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Comparison of the structure, crystallography and composition of eggshells of the guinea fowl and graylag goose

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ABSTRACT

The structure and composition of the eggshells of two commercial species (guinea fowl and greylag goose) have been studied. Thin sections and scanning electron microcopy show the similarity of the overall structure, but the relative thickness of the layers differs in these two taxa. Atomic force microscopy shows that the different layers are composed of rounded, heterogeneous granules, the diameter of which is between 50 and 100 nm, with a thin cortex. Infrared data and thermogravimetric analyses show that both eggshells are made of calcite, but differing on the quality and quantity when the organic component is considered. Chemical maps show that chemical element distribution is not uniform within a sample, and differs between the species, but with low magnesium content. Electron back scattered diffraction confirms the eggshells are calcite, but the microtexture strongly differs between the two species. Based on the chemical-structural differences, a species-specific biological control on the biomineralization is found, despite the rapid formation of an eggshell. Overall results indicate that to estimate the quality of eggshells, such as resistance to breakage, is not a straightforward process because of the high complexity of avian eggshell biomineralization.

1. Introduction

Calcified eggshells are used for systematic and phylogenetic purposes (Erben, 1970, 1972; Penner, 1984; Mikhailov, 1991; Adeyeye, 2009), to clean soils or water (Park et al., 2007) and to amend soils (Ahmad et al., 2012), for palaeoenvironmental reconstructions (Erben et al., 1979; Beacham and Durand, 2007; Goodman et al., 2013), biomimetic applications (Wei et al., 2009), and they are known from archaeological sites (Keepax, 1981; Beacham and Durand, 2007; Goodman et al., 2013). Their economic importance is also very relevant as bird eggs are a main food resource because of their high protein content. Chicken, duck, guinea fowl and goose eggs are consumed, with a large dominance of chicken eggs, and tens of billions of chicken eggs are produced every year. A chicken is able to lay one egg/day, but a goose lays only 15–30 eggs/year. The chicken eggshell is built in 20 hours at a temperature of 40 °C, and has about 10.000 pores, and the mechanical properties of the chicken eggshell are of commercial importance; for example, every year 1 billion of eggs show defects and are not sold (INRA, 2013). Also, it has been shown that the size and weight of the egg, and the thickness of the eggshell depend on the age of the bird. The thickness of the eggshell also varies in a single egg (Tyler, 1961) and the influence of the season, diet, and other factors has been demonstrated by Tyler and Geake (1960, 1961).

One of the first comparative analyses of the microstructures of eggshells using scanning electron micrographs was that of Erben (1970). Avian eggshells are composed of several layers made of organic compounds and a mineral phase (calcite) and, as noted by Nys et al. (2004), "the general structure of the eggshell is basically the same in all avian birds". Different descriptive nomenclatures have been used, and Fig. 1 illustrates the main terminology. Inner eggshell membranes are made of organic fibres and some organic cores are deposited on their surface. They are "seeding sites", where the calcification of the shell is initiated with calcite deposited around the mammillary knobs, followed then by the deposition of the spherolites and palisade layer. The organic cuticle is the last layer deposited (Fig. 1).

Chicken eggs are the most studied, both for their nutritional contents and shells. In contrast, few data are available on the guinea fowl and goose eggs, as well as other avian eggshells. Guinea fowl eggs are stronger than the chicken eggs despite a similar size and shape (Petersen and Tyler, 1966), but the origin of the difference of the strength is not yet unraveled. Brooks and Hale (1955) described

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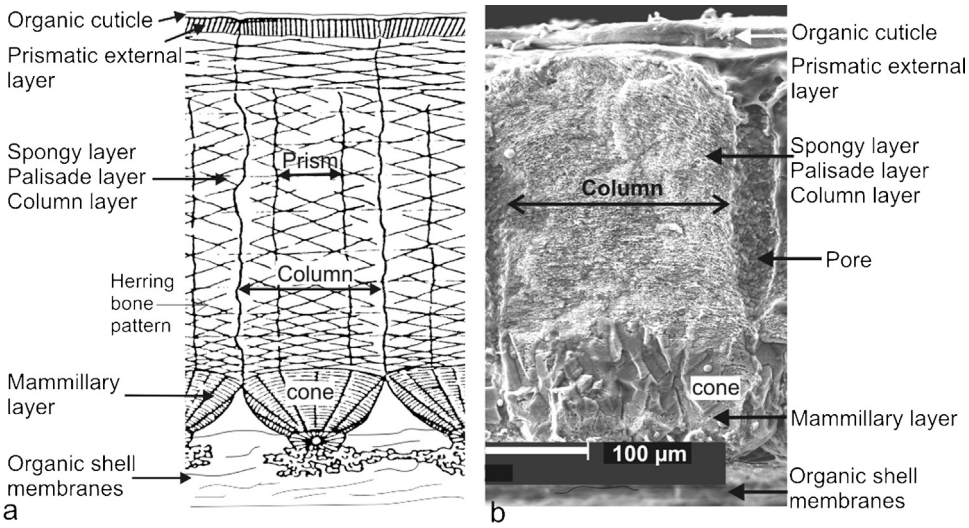

