Available online at www.sciencedirect.com
SCIENCE DIRECT®
C. R. Palevol 3 (2004) 549–562
COMPTES RENDUS PALEVOL

General Palaeontology (Palaeobiochemistry)

# Avian eggshell mineralization: biochemical and functional characterization of matrix proteins

Yves Nys [a,*], Joël Gautron [a], Juan M. Garcia-Ruiz [b], Maxwell T. Hincke [c]

[a] *Station de recherches avicoles, Institut national de la recherche agronomique, centre de Tours, 37380 Nouzilly, France*
[b] *Instituto Andaluz de Ciencias de la Tierra-CSIC, Campus de Fuentenueva. 18002 Granada, Spain*
[c] *Department of Cellular and Molecular Medicine, University of Ottawa, 451 Smith Road, Ottawa, Canada, K1H 8M5*

Received 30 January 2004; accepted after revision 26 July 2004

Available online 5 October 2004

Written on invitation of the Editorial Board

**Abstract**

The eggshell of the hen is a highly ordered and mineralised structure, which is sequentially deposited within an acellular milieu – the uterine fluid secreted by the distal oviduct. Spherulitic crystal growth of calcite is initiated on organic aggregates on surface of the eggshell membranes, followed by competition between radial crystallites for space to form a compact columnar biomineral. The exceptional mechanical properties associated with the well-defined eggshell ultrastructure and texture arise from the control of crystal morphology and growth by the organic matrix, and, amongst them, proteins specific to the uterus and eggshell (ovocleidins and ovocalyxins). The changes in uterine fluid constituents with stages of egg calcification, their effects on morphology of calcite grown in vitro, and the relationship between eggshell texture and mechanical properties point to this control of eggshell fabric. ***To cite this article: Y. Nys et al., C. R. Palevol 3 (2004)***.


**Résumé**

**Minéralisation de coquilles d'œuf d'oiseaux : caractérisation biochimique et fonctionnelle des protéines de la matrice.** La coquille de l'œuf de poule est une structure minérale parfaitement ordonnée, déposée dans un milieu acellulaire, le fluide utérin secrété par l'oviducte distal. La croissance sphérulique de calcite est initiée sur des sites organiques en surface des membranes coquillières et aboutit à une couche cristalline compacte par compétition pour l'espace entre sites adjacents. Les propriétés mécaniques exceptionnelles et la texture de ce biomatériau résultent d'un contrôle de sa fabrication par les constituants de la matrice organique, qui sont notamment composés de protéines utérines spécifiques à la coquille (ovocalyxines et ovocléidines). Cette hypothèse est étayée par la composition particulière du fluide utérin à chaque étape de calcification, les

* Corresponding author.
*E-mail address:* nys@tours.inra.fr (Y. Nys).

1631-0683/$ - see front matter © 2004 Académie des sciences. Published by Elsevier SAS. All rights reserved.
doi:10.1016/j.crpv.2004.08.002

modifications de morphologie de la calcite in vitro en présence de protéines de la matrice, et la relation entre texture et solidité de la coquille. ***Pour citer cet article : Y. Nys et al., C. R. Palevol 3 (2004)***.


*Keywords:* Bird; Uterus; Eggshell; Organic matrix; Calcite, Biomineralisation

*Mots clés :* Oiseau ; Utérus ; Coquille d'œuf ; Matrice organique ; Calcite ; Biominéralisation

## 1. Introduction

The eggshell is essential for propagation of all avian species; it is a sophisticated structure, whose properties reflect perfectly their crucial functions in reproduction. These functions are basically: (a) to protect the contents of the egg from the microbial and physical environment; (b) to control the exchange of water and gases through pores during the extra-uterine development of the chick embryo; (c) to provide calcium for embryonic development once the yolk stores are depleted. In order to meet these requirements, the eggshell must be a porous ceramic material. It must be as light as possible, and balances the requirement for strength to resist the impact of predators while permitting the hatching embryo to break through from the inner side to escape. For the same reasons, it must be of low chemical and biological activity on the outer surface, but easy to dissolve at the inner surface. This eggshell is rapidly formed at physiological temperatures ≤ 41 °C. All these features are simultaneously present in the remarkable eggshell, which seems to be designed ad hoc, but is certainly the result of an *evolutionary* process. All avian eggshells share the same mineral component, namely the trigonal phase of calcium carbonate ($CaCO_3$), known as calcite, which is the more stable polymorph at room temperature. The avian eggshell forms in a confined space, the distal segment of the hen oviduct, in an acellular uterine fluid that is supersaturated with respect to calcium and bicarbonate and contains the organic precursors of the shell matrix. Its distinctive features, as compared to bone or teeth, are the nature of the mineral deposit – calcium carbonate in the form of calcite, as well as the absence of cell – directed assembly during its fabrication upon organic cores present on the outer surface of the eggshell membranes. The thickness of the eggshell, the form and size of the whole eggshell and its structural elements, as well as features of the porous system varies among different species; however, the general structure of the eggshell is basically the same in all birds [9,52,59,68,73].

The aims of this review are to describe the structure and fabric of eggshell and current progress in identification and characterisation of eggshell matrix components, to present evidence supporting their involvement in shell calcification and to propose mechanisms by which their influence results in the unique mechanical properties of this highly regulated crystalline biocomposite ceramic. We focus this review on the chicken (hen) eggshell because most recent studies on eggshell components and their interaction with mineral during fabrication of the eggshell structure have been performed with this domestic specie.

## 2. Structure and formation of the eggshell

The existence of a perfectly defined structural polycrystalline organization throughout the calcified eggshell has been underlined since the earlier studies of Von Nathusius (1821–1899), whose papers were translated and edited by Tyler [73], and also in recent reviews [4,29,56,58]. It is usually considered that the avian eggshell is composed of six layers [58,72]. The innermost two layers are the uncalcified inner and outer shell membranes; each of them is made up of a network of fibres that envelops the albumen. The inner zone of the calcified shell is composed of irregular cones corresponding to the mammillary knob layer, the tips of which are penetrated by the outer membrane fibres. The palisade layer extends beyond the bases of the cones and ends in a thin vertical crystal layer where the crystallites are aligned perpendicular to the shell surface. The outer layer, the cuticle, is an organic layer deposited on the surface of the egg. It contains a thin film of hydroxyapatite crystals in its inner zone [15], and the bulk (2/3) of the superficial eggshell pigments

[57]. Many variations in the type, the number, and the thickness of these layers have been described for taxonomic purposes. See Mikhailov [52] for a clear descriptive review of these variations as seen by scanning electron microscopy.

The calcification of the eggshell is the result of a precipitation phenomenon occurring on the eggshell membrane, which takes place during passage of the egg through distinct regions of the oviduct [56,58,72]. It is amongst the most rapid mineralisation processes known, with a precise temporal and spatial control of its sequential formation. As the yolk travels down the oviduct, it progressively acquires the egg white in the magnum, followed by the eggshell membranes in the isthmus. In the distal red isthmus, organic aggregates known as mammillary knobs are deposited on the surface of the outer eggshell membranes at quasi-periodically, but randomly located sites, where heterogeneous nucleation of calcium carbonate occurs in the form of polycrystalline aggregation. In the next phase of shell formation (in the domestic chicken, five hours after ovulation), the egg enters the uterus and acquires its ovoid shape by albumen plumping. That is, fluid enters the albumen causing it to grow to its final size at laying. Calcite crystal growth subsequently occurs in the uterine fluid, an acellular milieu containing ionised calcium and bicarbonate greatly in excess of the solubility product of calcite [57], as well as the native and soluble organic precursors of the shell matrix [23]. The concentrations of these components vary during the sequential process of shell formation, i.e. initiation (five to ten hours post-ovulation in hen), linear deposition (10 to 20 h post-ovulation) and finally arrest of shell calcification [23,56,58]. The egg rotates during the linear deposition of calcium carbonate (0.33 g/h in hens) as the mammillary and palisade layers are sequentially formed. One and a half hour before oviposition (i.e. egg expulsion), mineralisation stops and the organic cuticle is deposited. Shell calcification halts prior to egg expulsion in a milieu that remains supersaturated relative to calcite. It is therefore likely that shell mineralisation is inhibited by a specific process or component of the uterine fluid at this stage.

