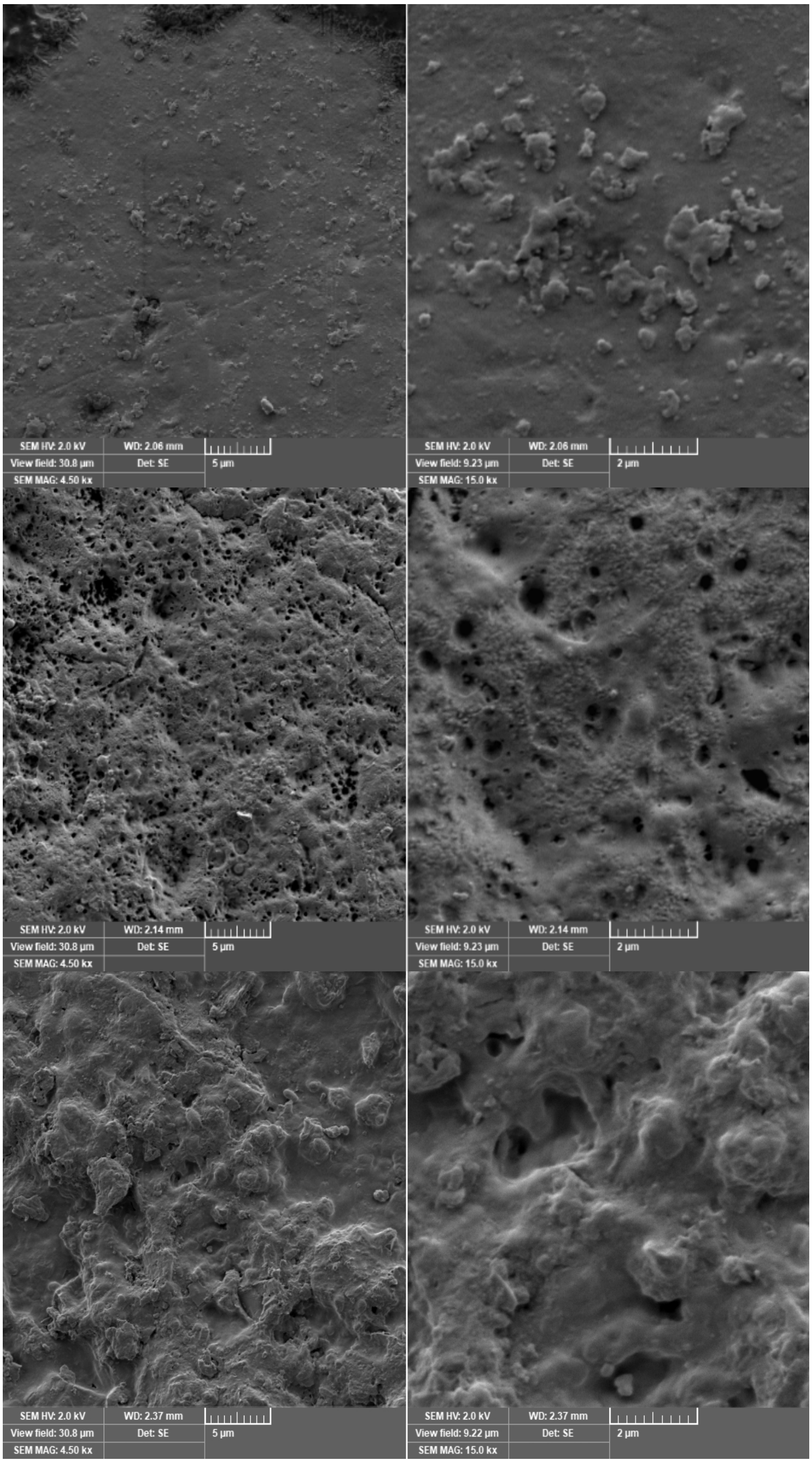


Fig.1. The concentration of calcium carbonate in different eggshells of various animal species.

Fig.2. Uncolored scanning electron micrograph (SEM) of eggshell surface (left side:4.5 kx, right side:15.0 kx): common ostrich (*Struthio camelus* (Linnaeus, 1758)) **(a)**, white laying hen (*Gallus gallus domesticus* (Linnaeus, 1758) **(b)**, giant African land snail (*Achatina fulica* (Bowdich, 1822)) **(c).**