Fig. 1. Structure of avian eggshells. (a) Schematic drawing showing the diversity of the descriptive terminology. (b) SEM image of the eggshell of *Gallus gallus* showing the main structural features.

an increase of the Mg/Ca ratio towards the outer part of the chicken eggshell and correlated this with the strength of the shell, while Dunn et al. (2012) discuss the relative thickness of the different layers and the crystal size.

The spherolitic - columnar structure of eggshells is usually described as "a typical case of competitive crystal growth" (García-Ruiz and Rodríguez-Navarro, 1994; Garcia-Ruiz et al., 1995). In contrast, it is also said that "the eggshell matrix components regulate eggshell mineralisation" (Nys et al., 2004), and that "It is highly unlikely that all 520 proteins... are involved in eggshell assembly" (Hincke et al., 2010). In this study, we examine the structure (micro- and nano), mineralogy, bulk and chemical compositions, and crystallinity of the eggshells of the guinea fowl (*Numida)* and greylag goose (*Anser*) to improve our knowledge of avian eggshell biomineralization.

2. Materials and methods

2.1. Materials

Eggs were collected from 38 weeks old guinea fowl hens (*Numida meleagris*) from the INRA farm (Nouzilly, France), and greylag goose (*Anser anser*) eggs were purchased from a local market.

2.2. Optical microscopy

Sections through the thickness of the shells (5–8 μm thick) were observed with polarized light microscopy.

2.3. Scanning electron microscopy (SEM)

Eggs were broken, fragments rinsed under tap water and air dried at room temperature. Some fragments were observed without additional preparation, while others were embedded in resin, grounded and polished using various grades of diamond paste down to a final 0.25 mm grade. Polished samples were cleaned with a detergent mixed with hot water for 1 min to remove any oil residue, and rinsed with tap water. Additional detailed procedures of the sample preparations are given in the figure legends. Au-Pd or C coated samples observations were conducted using a Philips 505 and a Philips XL30 SEM. Uncoated samples were examined using a PHENOM PROX in a back scattered electron mode.

2.4. Atomic force microscopy (AFM)

Samples were studied using a Nanoscope IIIa multi-mode scanning probe microscope operating in tapping mode. The tapping mode AFM utilizes an oscillating tip at amplitude of approximately several tens of nm when the tip is not in contact with the surface. The resolution of tapping mode AFM is in the order of a few nm. Phase imaging is a powerful extension that goes beyond simple topographical mapping to detect variations in chemical composition, friction and other physical properties. Compared with conventional secondary electron imaging SEM, AFM provides topographic direct height measurements and views of surface features since no coating is necessary. Moreover, three-dimensional AFM images are obtained without difficult sample preparation (decalcification, thinning...) that is necessary for TEM examination, and they have a similar resolution. Additional detailed procedures of the sample preparations are given in the figure legends.

2.5. Electron backscatter diffraction (EBSD)

For EBSD analyses, fragments of eggshell samples were embedded in epoxy resin to observe cross sections, from outer to inner surfaces, throughout the shell thickness. Samples were grounded and subsequently polished with alumina of 1 μm (5 min.) and 0.3 μm (5 min.) and finally with colloidal silica (0.06 μm; 10 min.). Before analysis, samples were coated with a thin layer (2.5 nm) of carbon (Pérez-Huerta and Cusack 2009) and the samples were surrounded by silver paint to avoid electron charging. The EBSD study was carried out with a Hikari EDAX camera mounted on a Field Emission Scanning Electron Microscope (FIB-FESEM) TESCAN LYRA located in the Central Analytical Facility (CAF) of The University of Alabama. EBSD data were collected with OIM 7.0 software at high vacuum, 30 kV, large beam intensity (20), and a resolution of 1 μm step size or less for crystallographic maps. Finally, data were analysed using OIM 5.3 from EDAX-TSL. In this study, EBSD data are represented by diffraction maps, crystallographic maps and pole figures, which represent the stereographic projection of crystallographic planes in reference to the {0001} calcite plane (see further details in Pérez-Huerta et al., 2011).

2.6. Thermogravimetric analyses (TGA)

Eggshell with the inner organic membranes was powdered. TGA were carried out in duplicate with a TGA 4000 (Perkin-Elmer). The