The weight and size of avian eggs vary within three orders of magnitude, for instance, from the 1.9 kg of the ostrich's egg to the 0.5 g of the egg of the hummingbird. The weight of the egg of the extinct bird *Aepiornis maximus* – thought to be the largest egg – is estimated at more than 10 kg. Across a wide number of bird species, the mass of eggshell is proportional to the egg's mass [3], representing 10–11% of the egg's weight. The bulk of the calcified zone consists of calcium carbonate (95% by weight) in the form of calcite, its most stable polymorph. Of the remaining material, 3.5% is an organic matrix consisting mainly of fibrillar proteins with disulphide cross-links and collagen types I, V and X in the eggshell membranes, and of proteoglycans and glycoproteins in the calcified layers [4,44,58].

The main function of the eggshell is to shelter the embryo from external aggression, a function that must be compatible with easy breakage from inside to allow hatching of the embryo. In addition, the eggshell structure must permit exchange of water and gases between the embryo and the environment during extra-uterine development, as well as being a source of calcium for the growing embryo. These requirements are fulfilled by the eggshell, because it is a ceramic material displaying a texture gradient. In the outer zone of the structure, there is a tough structure made of large crystals where the external impacts are absorbed by thin inter-crystalline organic layers that make intra-crystalline crack propagation difficult. However, the inner region of the eggshell is composed of microcrystals of calcite arranged with spherulitic texture, which facilitates the propagation of cracks during piping, when the embryo breaks out of the eggshell with its beak. Moreover, this facilitates the mobilization of calcium to nourish the embryo by dissolution of highly reactive calcite microcrystals. While the defined six-layer eggshell outlined above is a classical description, in fact, the eggshell is a single structure from the viewpoint of its mechanism of formation. It is thought that this high degree of control of size, shape and orientation of the crystals of calcite in avian eggshells, which is responsible for its unique ultrastructure and exceptional mechanical properties (in hen, egg breaking strength is 30 N for a mean eggshell thickness of 0.33 mm), results from competition for crystal growth between crystals belonging to the same and to adjacent nucleation sites [20] and from control of crystal morphology by matrix components interacting with calcium carbonate [4,21,39]. Under optical microscope, thin slides from the radial section of the eggshell (Fig. 1) show clearly its general structure: an inner organic membrane, the existence of a band of nucle-

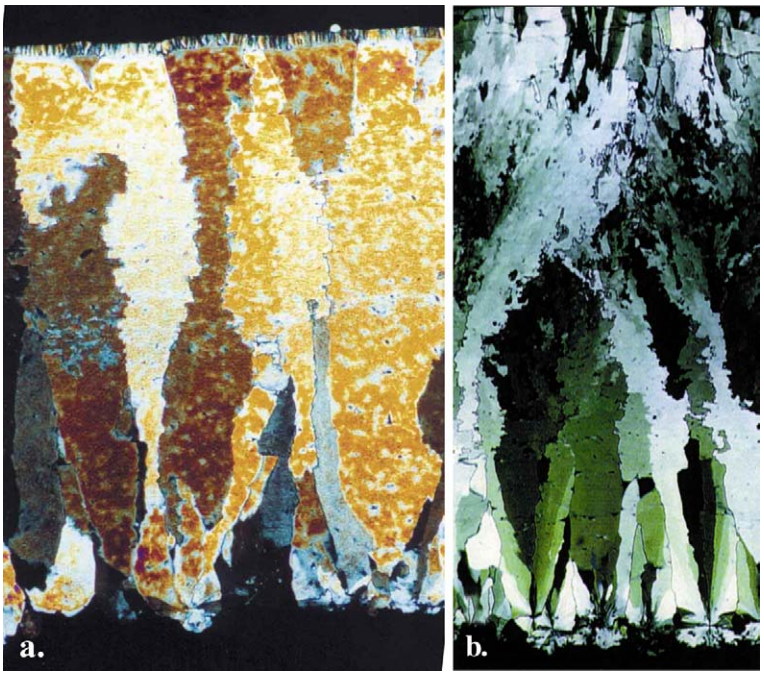


Fig. 1. Transverse section of eggshell viewed in cross-polarized light: (**a**) turkey, (**b**) guinea fowl.
Fig. 1. Section transversale de coquille, observée en lumière polarisée : (**a**) dinde, (**b**) pintade.

ation centres with radial structure (the so-called) mammillary cores and the existence of a dense crystalline region. These nucleation centres are hemispherical in shape and they are made of microcrystals of calcite with radial arrangement displaying concentric growth banding. During the initial phase of shell formation, the radial crystallites around the nucleation centres begin to compete for growth space and a columnar structure arises. Only those crystals oriented perpendicular to the substrate (outer eggshell membrane) will survive, the probability of surviving at a given thickness depending on how disoriented a crystal is with respect to this axis. Such a structure is particularly evident using crossed polarisers to reveal different crystallographic orientation of the columns (Fig. 2). From a simple two-dimensional model, it can be demonstrated that the number of surviving crystals is a function of the shell thickness scale with the power of 0.5 [63]. This can be verified with the orientation distribution function (ODF) obtained from X-ray diffraction textural studies at different levels of eggshell thickness. This correlation of ODF with thickness characterizes the eggshell. Thus, a simple geometrical law of competition for space explains the amazing textural gradient of the eggshell, with the exception of the cuticle. This model also provides a rational background to explain the different zones of the avian eggshell, as well as that seen in reptile eggshells including the aragonitic eggshell of turtles.

The texture of eggshells can be studied by different techniques. The size and orientation of crystals forming the eggshell can be determined directly by optical microscopy using thin-slices (< 30 μm) of radial sections and cross-polarisers. Information from optical microscopy is visual and accurate, but is also two-dimensional; it is local – lacking statistical meaning – and is limited to about 1-μm resolution. It also requires tedious sample preparation. On the contrary, most X-ray diffraction techniques do not require sample preparation and are suitable for systematic studies. Grain size, mosaicity (which correlates with density of crystalline defects, for instance due to protein incorporation) and crystal orientation (ODF) can be obtained from different X-ray diffraction techniques [67]. With X-ray techniques, it is possible to obtain three-dimensional information with a resolution at the scale of angstroms [65]. Only recently, a combination of both optical and X-ray techniques has been used for systematic studies of 3-D eggshell textures in order to

