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Biomineralization in modern avian calcified eggshells: similarity versus diversity

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ABSTRACT

Avian eggshells are composed of several layers made of organic compounds and a mineral phase (calcite), and the general structure is basically the same in all species. A comparison of the structure, crystallography, and chemical composition shows that despite an overall similarity, each species has its own structure, crystallinity, and composition. Eggshells are a perfect example of the crystallographic versus biological concept of the formation and growth mechanisms of calcareous biominerals: the spherulitic—columnar structure is described as "a typical case of competitive crystal growth", but it is also said that the eggshell matrix components regulate eggshell mineralization. Electron back scattered diffraction (EBSD) analyses show that the crystallinity differs between different species. Nevertheless, the three layers are composed of rounded granules, and neither facets nor angles are visible. *In-situ* analyses show the heterogeneous distribution of chemical elements throughout the thickness of single eggshell. The presence of organic matrices other than the outer and inner membranes in eggshells is confirmed by thermograms and infrared spectrometry, and the differences in quality and quantity depend on the species. Thus, as in other biocrystals, crystal growth competition is not enough to explain these differences, and there is a strong biological control of the eggshell secretion.

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Introduction

Calcified eggshells are classified as "leathery", semi-rigid and rigid depending of the abundance of the mineral part (1). Rigid eggshells are known from crocodiles since the Triassic period (about 150 My). The largest calcified eggs are assigned to a bird (*Aepyornis*), which are about 33-cm long and equivalent to 160 chicken eggs. Now, most rigid eggshells are known from birds, and the calcified eggshell is a reservoir for the bone formation of the embryo. Eggshells are used for systematic and phylogenetic purposes (2), palaeoenvironmental reconstructions, and biomimetic applications (3). Tyler (4) and Erben (5) have tried to simplify the descriptive nomenclature of the eggshell layers and a common pattern has been established. Eggs are the main food resource, but despite the large diversity of bird species, very few eggshells are studied, with chicken eggshell as the best-known example. Nevertheless, it is impossible to review all the published articles. For this, "old" articles and books should be consulted (6–8), most recent articles being dedicated to very detailed analyses of the structure or proteome. In this short contribution, we compare the main structural and compositional characteristics of various modern avian eggshells, followed by a discussion on eggshell biomineralization: is the nonbiogenic competitive crystalline growth or the organic matrix controlled mechanism the best explanation?

Materials and methods

Eggshells of *Gallus, Numida, Anser, Anas, Falco, Strix, Turdus, Uria, Phasianus, Columba, Casuarius, Dromaius, Rhea*, and *Struthio* were studied. Thin sections were observed using cross-polarized light; fragments were used for scanning electron and atomic force microscopy. Polishing and etching conditions were detailed in the legends of figures. The mineralogy and elemental chemical composition were analyzed using infrared spectrometry (FTIR) and thermogravimetry (TGA) on powdered samples and electron back scattered diffraction (EBSD) on polished surfaces. X-ray energy dispersive spectrometry (EDS) and X-ray absorption near edge structure spectroscopy (XANES) were performed on polished surfaces. More details on the materials and methods are available in previous articles (9–13).

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Results

Structure

Bird eggshells have a common structure: inner organic membranes, a mammillary layer, a columnar or palisade layer, and an outer prismatic layer covered by a thin organic cuticle (Figures 1A–E). The boundary between the mammillary and columnar layers is not clearly defined. This common structure, nevertheless, is not uniform and there is a large variation among avian eggshells. The relative thickness of the three calcified layers and the width/thickness ratio of the mammillae differ, depending on the species. Besides the layers, single or branched pores extend from the inner to the outer surface of the eggshell (Figure 1F), and detailed studies of such pores are known for ratite birds (2). The fibrous inner organic membranes are attached to the inner core of the mammillae (Figure 1G). Growth lines are more or less visible in the columnar layer, as well as the oblique herring-bone pattern (Figures 1H-I). The tabular arrangement of the columnar units is evidenced in the fracture of an eggshell of *Struthio* (Figure 1J). The thickness and structure of the outer prismatic layer differ depending on the taxa; for example, being thick and porous in *Dromaius* (Figure 1E) while it is thinner and compact in *Anser* (Figure 1K). Examination of the inner structure of the layers shows that they are made of mineral rounded particles (Figure 1L), embedded in an organic material (Figures 1M-N). The mineral particles are

