

The eggshell: structure, composition and mineralization

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1. ABSTRACT

The calcareous egg is produced by all birds and most reptiles. Current understanding of eggshell formation and mineralization is mainly based on intensive studies of one species - the domesticated chicken *Gallus gallus*. The majority of constituents of the chicken eggshell have been identified. In this article we review eggshell microstructure and ultrastructure, and the results of recent genomic, transcriptomic and proteomic analyses of the chicken eggshell matrix to draw attention to areas of current uncertainty such as the potential role of amorphous calcium carbonate and the specific nature of the molecules that initiate (nucleate) mammillary cone formation and terminate palisade layer calcification. Comparative avian genomics and proteomics have only recently become possible with the publication of the *Taeniopygia guttata* (zebra finch) genome. Further rapid progress is highly anticipated with the soon-to-be-released genomes of turkey (*Meleagris gallopavo*) and duck (*Anas platyrhynchos*). These resources will allow rapid advances in comparative studies of the organic constituents of avian eggshell and their functional implications.

2. INTRODUCTION

The calcareous egg of birds and reptiles, and formerly dinosaurs, is a successful reproductive adaptation to the terrestrial environment. The eggshell has been shaped through evolution to resist physical and pathogen challenges from the external environment, while satisfying the metabolic and nutritional needs of the developing embryo by regulating gas and water exchange, and serving as a calcium store. Animals that deposit their eggs in moist environments (turtles, crocodiles) produce eggs with shells that are incompletely calcified but that still function as a calcium reservoir. Eggshell ultrastructure varies considerably. Most generally, eggshell types can be categorized as membrane-like (snakes and lizards), pliable (most turtles) and rigid (some turtles, some gecko, all crocodile, all birds and dinosaurs) (1-3).

The avian egg is considered to represent the most advanced amniotic egg in oviparous vertebrates. The shell is a complex bioceramic that regulates the exchange of metabolic gases and water, and its properties are exquisitely fine-tuned to the environment of a given species

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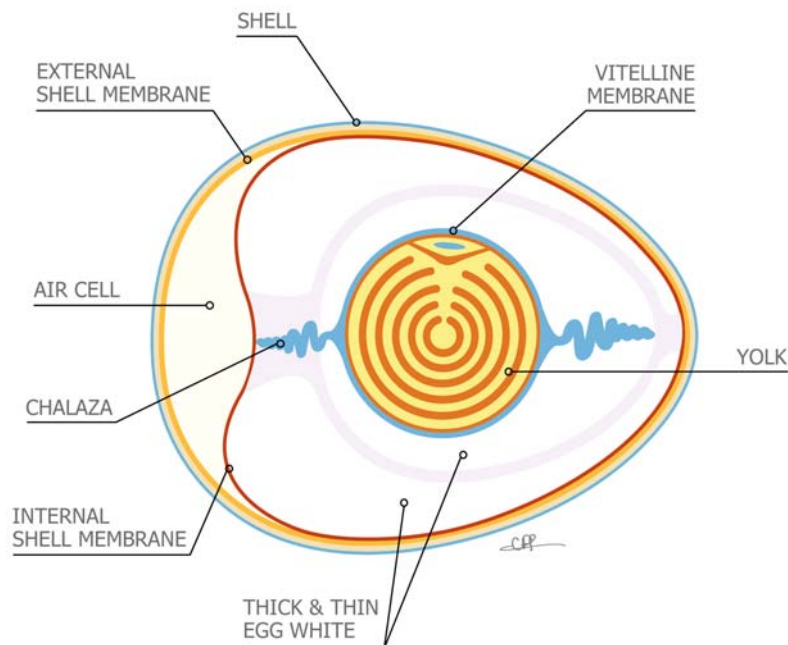


Figure 1. Longitudinal section to depict the interior contents of a chicken egg.

(4). Genetics controls the shell permeability, which depends on the characteristics of its pores - number, density, branching pattern and caliber. The shell resists the weight of the brooding hen and provides protection against physical damage, microbial invasion and predation by small animals.

Many physiological, biochemical, nutritional, structural and morphological studies have been conducted on avian eggs and most have utilized the egg of the domestic chicken. This is of course because of its ready availability and commercial importance as a nutritious food for human consumption. In contrast, there is much less known regarding eggs and eggshell of other birds or non-avian animals (snakes, lizards, turtles, crocodiles and dinosaurs). Hence, this review of eggshell biomineralization will emphasize the considerable body of research dealing with the chicken (*Gallus gallus*) eggshell which has provided insight into its structure, function and mineralization. Where possible, comparisons with eggshell from other avian species and with other animals will be made. We have incorporated the results of recent proteomic analyses of eggshell, with insight gained from avian transcriptomics and genomics, to draw attention to key features of eggshell formation and function.

The release of the chicken (*Gallus gallus*) genome sequence in 2004 was a tremendous advance, permitting molecular biology-based approaches to avian biology, physiology and breeding, amongst others (5). This database is also a fundamental resource to support proteomic studies of chicken tissues and egg compartments, including the eggshell. Comparative avian genomics and proteomics have only recently been possible with the publication of the *Taeniopygia guttata* (zebra finch)

genome (6). Highly anticipated are the soon-to-be-released genomes of turkey (*Meleagris gallopavo*) and duck (*Anas platyrhynchos*). These resources will allow rapid advances in comparative studies of the organic constituents of avian eggshell and their functional implications.

3. OVERVIEW OF EGGSHELL FORMATION AND STRUCTURE

The egg is composed of a central yolk surrounded by the albumen (egg white), eggshell membranes, calcified eggshell and cuticle (4) (Figure 1). The process of egg formation is well characterized in birds, particularly the domestic chicken, where the distinct spatial and temporal regulation of deposition of each egg compartment is known in detail (7,8). Each egg is individually shelled, followed by expulsion (oviposition) at intervals; for example, approximately 24 hr in chicken. This pattern is different in most reptiles, where multiple eggs are formed and acquire their shell within a single compartment of the oviduct, followed by simultaneous expulsion of the entire clutch (9). On the other hand, in crocodiles, like birds, the formation and shelling of eggs occur in different segments of the oviduct. However, the entire clutch is laid at the same time (10). The eggs of snakes and most lizards display floating crystals of calcite within single or multi-layered fibrous shell membranes (1).