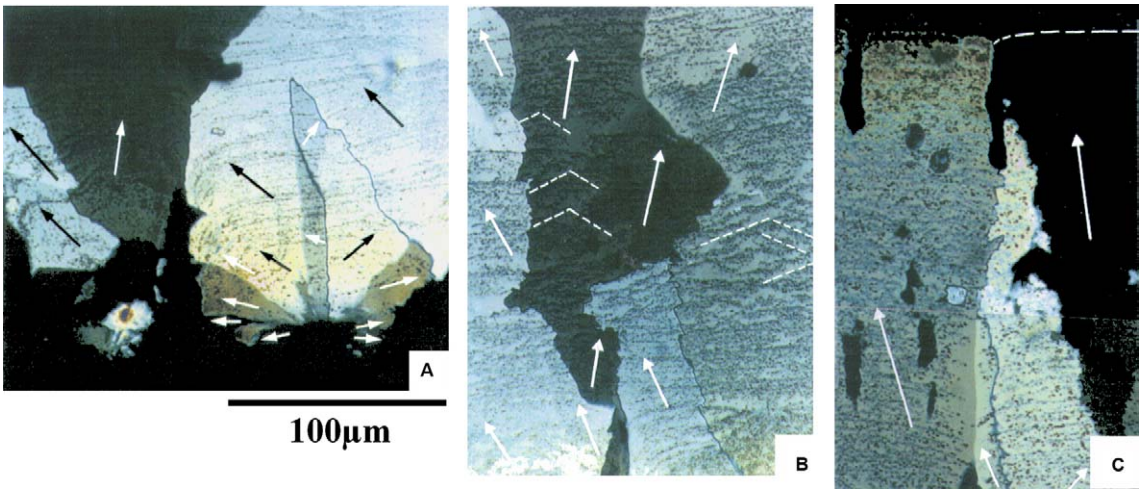


Fig. 2. Cross-section of an eggshell viewed in cross-polarized light. Arrows indicate the orientation of the *c* axis of calcite crystals. Scale bar is 100 µm. (**a**) Mammillary knobs, (**b**) mid palisade layer, (**c**) outer palisade.
Fig. 2. Vue transversale d'une coquille de poule en lumière polarisée. Les flèches indiquent l'orientation des cristaux de calcite. Échelle : 100 µm. (**a**) Couche des cônes, (**b**) couche palissadique à mi-distance et (**c**) en surface.

determine plausible correlation with mechanical properties and organic components [66]. In addition to these techniques, scanning and transmission electron microscopy as well as electron diffraction have been used.

Specific differences in shell ultrastructure within the mammillary layer and in crystallographic texture were observed among domestic bird species [59] and also in crystallographic texture [38]. Of particular interest is the eggshell of the guinea fowl, because of its exceptional breaking strength relative to that of the domestic hen. Such mechanical strength results from the larger ratio of eggshell weight to size in guinea fowl. This high ratio is due a shorter phase of crystal nucleation followed by a longer phase of eggshell deposition by crystallisation [60]. A correlation between breaking strength and shell thickness has been established for eggshell from numerous avian species [3]. This relationship indicates that the increased shell mass only accounts partially for the greater strength. Examination of ultrathin sections of guinea fowl shell reveals that the adjacent calcite crystals in the upper palisade layer demonstrate an intricate interlacing in contrast to that of hens where crystal columns remain separated. This crystal texture develops in the middle of the palisade layer, but tentative to associate with any changes in matrix secretion during the phase of rapid deposition has not been successful [60].

Fossils eggs, such as those from the Cretaceous theropod dinosaur *Troodon formosus*, show a porous eggshell structure reminiscent of those of avian birds with a mammillary and palisade layer [74]. Of particular interest is the observation in this fossil eggshell of non-branching pores, of spherulites in the mammillae, and of fibre tracks reminiscent of channels containing eggshell membrane fibres. In principle, many textural features should be preserved in fossils eggshells, but in most cases the degree of preservation does not allow quantitative studies [13]. This is also true for isotopic studies, which certainly are very informative for ecological reconstruction, but not for taxonomical purposes, because interspecies variations in eggshell tend to be smaller than intraspecies differences and those due to environmental factors. This assertion has been validated for modern [69] as well as for fossils eggshells [13,19].

As the model of competition for space shows, the actual ODF and its variation with thickness depend largely on the shape of crystals, i.e., on the relative growth rates of calcite crystal faces, which can be modulated by biomolecules in the crystallization milieu (the uterine fluid). The identification of different macromolecules in the uterine fluid as well as in organic extracts from decalcified eggshell is reported in the next section (3), while the mechanisms and interactions between matrix biomolecules and crystals are reviewed in section 4.

## 3. Characterization of eggshell matrix components

### 3.1. Eggshell membranes

Numerous authors have investigated the nature of the constituents of the shell membrane [5,11,44]. The fibrous material was initially identified as ovokeratin, but the amino-acid composition and the use of specific antibodies did not support this hypothesis. Similarly, the identification of desmosine and isodesmosine suggested the presence of elastin, but this was in disagreement with the low glycine content [11]. Finally, collagen was identified because of the presence of hydroxylysine and the observation of digestion of eggshell membranes by collagenase. This point was definitively confirmed by immunochemistry using antibodies against type-I, -V and -X collagens [6,75,77]. However, the differing amino acid composition of the membranes, as compared to other collageneous tissues, suggests that collagen is not predominant and that a unique protein containing lysine-derived cross-links may be present [44]. It is noteworthy that intact eggshell membranes are a prerequisite for shell calcification in laying hens, as shown by the detrimental effect that disruption of eggshell membrane crosslinking by Cu deficiency or aminopropionitrile has on shell structure [5,8,11]. In addition, shell membranes provide a barrier to prevent inward mineralisation. Type-X collagen may facilitate this inhibition, since chemical removal of this collagen induces in vitro calcium crystal formation on shell membrane fibres [4]. However, its localization in the core of the membrane fibres [6] does not support this hypothesis.

### 3.2. Eggshell matrix proteins and proteoglycans

Since 1990, numerous efforts have been carried out to identify and characterize the matrix molecules present in the mammillary and palisade layers of the eggshell. Mineral can be removed from the eggshell by decalcification with EDTA or acetic acid. However, the eggshell matrix proteins extracted from such shell exhibits aggregation, limiting their subsequent resolution by liquid chromatography. Use of denaturants alleviates this problem when pure proteins are needed for peptide sequencing, but limit the testing of functional properties of these components. An alternative and complementary source is the uterine fluid that contains the precursors of matrix proteins in their functional and native forms prior to incorporation into eggshell. The identification and characterization of numerous eggshell matrix proteins was initially achieved by amino acid microsequencing of PVDF-blotted bands after SDS-PAGE from eggshell extract and from uterine fluid, as well as generation of specific antibodies against these components after preparative SDS-PAGE [23–25,32]. These antibodies have been used to establish the presence of these proteins in the eggshell by western blotting, to localize them in the eggshell structure by immunofluorescence and by colloidal gold immunocytochemistry, and for studying their tissue and cellular origins to demonstrate tissue specificity. These antibodies have also been used to screen a cDNA library prepared from messenger RNA extracted from the uterus collected during eggshell mineralisation. This last approach has given insight into the genes coding for particular matrix proteins [24,33], and permitted the analysis of tissues involved in matrix synthesis using RT-PCR with primers corresponding to the sequenced cDNA. In addition, we have participated in the construction of single and multiple tissue cDNA libraries and in the program to produce expressed sequences tags (ESTs) for chicken reproductive tissues [12]. In parallel, purification of shell matrix protein by liquid chromatography allows microsequencing of N-terminus and internal peptides [24,50]. Database searching using tblastN with these peptide sequences allowed several corresponding ESTs to be identified in the public collections of avian tissue ESTs (>400 000). In some cases, EST sequences were assembled to obtain full-length cDNA sequences, whose conceptual translation product could be compared to peptides sequences to confirm the reality of the putative genes coding for the matrix proteins [24].

This combination of approaches led to identification of a variety of eggshell matrix proteins that can be subdivided into three groups: proteins that occur in other tissues of the body, egg white proteins, and uterine proteins unique to the process of eggshell formation (Fig. 3).