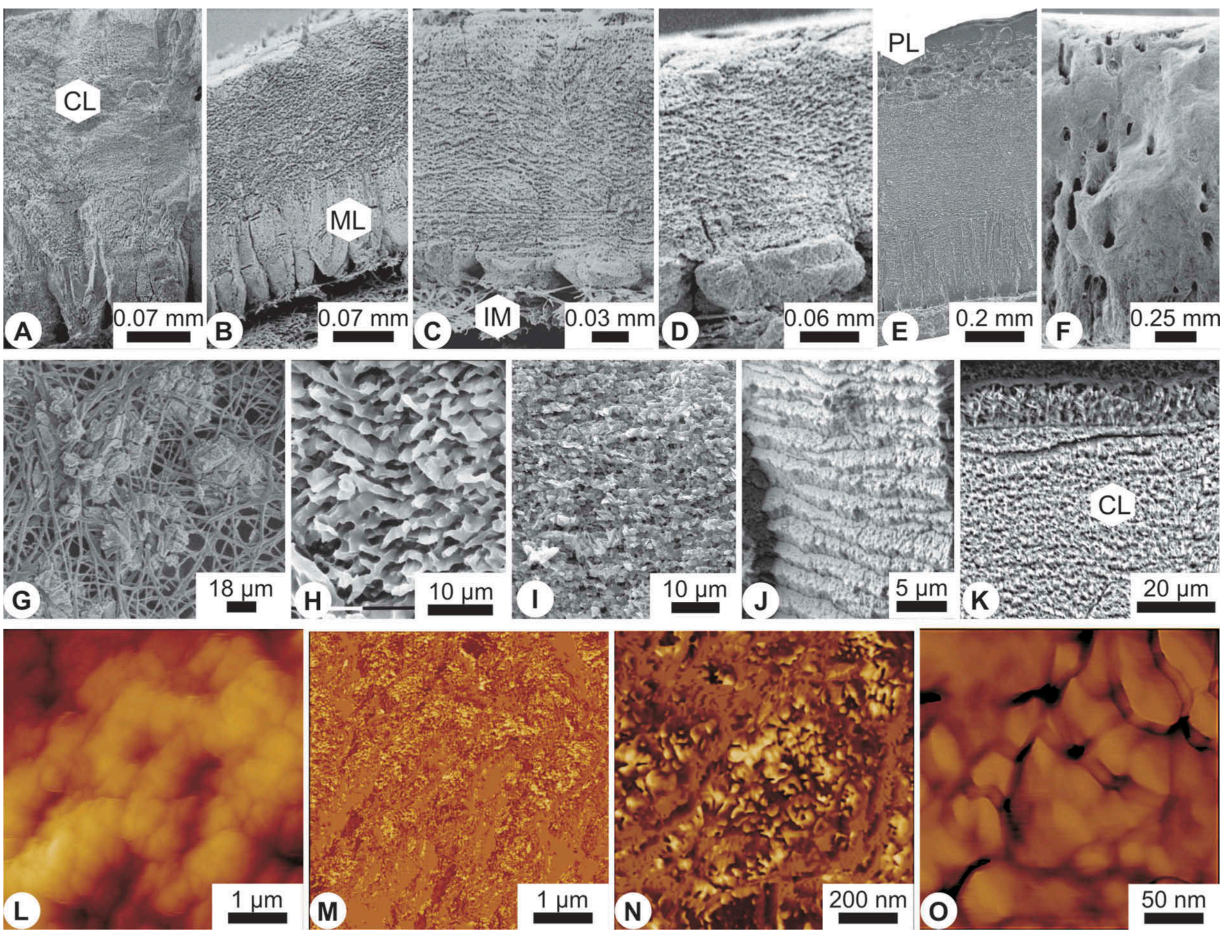

Figure 1. Micro- and nanostructures of some eggshells (A–D); Vertical sections showing the various shapes of the mammillary units and the differences in the thickness of the layers (A *Gallus*, B *Phasianus*, C *Strix*, D *Columba*, E *Dromaius*); (F) Etched vertical fracture of *Struthio* showing the complex pores (H_3PO_4 10% for 12 seconds); (G) Inner shell membrane fibers anchored in the spherolites of the mammillary layer (*Strix*); (H) Etched vertical fracture showing the herring-bone pattern (formic acid 10% for 30 seconds) (*Rhea*); (I) Etched vertical fracture showing the poor herring- bone pattern and growth lines in the columnar layer (*Gallus*) (formic acid 10% for 15 seconds); (J) Vertical fracture showing the tabular structure of the mammillary and columnar layer in *Struthio*; (K) Polished and etched vertical section showing the external part of the palisade/columnar layer (CL) and the thin prismatic outer layer (*Anser*); (L-M) Polished and etched vertical section in the inner part of the mammillary layer of *Struthio* (formic acid 10% for 10 seconds); L height image; (M) Phase image; (N) Detail of L, showing the organic matrix; (O) rounded nanogranules are surrounded by a thin cortex in the main layer of *Numida*; polished and etched section (formic acid 0.1% for 30 seconds). ML: mammillary layer, CL: columnar layer, PL: prismatic layer. A-K: Scanning Electron Microscope images, L-O: Atomic Force Microscopy images.