The process of egg formation is well-known for birds. Following ovulation, the yolk travels through specialized regions of the oviduct to collect specific components of the egg (Figure 2). The outer vitelline membrane and the albumen are deposited during passage through the infundibulum and magnum, respectively. Next, the yolk and albumen complex travel through a specialized

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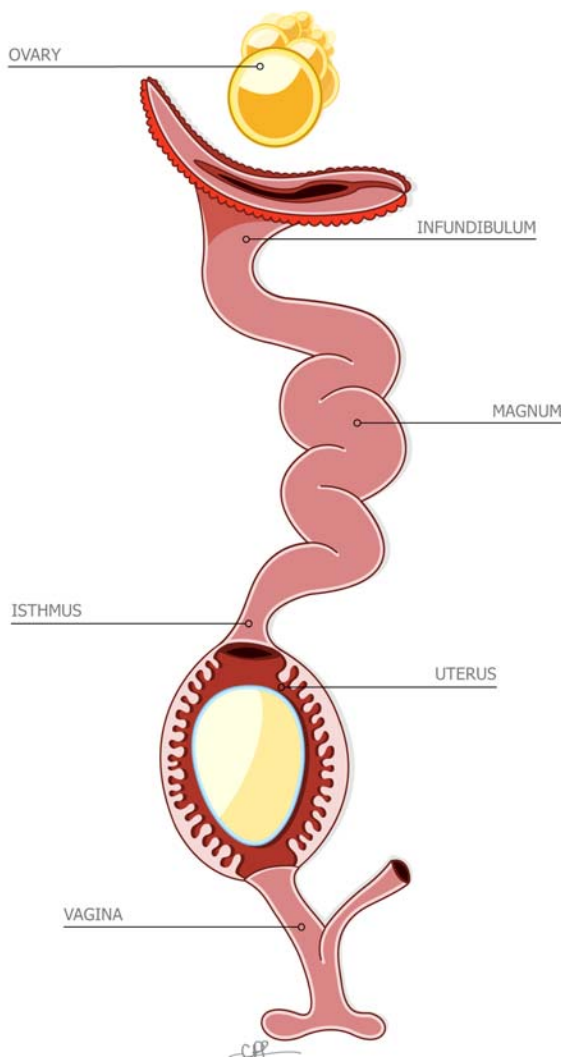


Figure 2. Stylized depiction of the reproductive system of the hen, containing an incomplete egg in the uterus.

segment of the oviduct known as the white isthmus. Here, the precursors of the eggshell membranes are secreted and assembled during approximately one hour. The resulting meshwork of interlaced fibres is organized into morphologically distinct inner and outer sheets that enclose the egg albumen. The membrane fibres are composed of roughly 10% collagen (types I, V and X) and 70-75% of other proteins and glycoproteins containing lysine-derived cross-links (11-14). The inner membranes remain uncalcified, while the fibres of the outer shell membrane become mineralized at discrete sites (8,15) (Figure 3A) and become incorporated into the base of the eggshell.

The first steps in eggshell mineralization are initiated when the forming egg enters the next oviduct region, the red isthmus (tubular shell gland). A quasi-periodic array of organic aggregates is deposited on the outer shell membrane. When the egg next enters the uterus (shell gland pouch) these sites subsequently become the

distinct crystal nucleation sites which are the origin of the mammillary knobs (8). The mechanisms that prevent calcification towards the inner membrane and albumen are not well understood. One proposal is that collagen type X prevents a generalized calcification of the shell membrane (15,16). It is well-known that any modification of the eggshell membranes due to inhibition of fibre formation or cross-linking alters eggshell formation and its mechanical properties. Thus, inhibition of the lysine-derived cross-linking of eggshell membrane by aminopropionitrile or by copper deficiency affects the pattern of eggshell structure and degrades its mechanical properties (16,17).

The eggshell forms by controlled precipitation of calcium carbonate on the outer membrane fibres, and occurs in the extracellular space between the dilated shell membranes that envelope the hydrated albumen and the mucosa of the uterine wall. Throughout all phases of mineralization, the incomplete shell is bathed in a uterine fluid containing 6 to 10 mM of ionized calcium and about 70 mM of bicarbonate ions, concentrations which are 80 - 120 times greater than the solubility product of calcite (18). Calcium carbonate precipitates spontaneously from this supersaturated milieu in the form of calcite (the most thermodynamically stable polymorph at body temperature and atmospheric pressure). Moreover, the organic constituents of the uterine fluid promote the formation of calcite, as opposed to other polymorphs of calcium carbonate (aragonite, vaterite) (19-21). The thickness of the resulting biomineralized structure may range from 0.052 mm (Palestine sunbird, *Nectarinia osea*) to 0.3-0.4 mm (chicken, *Gallus domesticus*) to 4.40 mm (the extinct elephant bird, *Aepyornis maximus*) (22,23), providing evidence for a wide range of scalability for eggshell formation. When complete, the avian eggshell has a well-defined structure that is described as follows from the inside (egg white side) to the outside (external surface): (i) the mammillae (or mammillary body / cone layer), (ii) the palisades (or palisade layer) comprising the thickest layer of the shell, and (iii) the transitional vertical crystal layer. Finally, a thin non-calcified cuticle layer coats the eggshell (24-26) (Figure 3A, 4). The transitional, inner zone of the cuticle contains spherical aggregates of hydroxyapatite (25).