### 3.3. Ubiquitous components

Osteopontin, a phosphorylated glycoprotein present at high concentration in bone and kidney but also in

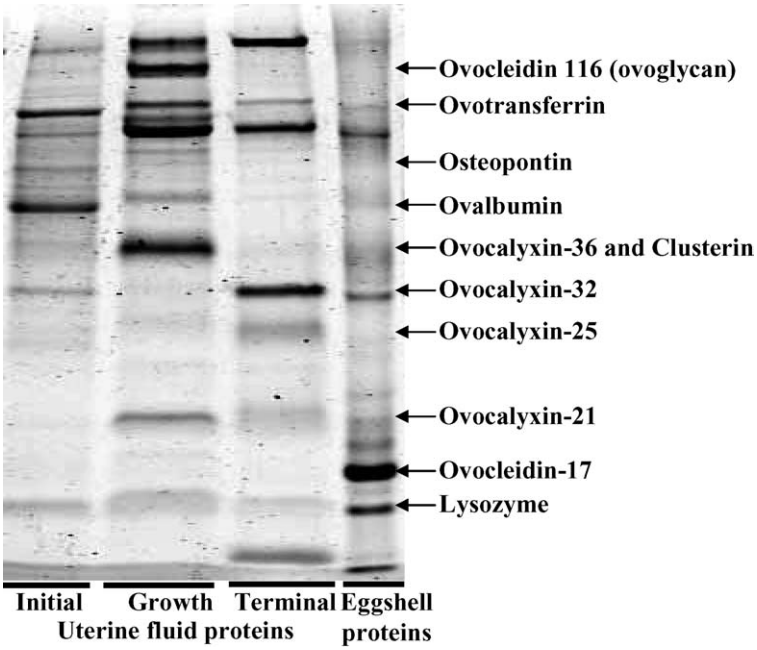


Fig. 3. Electrophoretic profile (SDS PAGE) of the uterine fluid collected at three stages of shell formation and of eggshell organic extract in hens. Coomassie blue staining.
Fig. 3. Profil électrophorétique (SDS PAGE) de fluides utérins collectés aux trois stades de formation de la coquille et d'un extrait organique de coquille de poule.

most body secretions is present in the eggshell [62]. In the domestic hen, the expression of osteopontin mRNA in uterus is upregulated by the entry of the egg into the uterus and the associated mechanical strain upon the uterine wall [42]. In the eggshell, osteopontin is localized in the core of the eggshell membrane fibres, at the bases of mammillae and in the outer palisade layer [18], but also throughout the palisade layer [51]. Partially purified eggshell osteopontin inhibits calcite crystal growth in an in vitro pH stat assay, and this inhibitory activity is lost after dephosphorylation of the protein by alkaline phosphatase [34]. Osteopontin purified from mammalian bone inhibits the formation of hydroxyapatite [36], whereas its renal form (uropontin) inhibits calcium oxalate crystal formation [71]. Furthermore, dephosphorylation of porcine bone osteopontin almost completely abolishes its ability to inhibit hydroxyapatite formation [36]. Therefore, chicken eggshell osteopontin is likely to be a potent and phosphorylation-dependent inhibitor of calcium carbonate precipitation. During eggshell formation, it may act as an inhibitor of calcite crystal growth or to modulate the speed of calcium carbonate precipitation from the supersaturated uterine fluid.

Comparison of gene expression by uterine cells when an egg is present or absent in the uterus reveals upregulation of a gene coding for the heparin-sulphate proteoglycan, glypican, previously identified in mouse [43]. Its expression is not specific to the uterus, but is observed in numerous chicken tissues. Its presence as a matrix protein in eggshell has not been determined, nor is its putative function in eggshell formation.

Recently, clusterin, a secretory disulphide-bonded heterodimeric glycoprotein was shown to be a component of eggshell matrix [49] and to be distributed throughout the mammillary and palisade layers. This protein, which is very similar to its mammalian homologues, is expressed in many tissues and may function as a secreted chaperone involved in stabilization and prevention of precipitations of proteins secreted under stress conditions. Clusterin could function in the uterine fluid to prevent the premature aggregation and precipitation of eggshell matrix components before

and during their assembly into the protein scaffold necessary for ordered mineralisation.

### 3.4. Egg white proteins

Ovalbumin was the first egg white protein revealed in shell matrix by N-terminal amino acid sequencing and immunochemistry [31]. Its presence in uterine fluid is predominant at the initial stage of eggshell formation [23], and it is localized in the mammillae of the eggshell [31]. Ovotransferrin and lysozyme are also present in eggshell membranes at high levels and are also elevated in uterine fluid during the initial stage of shell formation; their intramineral localization is limited to the mammillary knobs [25,35]. Ovalbumin, lysozyme, and ovotransferrin are major proteins of the egg white, representing 54, 3.5 and 12% of egg white, respectively. These proteins, therefore, may arrive via passive diffusion through the oviduct lumen. However, the uterus also synthesizes these proteins, as shown by RT-PCR and Northern blotting. Ovotransferrin and lysozyme modified the morphology of calcite crystals grown in vitro, suggesting their putative role in controlling calcium carbonate formation. However, it is likely that the predominant role of these three proteins, well known for their anti-microbial properties, is a chemical protective function during avian embryonic development [25,35,53].

### 3.5. Organic constituents unique to the process of shell calcification

This group is composed of shell proteins and proteoglycans that are only synthesised by tissues involved in eggshell calcification (red isthmus and uterus). These proteins are novel and specific to the eggshell mineralisation process, and have as yet, with few exceptions, only been identified in the domestic hen.

#### 3.5.1. Glycosaminoglycans

The presence of glycosaminoglycans (uronic acid, galactosaminoglycan, and hyaluronic acid) in the eggshell has been chemically demonstrated [44,54,55]. In hen eggshell, glycosaminoglycans are composed of equal ratios of hyaluronic acid and galactosaminoglycan, in which chondroitin sulphate and dermatan sulphate are predominant [54,55]. Dermatan and keratan sulphate glycosaminoglycans have been biochemically and immunohistochemically demonstrated and localised [4,10]. The appearance of a keratan sulphate proteoglycan, secreted by the isthmus gland cells [17], coincides with the formation of the mammillae 5.15 hrs post-ovulation and its location corresponds with the site of nucleation of the first crystals. This keratan sulphate proteoglycan may, therefore, play an important role during the deposition of the first crystals of the eggshell. During the following active phase of calcification, the secretion of a dermatan sulphate proteoglycan (named ovoglycan) predominates and is observed by immunofluorescence throughout the palisade layer of the eggshell [17]. This is in agreement with the observation of the localisation and regulation of expression of its core protein, ovocleidin-116 [33]. The dermatan sulphate glycosaminoglycan chain of ovoglycan is polyanionic and acidic, with high calcium affinity, and is likely to modulate crystal growth during palisade formation [17]. The observation of alteration in palisade formation after pharmacologically induced inhibition of ovoglycan sulphation [5,17], and the change in the crystal morphology observed in vitro in the presence of glycoaminoglycan [7] support this hypothesis.