surrounded by a cortex (Figure 1O), probably a mixture of amorphous calcium carbonate (ACC) and organic matrix, as shown by atomic force microscopy (AFM) phase imaging. It is noteworthy that there is a strong contrast between the tabular, geometric microstructural arrangement of the mammillary and prismatic units, and their rounded nanostructure.

Mineralogy and crystallography

Bird eggshells are calcitic, except in some pathological samples in which aragonite and vaterite are described (14). The organo-mineral composition, as well as the calcite polymorph, is demonstrated by infrared spectrometry (Figure 2). The wave number of the ν4 band of the calcite shows that the magnesium content is low, in all species. The intensity of amide I and A bands differs, showing that the organic mineral ratio varies. The full-width at half-maximum (FWHM) of the main band (ν3) is used as a crystallinity index (Figure 2).

The examination of thin sections using cross-polarized light shows the granular structure of the inner part of the mammillary layer (Figure 3A). Such sections also reveal the boundaries of the prismatic units in the columnar

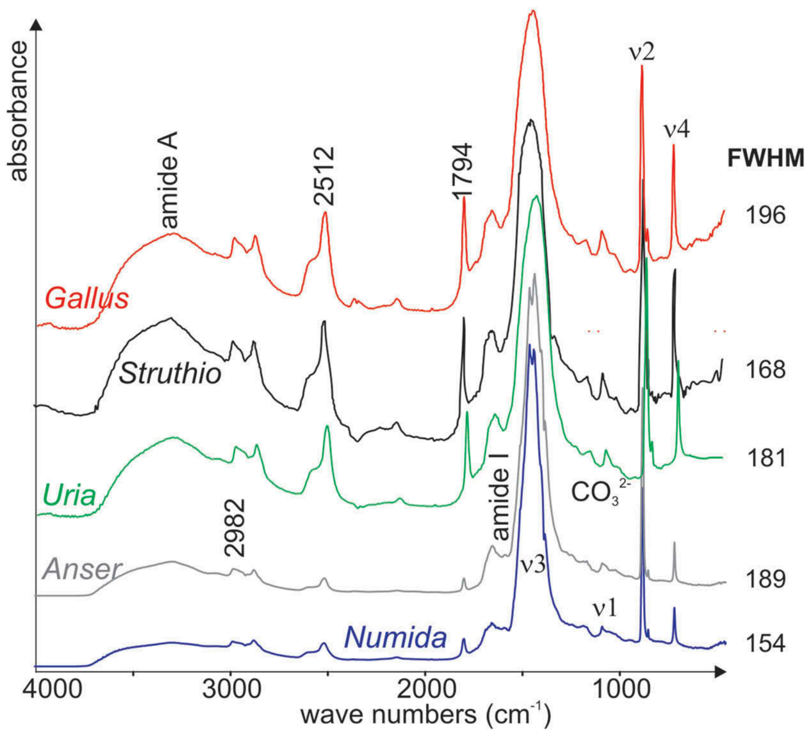

Figure 2. Fourier Transform Infra Red spectra of powdered eggshells, showing the low Mg calcite and the presence of organic components. FWHM: full-width at half-maximum of v3 band.