The mammillary layer is a regular array of cones or knobs, each with a core of concentrated organic material that was originally described as neutral mucopolysaccharide (27) and contains keratan sulfate (28,29). The corresponding uncharacterized proteoglycan has been termed "mammillan" (28). The individual fibres of the outer eggshell membrane are embedded into the mammillary cones. An important region of the mammillae - the calcium reserve body - contains microcrystals of calcite with spherulitic texture which facilitate the eventual dissolution of the mineral and mobilization of calcium to nourish the embryo. When embryonic development is complete, the weakened eggshell is more susceptible to propagation of cracks during pipping (hatching) (8, 26). The palisade layer is made up of groups of columns that are perpendicular to the eggshell surface and extend outwards from the mammillary cones (Figure 3, 4). This layer ends at

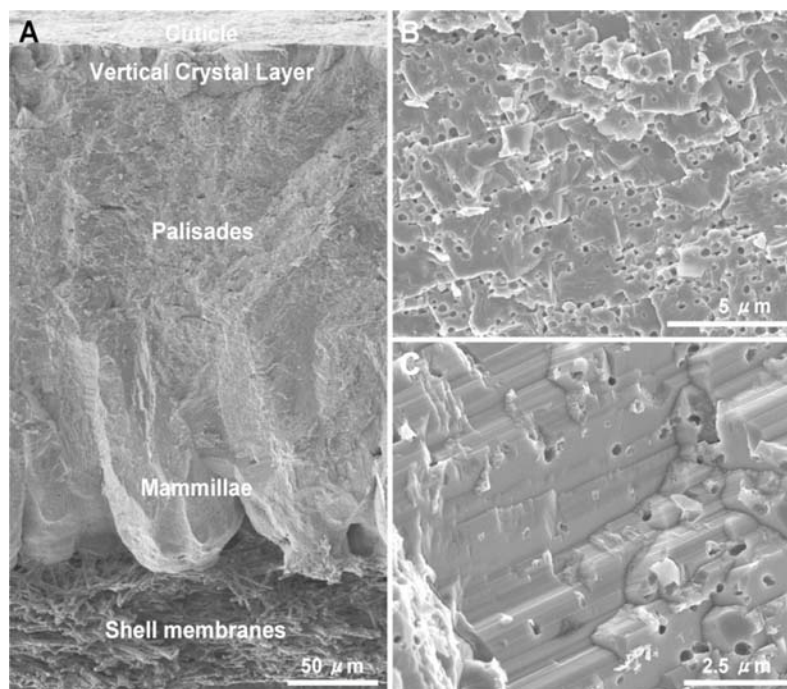


Figure 3. Calcified eggshell structure. (A) Scanning electron micrographs of avian eggshell showing overall structure and regions of the calcified eggshell that form on the shell membranes during the egg-laying cycle. At this low magnification, and starting adjacent to the shell membranes, eggshell consists of mammillae, palisades, vertical crystal layer, and cuticle. (B,C) Higher magnification of the palisades region shows extensive planes of cleaved calcite, and numerous small, spherical voids. Adapted with permission from 117.

the vertical crystal layer which has a crystalline structure of higher density than that of the palisade layer. The outer region of the palisade layer is a tough structure made of large crystals where the external impacts are absorbed by thin inter- and intra-crystalline organic layers that make intracrystalline crack propagation difficult (8). Pores that traverse the eggshell permit the diffusion of metabolic gases and water vapor. Cuticular material spans the pore opening and fills in the upper pore space.

3.1. Eggshell calcification

The avian eggshell is a porous bio-ceramic that is formed at body temperature in a cell-free environment. Its formation is one of the fastest calcifying processes known in biology. The completed chicken eggshell contains about 6 g of mineral which is deposited during its daily production cycle. Ion transport mechanisms across the uterine mucosa that underlie this process have been studied extensively (7,30,31). The mineralized shell is about 96% calcium carbonate. The remaining components include the organic matrix (2%) as well as magnesium, phosphorus and a variety of trace elements (8). The eggshell exhibits extensive intermingling of both its organic and inorganic phases (26). This type of organization is also observed in other calcified matrices (bone, cartilage, and tooth enamel, dentin and cementum), where occluded collagenous and noncollagenous elements are present in intimate contact with mineral (32). The precursors of the eggshell matrix are present in the acellular uterine fluid, from which they become incorporated into the calcifying shell.

Eggshell deposition occurs in three stages. The entire process lasts almost 17 hr in chicken breeds that are highly selected as layers, and is the lengthiest phase of egg formation (8). The first stage is about 5 hr in duration and corresponds to the initiation of mineralization. The first crystals of calcite are nucleated at the sites of the organic aggregates present on the surface of outer shell membranes. The composition of these aggregates is not understood, although a keratan sulfate proteoglycan has been implicated (28). The distribution of these nucleation sites is under genetic control and varies among species. The size of the mammillary cones, the cylindrical diameter of the palisades in the compact layer of the shell and, ultimately, the strength of the shell are determined by the spacing of these sites. During the first stage of mineralization, crystal growth is radial calcite. The nucleation sites become the origins of the mammillary cones. As they grow outwards, they gradually come together to form the bases of the palisade layer, at which point radial crystal growth is inhibited by mutual exclusion (8).

The second stage is the rapid growth of polycrystalline calcite to form the palisade layer. Formation of the palisades occurs into the available free space, producing crystals growing perpendicular to the surface of the forming shell, and there is a linear deposition of about 0.33 g / hr of calcium carbonate for about 10 hrs. The ultrastructure and crystallography of the compact mineral layer can be partially explained by a single model of competition for crystal growth: growth of crystals from the