#### 3.5.2. Proteins

Ovocleidin 17 was the first matrix protein to be purified using chromatographic techniques and characterized [32]. This protein is revealed by immunohistochemistry in the mammillary and palisade layers. It is also present in the uterine fluid at all stages of shell formation with the highest concentration being present during the growth phase [23]. This protein is 142 amino acids in length with a C-type lectin domain [46], and is secreted by the tubular gland cells [32]. It is a phosphoprotein that also occurs in a glycosylated form at a slightly higher molecular weight (23 kDa) [45]. The relative role of these forms is not clear. In goose, ansocalcin, a protein of 15 kDa with about 40% identity to ovocleidin-17 has recently been cloned and characterized [41]. Of interest is also the purification and amino sequencing of two C-type lectin proteins from ostrich eggshell, which have about 40% identity to each other [50]. Struthiocalcin-1 shows a 65% sequence identity with ansocalcin and 40% with ovocleidin-17. Stuthiocalcin-2 also showed features of the chicken and goose proteins, but less sequence iden-

tity. Emu eggshell is similar to that of ostrich, in that it contains two different C-type lectin-like proteins (Mann, in preparation). Ovocleidin-17 and related proteins in ostrich, emu and goose possibly act as framework proteins during matrix assembly. Ansocalcin alters in vitro the calcite morphology of crystals grown in vitro, but at rather high concentration. Other C-type lectin proteins have also been identified in numerous calcium carbonate biominerals, amongst them in mollusc (perlucin) [47], in sea urchin [76] and the mammalian pancreatic stone protein [14].

Ovocalyxin-21 and ovocalyxin-25 have recently been cloned [26] and are only detected in tissues where eggshell mineralisation takes place (uterus and red isthmus). Database analysis shows that ovocalyxin-25 has a WAP-type domain, which was also observed in lustrin A, a matrix protein from the nacreous layer of the molluscan shell and pearl [70].

Ovocalyxin-32 (32kDa) is present in uterine fluid during the growth phase, but is mainly present during the terminal phase of calcification, and consequently is localised in the outer region of the eggshell [24]. Database searches revealed that ovocalyxin-32 has limited identity (about 30%) to two unrelated mammalian proteins: latexin, a carboxypeptidase inhibitor, and TIG1, a protein encoded by a retinoic acid receptor-responsive gene. High-level expression of ovocalyxin-32 is limited to the isthmus and uterus tissues. It is secreted by surface epithelial cells as shown by immunocytochemistry at the light and electron microscope. In the eggshell, ovocalyxin-32 localizes to the outer palisade layer, the vertical crystal layer and the cuticle of the eggshell, in agreement with its demonstration by Western blotting at high levels in the uterine fluid during the termination phase of eggshell formation. Therefore, ovocalyxin-32 may be involved in processes associated with termination of shell mineralisation.

Ovocalyxin-36 has also been cloned. Its predicted amino acid sequence corresponds to the N-terminus and internal peptide sequences of a 36 kDa band found in eggshell extracts and in uterine fluid at high levels during the calcification phase of shell formation [27]. Ovocalyxin-36 is expressed only in uterine tissue and its expression is highly upregulated after the egg enters the uterus. This protein is therefore a promising candidate in the control of shell formation.

Ovocleidin-116 [33,48] is a major component of the chicken eggshell matrix observed throughout the palisade layer and most abundant in uterine fluid during the intense eggshell calcification phase. RT-PCR and Northern blotting indicate that it is expressed only in the uterus. It is secreted by the granular cells of the surface epithelium. The predicted sequence (742 AA) contains two N-glycosylation sites and two disulphide bonds [48]. Its N-terminal sequence corresponds to the core protein of a previously identified 190-kDa eggshell dermatan sulphate proteoglycan [10], named ovoglycan [17,18]. The core protein of this dermatan sulphate proteoglycan is modified by glycosylation (to about 116 kDa) and glycanation (to about 190 kDa) [10]. Both the 116-kDa and 190-kDa forms are present in uterine fluid and eggshell extract, but their relative roles remain unclear. This eggshell constituent is thought to play a primary role in the control of eggshell calcification.

An approach to globally characterize the matrix of various bird species and to contrast common and distinct features by SDS-PAGE and Western blotting across taxonomic groups shows that some matrix proteins are common to the eight domestic birds tested (ovocleidin-17, ovalbumin), and that there is a more restricted distribution for others (ovotransferrin, osteopontin). The distribution of proteoglycans at nucleation sites and within the palisade layer also varies between species [59]. The identification of the C-type lectin-like protein in four species (chicken, goose, emu, and ostrich) also supports the concept of a common process of eggshell formation, even if the precise role of this protein remains to be established.

## 4. Evidence of a role for organic constituents in eggshell mineralisation

A number of observations support the hypothesis that the eggshell matrix components regulate eggshell mineralisation. The first one is that the composition of the uterine fluid changes at different stages of shell formation: each phase of shell mineralisation (nucleation, rapid crystal growth and the completion of shell formation) is associated with a specific electrophoretic profile of biological macromolecules of the uterine fluid (Fig. 3, [23]). This is due to a sequential alteration in gene expression and/or protein secretion by the cells lining the uterus during eggshell formation. In addition, calcium aggregates spontaneously precipitate

from freshly collected uterine fluid, and these mineral pellets contain a specific subset of the uterine fluid proteins [23]. Finally, uterine fluid modifies the kinetics of calcium carbonate precipitation in vitro [16,23].

The induction time for calcium carbonate precipitation is reduced by the uterine fluid harvested during the formation of mammillary cores, suggesting that the macromolecular cocktail at this stage of the calcification of the egg promotes crystal nucleation. To a lesser extent, the uterine fluid collected during the growth phase also enhances precipitation kinetics. On the contrary, the total uterine fluid harvested at the end of calcification inhibits the precipitation of calcite [23]. This activity is retained after dialysis of the uterine fluid, indicating that the effect is due to macromolecules. Using model proteins (lysozyme, ribonuclease, myoglobin and α-lactalbumin), Hernández-Hernández et al. [30] have reported a biphasic effect of proteins on calcium carbonate precipitation. At low concentration, proteins promoted calcium carbonate precipitation, while at higher concentration they inhibited it. This behaviour is characteristic of crystallization inhibitors that have a strong affinity for target crystal surfaces. At low concentrations, these inhibitors act as substrates promoting nucleation and favouring precipitation due to stereochemical affinity with the crystal surface [40]. This can be explained by the so-called ionotropic effect [1]: the local elevation in $Ca^{2+}$ concentration in regions close to negatively charged patches on the protein surface would favour nucleation, even in the metastable zone. On the contrary, at higher concentrations, when there is an excess of inhibitors, they bind to crystal surface and block the growth sites, inhibiting precipitation. This inhibiting effect increases when the charge of protein is negative [30].

In vitro studies have also demonstrated that proteins affect dramatically the morphology of calcite crystals. Calcite grown from pure calcium carbonate solution displays the morphology of cleavage rhombohedra. Neutral to slightly charged proteins have a stronger effect on calcite morphology [37]. Proteins preferentially interact with those crystal faces that have the maximum density of carbonate groups and in which the carbonate groups are oriented perpendicular to the surface. The sequential inhibition of the growth of the {110}, {100}, and {001} faces could be caused by the combined effect of the density of carbonate groups and their orientation. The presence of soluble eggshell extract also affects the morphology of calcite crystals grown freely in solution [16] or upon avian eggshell membranes [78]. Similarly, the addition of small amounts of uterine fluid to a seeded metastable solution of calcium carbonate modifies the morphology and average size of calcite crystals. Such a modification depends on the dose and on the stage of uterine fluid and protein concentration. It may also favour aggregation of crystals at higher concentrations [22]. Some chromatographic fractions purified from the soluble fraction of eggshell extract induce morphological modifications of the rhombohedric calcite crystal at very low protein concentration. Purified ovocleidin-17 barely affects crystal morphology [28]. Its goose homologue, ansocalcin, has been shown to slightly alter calcite morphology and facilitates aggregation of crystals at higher levels (500μg/ml; [41]). Ovotransferrin reduces the size of the crystal and at 500 μg/ml promotes the development of elongated crystals with rough surface made of platelets with a V morphology most probably {018} faces [25]. Lysozyme at high concentration (>10 mg/ml) mainly affects the calcite faces parallel to the c axis, by inhibition of growth on {110} faces [35,64]. Finally, pure glycoaminoglycans also affect calcite morphology favouring elongation [7].