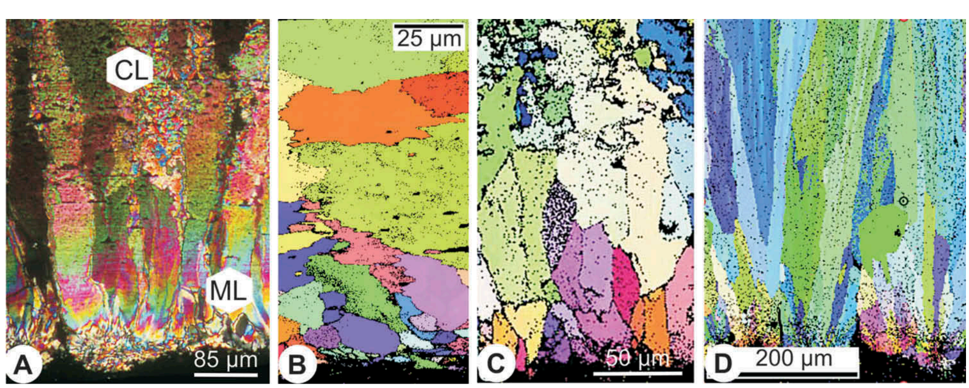

Figure 3. (A) Thin section (cross-polarized light) of the inner layers of *Anser* showing the prismatic units of the columnar layer; (B-D) Electron Back Scatter Diffraction maps showing the differences in the crystallinity in the inner mineralized layers (B *Anser*, C *Numida*, D *Struthio*).

layer, not always distinct in SEM images. The comparison of detailed EBSD maps of the mammillary layer of three species demonstrates that despite a common structural arrangement (divergent elongated units), the crystallographic pattern strongly differs (Figures 3B–D), and differences also exist in the palisade layer (13,15).

Composition

Chemical elements

The low Mg, P, K, and Na contents were described in some species (9,10,15,16). *In-situ* EDS analyses confirm these data in ratite and neognathid eggshells (Figure 4), but every species has its own composition; for example, *Uria* is rich in Fe, whereas *Phasianus* is rich in Fe and Si. It must be noticed that both *Uria* and *Phasianus* have colored eggs. Localized analyses and maps show that the elemental distribution is not homogeneous within an eggshell. Moreover, the distribution pattern of a given chemical element depends on the species, and for a given species, the elements have different patterns (Figure 5). EDS also allows performing quantitative analysis, but to precisely know the chemical speciation of the elements, X-ray microfluorescence and XANES are better techniques. For example, sulfur is associated with amino acids in the inner organic membranes; whereas it is mainly linked to sulfated polysaccharides in the mineralized layers (8,11,12).

Organic components

Organic components within the calcified layers were described as proteins (17). In decalcified, fixed and stained sections, the organic matrix is visible in all layers (18). Growth lines, herring-bone pattern, and the blocky structure of the palisade layer are preserved in these sections. As for other biominerals, the most studied components are proteins, especially those of chicken eggshells. A comparison of amino acid analyses

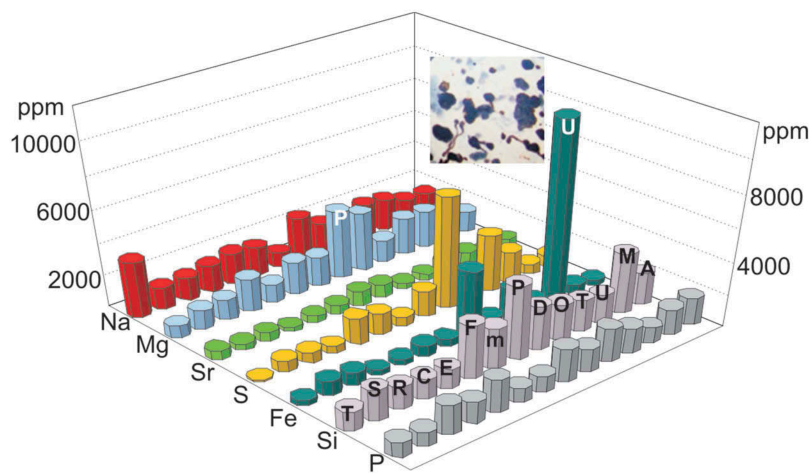

Figure 4. Elemental composition of some bird eggshells. T *Tinamou*, S *Struthio*, R *Rhea*, C *Casuarius*, E *Dromaius*, F *Falco*, m megapode, P *Phasianus*, D *Columba*, O *Strix*, T *Turdus*, U *Uria*, M *Anas*, A *Anser*. Insert: the dark spots of *Uria*.