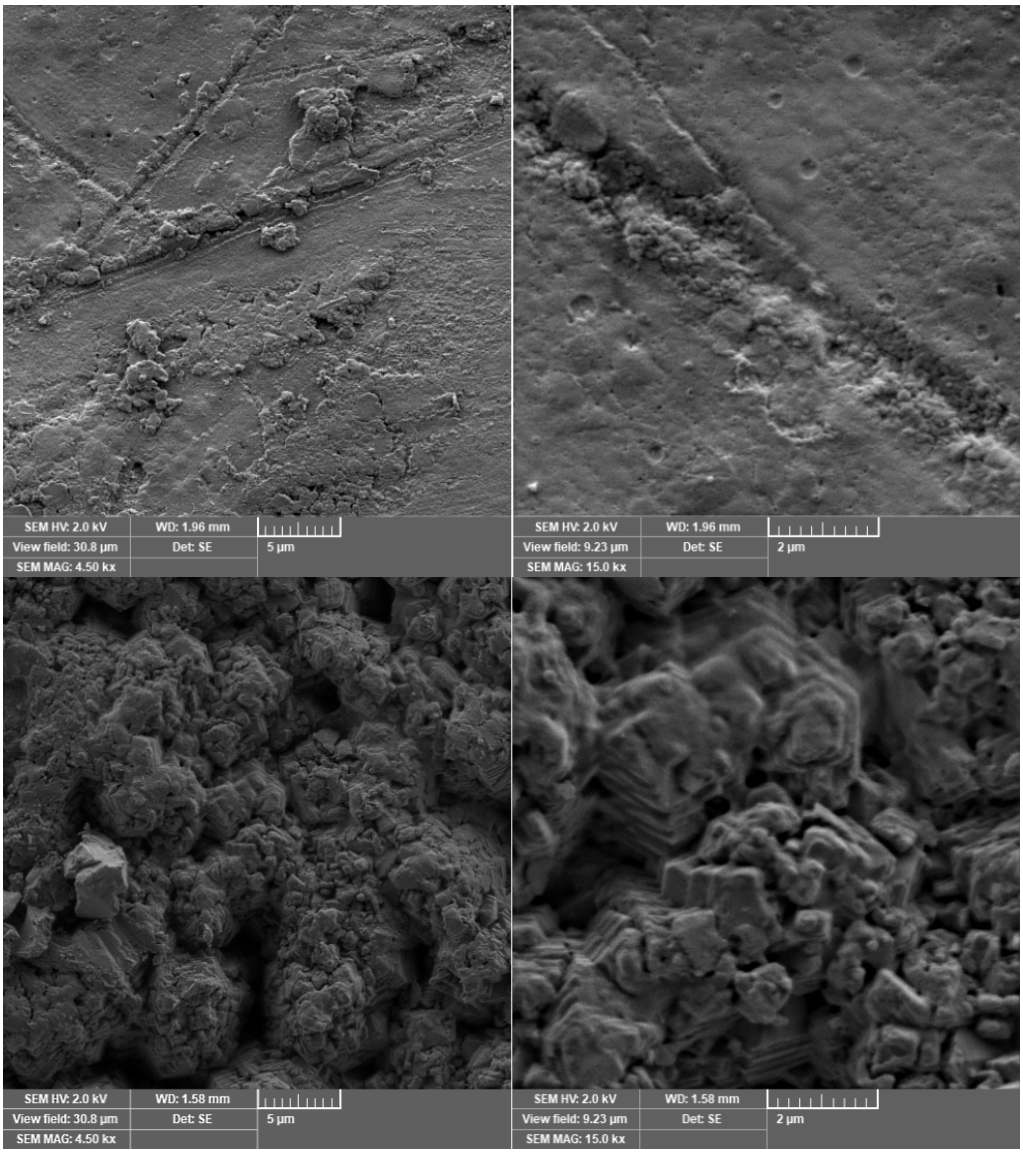


Fig.3. Uncolored scanning electron micrograph (SEM) of eggshell surface (left side:4.5 kx, right side:15.0 kx): European quail (*Coturnix coturnix* (Linnaeus, 1758), **(a)**, European pond turtle (*Emys orbicularis* (Linnaeus, 1758)) **(b)**.

Thicker eggshells at the sharp end of the egg can be explained with more calcium carbonate and other minerals [19]. The previous study has also shown that although the thickness is the main factor contributing to the mechanical strength of an eggshell, thicker does not guarantee stiffer or stronger eggs [20,25,26]. Moreover, it has been noted that increases in calcium levels improved the egg weight and eggshell thickness [13]. Additionally, increasing eggshell thickness means more mineral consumption from the feed [18,27].

IV. CONCLUSIONS

Calcium carbonate ($CaCO_3$) is one of the main factors contributing to eggshell quality that in turn is a feature of the obviously successful reproductive strategy. The comparative analysis of calcium carbonate ($CaCO_3$) content and structural surface characterization obtained from different laying hen eggshells and other animal species was studied by chemical analysis and scanning electron microscopy (SEM). Taken together, the data clearly showed that the eggshell of the white laying hens has less $CaCO_3$ content comparing with light-brown and dark-brown laying hens. Furthermore, the highest content of $CaCO_3$ and the strength of the eggshell were determined in the common ostrich. The results indicated that the least

$CaCO_3$ content was obtained in the eggshell of the African land snail. Our results about the structural surface characterization of various eggshells provide valuable clues towards further analyses of the physiological and genetic bases that may perhaps elucidate the causes of intraspecific variation.

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