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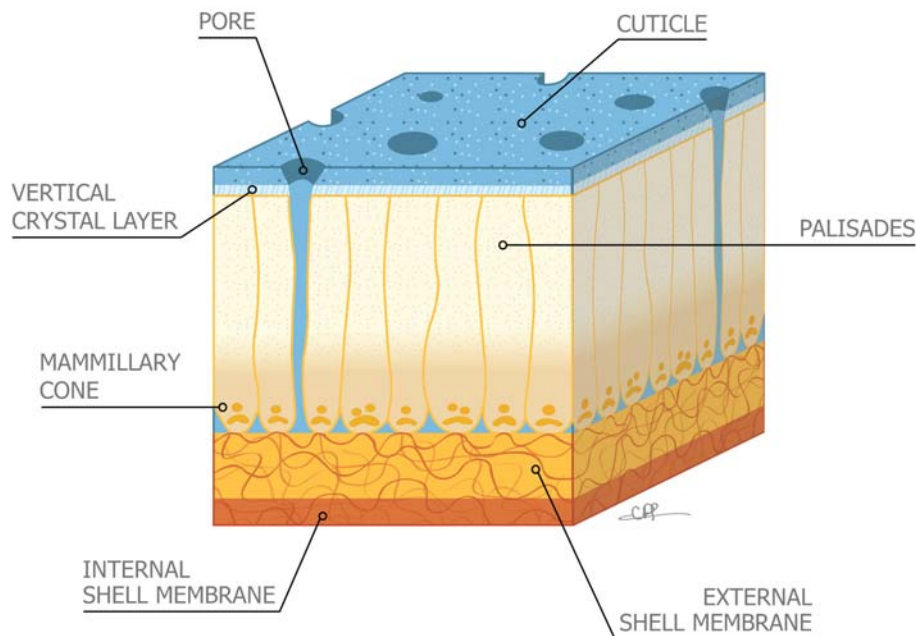


Figure 4. Artistic rendition of cross-sectional view of chicken eggshell. The major morphological features are labelled.

nucleation site occurs initially in all directions but, because of competition for space between adjacent sites of growth, only crystals growing perpendicular to the egg surface have space to grow. This model explains the appearance of preferred crystal orientation in the outer part of the eggshell, but is based on the hypothesis that the crystal growth is anisotropic. Anisotropy results from inhibition of crystal growth on the faces parallel to the *c*-axis and results in an elongation of the calcite crystal. This inhibition is thought to result from some organic components that are present in the uterine fluid, and then integrated into the outer eggshell (21). Another example of this phenomenon, which controls mineral texture, is the preferential localization of osteopontin on specific calcite crystal faces in the palisades (33).

The last stage corresponds to termination of calcification and lasts about 1.5 hr (8). The arrest of mineralization occurs in a uterine fluid that remains supersaturated in calcium and bicarbonate ions. The details are not well understood, but proteins are probably implicated since CaCO_3 precipitation is inhibited *in vitro* by high-molecular weight components of the terminal phase uterine fluid (34). Phosphorus is detected in the superficial layers of the chicken shell (35), and spherical aggregates of fine needlelike hydroxyapatite crystals are found in the outermost calcified layer of the chicken eggshell (25). Since phosphate anions can inhibit calcium carbonate precipitation (36), the relative contribution of inorganic phosphate and phosphoproteins to termination of calcification remains to be determined. Ovocalyxin-32, a major phosphoprotein of the eggshell matrix, is concentrated in the outer eggshell and cuticle and therefore is a potential candidate as a proteinaceous crystal growth inhibitor (37).

3.2. Eggshell microstructure and crystallographic texture

Thickness is the main factor contributing to the mechanical strength of the eggshell (23,38,39). However, the structural organization of the eggshell at different levels significantly influences its mechanical properties (40-43). Ultrastructure (the extent and disposition of major structural units) and microstructure or texture (the size of crystals, their shape and crystallographic orientation) are especially important. The ultrastructure of the chicken eggshell is extremely regular. It is a polycrystalline calcium carbonate ceramic consisting of only one polymorph, calcite. The mammillary cones are composed of calcite crystals that are small in size and are deposited without privileged orientation. The palisade layer of the chicken eggshell is about 200 micrometres thick, and is composed of irregular juxtaposed columns. In the palisade layer the crystals increase their size progressively and elongate along the calcite *c*-axis towards the eggshell surface. The lateral size (width) of crystals composing the eggshell increases with thickness from 20 micrometres at its inner part to about 80 micrometres at the outer shell (42).

Eggshell ultrastructure varies considerably between reptilia and aves. Excellent reviews describing differences amongst the rigid, fully mineralized eggshells are available (2,3), and can be summarized as follows: Testudoid (turtle) – radial aragonite ultrastructure with an organic core at its base; Geckoid – dense homogenous calcitic material with poor ultrastructural detail and vertically oriented crystallites in outer shell; Crocodiloid – tabular ultrastructure arranged in large wedges of calcite without an organic core; Ornithoid (avian) – the ultrastructure demonstrates a clear boundary between the lower (mammillary, with radiating calcite and organic core

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at its base) and upper (palisades, with squamatic ultrastructure) parts. Unfortunately, almost nothing is known about the differences in key physiological processes and eggshell matrix constituents that are responsible for these distinctive ultrastructures.

Microstructural characteristics (crystal size and orientation) can vary significantly from one eggshell to another, even within the same species. Because of important implications of eggshell strength for food safety of the table egg, this aspect has been extensively investigated in the chicken. In addition to defects or scratches which act as nucleation sites for crack formation, weaker eggshells are formed by crystals of abnormal sizes (generally larger) and shapes, which negatively affect their mechanical performance (42,44). Moreover, the preferential orientation of crystals has a strong influence on eggshell mechanical properties, since calcite is easily cleaved along specific crystallographic directions. Eggshells composed of smaller, less mutually aligned, calcite crystals are stronger than those formed by larger and highly oriented crystals. In most avian species, the majority of the crystals comprising the shell are progressively directed in a single privileged direction; the *c*-axis of calcite tends to a perpendicular orientation to the shell surface in its upper third. For example, this is true for chicken, quail, pheasant, turkey and ostrich eggshell, whereas exceptions have been noted for eggs of guinea fowl, duck and goose (45-47).