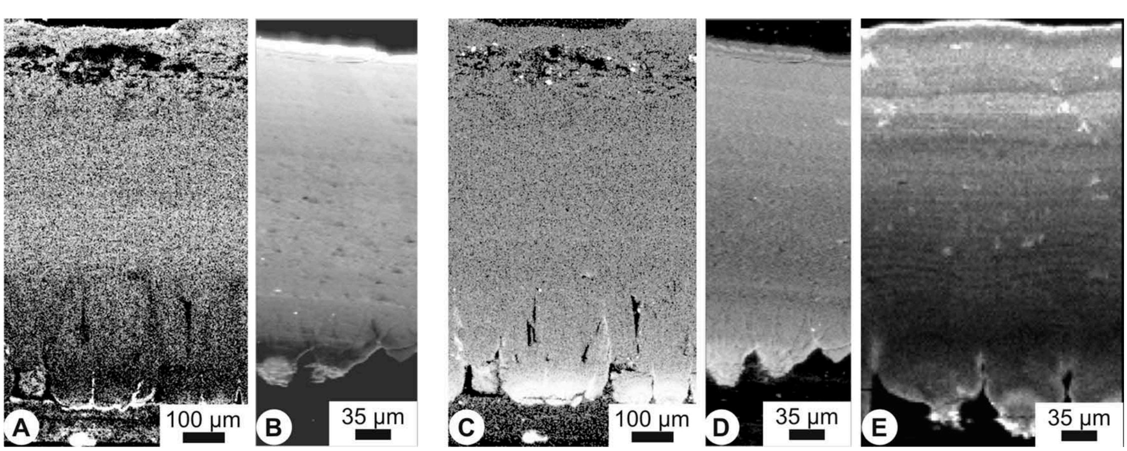

Figure 5. Elemental distribution maps in vertical section. (A-B) phosphorus maps. A *Dromaius*, WDS map; B *Anser*, XANES map; (C-E) magnesium maps. C *Dromaius*; D *Anser*; E *Gallus*.

is not easy because of the wide range of experimental procedures. Nevertheless, some trends appear to be common with high contents in aspartic and glutamic acids, serine, and glycine; but, every species has its own composition (Figure 6) (19–22). Both specific and common proteins have been evidenced in the soluble matrices (23). The components of the palisade layer are proteins (70%) and polysaccharides (GAG) (24). Also, it has been shown that some proteins are present in all mineralized layers (ovocleidin-17), whereas others are known in only one layer (25,26). Little attention has been paid to the insoluble matrix. Some proteins are present (27) and phospholipids and free fatty acids were identified in the ostrich eggshell (28). More than 500 proteins have been detected in chicken (29), 697 in turkey (30), and 475 in zebra finch (31). Strong similarities have been detected between chicken and turkey, but "there were important and unexpected differences" between these two species (32).

Discussion—conclusion

The comparison of eggshells from diverse taxa emphasizes the differences in structure and composition depending on the taxa. The interplay of the structure, crystallinity, and composition is a main factor responsible for the biomechanical properties of the eggshells. Nevertheless, how these parameters interact and their relative importance are not deciphered yet. The facts that the thickness of an eggshell varies and the average thickness depends on the age of the bird are only examples of the complexity of the question. Thus, to estimate the mechanical properties of an eggshell (mainly resistance to breakage) is difficult because of this variability. Another problem is the diversity of the used techniques, as well as their sensitivity (32–34). The contrast between the abundance of the literature dedicated to the proteins on one hand, and sugars and lipids on the other hand, is partially due to the availability and automatism of the techniques routinely used for proteins and proteomics, but not for lipids and sugars. Nevertheless, lipids and sugars play a role in the mechanical properties of the eggshells, the importance of which is still unknown.

Recognizing similarities and differences in structure and composition of eggshells, as briefly summarized in this contribution, is the first step to understand eggshell biomineralization. Precise mechanisms are not well