The nature of the interaction between matrix molecules and the mineralisation process is poorly understood. Protein adsorption is affected by protein surface properties (electrical charge density, conformation and hydrophobicity) as well as solution conditions (pH, ionic strength, etc.). These parameters affect the protein structure. Adsorption is enhanced by the hydrophobicity of the protein. Also, electrostatic interaction plays an important role, especially with hydrophilic surfaces such as calcite as shown by investigating the effect of a group of globular proteins of similar size and conformation, but with different isoelectric points [30]. Therefore, at a given pH, their surface charges are different. The experimental results suggest that the dominant mechanisms accounting for adsorption of this tested group of proteins are hydrophobic and electrostatic interactions, these proteins adsorbing to different faces in accordance with their net electrostatic attractions (or repulsions) to calcite surfaces. For charged proteins, their morphological effect is more pronounced when proteins are neutrally or negatively charged. When proteins are neutral or low charged,

hydrophobic interactions govern the effect. Under this condition, the amount of proteins adsorbed on the surface is maximum, in such a way that proteins become dehydrated by their adsorption onto surface and calcite morphology is strongly modified [64]. These hypotheses are also supported by the importance of non-collagen proteins with carboxyl groups in bone calcification. In eggshell, highly sulphated proteoglycans such as ovoglycan are likely to influence mineralisation by electrostatic interactions [7]. Protein phosphorylation is another post-translational modification that may be crucial, as shown by Hincke and St Maurice [34]. The dephosphorylation of eggshell osteopontin is associated with loss of inhibition of calcium carbonate precipitation when tested in vitro using the pHstat method.

If eggshell matrix proteins participate in establishing the morphology of calcite crystals, it would affect the texture of the eggshell and therefore influence its mechanical properties. This hypothesis leads to the prediction that differences in the total amount of eggshell matrix and/or relative composition of the matrix would correlate with variations in eggshell strength. This proposal was tested by micro-extraction, SDS-PAGE electrophoresis and quantification by ELISA of the matrix proteins in eggshell samples. The concentration of three proteins (ovotransferrin, ovalbumin and ovocleidin-17) were analysed in shell from eggs laid at initiation and at the end of the laying year [61] and after moulting of the hens [2]. As anticipated, a significant improvement (20%) in eggshell breaking strength was observed after moulting. Interestingly, neither the eggshell thickness nor the amount of organic matter varied significantly after moulting. However, a decrease in grain size was measured by optical microscopy (from about 72 to 58 μm), while crystal orientation remained unchanged. This reduction in grain size could explain the observed improvement in mechanical properties. In fact, a good correlation between grain size and breaking strength was observed. Although some trace elements modify the morphology of calcite crystals grown in vitro, no change in the concentration of the trace elements analysed (Al, Cr, Fe, Co, Ni, Cu, Zn, As, Se, Sr, Mn, Cd, Ba, Pb) was observed in eggshell after moulting. On the other hand, the composition of the organic matrix changes after moulting [2] as revealed by change in staining intensity of various electrophoretic bands (lower level of ovocleidin-116, ovotransferrin) and by ELISA, confirming a notable decrease in ovotransferrin. It has been observed that whole extract and specific components such as ovotransferrin affects calcite crystal growth, suggesting that the change in these protein concentrations may be associated with the observed variation in crystal size that is responsible for the improved mechanical properties after moulting [67].

It is clear from all the above-reviewed results that the matrix components play an active role in the control of growth kinetics and of crystal morphology. Consequently, coupled with competition for crystallisation space, the organic matrix regulates the textural organization within the eggshell. However, additional information is needed to better understand the nature of the interactions between macromolecules and growing calcite crystals, to learn to emulate in vitro actual crystal morphology in eggshells, and to know the relative importance of different matrix components. The eggshell, however, due to its spatial and temporal sequence of formation, as well as to the emerging relationship between textural structure and mechanical properties that is seen between species and at different physiological stages, constitutes a valuable model to better understand the calcitic biomineralisation that is found in diverse organisms.

## References

[1] L. Addadi, S. Weiner, Control and design principles in biological mineralization, Angew. Chem. Int. Ed. Engl. 31 (1992) 153–169.

[2] A.M.H. Ahmed, A. Rodriguez, M.L. Vidal, J. Gautron, J.M. Garcia-Ruiz, Y. Nys, Quantification of eggshell matrix proteins before and after moulting, in: Proc 16th Eur. Symp. on the Quality of Poultry Meat and 10th Eur. Symp. on the Quality of Eggs and Egg Products, Vol. III, , 2003, pp. 63–69.

[3] A. Ar, H. Rahn, C.V. Paganelli, The avian egg: mass and strength, Condor 81 (1979) 331–337.

[4] J.L. Arias, D.J. Fink, S. Xiao, A.H. Heuer, A.I. Caplan, Biomineralization and eggshells: cell-mediated acellular compartments of mineralized extracellular matrix, Int. Rev. Cytol. 145 (1993) 217–250.

[5] J.L. Arias, M. Cataldo, M.S. Fernandez, J.J. Wu, E. Kessi, Effect of beta-aminoproprionitrile on the eggshell mineralisation, Br. Poult. Sci. 38 (1997) 351–356.

[6] J.L. Arias, O. Nakaruma, M.S. Fernandez, J.J. Wu, P. Knigge, D.R. Eyre, et al., Role of type-X collagen on experimental mineralization of eggshell membranes, Connect. Tissue Res. 36 (1997) 21–31.

[7] I.L. Arias, C. Jure, J.P. Wiff, M.S. Fernandez, V. Fuenzalida, J.L. Arias, Effect of sulfate content of biomacromolecules on the crystallization of calcium carbonate, Mater. Res. Soc. Symp. Proc. 711 (2002) 243–248.

[8] S. Baumgartner, D.J. Brown, E. Salevsky, R.M. Leach, Copper deficiency in the laying hen, J. Nutr. 108 (1978) 804–811.

[9] R.G. Board, Properties of avian eggshells and their adaptive value, Biol. Rev. 57 (1982) 1–28.

[10] D.A. Carrino, J.P. Rodriguez, A.I. Caplan, Dermatan sulfate proteoglycans from the mineralized matrix of the avian eggshell, Connect. Tissue Res. 36 (1997) 175–193.

[11] S.D. Chowdhury, Shell membrane system in relation to lathyrogen toxicity and copper deficiency, Worlds Poult. Sci. J. 46 (1990) 153–169.

[12] L.A. Cogburn, W. Carre, X. Wang, T.E. Porter, Y. Nys, J. Tang, E. Bernberg, R. Morgan, J. Burnside. Chicken gene discovery: sequencing and CAP3 analysis of 42 870 ESTs from single and multiple tissue c DNA libraries (submitted).

[13] I. Cojan, M. Renard, L. Emmanuel, Palaeoenvironmental reconstruction of dinosaur (Maastrichtian, Provence Basin, France), Palaeogeogr. Palaeoclimatol. Palaeoecol. 191 (2003) 111–138.

[14] M. De Reggi, B. Gharib, Protein-X, pancreatic stone-, pancreatic thread-, reg-protein, P19, lithostathine: characterization, structural analysis and putative function(s) of the major non-enzymatic protein of pancreatic secretions, Curr. Protein Pept. Sci. 2 (2001) 19–42.