4. REGULATION OF CALCIFICATION BY MATRIX CONSTITUENTS

The soluble matrix proteins of calcitic biomaterials can modify crystal growth, and in this manner regulate the macroscopic properties of the resulting bioceramic. In formation of the mollusc shell, specific proteins seem to control phase switching between the polymorphs of calcium carbonate, resulting in the outer calcitic and inner aragonitic (nacre) layers of the shell (48,49). For *Gallus gallus*, a number of experimental observations support the role of eggshell matrix proteins in determining the fabric of the eggshell and therefore its mechanical properties. Egg calcification takes place in the uterine fluid over three distinct phases (initiation, active calcification, and termination of shell calcification). The uterine fluid displays distinct protein electrophoretic profiles at each phase of shell mineralization, suggesting specific roles for different organic constituents during deposition of the mammillary bodies, palisades and the terminal zone (34). The nature of the interactions between the mineral phase and eggshell matrix proteins has been intensely investigated, but mechanistic details remain unknown. Whole uterine fluid modifies calcium carbonate precipitation kinetics, favours development of the calcite polymorph and alters the size and the morphology of calcite crystals grown *in vitro* (19,50). The lag time for calcium carbonate precipitation is reduced by the uterine fluid harvested during the initial and growth stages of eggshell mineralization, suggesting that these matrix precursors promote crystal nucleation. To a lesser extent, the uterine fluid collected during the growth phase also

enhances precipitation kinetics. In contrast, the total uterine fluid harvested at the terminal stage of calcification inhibits calcite precipitation (51). In agreement with these observations, partially purified eggshell matrix proteins inhibit calcium carbonate precipitation and alter patterns of calcite crystal growth, leading to morphological modifications of rhombohedral calcite crystals grown *in vitro* (21).

4.1. Purified eggshell matrix components

In chicken eggs, ovocleidin-17 (OC-17) is an abundant matrix protein that is concentrated in the mammillary layer. Calcite crystals grown in the presence of OC-17 are twinned (10-100 $\mu\text{g/ml}$) or display a protein concentration-dependent aggregation (50-200 $\mu\text{g/ml}$) (52,53). *In vitro* crystal growth experiments with ansocalcin, the goose eggshell analogue of OC-17, demonstrate that low levels of the protein (up to 10 $\mu\text{g/ml}$) induce calcite crystals with screw dislocations, while at higher concentrations ($> 50 \mu\text{g/ml}$), polycrystalline calcite aggregates are nucleated (53). Elevated concentrations of egg white proteins affect calcite crystal growth. Ovotransferrin (0.5 mg/ml) leads to smaller crystals and promotes the development of elongated crystals (54). Lysozyme at high concentration ($>10 \text{ mg/ml}$) mainly affects the calcite faces parallel to the *c*-axis, by inhibition of growth on {110} faces (55-57). Ovalbumin stabilizes amorphous calcium carbonate when present at the early phase of mineralization (58-60). Synergistic effects on crystal growth due to mixtures of these proteins have not been systematically explored.

Pure glycosaminoglycans inhibit calcium carbonate precipitation and affect calcite morphology leading to crystal elongation (61-63). Sulfated proteoglycans such as ovoglycan (ovocleidin-116, containing dermatan sulfate) and mammillan (a putative keratan sulfate proteoglycan) are likely to influence mineralization by electrostatic interactions (14,28). Protein phosphorylation is another post-translational modification that may be crucial. Partially purified eggshell osteopontin strongly inhibits calcium carbonate precipitation in a phosphorylation-dependent manner, suggesting that it could be a potent regulator of eggshell calcification (33,64). The major phosphorylated eggshell matrix proteins are osteopontin, ovocleidin-17, ovocleidin-116 and ovocalyxin-32 (65). Ovocleidin-17 is phosphorylated on two possible sites, Ser-61 and -67 (66), and can also be glycosylated at Asn-59 to yield a 23 kDa form (ovocleidin-23) (67). The function of these modifications remains unknown but the phosphorylation sites are preserved in closely related eggshell proteins isolated from other avian species (see below), suggesting their importance.

To date, we are aware of only one non-avian eggshell matrix protein that has been isolated and characterized. Pelovaterin is an anionic polypeptide (42 amino acids) purified from the aragonitic eggshells of the soft-shelled turtle, an animal so-called because its carapace is leathery and pliable (68). Its eggshell consists of a fibrous shell membrane and a calcareous layer composed of the aragonite polymorphic form of calcium carbonate. The

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amino acid sequence of pelovaterin shows no homology to any known protein. Purified pelovaterin induces the formation of the vaterite polymorph of calcium carbonate, alters crystal morphology and increases the mineral growth rate (68). Under certain conditions Pelovaterin can induce and stabilize aragonite as the dominant polymorph (69).

In vivo observations are in line with the results of *in vitro* experiments. Variations in nutritional, genetic or physiological characteristics of the domestic chicken affect the strength of the eggshell. The well-known reduction in shell strength associated with eggs from aged hens coincides with a change in the relative proportions of matrix proteins in the shell (70,71). Moulting, which rejuvenates hen reproductive physiology, restores the strength of the shell and reverses the changes previously observed for matrix composition and crystalline texture of the shell (44). Association studies between polymorphisms of genes encoding shell proteins and shell characteristics revealed that certain alleles are correlated with shell hardness (osteopontin), elasticity (ovocleidin-116), overall shell thickness (ovocleidin-116) and thickness of the mammillary layer (ovocalyxin-32) (72).

4.2. Amorphous calcium carbonate

Amorphous calcium carbonate (ACC) is now recognized as a transient non-crystalline precursor phase of calcite or aragonite in the calcified structures produced by many invertebrates. It represents a common biological mechanism for fabrication of biominerals, and allows the growth of single crystals with very complex shapes, for example, sea urchin spicules (73). There is still little direct evidence regarding the presence of ACC in eggshell mineralization. In this regard, however, circular voids or cavities have been described by many observers in the calcitic palisade layer of the eggshell (Figures 3B,C) (26,38-41,74). It has recently been recognized that similar cavities are produced during *in vitro* crystallization of amorphous calcium carbonate, possibly compensating for the volume decrease from ACC to calcite, with an impact on the kinetics of the crystallization process (75). *In vitro* work suggests that quail shell matrix extracts can induce the precipitation of ACC (76). Egg white proteins are well-described constituents of the eggshell matrix (77). Purified ovalbumin stabilizes ACC *in vitro*, while lysozyme does not (60). Ovocleidin-17 (OC-17) is one of the most abundant chicken eggshell matrix proteins. Computational predictions based on its X-ray structure suggest that OC-17 can catalyze transformation of ACC into calcite (78). While the role of ACC in eggshell mineralization is for the moment speculative, this is a field of intense activity.