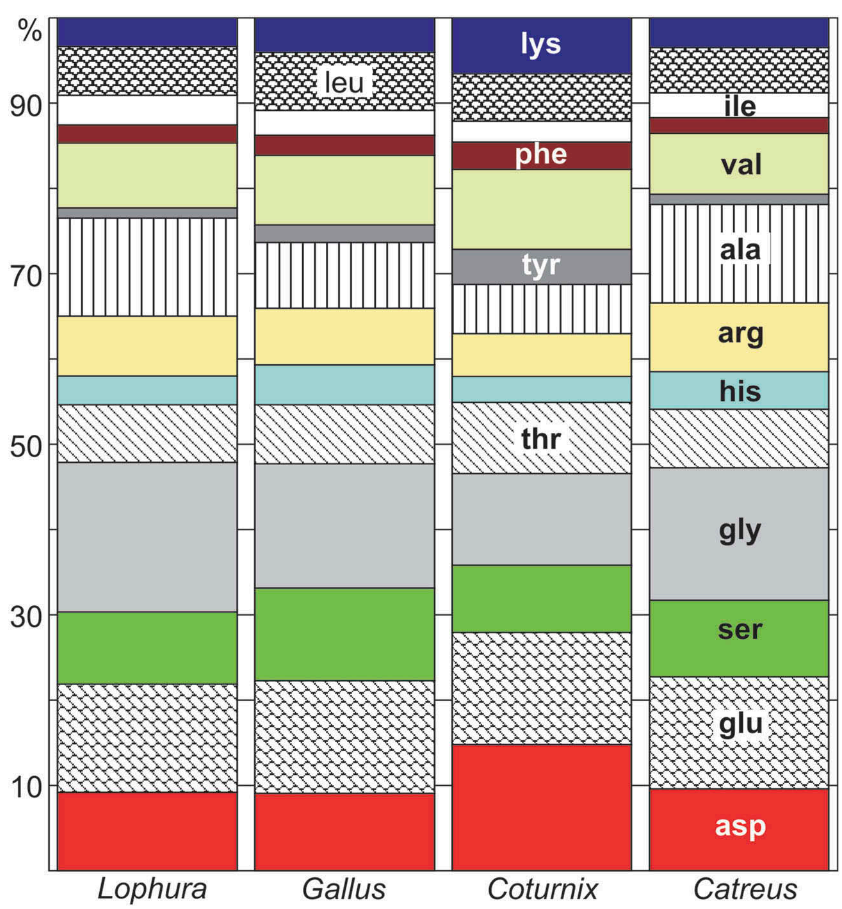

Figure 6. Amino acid composition of the matrix extracted from eggshells.

understood; however, two competing models are evoked to explain the formation of eggshells. The first model highlights the biological control, and the role of the organic matrix ["The eggshell matrix components regulate eggshell mineralisation" (35–37)]. In contrast, the alternative model advocates for more inorganic control (38,39), including the idea that the spherulitic—columnar structure of eggshells is "a typical case of competitive crystal growth" (39).

Independent of these models, several facts are precisely known about eggshell biomineralization such as the timing and sequence of the chicken eggshell formation (26,35–37). The role of the inner organic membranes is of major importance in the beginning of the process (25,40). The first mineral deposits are amorphous $CaCO_3$ particles, which progressively changed into calcite crystals (41). Previous authors have shown that the proteins present in the calcifying uterine fluid differ at the different stages of the eggshell formation and thus, affect the mineralization process (26,35,36). Also, not just the presence, but the concentration of proteins influences such a process. The marked influence of organic matrix components and inner membranes in eggshell biomineralization reinforces the biological control in opposition to pure inorganic mechanisms involved in calcification.

The "inorganic model" tends to neglect the role of organics and the onset of biomineralization by focusing mainly on the structure of the mammillary cones and the palisade layer. However, the structural-compositional analysis of all layers in eggshells highlights the biological control on mineralization. AFM images indicate that the nanostructure of layers is made up by rounded granules, surrounded by a nonmineralized cortex. This "nanosphere particle morphology" is characteristic of mature biogenic crystals (42) and can be found in biomineral structures produced by a wide array of organisms (43). At microstructural level, the diversity of crystallographic textures found in eggshells (13,15), as well as the presence, the spatial distribution and the shapes of pores cannot be explained by invoking crystal competition, and even the role of organic matrix components is observed for the formation of calcite crystals in the mammillary and palisade layers. Furthermore, differences in the elemental and amino-acid composition of eggshells do not support simple "abiogenic mineralization models" for their formation. In summary, our current knowledge of the timing and sequence of the formation of chicken eggshells and the structure-composition of eggshells from different species indicate that they cannot be viewed as mere bioceramic composites (44). In contrast, the strong link between inorganic and organic phases suggests a type of biomineralization mechanism similar to other calcified matrices, such as bone or tooth enamel.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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