[15] J.E. Dennis, S.Q. Xiao, M. Agarval, D.J. Fink, A.H. Heuer, A.I. Caplan, Microstructure of matrix and mineral components of eggshells from white leghorn chicken (*Gallus gallus*), J. Morphol. 228 (1996) 287–306.

[16] J.M. Dominguez, J. Gautron, J.M. Garcia-Ruiz, Y. Nys, The effect of avian uterine fluid on the growth behaviour of calcite crystals, Poult. Sci. 79 (2000) 901–907.

[17] M.S. Fernandez, A. Moya, L. Lopez, J.L. Arias, Secretion pattern, ultrastructural localization and function of extracellular matrix molecules involved in eggshell formation, Matrix Biol. 19 (2001) 793–803.

[18] M.S. Fernandez, C. Escobar, I. Lavelin, M. Pines, J.L. Arias, Localization of osteopontin in oviduct tissue and eggshell during different stages of the avian egg laying cycle, J. Struct. Biol. 143 (2003) 171–180.

[19] G. Garcia, M. Vianey-Liaud, Dinosaur eggshells as biochronological markers in Upper Cretaceous continental deposits, Palaeogeogr. Palaeoclimatol. Palaeoecol. 169 (2001) 153–164.

[20] J.M. García-Ruiz, A. Rodríguez-Navarro, Competitive Crystal Growth: The avian eggshell model, in: D. Allemand, J.-P. Cuif (Eds.), Biomineralization 93, Musée Oceanographique de Monaco, 1994, pp. 85–94.

[21] J. Gautron, Y. Nys, Protéines du fluide utérin : caractérisation et implication dans la minéralisation de la coquille de l'œuf, in: Y. Nys (Ed.), C. R. 5e Symp. eur. sur la qualité de l'œuf et des ovoproduits, Tours, 1993, pp. 134–140.

[22] J. Gautron, M. Bain, S. Solomon, Y. Nys, Soluble matrix of hen's eggshell extracts changes in vitro the rate of calcium carbonate precipitation and crystal morphology, Br. Poult. Sci. 37 (1996) 853–866.

[23] J. Gautron, M.T. Hincke, Y. Nys, Precursor matrix proteins in the uterine fluid change with stages of eggshell formation in hens, Connect. Tissue Res. 36 (1997) 195–210.

[24] J. Gautron, M.T. Hincke, K. Mann, M. Panhéleux, M. Bain, M.D. McKee, S.E. Solomon, Y. Nys, Ovocalyxin-32, a novel chicken eggshell matrix protein: Isolation, amino acid sequencing, cloning and immunocytochemical localization, J. Biol. Chem. 276 (2001) 39243–39252.

[25] J. Gautron, M.T. Hincke, M. Panhéleux, J.M. Garcia-Ruiz, T. Boldicke, Y. Nys, Ovotransferrin is a matrix protein of the hen eggshell membranes and basal calcified layer, Connect. Tissue Res. 42 (2001) 255–267.

[26] J. Gautron, A. Vignal, E. Murayama, M.D. McKee, M.L. Vidal, Y. Nys, M.T. Hincke, Cloning of ovocalyxin, a novel chicken matrix protein related to lysopolysaccharide binding protein (LBP), bactericidal permeability increasing protein (BPI) and to Plunc family protein. (Submitted to publication).

[27] J. Gautron, M.T. Hincke, E. Murayama, M. McKee, Y. Nys (in preparation).

[28] J. Gautron, M.T. Hincke, Y. Nys, J.M. Garcia-Ruiz (unpublished).

[29] R.M.G. Hamilton, The microstructure of the hen's eggshell: a short review, Food Microstruct. 5 (1986) 99–110.

[30] A. Hernández-Hernández, A.R. Navarro, J.M. García-Ruiz, Influence of model proteins on the precipitation of calcium carbonate, in: Proc. 16th Eur. Symp. on the Quality of Poultry Meat and 10th European Symposium on the Quality of Eggs and Egg products. Saint-Brieuc, France, Vol. III, 2003, pp. 28–34.

[31] M.T. Hincke, Ovalbumin is a component of the chicken eggshell matrix, Connect. Tissue Res. 31 (1995) 227–233.

[32] M.T. Hincke, C.P.W. Tsang, M. Courtney, V. Hill, R. Narbaitz, Purification and Immunochemistry of a soluble matrix protein of the chicken eggshell (ovocleidin-17), Calcif. Tissue Int. 56 (1995) 578–583.

[33] M.T. Hincke, J. Gautron, C.P.W. Tsang, M.D. McKee, Y. Nys, Molecular cloning and ultrastructural localization of the core protein of an eggshell matrix proteoglycan, ovocleidin-116, J. Biol. Chem. 274 (1999) 32915–32923.

[34] M.T. Hincke, M. St Maurice, Phosphorylation-dependent modulation of calcium carbonate precipitation by eggshell matrix proteins, Chemistry and Biology of Mineralized Tissues, in: Proc. 6th Int. Conf. Vittel, France, Am. Ac. Orthop. Surg. Ed, 1998, pp. 13–17.

[35] M.T. Hincke, J. Gautron, M. Panhéleux, J.M. Garcia-Ruiz, M.D. McKee, Y. Nys, Identification and localization of lysozyme as a component of the eggshell membranes and shell matrix, Mater. Biol. 19 (2000) 443–453.

[36] G.K. Hunter, C.L. Kyle, H.A. Goldberg, Modulation of crystal formation by bone phosphoproteins: structural specificity of the osteopontin-mediated inhibition of hydroxyapatite, Biochem. J. 300 (1994) 723–728.

[37] C. Jimenez-Lopez, A. Rodríguez-Navarro, J.M. Dominguez-Vera, J.M. García-Ruiz, Influence of lysozyme on the precipitation of calcium carbonate: a kinetic and morphological study, Geochim. Cosmochim. Acta. 67 (2003) 1667–1676.

[38] O. Kälin, A. Rodríguez-Navarro, J.M. García-Ruiz, Estudio de los cascarones de Aquila adalberti, Informe Final del Proyecto para la Agencia del Medio Ambiente de la Junta de Andalucía, 1996, 101 p.

[39] G. Krampitz, G. Graser, Molecular mechanisms of biomineralization in the formation of calcified shells, Angew. Chem. Int. Ed. Engl. 27 (1988) 1145–1156.

[40] L.M. Lahav, L. Leiserowitz, Tailor-made auxiliaries for the control of nucleation, growth and dissolution of 2-dimensional and 3-dimensional crystals, J. Phys. D Appl. Phys. 26 (1993) B22–B31.

[41] R. Lakshminarayanam, S. Valiyaveettil, V.S. Rao, R.M. Kini, Purification, characterization, and in vivo mineralization studies of a novel goose eggshell matrix protein, ansocalcin, J. Biol. Chem. 31 (2003) 2928–2936.

[42] I. Lavelin, N. Yarden, S. Ben-Bassat, A. Bar, M. Pines, Regulation of osteopontin gene expression during eggshell formation in the laying hen by mechanical strain, Matrix Biol. 17 (1998) 615–623.

[43] I. Lavelin, N. Meiri, M. Einat, O. Genina, M. Pines, Mechanical strain regulation of the chicken glypican-4 gene expression in the avian eggshell gland, Am. J. Physiol. Regul. Integr. Physiol. 283 (2002) R853–R861.

[44] R.M. Leach, Biochemistry of the organic matrix of the eggshell, Poult. Sci. 61 (1982) 2040–2047.