5. EGGSHELL MATRIX PROTEINS

5.1. Eggshell matrix proteome

The protein constituents of the uterine fluid differ between the 3 stages of the eggshell calcification process (initial, growth and terminal) (34); they become progressively incorporated into the mineralizing eggshell resulting in their differential distribution throughout the eggshell zones (4). Thus a complex array of distinct proteins is released by eggshell demineralization (51,79).

These eggshell matrix proteins comprise an organic matrix of soluble and insoluble proteins, glyco- and phosphoproteins, and proteoglycans, which represent about 2% by weight of the calcified eggshell.

A large number of eggshell proteins (>500) have been identified by recent proteomic approaches (80,81). A small number of the most abundant of these had already been identified by classic characterization approaches: *Eggshell-specific proteins* were identified during investigation of abundant constituents of the eggshell and uterine fluid. These novel proteins were termed Ovocleidins (ovo, Latin – egg; kleidoun, Greek – to lock in, implying a functional role) or Ovocalyxins (ovo, Latin – egg; calyx, Latin – shell, referring to their shell location). Two possible roles for the Ovocleidins and Ovocalyxins have been proposed in avian reproduction: regulation of eggshell mineralization and anti-microbial defence (37,74,82,83). *Egg white proteins* ovalbumin, lysozyme and ovotransferrin are also present in the uterine fluid, and are primarily localized in the innermost regions (shell membranes and mammillary cone layer) of the eggshell (54,55,84). Lastly, Osteopontin, which seems to be an invariant feature of biological calcification in birds and mammals, is found in avian bone and eggshell (26,33,64,85-88). Matrix proteins are sequentially incorporated into the calcifying eggshell, which results in their distinct localization patterns in the inner (mammillary) and outer (palisade) layers of the mineralized shell (79).

Proteomic analysis allows minute amounts of biologically active proteins in tissue or fluid to be identified. The eggshell proteome released by decalcification is a complex mixture of uterine-derived proteins, including proteins originating from degraded cells or basement membranes, as well as those associated with the proximal oviduct (*i.e.* egg white, egg yolk and vitelline membrane proteins) (80). The number of eggshell proteins identified by mass spectrometry (>500 proteins) is almost 4-5 times greater than those found in other egg compartments (*i.e.* 148 proteins in egg white, 137 in the vitelline membrane and 316 in egg yolk) (65,89-93). However, many proteins released from the eggshell matrix by decalcification are low in abundance, do not exhibit a signal peptide required for regulated secretion and are normally viewed as intracellular constituents. It has been suggested that these eggshell matrix constituents arise from non-specific breakdown of the cells lining the oviduct due to abrasion or normal turnover during the lengthy mineralization process (80). This interpretation is in line with reports that maternal nuclear and mitochondrial DNA are present within the chicken calcified eggshell (94), suggesting that a variety of cellular constituents are passively incorporated into the eggshell during its formation.

The “Eggshell-Specific” proteins that are highlighted in this review are abundant components of the eggshell matrix and therefore are likely to be relevant to eggshell function. Supportive evidence for an eggshell-specific role would be restricted high level expression in a limited oviduct segment, up-regulation of expression in

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synchrony with movement of the forming egg through the oviduct, demonstration of a secretory process (*i.e.* presence of signal peptide or secretion granule localization demonstrated by colloidal gold immunocytochemistry) and secretion during eggshell formation, and finally, evidence for a functional role in eggshell (*i.e.* calcification, antimicrobial protection). Many of these criteria have been met by the Ovocleidins and Ovocalyxins.

5.2. Transcriptomics

Global gene expression profiling of the hen's oviduct during sexual maturation and eggshell formation have revealed a large number of differentially expressed genes (31,95,96). Upregulation of genes coding for eggshell-specific matrix proteins (ovocleidin-116, ovocalyxin-32 and ovocalyxin-36) occurs during both of these physiologically distinct processes, while osteopontin expression is only upregulated in uterus during eggshell calcification. This approach complements earlier focused proteomic analysis of the eggshell (65,80) that revealed more than 500 eggshell proteins. However, less than 10% of the identified proteins were common to both strategies. The characterization of all proteins in the eggshell is a prerequisite for exploration of functional properties and regulation of uterine proteins involved in fabrication of the eggshell. Additional biochemical studies are needed to confirm the biological activity of these proteins and to understand their roles in providing nutrients and protection for the developing embryo. Genes involved in the physical or chemical defense of the egg are functional candidates for marker-assisted selection to improve egg and eggshell quality. For example, the ovocalyxin-32 gene is expressed at higher levels in a low-egg production strain, as compared to a high-egg production strain, of Taiwanese country chickens (96).

5.3. Genomics

As expected, there is a high degree of synteny between the two avian genomes that have been sequenced to date: *Gallus gallus* and *Taeniopygia guttata* (zebra finch) (5,6). This is in spite of an estimated 100 Myr of evolutionary distance between Galloanserae (comprising galliform: chicken-like and anseriform: goose-like) and Neoaves (all other modern birds, such as zebra finch). By comparison, the evolutionary divergence of the ancestor of mouse and human species is estimated to have occurred 65-75 million years ago (97). Inspection of the *Gallus gallus* and *Taeniopygia guttata* genomes reveals that the chromosomal localization of ovocleidin-116 (OC-116) is adjacent to that of osteopontin on chromosome 4, and, moreover, that these two genes are contiguous with other mineralization-specific genes (bone sialoprotein, dentin matrix protein 1). These genes form the well-known SIBLING (small integrin-binding ligand, N-linked glycoprotein) mineralization gene locus which is present in both avian and mammalian genomes (4). Based on its position within this gene locus, OC-116 is predicted to be the avian ortholog of mammalian MEPE (matrix extracellular phosphoglycoprotein) (4,98,99).

The evolutionary genetics of vertebrate tissue mineralization suggest that OC-116 and other SIBLING proteins are members of the secretory calcium-binding

phosphoprotein (SCPP) family that functions in tetrapod mineralization (100,101). Proteins originating from the SCPP genes have a common characteristic: they bind calcium ions via acidic amino acids such as glutamate, aspartate and phospho-serine (100). One member – osteopontin (OPN) – regulates hydroxyapatite calcification in vertebrate mineralized tissues such as bone and teeth (102). Osteopontin purified from eggshell inhibits calcium carbonate precipitation (64). Purified osteopontin interacts specifically with the {104} calcite crystal face that may regulate eggshell calcification (26,33). Another SIBLING member – dentin matrix protein 1 (DMP1) – is also an eggshell matrix protein (80,103) but its influence upon calcite crystallization is not yet known. Of these proteins, OC-116 is highly specialized to function in calcitic mineralization of the avian shell. Further study of common features of OC-116 from different avian species should help to define its structural motifs that are specific for calcitic mineralization (98).

A complementary approach to establish the role of matrix proteins in the variability of the eggshell physical and mechanical properties has been taken using genetic and genomic approaches. This study reveals a number of significant associations between alleles of certain candidate genes (OC-116, OCX-32, OPN and ovalbumin) and measurements of eggshell biomechanical properties (72). Single nucleotide polymorphisms (SNPs) in the OC-116 gene are significantly associated with eggshell elastic modulus and thickness, and egg shape, whereas OPN was associated with eggshell fracture toughness. OCX-32 SNPs were found to be significantly associated with mammillary layer thickness.

5.4. Ovocleidin-17

OC-17 is an abundant matrix protein (40 µg/g of shell) which is distributed throughout the shell matrix, but is concentrated in the mammillary bodies (66,82). It is a member of a family of homologous eggshell matrix proteins that have been identified in goose (ansocalcin), ostrich (struthiocalcin: SCA-1 & -2), emu (dromaiocalcin: DCA-1 & -2) and rhea (rheacalcin: RCA-1 & -2). These proteins form a family of two related groups based on sequence identity, patterns of serine phosphorylation and conservation of cysteine residues (66,104-106). These sequence similarities have persisted during more than 100 million years of evolution separating the Palaeognathae (ratites) and Neognathae (all other birds), implying an important and conserved role in eggshell function. Therefore, it is likely that homologous proteins are found in the shells of all bird species. OC-17 and the other members of this family contain a single C-type lectin-like domain (CTL), and therefore resemble mammalian Reg (Regenerating islet-derived) proteins such as lithostathine, fish Type II antifreeze proteins and snake venom anticoagulant proteins (107). The X-ray structure of OC-17 reveals a mixed alpha helix / beta sheet structure and verifies the C-type lectin-like domain (52,108). Computational modelling based on its X-ray structure leads to the prediction that OC-17 could catalyze transformation of ACC into calcite (78). This is a provocative prediction, which has not yet been tested experimentally.

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OC-17 was the first eggshell matrix protein to be isolated and to be directly sequenced (66,82). However, in spite of significant efforts, it has not yet been cloned. Although the chicken genome is approximately 95% complete (Ensembl release 60 - Nov 2010), the complete OC-17 gene has not yet been identified. Nothing is yet known concerning regulation of OC-17 gene expression or tissue specificity of its mRNA synthesis.

5.5. Ovocleidin-116

OC-116 was the first eggshell matrix protein to be cloned, by expression screening a uterine library using an antibody raised to the abundant 116-kDa protein observed in hen uterine fluid during the active calcification phase of shell formation (74). OC-116 is the most abundant eggshell matrix protein, estimated at 80 µg/g of shell (109). It is not absolutely eggshell specific, but is also present in embryonic chicken osteoblasts and osteoclasts, as well as young chick cortical bone, laying hen medullary bone and growth plate hypertrophic chondrocytes (98,103). This suggests an additional role for this protein in bone mineralization, a role which may be similar to that of its mammalian ortholog MEPE (110). The N-terminus of the mature protein and conceptual translation product from cDNA correspond to that previously reported for a 200 kDa eggshell matrix proteoglycan that is converted to 120 kDa by chondroitinase ABC treatment (111). Therefore, OC-116 is the core protein (predicted to be 75 kDa) which is common to the doublet bands of an eggshell dermatan sulfate proteoglycan (116 kDa and 180 kDa). It is hypothesized that the highest molecular weight form of OC-116 corresponds to the N-glycosylated core protein with attached glycosaminoglycans, while the 116 kDa form corresponds to the protein without glycosaminoglycans (74). Sequencing of peptides purified from protease-treated eggshell extract reveal that both predicted N-glycosylation sites are modified; however, while Asn-62 is entirely glycosylated, Asn-293 is only marginally occupied (110). Detailed analysis of the carbohydrate structures attached to Asn-62 revealed 17 different oligosaccharide structures (112). On the other hand, the putative glycosaminoglycans associated with OC-116 have only been characterized in a preliminary fashion (111). OC-116 is phosphorylated to a variable and partial extent on at least 22 serine and threonine residues. Two sites that were frequently identified with different cleavage methods were Ser-444 and Thr-664 (65). The N-terminal amino acid sequence of a quail 116 kDa eggshell matrix protein reveals 75% identity to the N-terminus of chicken OC-116 (113), while zebra finch and chicken OC-116 proteins possess 40% overall identity (77).

Ultrastructural immunocytochemistry indicates that OC-116 is synthesized and secreted from the granular cells of the uterine epithelium, and becomes widely distributed throughout, the palisade layer of the calcified eggshell (74). Transmission electron microscopy (TEM) of the organic matrix of the avian eggshell reveals two structural features within the palisade layer; vesicular structures with electron-lucent cores intermingle between flocculent sheets of organic material. OC-116 is predominately associated with the periphery of the

vesicular structures that probably correspond to the walls of microvesicular holes (cavities) in the calcitic eggshell (See section 4.2, Figures 3B,C) (74). Such localization studies have not distinguished between the differentially phosphorylated, N-glycosylated or glycanated forms of OC-116. Single nucleotide polymorphisms (SNPs) in the OC-116 gene are significantly associated with eggshell elastic modulus and thickness, as well as egg shape (72).