[45] K. Mann, Isolation of a glycosylated form of the chicken eggshell protein ovocleidin and determination of the glycosylation site, Alternative glycosylation/phosphorylation at an N-glycosylation sequon, FEBS. Lett. 463 (1999) 12–14.

[46] K. Mann, F. Siedler, The amino acid sequence of ovocleidin-17, a major protein of the avian eggshell calcified layer, Biochem, Mol. Biol. Int. 47 (1999) 997–1007.

[47] K. Mann, I.M. Weiss, S. André, H.-J. Gabius, M. Fritz, The amino-acid sequence of the abalone (*Haliotis laevigata*) nacre protein perlucin. Detection of a functional C-type lectin with galactose/mannose specificity, Eur. J. Biochem. 267 (2000) 5257–5264.

[48] K. Mann, M.T. Hincke, Y. Nys, Isolation of ovocleidin-116 from chicken eggshells, correction of its amino acid sequence and identification of disulfide bonds and glycosylated asn, Matrix Biol. 21 (2002) 383–387.

[49] K. Mann, J. Gautron, Y. Nys, M.D. McKee, T. Bajari, W.J. Schneider, M.T. Hincke, Disulfide-linked heterodimeric clusterin is a component of the chicken eggshell matrix and egg white, Matrix Biol. 22 (2003) 397–407.

[50] K. Mann, F. Siedler, Ostrich (*Struthio camelus*) eggshell matrix contains two different C-type lectin-like proteins. Isolation, amino acid sequence, and posttranslational modifications, Biochim. Biophys. Acta, Proteins & Proteomics 1696 (1) (2004) 41–50.

[51] M. McKee, M.T. Hincke (in preparation).

[52] K.E. Mikhailov, Avian eggshells: an Atlas of scanning electron micrographs, British Ornitologists' Club Occasional Publications, 1997, n°3, 88 p.

[53] Y. Mine, C. Oberle, Z. Kassaify, Eggshell matrix proteins as defence mechanism of avian eggshell, J. Agric. Food Chem. 51 (2003) 249–253.

[54] T. Nakano, N. Ikawa, L. Ozimek, Extraction of glycosaminoglycans from chicken eggshell, Poult. Sci. 80 (2001) 681–684.

[55] T. Nakano, N. Ikawa, L. Ozimek, Galactosaminoglycan composition in chicken eggshell, Poult. Sci. 81 (2002) 709–714.

[56] Y. Nys, La coquille d'oeuf : un biomatériau composite, Pour la Science 289 (2001) 48–54.

[57] Y. Nys, J. Zawadszki, J. Gautron, A.D. Mills, Whitening of brown-shelled eggs: Mineral composition of uterine fluid and rate of protoporphyrin deposition, Poult. Sci. 70 (1991) 1236–1245.

[58] Y. Nys, M. Hincke, J.L. Arias, J.M. Garcia-Ruiz, S.E. Solomon, Avian eggshell mineralization, Poult. Avian. Biol. Rev. 10 (1999) 143–166.

[59] M. Panhéleux, M. Bain, M.S. Fernandez, I. Morales, J. Gautron, J.L. Arias, et al., Organic matrix composition and ultrastructure of eggshell: a comparative study, Br. Poult. Sci. 40 (1999) 240–252.

[60] M. Panhéleux, O. Kälin, J. Gautron, Y. Nys, Features of eggshell formation in guinea fowl: kinetics of shell deposition, uterine protein secretion and uterine histology, Br. Poult. Sci. 40 (1999) 632–643.

[61] M. Panhéleux, Y. Nys, J. Williams, J. Gautron, T. Boldicke, M.T. Hincke, Extraction and quantification of eggshell matrix proteins (ovocleidins, ovalbumin, ovotransferrin) in shell from young and old hens, Poult. Sci. 79 (2000) 580–588.

[62] M. Pines, V. Knopov, A. Bar, Involvement of osteopontin in eggshell formation in the laying chicken, Matrix Biol. 14 (1995) 765–771.

[63] A. Rodríguez-Navarro, J.M. García-Ruiz, Model of textural development of layered crystal aggregates, Eur. J. Mineral. 12 (2000) 609–614.

[64] A. Rodríguez Navarro, R. Messier, C. Jimenez-Lopez, J.M. Garcia-Ruiz, Importance of electrostatic interactions between proteins and calcite surfaces, in: P. Li, P. Calvert, R. Levy, T. Kokubo, C. Scheid (Eds.), Mineralization in Natural and Synthetic Biomaterials, Mater. Res. Soc. Symp. Proc, , 2000, pp. 599–606.

[65] A. Rodriguez-Navarro, C.S. Romanek, Mineral fabrics analysis using a low-cost universal stage for X-ray diffractometry, Eur. J. Mineral. 14 (2002) 987–992.

[66] A. Rodriguez-Navarro, O. Kalin, Y. Nys, J.M. Garcia-Ruiz, Influence of the microstructure and crystallographic texture on the fracture strength of hen's eggshells, Br. Poult. Sci. 43 (2002) 395–403.

[67] A. Rodríguez-Navarro, J. Gautron, Y. Nys, A. Hernández-Hernández, J.M. García-Ruiz, Characterization of eggshell microstructure and its influence on mechanical properties, in: Proc. 16th Eur. Symp. on the Quality of Poultry Meat and 10th European Symposium on the Quality of Eggs and Egg products, Vol. III, , 2003, pp. 63–69.

[68] A.L. Romanoff, A.J. Romanoff, The Avian Egg, John Wiley & Sons Inc, New York, 1949, 918 p.

[69] F.C. Schaffner, P.K. Swart, Influence of diet and environmental water on the carbon and oxygen isotopic signature of seabird eggshell carbonate, Bull. Mar. Sci. 48 (1991) 23–38.

[70] X. Shen, A.M. Belcher, P.K. Hansma, G.D. Stucky, D.E. Morse, Molecular cloning and characterization of lustrin A, a matrix protein from shell and pearl nacre of *Haliotis rufescens*, J. Biol. Chem. 272 (1997) 32472–32481.

[71] H. Shiraga, W. Min, W.J. VanDusen, M.D. Clayman, D. Miner, C.H. Terrell, et al., Inhibition of calcium crystal growth in vitro by Uropontin: another member of the aspartic acid-rich protein superfamily, Proc. Natl Acad. Sci. USA 89 (1992) 426–430.

[72] S.E. Solomon, Egg and Eggshell Quality, Wolfe Publ. Ltd, London, 1991.

[73] C. Tyler, Wihhelm von Nathusius 1821–1899 on avian eggshells, University of Reading, UK, 1964.

[74] D.J. Varricchio, J.R. Horner, F.D. Jackson, Embryos and eggs for the Cretaceous theropod dinosaur *Troodon formosus*, J. Verteb. Paleontol. 22 (2002) 564–576.

[75] X. Wang, B.C. Ford, C.A. Praul, R.M. Leach, Collagen X Expression in oviduct tissue during the different stages of the egg laying cycle, Poult. Sci. 81 (2002) 805–808.

[76] F.H. Wilt, Matrix and mineral in the sea urchin larval skeleton, J. Struct. Biol. 126 (1999) 216–226.

[77] M.M. Wong, J.C. Hendrix, K. Von der Mark, C. Little, R. Stern, Collagen in the eggshell membranes of the hen, Dev. Biol. 104 (1984) 28–36.

[78] T.M. Wu, J.P. Rodriguez, D.J. Finck, D.A. Carrino, J. Blackwell, A.I. Caplan, et al., Crystallization studies on avian eggshell membranes: implication for the molecular factors controlling eggshell formation, Matrix Biol. 14 (1994) 507–513.