5.6. Ovocalyxin-32

OCX-32 was originally identified as a 32 kDa uterine fluid protein that is abundant in the terminal phase of shell mineralization (37,114). Sequencing of peptides derived from the purified protein allowed Expressed Sequence Tag sequences (ESTs) to be identified that were assembled to yield a full-length composite sequence whose conceptual translation product contained the complete amino acid sequence of OCX-32. OCX-32 is expressed at high levels in the uterine and isthmus regions of the oviduct (37). The timing of OCX-32 secretion into the uterine fluid has been interpreted to suggest that it plays a role in the termination of eggshell calcification (4,34). This hypothesis is supported by observations of morphological changes in calcite crystals by uterine fluid collected during the terminal phase of calcification, and by the co-precipitation of OCX-32 with calcium carbonate *in vitro* from fresh uterine fluid (19,51). In the eggshell, OCX-32 localizes to the outer palisade layer, the vertical crystal layer, and the cuticle of the eggshell (37). OCX-32 is phosphorylated at serines and threonines between positions 257 and 268 (65).

5.7. Ovocalyxin-36

OCX-36 is a prominent 36 kDa protein present in the uterine fluid collected during the active calcification stage of shell mineralization (83). The protein is only detected in the regions of the oviduct where eggshell formation takes place (isthmus and uterus). Moreover, the uterine OCX-36 message, quantified by real time RT-PCR, is strongly upregulated during eggshell calcification (83). OCX-36 localizes to the calcified eggshell (predominantly in the inner part of the shell), and is abundant in the shell membranes. The OCX-36 protein sequence displays significant identity with mammalian proteins such as lipopolysaccharide-binding proteins (LBP), bactericidal permeability-increasing proteins (BPI) and palate, lung and nasal epithelium clone (Plunc) family proteins that are key components of the innate immune system and act as the first line of host defense (99,115). LBP proteins initiate the inflammatory host response upon the detection of a pathogen (116). OCX-36 may therefore participate in natural defense mechanisms that keep the egg and oviduct free of pathogens. However, the impact of the purified protein upon calcite crystallization has not yet been evaluated.

5.8. Osteopontin

OPN is a phosphoglycoprotein associated with normal and pathological calcium mineralization (102). In the chicken, OPN is found in both bone and eggshell (64,86,117). The oviduct expression of OPN is entirely uterine-specific and is temporally associated with eggshell calcification through coupling of physical distension of the

uterus to osteopontin gene (Spp1) expression (87). Localization studies show that OPN is concentrated in the palisade layer of the eggshell (26,33,88,117). OPN exists as two to three predominant forms in both eggshell and bone that however appear to differ in their posttranslational modifications (117). A number of phosphorylated residues in chicken eggshell and osteoblast OPN have been identified, which partially overlap between the two tissues (65,118). Dephosphorylation of eggshell OPN greatly diminishes its ability to inhibit precipitation of calcium carbonate from a supersaturated solution (64).

After decalcification and processing of the eggshell for transmission (TEM) and scanning (SEM) electron microscopy, an extensive organic matrix network is observed throughout all regions, which includes interconnected fibrous sheets, irregularly shaped aggregates, vesicular structures, protein films, and isolated protein fibres. OPN is associated with protein sheets in the highly mineralized palisade layer, but not with the vesicular structures (26,33,117). The association of OPN with parallel sheets of matrix, and more diffusely with the {104} crystallographic faces of eggshell calcite, may function in regulating palisades growth by orienting calcite crystals and by regulating the speed of mineralization. The elongated calcite crystals in the palisade layer tend to be preferentially orientated with the (001) planes parallel (c-axis perpendicular) to the shell surface, which orients the {104} plane at 44° tangential to the surface (42,119). The {104} calcite face is the natural cleavage plane, and specific OPN binding to this growing crystal face during mineralization could modify the resistance of the shell to fracture along this plane. The finding of an interaction between OPN and the {104} eggshell calcite faces was confirmed by *in vitro* studies of synthetic calcite growth where inhibition by added OPN was observed at the {104} faces (33).

Both OPN and OC-116 are synthesized and secreted by the granular epithelial cells of the shell gland (74,86,88,117). Unusual patterns of uterine OPN expression may underlie certain defects in eggshell mineralization (120). A candidate gene association analysis with eggshell matrix genes recently revealed that OPN SNPs were associated with eggshell fracture toughness (72).

6. SUMMARY AND PERSPECTIVE

There are about 4260 species of mammals known on this planet, out-numbered by reptiles (>6700 species) and birds (>9700 species). One defining characteristic for all birds and most reptiles is the calcareous eggshell, which is a feature of their obviously successful reproductive strategies. This review has attempted to integrate current knowledge of eggshell formation and structure with recent genomic, transcriptomic and proteomic analyses for the chicken *Gallus gallus*, the best known and the only well-studied example. The constituents of the eggshell matrix have been mainly identified, and we are beginning to understand the role of key molecules (such as osteopontin, ovocleidins and ovocalyxins) in mineralization. In spite of this

knowledge, major uncertainties remain, such as the possible role of amorphous calcium carbonate in fabrication of the calcitic eggshell. Moreover, the precise features of the eggshell membranes and mammillary cones that provide a template for initiation of calcification at an array of nucleation sites are poorly understood, although this is a key event in shell mineralization. The protein constituents of the outermost zones of eggshell (vertical crystal layer and the cuticle), and their role in termination of shell mineralization, also remain poorly characterized. Comparative avian genomics and proteomics, recently possible with the *Taeniopygia guttata* (zebra finch) genome, and soon for turkey (*Meleagris gallopavo*) and duck (*Anas platyrhynchos*), should provide significant insight into protein function. For example, comparisons of protein sequence between zebra finch and chicken for osteopontin (68% identity) and ovocleidin-116 (40% identity) are likely to point to key structural features that regulate calcitic biomineralization. At the same time, a challenge remains to extend our detailed level of understanding to the distinctive features of the reptilian eggshell.

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